NOTE TO USERS

Humoral Response to *M. tuberculosis* Antigens in Patients with Tuberculosis in the Gambia

Chris Greenaway
Department of Epidemiology,
McGill University, Montreal
August 2003

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science (M.Sc.)

Your file Votre référence
ISBN: 0-612-98651-9
Our file Notre référence
ISBN: 0-612-98651-9

Table of Contents

Table of Contents /2

Table of Contents /3

List of Tables

List of Tables cont...

List of Figures

Acknowledgements

Dr. Dick Menzies my MSc thesis supervisor for his endless support, enthusiasm, optimism and patience throughout the long process (helping write my first grant, the numerous emails while in the Gambia troubleshooting the study, providing financial support for the laboratory materials, helping with the analysis of the study and constructive and insightful comments on the manuscript) that produced the final draft of this thesis.

Dr. Christian Lienhardt for generously allowing me to use the data from the TB GENEV study and access to the stored sera of the study participants.

Dr. Richard Adegbola for helping resolve the difficulties of optimizing the ELISA technique in the Gambia.

Dr. Keith McAdam for believing in the study and facilitating the relocation of myself and my family to the MRC Laboratories in the Gambia for a year.

Dr. Marila Gennaro for the initial training in the ELISA technique, providing the antigens free of charge, and for technical support in the in the field.

Arnaud Marchand for providing the space in his laboratory at the MRC Laboratories in the Gambia, and for his support of the ELISA technique in the field.

The TBGENEV staff for introducing me to the challenges of field work and the culture of the people of the Gambia.
MRC Staff for making our year in the Gambia enjoyable and memorable.
The people of the Gambia for participating in the study

The MRC of Canada for providing salary support for a research fellowship year

Finally and most importantly, heartfelt appreciation to Mark, Adam, Michael and Nathan for their encouragement and support while I was taking the MSc courses and working full-time, for accompanying me to the Gambia and sharing this unforgettable experience, and for their endless patience with all the nights and weekends away to complete the analysis and writing of this thesis.

1. ABSTRACT

New tests to diagnose active tuberculosis (TB) that are simple, rapid and inexpensive, yet sensitive and specific are urgently needed. We assessed the sensitivity and specificity of seven different *M. tuberculosis* antigens for the diagnosis of active pulmonary TB in The Gambia. Three of the antigens tested were restricted, i.e. absent from BCG and some non-tuberculous mycobacteria (ESAT6, CFP-10 and Rv3871), and four shared, i.e. common to most mycobacteria (38kDa, GLU-S, 19kDa and 14kDa). Sera from 100 patients with active pulmonary TB, 100 household contacts, and 100 healthy neighborhood controls, in the Gambia, were tested by ELISA for antibodies to these 7 antigens. The sensitivity and specificity of both the shared and the restricted antigens were unacceptably low. In countries with high rates of TB, such as the Gambia, the clinical utility of serologic testing to diagnose active tuberculosis remains limited.

RÉSUMÉ

Il existe un besoin urgent pour de nouveaux tests diagnostiques simples, rapides, peu coûteux, mais néanmoins sensibles et spécifiques pour la tuberculose active. Nous avons évalué la sensibilité et la spécificité de sept antigènes différents de *M. tuberculosis* pour le diagnostic de la tuberculose pulmonaire active en Gambie. Trois de ces antigènes étaient caractéristiques pour le complexe tuberculose, car ils sont absents du BCG (ESAT6, CFP-10 et Rv3871) et quelques mycobactéries non tuberculeuses. Les quatre autres antigènes étaient partagés, c'est-à-dire communs avec d'autres mycobactéries (38kDa, GLU-S, 19kDa et 14kDa). Les sérums de 100 patients avec tuberculose pulmonaire active, de 100 contacts domestiques et de 100 cas contrôles (voisins sains), tous prélevés en Gambie, ont été testés par ELISA pour les anticorps à ces sept antigènes. La sensibilité et la spécificité de tous ces antigènes, aussi bien les spécifiques que les communs, étaient trop basses. Dans les pays à haute prévalence de tuberculose comme la Gambie, l'utilité clinique de tests sérologiques pour le diagnostic de la tuberculose active reste limitée.

2. ABBREVIATIONS

AFB	Acid Fast Staining
BCG	Bacille Calmette-Guérin Vaccination
CDR	Case Detection Rate
ELISA	Enzyme-linked immunosorbant assay
EPI	Extended Program on Immunization
GNP	Gross National Product
HIV	Human Immunodeficiency Virus
LAM	Lipoarabinomannan
MAC	*Mycobacterium avium complex*
MTB	*Mycobacterium tuberculosis*
NTP	National Tuberculosis Program
NLTP	National Leprosy and Tuberculosis Program
NTM	Non-tuberculous Mycobacteria
PPD	Purified Protein Derivative
ST-CF	Short Term Culture Filtrate
TST	Tuberculin Skin Test
TB	Tuberculosis
WHO	World Health Organization

3. INTRODUCTION

Mycobacterium tuberculosis (MTB) infects one third of the world's population and causes an estimated 1.9 million deaths and 8 million new cases annually.[1] Despite the fact that tuberculosis (TB) is preventable and treatable, the number of cases continues to rise at a rate of 0.4% per year globally and 6% per year in Sub-Saharan Africa.[2, 3] Only 27% of the estimated 8 million new cases of TB each year are detected, primarily due to the fact that presently available rapid diagnostic tests lack sensitivity and are technically cumbersome.[4] New diagnostic methods that are inexpensive, simple, rapid, highly sensitive and specific, and easily adaptable to resource poor countries are urgently needed.[5] A new test that could be used in resource poor countries (which have 95% of the burden of tuberculosis) to detect active pulmonary TB would benefit the individual and provide a tool to improve the tuberculosis control program in these communities.

Rapid diagnosis of active TB is primarily limited to acid fast (AFB) staining of respiratory secretions. Under optimal conditions approximately 70% of cases of pulmonary TB are smear positive. In developing countries the sensitivity of AFB staining ranges from 20%-50%. This is because smears are usually performed on unconcentrated sputum, and that in many countries with increasing rates of TB the number of specimens have overwhelmed the capacity of the laboratory.[6-8] Despite the limitation in sensitivity, the specificity of sputum AFB smear is greater than 95%. Chest radiographs, when available, have similar sensitivity to AFB smear but have poor specificity (70-80%) in diagnosising active tuberculosis.[9, 10] Nucleic acid amplification methods have better sensitivity than AFB smears (60-95%) and excellent specificity (>95%), but cannot be widely implemented in most developing countries as they are expensive and require sophisticated equipment.[11]

An enormous amount of work has been invested over the years to develop a serological test to diagnose TB, as this technology would be easily adaptable to the developing world setting. Since the mid 1990s, new restricted antigens have been described and have provided hope that they may lead to new tests that are more specific. Improved sensitivity and specificity have been achieved over the past 10

years with the use of purified and recombinant antigens and an enzyme-linked immunosorbent assay (ELISA) method of detection. The overall performance of serologic tests remain sub optimal in that single antigens have a sensitivity of 70% in smear positive active pulmonary TB and less than 50% in extrapulmonary TB, in children with tuberculosis, and in persons co-infected with human immunodeficiency virus (HIV).[12, 13] Specificity ranges from 50%-100% and is best in healthy controls from low TB incidence countries.[14-17] Specificity is unacceptably poor in clinically appropriate controls such as persons with inactive TB, other respiratory conditions, and those with non-tuberculous mycobacterial disease.[18-20] There is very little data on the serologic response to these new restricted antigens in countries with high rates of tuberculosis. We performed a study in the Gambia, a country with high rates of tuberculosis, to evaluate the sensitivity of 7 different mycobacterial antigens (4 shared i.e. common to all mycobacteria and 3 more restricted to MTB) to diagnosis active pulmonary tuberculosis and to determine the specificity in health community controls. The objective was to determine if the sensitivity and specificity of the 3 restricted mycobacterial antigens would be better than the 4 shared antigens.

4. BACKGROUND

4.1 Epidemiology of Tuberculosis

4.1.1. Global Burden of Tuberculosis

Tuberculosis infects one third of the world's population (1.86 billion persons) and is a leading infectious cause of death worldwide. In 1997 tuberculosis caused 1.9 million deaths and 8 million new cases, at least 44% of which were infectious pulmonary disease (smear-positive).[1] The global burden of tuberculosis remains very high and despite present efforts the number of cases continues to rise at a rate of 0.4% per year worldwide and 6% per year in Sub-Saharan Africa.[2, 3] The escalating tuberculosis epidemic is due to poor control of the disease in Southeast Asia, Sub-Saharan Africa, and Eastern Europe, and is fueled by a lack of resources and government commitment, the deterioration of public health systems, and high rates of HIV co-infection.

In 1993, the World Health Organization (WHO) declared tuberculosis to be a global emergency [21] stating that "TB does not stop at national borders and that control of TB in industrialized nations will be impossible until it is reduced as a health threat in Africa, Asia and Latin America." In the same year the WHO launched a new global TB program aimed at cutting the 3 million deaths per year to 1.6 million per year within the following 10 years. The focus of the WHO control program is to ensure the rapid detection and cure of infectious cases by the National Tuberculosis Programs (NTP). It suggested that each NTP work towards two objectives by the year 2000: 1) to successfully treat 85% of detected smear-positive cases, and 2) to detect 70% of all such cases by the introduction of an effective framework for TB control.[22]

4.1.2. The Burden of Tuberculosis in the Gambia

The Gambia is a country with a high incidence of tuberculosis. In 1998, 1,631 new cases of active tuberculosis were reported, for a case detection rate (CDR) of 138 per 100,000 population. This figure has been steadily increasing since the mid 1990s (CDR of 84/100,000 in 1994 and 109/100,000 in 1996). The male-female ratio of smear positive pulmonary cases in 1998 was 2.3, and most cases were in young

productive individuals (76% in those between the ages of 15 and 44 years and 34% in the 25-34 year age-group).[23] The seroprevalence of HIV-1 in the general population in the Gambia has been slowly increasing over the past 10 years. In a survey of pregnant women done between 1993-1995 the seroprevalence for HIV-1 was reported to be 0.51% (HIV-2 was 1.1%) and in 2001 it had increased to 1.95%.[24, 25] The seroprevalence of HIV in TB patients remains relatively low due to the low HIV seroprevalence in the general population. In 1995, 7% of TB cases were HIV positive and in 1998 8-10% of TB cases admitted to the hospital at the Medical Research Council Laboratories in the Gambia were HIV positive.[23]

Fortunately, the level of drug resistance in *M.tuberculosis* is still low in the Gambia. In a nationwide survey from June to December 1999, only 4% of 225 strains were found to have any resistance.[26] The WHO Extended Program on Immunization (EPI) began in the Gambia in 1979 and since then, Bacille Calmette-Guérin (BCG) vaccination has been administered to all newborns, with an overall coverage rate of 96%. Before the EPI program BCG was used sporadically in the Gambia.

4.1.3. The National Leprosy and Tuberculosis Program in the Gambia

In 1999, the functioning and efficacy of the National Leprosy and Tuberculosis Program (NLTP) of the Gambia was found to be poor and had deteriorated since 1996 [as assessed by the Royal Netherlands Tuberculosis Association (KNCV)].[23] Several areas of weakness were identified including poor managerial capacity of the program, insufficient supervision and training of NLTP staff, and poor case holding with high default rates in many treatment centres. The program fell short of the targets set by the WHO with a 55% detection rate of smear positive cases, and 73% successful treatment of smear positive cases (both substantially less than the 70% and 85% targets). These shortcomings were felt to be primarily due to poor case finding, and poor case holding and defaulter-tracing. In 1998, some centres reported that as many as 16%-30% of cases defaulted and that tracing of these cases often did not occur until after 2 or more weeks of treatment had been missed. In addition, the retreatment policy in the TB sanatorium did not conform to the NLTP manual. Retreatment

outcomes were poor, with a cure rate of only 29%. No information on the outcome of retreatment was available for 49% of cases, as they had been transferred during the course of treatment. (Table 1)

Table 1: TB Program Indicators[23, 27]

	WHO target	Africa (1998)	The Gambia (1998)
Successful treatment of smear positive pulmonary TB	85%	62%	73%
Case detection rate of smear positive pulmonary TB (CDR)*	70%	50%	55%
DOTS coverage	100%	77%	90%
Treatment success and outcome of smear positive pulmonary TB (1998)			
Cured	85%	62%	73%
Died		6.5%	5%
Failure		1.4%	3%
Interrupted treatment (defaulted)		11.8%	14%
Transferred out		5.7%	4%
Retreatment success 1997 (N=66)			
Cured		55.8%	29%
Defaulted		11%	10%
Transferred out or no results		5.1%	49%
Died		7.5%	15%

* CDR= annual new smear positive notifications/estimated annual new smear positive incidence

4.2 The Gambia- The Country, its People and its Economy

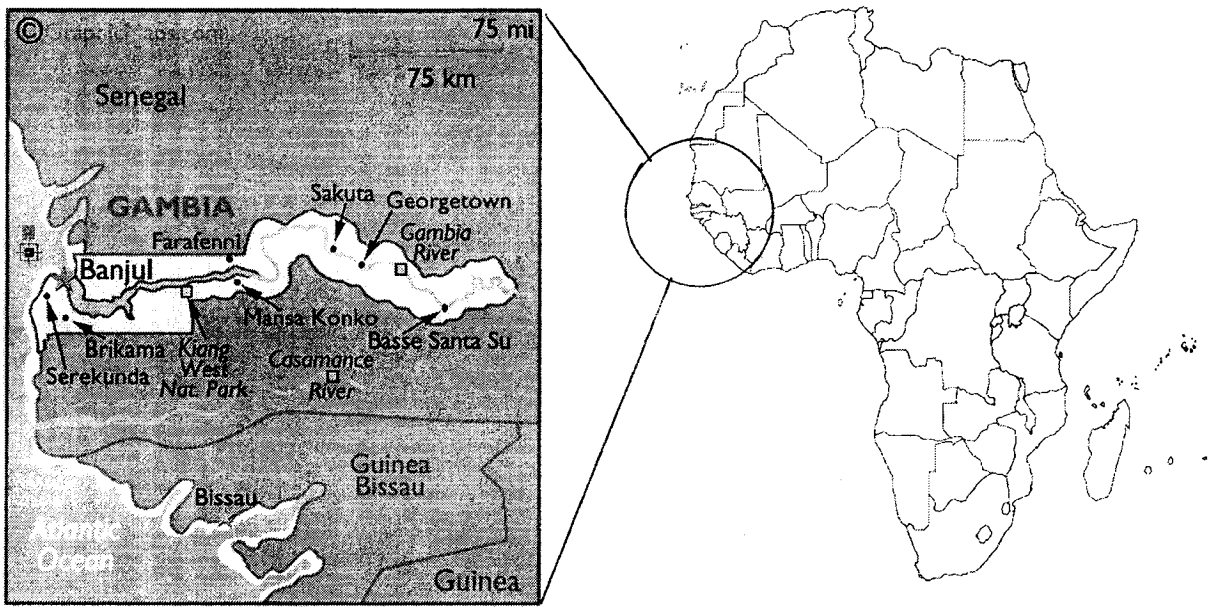


Figure 1: Map of the Gambia, map of Africa

The Gambia is a tiny finger-shaped country on the west coast of Africa completely surrounded by Senegal except for 80km of western coastline. It is the smallest country in Africa situated at 13° 28' N (latitude) and 16° 34' W (longitude), and has a total land area of 11,300 sq km (4400 sq mi). The population of the Gambia is 1.5 million (2003) with over 70% of the population living in rural areas.[25] The annual population growth is 3.3% and the population density is 134 persons per square km, one of the highest in Africa. It was previously a British colony and became an independent country in 1965. The Gambia was at the centre of the slave trade in the 1700s and is the birthplace of Kunta Kinte, slave ancestor of American Author Alex Haley. Banjul is the capital city. The major ethnic groups are Black Africans 99% [major tribes include Mandinka (42%), Fula (18%), Wolof (16%), Jola (10%), Serahuli (9%), Aku (<1%)] while Lebanese and Europeans make up the remaining 1% of the population. The Aku are a minority group who are descendants of repatriated slaves and are an important sector of the local elite. Non-Gambian

residents, primarily Mauritanians, Lebanese, Moroccans and Chinese make up the bulk of the successful traders and businessmen. English is the official language, and the main African languages are Wolof (the main trading language), Mandinka and Fulani. Close to 90% of the population is Muslim, 9% are Christian, and 1% follow indigenous beliefs.

The Gambia River runs through the centre of the country in an east-west direction. Most of the country has savannah-type vegetation and secondary forests with dense mangrove swamps along the riverbanks. The Gambia has a tropical climate with two distinct seasons. The rainy season starts in July, peaks in mid-late August, and ends in October. During this season the relative humidity is high. The longer dry season extends from November to June with daytime temperatures varying between 30°C and 40°C, while the relative humidity stays between 30% and 60%. Temperatures along the coastal areas are generally lower than the country average, whereas inland temperatures are higher than the country average.[28]

The Gambia is a poor country ranking among the 30 poorest countries in the world. It has a gross national product (GNP) per capita of $US $330, and 59% of the population has an income of less than $1 per day.[25] The Gambia has no important mineral or other natural resources and has a limited agricultural base. The Gambia has a rural economy and agriculture accounts for 58% of the GNP. The primary cash crop is groundnuts (peanuts) and farmers produce various other subsistence crops. Tourism is the second major industry, accounting for 10-15% of the GNP. Adult literacy is low with a literacy rate in males of 44% and in females of 30%. The general health of the people is poor. The life expectancy is 47 years and the under-five mortality is 126 per 1,000 live births.[25, 28] (Table 2)

Table 2- The Gambia, Economic and Health Indicators in Comparison to Those of Sub-Saharan Africa and Canada

Indicators	Sub-Saharan African Region	The Gambia	Canada
Economy and Population			
GDP per capita (US$ in 2001)	519	330	21,340
Percent of population below $1 per day	43	59	-
Total Population (thousands in 2001)	633,831	1,337	31,015
Percent of population urbanized	35	31	79
Population annual growth rate (%)	2.6	3.3	1.0
Annual Crude Birth rate (per 1,000 population)	41	38	11
Annual Crude Death rate (per 1,000 population)	16	17	8
Education			
Total Adult Literacy Rate (% of population >15 years)	61	37	
Literacy Male	69	44	
Literacy Female	54	30	
Net Primary school enrolment/attendance (% 1995-2001)	57	46	99
Health			
Life Expectancy at birth (in years)	48	47	79
Infant mortality rate per 1,000 live births (under 1 year)	107	91	5
Under-five mortality ranking (of 193 countries) [1]		35	161
Under five mortality rate (per 1,000 live births)	173	126	7
Percent under five years moderately/severely underweight[2]	29	17	-
Percent children immunized BCG in 2001	73	99	-
Percent children immunized DPT3 in 2001	54	96	97
Percent children immunized Polio3 in 2001	52	87	89
Percent children immunized Measles in 2001	58	90	96
Percent population with access to safe water	57	62	100
Percent population with access to sanitation	53	37	100
Prevalence (%) Adult HIV/AIDS[3]	8.6	1.6	.31
People (0-49 years) living with HIV/AIDS, end 2001		8,400	
Women			
Total Fertility Rate in 2001[4]	5.6	4.9	1.6
Literacy Rate % of males	78	68	

Source: UNICEF – State of the World's Children 2003[25]

1. 41 of the top 50 countries are in Sub-Saharan Africa
2. Below minus two standard deviations from median weight for age of reference population.
3. Adult prevalence rate (15-49 years) in 2001
4. The number of children that would be born per women if she were to live to the end of her child-bearing years and bear children at each age in accordance with prevailing age-specific fertility rates

4.3 Structure of Mycobacteria

Mycobactium tuberculosis is a slow growing (generation time 12-14 hours) gram positive, rod-shaped organism that is typically 1-4 mm in length and 0.3-0.6 mm in diameter. It has a characteristic "acid-fast" nature which is due to the unique characteristics of its cell wall, which contains high quantities of mycolic acid that resist decolouration. In smears from broth cultures, *M. tuberculosis* characteristically produces cords or dense clusters of bacilli in alignment. This is due to the production of trehalose 6,6'-dimycolate or "cord factor". [29]

The mycobacterial cell wall has the following components: 1) plasma membrane, 2) a complex polymer associated with the plasma membrane which is composed of peptidoglycans, arabinogalactan, and mycolic acids, 3) a second bilayer composed of glycolipid surface molecules associated with mycolic acids, 4) lipoarabinomannan (LAM) which traverses the entire depth of the cell wall and 5) several proteins interspersed within the plasma membrane, and between the plasma membrane and the peptidoglycan layer. Many of the components of the cell wall such lipoarabinomannan, arabinogalactan, and "cord factor" have been antigen targets for antibody testing. [30, 31] During growth and stress, mycobacteria secrete proteins into the extracellular space. These secreted proteins have also been the targets for antibody testing.

4.4 Immune Response to Tuberculosis

4.4.1. Glossary [30]

Cytokines: Low-molecular weight proteins that influence cell growth, inflammation, immunity and repair. Among these molecules are interleukins, interferons, tumor necrosis factors, and growth factors.

Interleukins: A subset of cytokines that modulate interactions between leukocytes.

Pro-Inflammatory Cytokines

IL-1: Released by alveolar macrophages and other antigen presenting cells; attracts and stimulates CD4 lymphocytes.

IL-2 : Released by CD4 lymphocytes after antigen presentation; attracts and activates other lymphocytes.

IL-12 : Produced by macrophages; plays a major role in immunity by fostering the TH1-pathyway lymphocyte differentiation

INF-γ : Produced by CD4, CD8, NKcells and γ/δ; plays a major role in stimulating the production of TNF-α, antigen processing, and regulation by macrophages.

TNF-α: Produced by infected macrophages; attracts and activates T lymphocytes.

Pro-Inflammatory Cascade: The collective effect of cytokines IL-1, IL-2, IL-12, INF-γ and TNF-α and whose ultimate goal is containing and killing *M.tuberculosis* bacilli.

Anti-Inflammatory Cytokines

IL-4 : Produced by macrophages; inhibits T-cell functions and promotes eosinophil and B-cell functions.

IL-10 : Produced by macrophages; works with IL-4 and transforming growth factor-β to stimulate CD4 and B-cell function.

TGF-β: Is produced by macrophages to suppress CD4 lymphocyte function; is a modulator of inflammation. Excessive TGF-β production may impair host defense and interferes with IL-2, Il-12 and IFN-γ

Anti-inflammatory Cascade: The collective effect of cytokines IL-4, IL-10, and TGF-β and has an ultimate goal to suppress the growth of, and to contain and kill *M.tuberculosis* bacilli.

Lymphocytes

B lymphocytes: Cells derived from embryonic bursal or bone marrow-derived cells that produce antibodies. They are central to the humoral immune response.

T lymphocytes: Thymus-derived lymphocytes and play a predominant role in cell mediated immunity and are central in the immune response to mycobacteria.

T-helper (Th) (CD4) cells: Lymphocytes that interact with the T cell receptor through the Major Histocompatibility (MHC) II protein and inhibit mycobacteria within macrophages. Depending on the pattern of antigenic stimulation, the CD4 lines will evolve into different subtypes of lymphocytes that have specific patterns of cytokine production; Th0, Th1 or Th2 subtypes.

T-cytotoxic (Tc) (CD8) cells: Lymphocytes that interact with the MHC Class I molecule on antigen presenting cells and have very potent cytotoxic functions. CD8 cells can also evolve into different subtypes of lymphocytes that have specific patterns of cytokine production; Tc0, Tc1 or Tc2.

Th1 and Tc1: Lymphocytes that produce IL-2, IL-12 and interferon-γ (IFN- γ). These cytokines are pro-inflammatory, and promote macrophage activation, cell mediated immunity and delayed type hypersensitivity (DTH).

Th2 and Tc2: Lymphocytes produce IL-4, IL-6 and IL-10, are anti-inflammatory, and inhibit macrophage function and promote expansion of and differentiation of B-lymphocyte populations.

Th0 and Tc0: Those lymphocytes that remain undifferentiated or as a mixed population of lymphocytes, and produce a mixture of the above cytokines.

Table 3: Cytokines Involved in TH1 TH2 Response

	TH1	**TH2**
Arm of the immune system	**Cell mediated**	**Humoral Immunity**
Cytokines	IFN γ IL12 IL2 TNF α	IL4 IL5 IL6 IL10 IL13

4.4.2. Pathophysiology

M. tuberculosis (MTB) is a facultative intracellular organism that requires aerobic conditions for growth and has a predilection for invading phagocytic monocytes. Classic studies have demonstrated immunity to TB to be cell mediated. The interaction between T lymphocytes and activated macrophages results in granuloma formation and subsequent killing of intracellular tubercle bacilli. [32] The ability of the host immune response to successfully contain and kill *M. tuberculosis* is a dynamic balance between the host (interaction between T lymphocytes and macrophages) and bacterial factors. Although antibodies are not thought to play an important role in protective immunity, TH2 lymphocytes and B cells are involved in the overall immune response, and antibodies are produced to several antigens.[30] Tubercle bacilli do not elaborate products that are inherently toxic but, ironically, the injurious effects of tuberculosis are largely due to the host defense mounted in response to the bacilli in its tissues.

Inhaled tubercle bacilli that reach alveoli are ingested by alveolar macrophages. The ability of macrophages to destroy the tubercle bacillus depends on the innate microbicidal capacity of the host macrophages and the virulence factors of the mycobacteria. Mycobacteria that evade intracellular destruction are able to multiply within macrophages, a process which eventually leads to disruption of the macrophage. Tubercle bacilli (protected inside macrophages) are transported to the hilar lymph nodes and are then systemically disseminated to the apices of the lungs, kidneys, bone growth plates and vertebrae. Ruptured macrophages attract blood monocytes and other inflammatory cells. The new monocytes differentiate into macrophages, which ingest but do not destroy the mycobacteria. During this stage bacilli multiply logarithmically. The struggle between bacilli and macrophages results in the release of cytokines and chemokines that attract other immune-effector cells (dendritic cells, peripheral blood monocytes (PBMs), lymphocytes and neutrophils), and serve to activate macrophages. New activated macrophages are recruited to the area and are intrinsically more capable of limiting mycobacterial replication (tuberostatic) and promote intracellular killing (tuberocidal). The cell-mediated

response produces granulomas (composed of activated macrophages, T lymphocytes and epitheliod cells), which serves as a physical barrier to contain bacilli and provide an anaerobic environment where TB cannot thrive. Development of cell-mediated immunity takes 3 to 8 weeks. The greatest "virulence" factor of *M.tuberculosis* appears to be its ability to evade destruction by macrophages.[30, 32, 33]

In immunocompetant hosts the fully developed cellular immune response halts bacterial proliferation and kills the majority of the bacillary population, resulting in involution of primary lesions and metastatic foci of infection. In immunocompromised hosts such as, infants, persons with AIDS, and other vulnerable hosts, the immune response is insufficient to contain the infection and the patient experiences progressive lung or extrapulmonary disease. Most individuals initially contain the infection. Some reactivate at a later date when the immune system becomes weakened (with older age or an immunosuppressive illness).[30, 32, 33]

4.4.3. Cellular Immune Response to *Mycobacterium tuberculosis*

The initial immunologic response to *M.tuberculosis* is a vigorous TH1 type cell mediated response. This is characterized by the production of the pro-inflammatory cytokines IL-2, IL-12, IFN-γ and TNF-α. Infected macrophages initiate the immune response by processing antigens for presentation to the T-lymphocytes and by releasing cytokines IL-1 and IL-12.[30, 34, 35] T-lymphocytes are attracted to the area of inflammation and produce IL-2 and IFN-γ. These cytokines further heighten the immune response by attracting more CD-4 lymphocytes and stimulate their differentiation into TH-1 subsets.

The most crucial cytokines in the pathogenesis of tuberculosis are IL-12, interferon-γ (IFN-γ) and Tumor Necrosis Factor α (TNF-α). IFN-γ and TNF-α are both crucial cytokines as they are directly involved in macrophage activation. Mice with disrupted IFN-γ genes are unable to control mycobacterial infection and die very rapidly with widespread dissemination, caseous necrosis, and large abscesses.[36, 37]. TNF-α is thought to play an important role in the response to infection with *M.tuberculosis*. It is produced by infected macrophages when they are exposed to the

cell wall product (of *M.tuberculosis*) lipoarabinomannan. TNF-α assists in granuloma formation by attracting more monocytes to the site of inflammation, and promotes their transformation into epitheliod and giant cells. IL-12 is also a crucial cytokine and has been found in lung infiltrates, pleural fluid, granulomas, and in lymph nodes. The protective role of IL-12 can be inferred from experiments with IL-12 knock-out mice that are highly susceptible to mycobacterial infection. [33]

There is accumulating evidence that mycobacteria may inhibit the release of the pro-inflammatory cascade of cytokines and stimulate the release of anti-inflammatory Th-2 type cytokines (TGF-β, IL-4 and IL-10) in order to evade host immune destruction. IL-4 and IL-10 are important suppressors of the TH-1 response and likely promote infection with intracellular pathogens due to their inhibition of macrophage activation. Interestingly, lipoarabinomannan from virulent mycobacteria selectively induces TGF-β which suppresses cell-mediated immunity by inhibiting the release of pro-inflammatory cytokines.[38]

Several groups have shown high levels of Th2-type cytokines in patients with active TB that increase with disease severity (particularly in cavitary disease).[39-43] This is not a consistent finding, however, as other groups have not been able to substantiate this.[44-47] The balance between Th1 and Th2 cells is decisive for the outcome of other mycobacterial disease, as illustrated by the spectrum of disease manifestation of leprosy.[30] It seems plausible that similar mechanisms may apply to *M.tuberculosis*. The relevance of the Th-1-Th-2 concept in the susceptibility to, or the clinical deterioration from (associated with rising antibody levels) tuberculosis remains uncertain.[30, 33] It remains to be resolved whether the TH2 cytokines detected in cases of active tuberculosis actually lead to active tuberculosis, or if they are present as a consequence of uncontrolled mycobacterial infection.[30, 33]

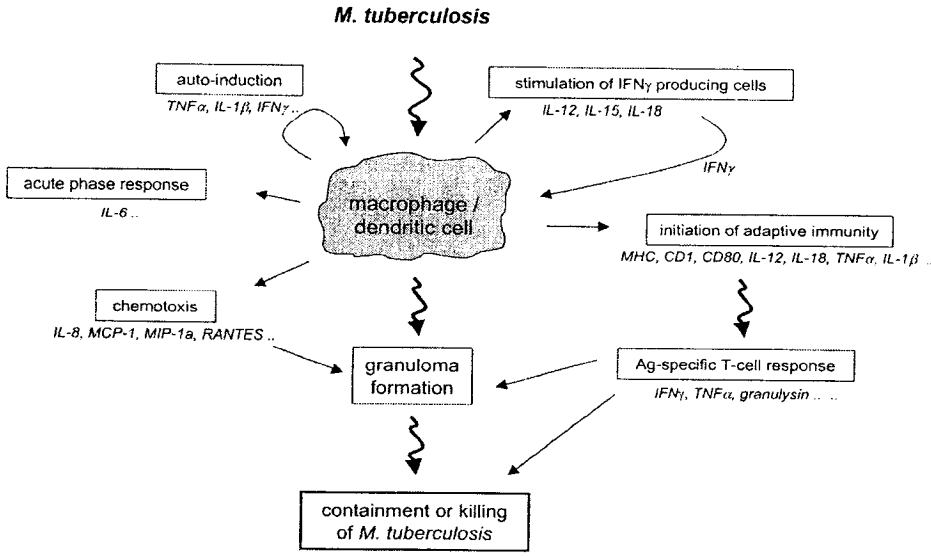


Figure 2 : Inflammatory response of phagocytic cells on activation by *M.tuberculosis*. Reproduced with permission from Crevel et al. Clin Micro Review. 2000;15(2):299[33]

4.4.4. Humoral Immune Response to *Mycobacterium tuberculosis*

Antibodies are thought not to play an important role in protective immunity to *M.tuberculosis* but are produced to several different antigens at various stages of disease. A detailed discussion of the humoral immune response is discussed in Section 4.6.3. Humoral Response (page 44).

4.5.Diagnostic Testing for Tuberculosis

4.5.1. Historical Development and Limitations of Present Diagnostic Tests

The search for a rapid, accurate, yet inexpensive test for the diagnosis of active tuberculosis began almost a century ago. Of all the tests currently licensed for the diagnosis of active TB (acid-fast stains, culture, chest radiographs, tuberculin skin tests, molecular amplification, and serology) all but the molecular techniques were introduced more than 70 years ago. All of these tests lack sufficient sensitivity and specificity. This results in delayed diagnosis and subsequent new transmission of disease, and to over diagnosis and unnecessary empiric drug therapy (with the attendant costs and risk of adverse events). New diagnostic tests must be developed if global control of tuberculosis is to be achieved.

Table 4: Currently Available Diagnostic Tests for Active Tuberculosis

	Year Introduced	Sensitivity (%)	Specificity (%)	Time to Result
Tuberculin Skin Test	1913	50-90	Depends on BCG and NTM	48-72 hours
CXR	1905	50-75	60-80	< 1 hour
AFB smear	1885	30-80	80-90	1 – 4 hours
TB Culture	1910	80-90	98	2-8 weeks
Serology	1898	60-80	60-80	1-4 hours
Nucleic Acid Amplification	1990	60-95	98	4-6 hours

4.5.2. Ideal Characteristics of a New Diagnostic Test for Active Tuberculosis

The acid fast stain is the cornerstone of case detection in the WHO TB control program. As outlined below the acid fast smear is limited by poor sensitivity (ranging from 20%-70%) in resource poor countries, is labour intensive, and requires at least 3 visits to the health centre before a diagnosis is made. Experts at a recent WHO convened workshop stressed that there was an urgent need to develop new tests to improve case detection (to replace the AFB smear), particularly in developing countries. This group proposed that an ideal new test should have the following

characteristics; sensitivity >70%, specificity >98%, require only one visit to the health facility, require no equipment or refrigeration, that minimal training would be needed to perform the test, that reagents would be supplied with the kit, have a stable shelf life of greater than 2 years, and be inexpensive with a cost as low or lower than the AFB smear. [48]

4.5.3. AFB Smears

At present, the most widely used rapid test is the direct microscopic exam of a smear of sputum for acid-fast bacilli (AFB). AFB smears are inexpensive, can be accomplished under field conditions, and can be performed even in the absence of electricity as slides can be examined with a light microscope using reflected sunlight. Smears, however, are labour intensive as a minimum of 300 fields should be examined to call a smear negative, and have sub-optimal sensitivity particularly in the developing world setting.[6, 11, 49]

The sensitivity of the AFB sputum smear depends on the bacillary load in the sputum, and has a threshold of detection of about 10,000 bacilli/ml of sample.[50] Smears therefore, will detect patients with advanced disease but not those with minimal or early disease. Sensitivity ranges from 20% to 80%, depending on the setting.[6, 50-53] Sensitivity is as low as 25% and reproducibility is poor in resource-poor countries where there has been an upsurge TB. This is because smears are done on unconcentrated specimens and because the number of specimens have overwhelmed smear microscopy services.[7, 8] Florescent microscopy reduces technician reading time, but the cost of the equipment and reagents is too high for most resource poor countries. Microscopists require proper training in reading AFB slides, a procedure that requires both skill and practice. Many TB control programs do not have good quality control built into their programs.[6] The specificity of AFB smears is very high when done in high incidence countries because very few patients with clinical signs of tuberculosis have illnesses caused by mycobacteria other than *M. tuberculosis*. [52, 54]

The AFB smear is the cornerstone of the WHO Directly Observed Therapy Short Course (DOTS) program as it detects those cases that are the most likely to

transmit infection to their close contacts. Even though this is the most appropriate test available for the developing world setting it has significant limitations. Many smear positive patients will be missed with this technique in resource poor countries, because of poor sensitivity of AFB smears. Despite the fact that the capital cost for equipment for AFB smears is low, and that materials are inexpensive (less than $1 per slide), these costs make up a significant portion of the start-up costs for a tuberculosis control program in developing countries and may not be affordable in these countries. At least three visits to the heath center are required before a diagnosis is made and treatment is started because three separate sputum samples must be collected over 2 days. This can be an enormous burden to the patient with tuberculosis. [55, 56]

4.5.4. Culture

Culture is considered the gold standard for the diagnosis of active pulmonary tuberculosis but is available only in specialized reference laboratories in most developing world settings. Detection of TB takes up to 8 weeks on solid media such as Lowenstein-Jensen or Middlebrook Agar. This can be reduced to approximately 2 to 3 weeks when liquid media systems such as the BACTEC 460 AFB system (Becton Dickenson Diagnostic Instruments, Sparks, Md) or the Mycobacteria Growth Indictor Tube; MIGIT System (Becton Dickenson Microbiology Systems, Cockeysville, Md) are used, but these systems are much more expensive than solid media.[49] The sensitivity of culture is almost 100% when sputum smears are positive. When smears are negative, however, the sensitivity of culture is as low as 50% for the first specimen, and increases to 90% with three specimens and 100% with six specimens.[50] Specificity of culture is excellent with only 1 to 2% false positive results.[57] In the developing world, culture is reserved for cases where initial therapy has failed or for surveillance to estimate the level of drug susceptibility in the country.

4.5.5. The Tuberculin Skin Test (TST)

The TST is easy to perform, is relatively inexpensive, and has fair reproducibility, with a standard deviation with each measurement of 2 to 3 mm.[58] Results of a TST require that the patient return 48 to 72 hours after it has been planted. The sensitivity of the test is reduced to 53%-90% in patients with newly diagnosed active TB [59-61] and is more likely to be falsely negative in those that are malnourished [62], HIV-infected,[63, 64] or have extensive disease. This is temporary, however because after a month or more of effective treatment greater than 95% of patients have a positive TST.[65] The key problem of the TST however, is poor specificity, as it does not distinguish between active tuberculosis and latent TB infection. It can be falsely positive as a result of exposure to non-tuberculous mycobacteria or vaccination with Bacille Calmette-Guérin (BCG). The tuberculin skin test is not useful in diagnosing active TB in the developing world where a large segment of the population is latently infected with *M.tuberculosis* and where there are high background rates of exposure to non-tuberculous mycobacteria.

The major drawbacks of TST testing, therefore, are the need for a return visit to allow reading (48-72 hours later), the inability to distinguish latent infection from active disease, problems in interpretation due to cross-reactivity with other mycobacterial species, and false-negative results because of intercurrent immunosuppression, as well as the variability inherent in application and reading of the test.

4.5.6. The Chest Radiograph

The chest radiograph is neither sensitive nor specific for the diagnosis of pulmonary tuberculosis (each ranging from 64% to 80%).[9, 10, 50] Chest radiographs, however, remain part of the WHO diagnostic algorithm in the work up of suspected pulmonary tuberculosis.[55] Disadvantages of the chest radiograph other than sub-optimal sensitivity and specificity are the initial capital costs for equipment, and recurrent costs for reagents, films equipment, maintenance, and technician time.

4.5.7. Nucleic Acid Amplification and Hybridization Methods

There are several molecular methods available for the direct detection of mycobacteria in clinical specimens. These include the Amplified *Mycobacterium tuberculosis* Direct (MTD2) Test (GenProbe), AMPLICOR Mycobacterium tuberculosis (MTB) Test (Roche Diagnostics Corp., Indianapolis, Ind), LCx MTB Assay (Abbott LCx Probe System, Abbott Park, Ill), and the BD ProbeTec ET System (Becton Dickinson Biosciences Microbiology Products). These tests were introduced approximately 10 years ago and most can be used on direct specimens for rapid diagnosis, to distinguish smear positive sputa due to non-tuberculous mycobacteria from those due to *M.tuberculosis,* or for culture confirmation. [49, 66] Reproducibility of these tests is excellent and sensitivity exceeds 95% for AFB smear positive pulmonary disease. Sensitivity in smear negative culture positive specimens, however, is only 50-70%. [18, 49, 66-70] Specificity of the amplification and hybridization tests in almost all studies exceeds 98%, although specificity can be much worse as a result of cross-contamination within the laboratory unless recommended procedures are strictly followed.[49, 66] Molecular amplification and hybridization methods have good sensitivity and excellent specificity, and a rapid turn around time of 4-6 hours, but could not be widely implemented in the developing world as they are expensive, require sophisticated equipment, and highly trained technicians.[11]

4.6 Serologic Tests

4.6.1. History

The earliest detection of circulating antibodies to mycobacterial antigens in patients with TB was demonstrated in a study by Arloing published in 1898, only 16 years after Koch identified the tubercle bacillus.[71] Arloing developed an agglutination test using Koch's old tuberculin and reported a sensitivity of 57% in patients with pulmonary TB, but found that 11% of healthy controls and patients sick with other illnesses were falsely positive.[71] Despite concerted efforts over the past 100 years to develop improved serologic tests, the limitations of poor sensitivity and specificity have still not been resolved. The sensitivity and specificity of humoral testing is determined primarily by the choice of the antigen tested, its purity and cross reactivity with other mycobacterial species, and the method of antibody detection used.

4.6.2 Mycobacterial Antigens

In the past 100 years many different mycobacterial antigens have been tested. These include crude preparations containing many different antigens, and single antigens from secreted structural proteins, cell wall components, cording factor and mycobacterial enzymes. The antigens most studied up until the early 1990s have been the 38kDa, 19kDa, 14kDa, Antigen 85B and lipoarabinomannan (LAM). A list of selected antigens is shown in table 5 and are discussed in the following text.

Table 5: Selected Mycobacterial Antigens

Shared Antigens (Common to Most Mycobacteria)	**Restricted Antigens.** (Absent in BCG and Some Non-Tuberculous Mycobacteria)
Crude/Mixed Antigens **Mycobacterial Sonicates** Purified protein derivative (TST) Antigen A60 antigen Antigen Kp90 **Cell Wall Components** Liproarabinomanan (LAM) Glycolipids Cord Factor (trehalose dimycolate) **Secreted Antigens** 38 kDa (Antigen 5) 19 kDa 85B antigen (30/31 kDa antigen) MPT 51 MPT 53 MPT 63 MPB 70 **Heat Shock Proteins** 14 kDa DnaK (70kDa) GroEL (65kDa) GroES (10kDa **Enzymes** SodA GluS	**RD-1 Region** Absent in all BCG, present in most non-tuberculous mycobacteria* ESAT-6 CFP-10 **RD-2 Region** Absent in Some BCG** MPT 64

* present in *M.leprae, M. africanum, M. kansasii, M. marinum, M. szulgai and M. bois*, and several non pathogenic environmental species such *M.flavescens, M.smegmatis and M.gastri.*

** absent in BCG-Danish, Prague, Glaxo, Frappier, Connaught, Phipps, Tice, Pasteur

4.6.2a) General Information

Historically, the order in which different *M. tuberculosis* proteins have been isolated and described has been based on their abundance in various mycobacterial preparations, or due to the availability of monoclonal antibodies defining the molecule. Since the 1990s, the understanding of the central role secreted proteins play in the immune response to tuberculosis and the ability to predict and produce antigens limited to fewer Mycobacterial species (as a result of the knowledge of the genomic sequence of *M.tuberculosis* and comparative genomics of BCG) has influenced the choice of new mycobacterial antigens that have been developed.

It has been recognized since the late 1980s that proteins secreted from actively growing *M.tuberculosis* stimulate a stronger immunologic response than proteins from dead bacilli.[72, 73] During the initial infection mycobacteria multiply freely within macrophages. The protein products produced during this active growth phase are thought to be the first antigens that elicit a protective immunologic response. Structural proteins released after dead or degraded bacteria are processed are thought to elicit an immunologic response much later in the pathogenesis of tuberculosis. Short-term culture filtrate (ST-CF), the effluent collected from cultured bacteria during the first phase of mycobacterial growth (before proteins from dead and dying bacteria are released), is thought to be the closest approximation of the mixture of antigens that the host first encounters after infection with *M.tuberculosis*. The composition of ST-CF is influenced by culture conditions such as cultivation time, temperature, and shaking of the cultures. This suggests that the relative proportions of proteins that the host is exposed to may be influenced by the growth conditions of the mycobacteria.[32, 74] Short-term culture filtrate contains a mixture of approximately 100 shedded outer wall and secreted proteins (many highly immunogenic), but no structural proteins. Some of the major proteins found in ST-CF are heat shock proteins DnaK (70kDa) and GroES (10kDa), Proline rich complexes (45-47kDa), L-alanine dehydrogenase (40kDa), 38kDa Ag, Antigen 85 complex (30-32 kDa), MPT51

(27kDa), MPT64 (26kDa), super oxide dismutase (SodA, 23kDa), 19kDa lipoprotein, alpha-crystalline (12/16 kDa), and ESAT-6.[32, 75]

The specificity of antigens in serologic testing for *M.tuberculosis* is determined by the amount of cross reactivity with other mycobacterial species. Recent advances in *M.tuberculosis* genomics using subtraction hydrization and DNA micro arrays have identified a genomic segment, RD1, that was initially described as present in *M.tuberculosis complex* and the following non-tuberculous mycobacteria; *M.kansasii*, *M. szulgai, M.flavescens*, and *M. marinum* but absent from all strains of *M.bovis* BCG.[76] In 1995, ESAT-6, a highly immunogenic, early secreted 6-kD antigen, encoded in the RD1 region, was isolated from short term culture filtrate.[77] At the time of conception of this project ESAT-6 held promise to be a highly specific antigen as it was believed to be absent from *M.bovis* BCG, and most non-tuberculous mycobacteria. Other proteins in the ESAT-6 family encoded in the RD-1 region, such as CFP-10, have been produced and are undergoing testing.[78, 79] Since the completion of this project, data has been published showing that, contrary to what was originally believed, the genes encoding ESAT-6 and CFP-10 are widely distributed in most mycobacteria. These genes have been found in pathogenic mycobacteria such as *M. leprae, M. africanum, M. kansasii, M. marinum, M. szulgai* and *M. bovis*, and several non-pathogenic environmental species such *M. flavescens, M. smegmatis and M. gastri*.[80, 81]

Another region of the genome that has attracted attention is the RD2 region, which is absent in many strains of BCG including BCG-Danish, Prague, Glaxo, Frappier, Connaught, Phipps, Tice, and Pasteur. The focus on antigen development in the past decade has been to characterize and test antigens found in culture filtrate as well as those to be restricted to *M.tuberculosis* i.e. those encoded in the RD1 and RD2 regions of the genome.

4.6.2b) Antigens Shared by Many Mycobacteria

i) Crude/Mixed Antigens

Old Tuberculin/Purified Protein Derivative (PPD)

Old tuberculin was first described by Koch in 1890 and consisted of the heat-inactivated constituents present in a glycerol broth in which mycobacteria had been grown for 6-8 weeks. This antigen mixture was used in the first skin test. Purified Protein Derivative (PPD) was derived from Koch's mixture after the bulk of the carbohydrate material had been removed by precipitation with ammonium sulfate or trichloroacetic acid.[32] PPD was used in many of the early serologic tests, but had low specificity due to cross-reactivity with other mycobacteria, as it is composed of many different common antigens shared by all mycobacteria.[71, 82]

A60 Antigen Complex

A60 antigen complex is an mixture of at least 30 different immunogenic antigens extracted from *M.bovis* BCG and *M.tuberculosis.*[83, 84] It was first described by Cocito and Vanlinden in1986 and is composed primarily of lipoarabinomannan, but also contains both free and bound lipids, polysaccarides, and proteins.[84] This is the antigen used in the commercial test Anda TB produced by Anda Biologicals, Stasbourg, France. The performance of this antigen has generally attained good sensitivity ranging from 66% to 88% and fair specificity ranging from 91% to 98%. Specificity decreased to 75% when controls were infected with non-tuberculous mycobacteria.[85, 86, 87, 88]

Antigen P-90

Antigen P-90 is prepared from sonicated, broken *M. bovis* BCG bacilli. This has formed the basis of a commercial test by Kreatech Diagnostics (Amerstdam, Netherlands) in which IgA antibodies to mycobacterial antigen P-90 are measured. When tested in both Italy and Turkey the sensitivity for pulmonary TB was 68%-83%,

but specificity was as low as 86% when tested in persons with other pulmonary diseases.[19, 89]

ii) Cell Wall Components

Lipoarabinomannan

Lipoarabinomannan is a major component of the mycobacterial cell wall of *M.tuberculosis* and *M.leprae* and has been shown to be immunogenic. In HIV negative patients with culture positive pulmonary tuberculosis the sensitivity of this antigen ranges from 22% to 89%, but was much lower in HIV positive patients (values ranging from 7%-40%).[83] The specificity was high in healthy populations from non-endemic countries but was only 84% in Tanzania in hospital controls.[90] In Mexico, Sada found that 40% of patients with histoplasmosis and the one patient with paracoccidomycosis had anti-LAM antibodies, suggesting cross-reactivity with these fungi. [91] This is also supported by the report by Wheat et al where some patients with tuberculosis had falsely positive serologic results when tested with *Histoplasma capsulatum* antigens.[92] Cross-reactivity of these antigens with these fungi is obviously of concern in areas where histoplasmosis and paracoccidiomycosis are endemic.

Glycolipids

Mycobacterial cell wall glycolipids such as diacyl trehaloses (DAT1 and DAT2), 2,3,6-triacyltrehalose (TAT), phenolglycolipid (PGL-Tb1), trehalose-6,6'-dimycolate (cord factor) and mannophosphoinosotides have been found to be immunogenic. A study from Spain described the humoral response with 3 classes of immunoglobulins (IgG, IgM and IgA) to several antigens (DAT, TAT, SL-1 and cording factor). In this study the sensitivity in smear positive pulmonary TB for these antigens to IgG was 69% (range, 57%-83%) and to IgA was 71% (range, 38%-88%). In patients with smear negative pulmonary TB, the sensitivity was lower, with a mean sensitivity of 64% (range, 50%-81%) for IgG and 41% (range, 19%-50%) for IgA. The mean specificity of these antigens when tested in patients with non TB pulmonary disease was 56% (range 42%-75%) for IgG and 66% (range, 48%-96%) for IgA.[93]

The utility of ELISA testing with these antigens is limited by extremely poor specificity.

iii) Secreted Antigens

38kDa

The 38kDa antigen has been the most extensively studied antigen in serologic assays. In 1978, Daniel and Anderson isolated a protein by immunoabsorbent affinity chromatography, which they referred to as antigen 5 (also known as 38kDa, antigen b, PhoS, CIE Ag78, 3T).[71] This original antigen was likely contaminated with lipoarabinomannan and has been further purified into the antigen known as the 38kDa. This is a secreted antigen that is mainly localized to the outer cell wall of *M.tuberculosis*, and is only released in small amounts in the culture filtrate. It is highly antigenic in the first phase of illness.[32] Homologous genes for this protein are found in *M.bovis* as well as *M.avium intracellularae*. BCG culture fluids have about 1/10 the concentration of this protein as compared to culture fluids from MTB. [94] It is therefore at least a quantitatively specific antigen.

Overall, the 38kDa antigen has been the best single antigen for the diagnosis of active pulmonary disease, with sensitivity ranging from 58%-85% in those with smear positive pulmonary TB, and 15%-70% in those with smear negative TB. As described with other antigens individuals with more extensive disease are more likely to have a positive serologic test and the intensity of the antibody response increases with increasing disease severity.[95-99] Specificities are generally high (>95%), but decrease to <90% in persons with latent TB or those infected with non-tuberculous mycobacteria.[15, 17, 71, 95, 96, 100-109] In a study in Tanzania, specificity was poor and was reported to be 70% in HIV negative hospitalized controls and only 50% in HIV positive hospitalized controls.

A commercial kit (ITC Tuberculosis, AMRAD-ITC, Sydney, Australia) made up of only recombinant 38kD looked promising.[97, 98] When it was combined with 4 other recombinant antigens and peptides however, different groups have reported widely variable sensitivity and specificity in different populations.[99, 110-113]

19 kDa

The 19kDa antigen is a secreted cell wall associated antigen.[32] Although initially thought to be a promising antigen in smear negative pulmonary TB this has not been confirmed in more recent studies. Interestingly, it has been negatively correlated with cavitation in one study.[14] There is also marked geographic variation in the specificity of this antigen. The specificity was very good in London[15, 104] but was much poorer in community controls in India, possibly due to cross reactions with non-tuberculous mycobacteria or due to latent tuberculosis.[114] Sensitivity has ranged from 8% to 70% in smear positive pulmonary disease and 7%-61% in smear negative disease. [15, 100, 104, 107, 108, 115]

Antigen 85 complex

Antigen 85 complex is a group of three distinct but markedly cross-reacting, secreted, outer cell wall antigens each encoded by separate genes. They have been referred to by several different names including, 85A (31kDa, P32 antigen, MPT44), 85B (30kDa, MPB/MPT59, alpha antigen, antigen a_2, antigen 6), and 85C (31.5 kDa, MPT45). They show varying degrees of fibronectin binding, suggesting that they play an important role in macrophage uptake.[32]

This complex appears to be present in *M.tuberculosis*, BCG, *M.bovis* and *M.kansasii*. The 85B antigen elicits good antibody responses in those with smear positive pulmonary TB with sensitivities ranging from 23%-86%.[17, 107, 116, 117] In smear negative pulmonary disease sensitivity is lower, ranging from 19-49%.[12] Specificity has generally been >95% but in a recent study in Chile, the specificity of Ag85B was only 85% in healthy controls, and decreased to 80% when the antigen was combined with the Ag85 Complex.[117]

MPT51, MPT53 and MPT 63

These proteins have been found to be a major component of the short term culture filtrate (ST-CF).[32] Antibody responses to antigens MPT51 and MPT63,

however, have had very poor sensitivity ranging from 3%-23% in patients with culture positive pulmonary disease.[107, 108]

MPT70

MPT70 is a secreted protein found in ST-CF and produced in varying concentrations by different BCG strains. High concentrations of this antigen are produced by BCG Tokyo, Moreau, Russia and Sweden whereas much lower concentrations (1% of levels of BCG Tokyo) are produced by BCG Pasteur, Copenhagen and Tice.[118, 119] Antibody responses in two studies have shown very poor sensitivity (7%[108] and 10%[120]).

iv) Heat Shock Proteins

14 kDa (12/16 kDa- α-crystalin)

Heat Shock Proteins (stress proteins) are widely distributed in nature and are highly conserved with substantial structural similarities across taxonomic lines. They are produced in large quantities by cells growing under stressful culture conditions such as high temperature, and are thought to have important functions in maintaining cell integrity.

14kDa is a small protein that belongs to the family of heat shock proteins. The way that this molecule reaches the surroundings is not clear. It is probably found on the external side of the cell wall.[32] The 14kDa antigen can be recovered from disrupted tubercle bacilli but is not readily found in culture supernatants.

Accumulating evidence suggests that the 14kDa may be a marker for latent tuberculosis or those with an enhanced immune response that have successfully contained the disease.[14, 104, 109] It has had variable sensitivity in smear positive pulmonary disease (21% to 72%) and in smear negative pulmonary tuberculosis (23%-79%).[14, 15, 17, 100, 104, 107-109] It is one of the antigens most likely to be positive in contacts of active cases of TB[15] or in people with inactive tuberculosis. [14, 104, 109] Jackett showed that 28% of household contacts of infectious cases had antibodies to the 14kDa antigen in comparison to 11% for the 38kDa and 6% for the 19kDa.[15]

Bothamley et al also showed that antibodies levels to the 14kDa Ag were significantly higher in health care workers than in factory workers in Indonesia.[121]

DnaK (70kDa), GroEL (65kDa) and GroES (10kDa

The DnaK (70kDa) and the GroES (10kDa) molecules are important mycobacterial heat shock proteins. Studies with monoclonal antibodies have shown that this protein has multiple epitopes, many of which are widely shared between mycobacteria while some appear to be species specific.[122] Even though they contain some species-restricted epitopes they have been of little diagnostic value.[12]

v) Enzymes

Superoxide Dismutase (SodA)

Superoxide dismutase is an enzyme found in very early samples of ST-CF and may therefore, play an important role in the initial immune response to *M.tuberculosis*. This protein may protect bacteria from the toxic effects of super oxide radicals by breaking down oxygen free radicals generated during the oxidative burst in the macrophage. It is unclear if this enzyme is secreted.[32] There is little data on the immunogeniticity of this protein for either cellular or humoral responses.

Glutamine Synthatase (GLU-S)

Glutamine synthatase is an enzyme also found in large quantities in the early ST-CF and is thought to have two potentially important functions; synthesis of the glutamate/glutamine polymer that makes up some of the cell envelope, and inhibition of phagosome-lysome fusion in infected monocytes.[123] In a study of different mycobacteria, this enzyme was found in the culture filtrate of *M.tuberculosis* and *M.bovis BCG,* but not in the culture filtrate of *M.kansasii*, *M.fortuitum*, *M. phlei* and *M. smegmatis,* suggesting that it may be more specific to pathogenic mycobacteria. [124] There are no studies reporting the humoral response to this enzyme.

4.6.2c) Antigens Restricted to Certain Mycobacteria

i) RD-1 Region (absent in BCG and from Some Non-Tuberculous Mycobacteria)

ESAT-6 (Early Secreted Protein, 6kDa)

ESAT-6 a highly immunogenic, 6-kDa protein found in large quantities in the culture filtrate, encoded in the RD-1 region that was first described in 1995. This antigen was initially thought to be a highly specific antigen because it was believed that it was absent from most non-tuberculous mycobacteria as well as all strains of *M.bovis BCG.* Recent studies however, have shown that the ESAT-6 gene is widely distributed in many mycobacteria and has been described in pathogenic mycobacteria such as *M.leprae, M. africanum, M. kansasii, M. marinum, and M. szulgai* and *M. bois*, and several non-pathogenic environmental species such *M.flavescens, M.smegmatis and M.gastri.*[80, 81]

This antigen has been found to be highly immunogenic in several situations and the cell-mediated response to ESAT-6 has been associated with recent contact, conversion, increased risk of disease, and active disease. [125-129] Positive cellular responses to ESAT-6 however, have also been found in a large proportion of asymptomatic individuals living in TB endemic countries (30% in the Gambia[125] and 56% of in India[130]). This suggests that there is either cross-reactivity of ESAT-6 with other mycobacteria or that this represents latent tuberculosis.

Recent studies have demonstrated that the humoral response to ESAT-6 is poor in those with active TB, with sensitivity ranging from 13%-27%. Specificity in assymptomatic controls living in low TB endemic countries was greater than 93%.[107-109] Studies that use controls living in TB endemic countries will need to be done to determine the utility of using ESAT6 as a diagnostic test in the developing world. Interesting ESAT6 has been found in as many as 57% of persons with inactive tuberculosis (stable fibrosis on chest X-ray for > 2 months, TST > 5mm, and smear and culture negative). This suggests that the antibody response to ESAT-6 may be an important marker for those with an enhanced immune response that have successfully contained the disease, but who are at risk for reactivation.[109]

CFP 10, TB 10.4 and the ESAT-6 Family

Two other members of the ESAT-6 family encoded in the RD1 region, (TB10.4 and CFP10) have also been found to be immunodominant in humans.[131] Preliminary data on cellular immune response to CFP-10 and ESAT-6 suggest that they may be useful in diagnosing active TB in countries with a low incidence of TB.[128, 129] In a study in India however, 56% of healthy individuals living in Bombay had a cellular immune response to ESAT-6 and 76% had a response to CFP-10 suggesting that there was cross-reactivity of these antigens with environmental non-tuberculous mycobacteria or that the responses were due to latent TB infection.[130] When used as a skin test reagent, CFP-10 and ESAT-6 are more specific than PPD.[132] The antibody response to CFP-10 in those with active TB was poor, with a sensitivity of only 16% in one study.[133] The utility of these antigens as serologic diagnostic agents will be determined by their specificity in populations living in TB endemic countries.[127]

New RD-1 Protein Products of Genes Rv3871, Rv3872, Rv3878

These antigens are encoded by genes in the RD-1 region and have shown poor sensitivity (4%, 3% and 7%) to detect antibodies in humans with active tuberculosis.[133]

ii) RD-2 Region (Absent in Some BCG)

MPT64

This is a secreted protein that makes up a large portion of the Short term Culture Filtrate. Its is encoded in the RD2 deletion area and therefore is absent in BCG Danish, Pasteur, Glaxo and Tice but found in some substrains of BCG such as the Tokyo, Morau, Russia and Sweden strains.[134, 135] Sensitivity of the antibodies to MPT64 to detect active TB was only 7% in HIV negative individuals and 5% in HIV positive individuals.[107, 120] MPT64 performed well in skin testing of guinea pigs but was disappointing in humans.[136, 137]

4.6.3 Summary of Humoral Response

a) General Kinetics

Even though the humoral response does not play a central role in the protective immunity against mycobacteria, antibodies in all immunoglobulin classes (IgM, IgG and IgA) have been demonstrated against many different mycobacterial antigens. In general, IgG antibodies appear to be indicative of active disease and the percent of positive subjects and intensity of the antibody response increases with more advanced disease. IgM antibodies develop early, never reach high titer, and are directed chiefly against polysaccharide antigens. IgM antibodies do not correlate well with active disease.[71, 138] IgA antibodies have good sensitivity to both protein and glycoprotein antigens but specificity is poorer than for IgG antibodies, ranging from 48%- 94%.[87-89, 93, 110] During the course of treatment, antibody levels rise during the first 1 or 2 months, fall subsequently, and remain positive in many patients for up to 24 months or more after treatment.[16, 122]

b) Sensitivitiy and Specificity

Sensitivity for the purified and recombinant antigens have ranged from 13% to 94%. Patients with extensive, smear positive disease have the highest sensitivity.[14, 17, 99, 139] It is much lower in patients with pauci-bacillary pulmonary, [68, 98] in extra-pulmonary disease,[116] in HIV infected individuals,[140] and in children.[20, 140] Sensitivity of serologic testing is generally higher in areas that have a high incidence of tuberculosis [96, 98, 101, 102, 111, 113] as compared to areas with a low incidence of tuberculosis.[95, 99, 110, 112] This likely reflects chronicity or extent of disease, but could be due to previous exposure to non-tuberculous mycobacteria.[71]

Specificity has ranged from 50%-100% and is highest when the control population is comprised of normal healthy volunteers from non-endemic countries.[15, 17, 104, 141] It remains unacceptably poor when tested in more appropriate control populations such as individuals with inactive TB, other respiratory conditions, or non-tuberculous mycobacterial disease. [68, 90, 106, 109]

A summary of the antibody responses to different antigens in the different spectra of disease are outlined for smear positive TB (table 6), smear negative TB (table 7), extrapulmonary TB (table 8), inactive TB (table 9), non-tuberculous mycobacteria (table 10) and for each antigen by disease stage (table 11). Several commercial serologic tests have been developed over the years and are outlined in table 12.

Table 6: Average Serologic Response in Studies of Smear Positive Patients to Different Antigens

Antigen	Sensitivity mean (range)	Specificity mean (range)	Reference
Crude			
Sonicates/filtrates	74% (49%-100%)	96% (86%-100%)	82
PPD	69% (32%-86%)	90% (79%-100%)	82
Semi-Purified			
Antigen 5	75% (49%-89%)	94% (88%-100%)	82, 95, 96, 101-103
A60 Antigen	84% (48%-100%)	87% (71%-100%)	82, 85-88, 142, 143
P-90	34%(75%-83%)	88% (86%-90%)	19, 89
Cell Wall			
Glycolipids	64% (57%-75%)	97% (92%-99%)	82
Plasma membrane	89% (75%-93%)	96% (92%-97%)	82
LAM[1]	56% (26%-81%)	92% (84%-100%)	12, 82, 90, 91, 104
Purified Epitopes Mab[2]			
38 kDa	72% (53%-85%)	92% (70%-100%)	12, 15, 82, 100, 104-106
38kDa (Recombinant)	52% (17%-91%)	92% (81%-100%)	17, 97, 98, 107-110
19 kDa	51% (8%-70%)	96% (91%-98%)	12, 15, 82, 100, 104, 114
19 kDa (Recombinant)	25% (17%-32%)	99% (98%-100%)	107, 108
14 kDa	47% (21%-72%)	96% (74%-100%)	12, 15, 82, 104, 105
14 kDa (Recombinant)	28%	99% (98%-100%)	107, 108
Ag85B	62% (46%-70%)	98%	12
Ag85B (Recombinant)	30% (12%-70%)	94% (85%-100%)	107, 108, 117
ESAT-6 (recombinant)	18% (13%-27%)	94% (85%-100%)	107-109
Combined Antigens			
ITC Combination test[3]	53% (20%-87%)	83% (79%-88%)	99, 110-113
Detect TB[4]	84%	85%	141

1. Lipoarabinomannan
2. Defined by epistopes with a monoclonal antibody
3. ITC (AMRAD-ITC, Sydney, Australia) Is a combination of the 38kDa and 4 Recombinant Antigens
4. Detect TB (Biochem Immunosystems, Montreal) contains 3 recombinant antigens and 2 synthetic peptides
5. Antigens in this table are purified unless otherwise specified.

Table 7: Average Serologic Response in Studies of Smear Negative Patients to Different Antigens

Antigen	Sensitivity mean (range)	Specificity	Reference
38 kDa	50% (52%-90%)	88%	12, 15, 100, 104, 106
38 kDa (Recombinant)	25% (16%-33%)	93%	107, 109
19kDa	43% (7%-61%)	97%	12, 15, 100, 104
19kDa (Recombinant)	42%	97%	107
14 kDa	27% (13%-54%)	93% (89%-95%)	15, 100, 104
14kDa (Recombinant)	32% (13%-50%)	86% (69%-100%)	107, 109
Ag 85B	19%	97.5%	12
Ag 85B (Recombinant)	13%	97.5%	107
LAM	32% (7%-58%)	91% (84%-95%)	12, 90, 91, 104
ESAT-6 (Recombinant)	20% (13%-27%)	94% (91%-97.5%)	107, 109
Combined Antigens			
ITC- combination[2]	41% (0%-86%)	84% (79%-88%)	110, 112, 113
Detect TB[3]	65%	85%	141

1. Lipoarabinomannan
2. ITC (AMRAD-ITC, Sydney, Australia) Is a combination of the 38kDa and 4 Recombinant Antigens
3. Detect TB (Biochem Immunosystems, Montreal) contains 3 recombinant antigens and 2 synthetic peptides
4. Antigens in this table are purified unless otherwise specified.

Table 8: Average Serologic Response in Studies of Patients with Extrapulmonary TB to Different Antigens

Antigen	Sensitivity mean (range)	Specificity mean (range)	Reference
38 kDa	73% (72%-75%)	80% (70%-90%)	100, 106
38 kDa (Recombinant)	18%	91% (86%-96%)	110
Ag60	83% (68%-94%)	88% (75%-96%)	86-88
19kDa	19% (17%-21%)	97.5%	100, 114
14 kDa	19%	89%	100
Ag 85B	18%	100%	116
LAM	59%	93%	91
ITC-combination[2]	43% (18%-65%)	83% (79%-88%)	110-112

1. Antigens in this table are purified unless otherwise specified.
2. ITC (AMRAD-ITC, Sydney, Australia) Is a combination of the 38kDa and 4 Recombinant Antigens

Table 9: Percent Positive Serologic Responses to Different Antigens in Patients with Inactive TB.

Antigen	Percent positive	Reference
38 kDa (Recombinant)	25%	109
14 kDa	71%	109
ESAT-6	53%	109

Antigens in this table are purified unless otherwise specified.

Table 10: Percent Positive Serologic Responses to Different Antigens in Patients with Non-Tuberculous Mycobacteria Disease.

Antigen	Percent positive	Reference
38 kDa (Recombinant)	0%	109
14 kDa	31%	109
ESAT-6	15%	109

Antigens in this table are purified unless otherwise specified.

Table 11: Sensitivity and specificity for Different Sites and Extent of Tuberculosis by Antigen and Method of Production

Antigen	Sensitivity mean (range)	Specificity mean (range)	Reference
38kDa (Purified)			
Smear positive Pulm	72% (53%-85%)	92% (70%-100%)	12, 15, 82, 100, 104-106
Smear negative Pulm	50% (52%-90%)	88%	12, 15, 100, 104, 106
Extrapulmonary	73% (72%-75%)	80% (70%-90%)	100, 106
38kDa (Recombinant)			
Smear positive Pulm	52% (17%-91%)	92% (81%-100%)	17, 97, 98, 107-110
Smear negative Pulm	25% (16%-33%)	93%	107, 109
Extrapulmonary	18%	91% (86%-96%)	110
Inactive Pulm	25%	89%	109
19 kDa (Purified)			
Smear pos Pulm	51% (8%-70%)	96% (91%-98%)	12, 15, 82, 100, 104, 114
Smear neg Pulm	43% (7%-61%)	97%	12, 15, 100, 104
Extrapulmonary	19% (17%-21%)	97.5%	100, 114
19 kDa (Recombinant)			
Smear Pos Pulm	25% (17%-32%)	99% (98%-100%)	107, 108
Smear Neg Pulm	42%	97%	107
14 kDa (Purified)			
Smear Pos Pulm	47% (21%-72%)	96% (74%-100%)	12, 15, 82, 104, 105
Smear Neg Pulm	27% (13%-54%)	93% (89%-95%)	15, 100, 104
Extrapulmonary	19%	89%	100
14 kDa (Recombinant)			
Smear Pos Pulm	28%	99% (98%-100%)	107, 108
Smear Neg Pulm	32% (13%-50%)	86% (69%-100%)	107, 109
Inactive Pulm	71%	79%	109
Ag85B (Purified)			
Smear Pos Pulm	62% (46%-70%)	98%	12
Smear Neg Pulm	19%	97.5%	12
Extrapulmonary	18%	100%	116
Ag85B (Recombinant)			
Smear Pos Pulm	30% (12%-70%)	94% (85%-100%)	107, 108, 117
Smear Neg Pulm	13%	97.5%	107
ESAT-6 (recombinant)			
Smear Pos Pulm	18% (13%-27%)	94% (85%-100%)	107-109
Smear Neg Pulm	20% (13%-27%)	94% (91%-97.5%)	107, 109
Inactive	53%	93%	109
LAM (Purified)			
Smear Pos Pulm	56% (26%-81%)	92% (84%-100%)	12, 82, 90, 91, 104
Smear Neg Pulm	32% (7%-58%)	91% (84%-95%)	12, 90, 91, 104
Extrapulmonary	59%	93%	91
ITC Combination test (Purified)			
Smear Pos Pulm	53% (20%-87%)	83% (79%-88%)	99, 110-113
Smear Neg Pulm	41% (0%-86%)	84% (79%-88%)	110, 112, 113
Extrapulmonary	43% (18%-65%)	83% (79%-88%)	110-112
Detect TB (Recombinant)			
Smear Pos Pulm	84%	85%	141
Smear Neg Pulm	65%	85%	141

Table 12: Commercial Kits Available for Serologic Diagnosis of Tuberculosis

Name of Kit	Manufacturer	Antigens Used	Method Used	Turn Around Time	References
ITC Tuberculosis	(AMRAD-ITC, Sydney, Australia)	38 kDa, 4 other purified secreted proteins	IgG , Western Blot		[98, 110, 113]
Rapid Test TB	(Quorum Diagnostics, Vancouver, Canada)	Recombinant 38 kDa	Test strip added to tube, coloured bands appear	15 mins	[110, 144]
Tuberculosis IgA EIA	Kreatech Diagnostics (Amsterdam, Netherlands)	Mycobacterial P-90 antigen	IgA, microtitre plate EIA	~ 3 hours	[110, 144]
Pathozyme-TB complex	Omega Diagnostics (Alloa, Scotland)	Recombinant 38 kDa	IgG, microtitre plate EIA	~ 3 hours	[110, 144]
Pathozyme-Myco	Omega Diagnostics (Alloa, Scotland)	Recombinant 38 kDa Liparabinomannan	IgG, microtitre plate EIA IgM IgA	~ 3 hours	[110]
Anda TB	Anda Biologicals (Stasbourg, France)	Antigen 60 prepared from the cytoplasm of M.bovis-BCG	IgG, IgM, IgA antibodies on a microtitre plate EIA	2-3 hours	[110]
Detect-TB	BioChem ImmunoSystems, (Montreal, Quebec)	5 recombinant antigens			[68, 141]
MycoDot	DynaGen Inc, (Cambridge, MA, USA)	Liparabinomannan (LAM)	IgG, Nitrocellulose strip	20 mins	[90]

c) Relationship of Humoral Response to Disease Stage

Several studies have shown that the sensitivity of antibody responses (higher percent positive) and the intensity of antibody response increases with increasing disease severity and duration of symptoms. [14, 17, 99, 139] Antibodies are lowest in primary disease, in paucibacillary disease, or well controlled disease.[98, 145, 146] This likely reflects the fact that in early infection a Th1 cellular response predominates, and as disease progresses, a Th-2 cellular response predominates.[39-43] These data suggest that for paucibacillary disease and stages of disease with a high Th1 cellular response that serologic testing is unlikely be of diagnostic benefit. These are the same populations for which all diagnostic tests have low sensitivity.

The most sensitive antigen to detect a serologic response changes as disease progresses. The 38kDa, 85B Ag, and 19kDa are the most sensitive in those with advanced disease, the 38kDa and liproarabinomannan are the most sensitive antigens in extrapulmonary disease, and ESAT-6 and 14kDa are most commonly positive in latent tuberculosis or inactive TB (table 11, page 49). This suggests that a serologic test designed to detect all stages of tuberculosis should contain all of the antigens mentioned above. Alternatively stage specific tests (i.e. advanced pulmonary, smear negative pulmonary, extrapulmonary and inactive or latent TB) could be developed which would contain the best antigens for each stage of disease.

d) Effect of Non-tuberculous Mycobacteria on Serologic Testing

i) Geographic Distribution of Non-tuberculous Mycobacteria

The presence of different non-tuberculous mycobacteria (NTM) varies from one geographical region to another and are generally most common in tropical (Asia, Africa, South America)[147-151] and subtropical areas (Australia and the southern US),[152] and less common in colder climates.[147-150, 153-157] Different non-tuberculous mycobacterial species also vary from region to region within a country, influenced by temperature, soil and water pH, and the degree of humidity.[147, 151, 152,

[158] Table 14 outlines the variation in skin test reactivity in different populations to different species of NTM. It is felt that sensitization by NTM (particularly rapid-growers) produces some immunity to mycobacteria, thereby decreasing susceptibility to tuberculosis or leprosy. This is supported by the observation that rapid growing non-tuberculous mycobacteria are more common in moist climates of a country where the rates of tuberculosis and leprosy are lower than they are in drier areas of the same country.[150, 158] As in most tropical countries, the skin test sensitivity to NTM in the Gambia is very high and varies from region to region. Rapid growers were more common in the western part of the country where the humidity is highest (Table 15).

ii) Effect of Non-Tuberculous Mycobacteria on Humoral Response

The specificity of serologic tests in different settings depends on the composition of mycobacteria in the environment to which the individual is exposed, whether or not the antigen tested cross reacts with the NTM found in the environment, and if the NTM elicits an antibody response. There is moderate cross reactivity between *M.tuberculosis* and non-tuberculous mycobacteria. Twenty-nine percent of Japanese patients and 57% of Californian patients with active TB had a positive antibody response to crude *Mycobacterium avium-intracellulare* (MAI). [159] [160] When antibodies to *M.tuberculosis* antigens were measured in individuals infected with non-tuberculous mycobacteria, the 38kDa and the 19kDa had the best reported specificities, both ranging from 97.5%-100%. [100, 107, 109] When the antibodies to the 14kDa and ESAT-6 were measured specificities were very poor (69% and 85% respectively).[109]

Table 13: Percent of Population with Positive Skin Test Reactions to Various Non-Tuberculous Mycobacterial antigens in Different Geographic Regions

Author Reference	Corrah [151]	Fine [150]		Paul [147]		Brickman [154]	Davignon [155]		Von Reyn [149]				Kardjito [148]	Jeannes [153]
Age	6-18 yrs	Adults/Children		School age		First grade	15-19 yrs		Adults				Adults	15-60 yr
TST cutoff	≥2 mm	≥5mm	≥10mm	≥5mm		≥5mm	≥10mm		≥10mm				≥5mm	≥5mm
Country	Gambia	Malawi		Kenya BCG	Kenya No BCG	Canada Montreal	Canada Montreal	Canada Quebec	US	Finland	Trinidad	Kenya	Indonesia	Ontario
PPD		59	48	76	24	1	3	1	7	7	26	35	88	
Leprosin													79	
Slow Growers														
M.avium (A)	87	58	42	62	34	3			12	3	14	30		41
M.avium (B)	25	32	18	58	19									
M.gordonae		14	7	59	34	5								
M.intracellulare		77	60											
M.intracellulare (Battery or PPD-B)						2	2	1						38.5
M. Kansasii	11	59	44			2	1	0.2						
M. marinum	43	59	44											
M.scrofulaceum	40	77	66										88	26.8
M. Xenopi		11	6											
Rapid Growers														
M.chitae	59	47	29											
M.flavescens														
M.fortuitum (A)		44	31											
M.fortuitum (1)		24	8											
M.fortuitum (2,3,4)		71	53											
M.neoaureum	51	43	31		25	22								
M. nonchromo-geniucum	28	50	34		11	6								
M. vaccae	33	43	33		24	14							97	

Table 14: Skin Test Reactions to Non-Tuberculous Mycobacteria in the Gambia

Reagent	No. tested	Bank	Banjul	Farafenni	Georgetown	Basse
Avium A	499	N	76%	63%	49%	67%
		S	86%	62%	70%	72%
Avium B	499	N	24%	9%	19%	4%
		S	25%	18%	29%	41%
Avium C	499	N	92%	91%	81%	69%
		S	46%	82%	85%	96%
Kansasin	446	N	21%	6%	29%	24%
		S	11%	4%	23%	27%
Marinin	560	N	18%	17%	36%	23%
		S	43%	22%	28%	36%
Scrofulin	746	N	51%	38%	48%	32%
		S	40%	53%	77%	51%
Chitin	480	N	17%	45%	57%	63%
		S	59%	33%	42%	63%
Neoaurumin	560	N	33%	21%	42%	52%
		S	51%	33%	34%	43%
Nonchromogen	484	N	31%	46%	41%	30%
		S	28%	19%	38%	31%
Vaccin	746	N	67%	65%	59%	45%
		S	33%	45%	45%	56%

Adapted from reference 139. Corrah PT. Studies of Tuberculosis in the Gambia. PhD Thesis. Medical Research Council, Fajara, the Gambia: University College, London, 1994.

e) Effect of BCG on Serologic Testing

Results from studies on the effect of BCG on serologic testing have been contradictory. After an intravenous challenge with BCG, animals develop a short-lived IgM antibody response that lasts between 2 and 8 weeks.[138] In one human study, no antibodies were detected 8 weeks after BCG vaccination, whereas in another small study low levels of antibody were detectable for up to 8-16 weeks post vaccination.[161-163]

Human studies using a variety of antigens have shown widely varying results. Kardjito found [with *M.bovis* BCG Glaxo as the antigen] that there was no effect on antibody levels in patients or controls regardless of the number of years since vaccination.[164] Zhou found [with the ITC Tuberculosis test (AMRAD-ICT, Sydney, Australia) which contains 5 recombinant antigens] that 9% of children vaccinated within 10 years and 2% of adults vaccinated 20 years prior had a positive serologic response. [98, 164] Gevaudin found that 10/148 (7%) of BCG vaccinated as compared to none of the BCG unvaccinated people had antibody responses to A60 antigen complex [a mixture of antigens extracted from *M.bovis* BCG and *M.tuberculosis*] several years after BCG vaccination.[86, 164]

The antibody response appears to be enhanced in BCG vaccinated individuals if rechallenged with BCG or subsequently exposed to *M.tuberculosis*. School children, who were revaccinated with BCG had more rapid and higher titres of antibodies than when they were initially vaccinated with BCG.[163] In a study by Jackett et al, none of the BCG vaccinated healthy controls had detectable antibodies to the 14kDa antigen. BCG vaccinated household contacts of active TB cases however, were more likely to have antibodies to the 14kDa as compared to non BCG vaccinated household contacts.[15] This suggests that BCG vaccinated individuals have an enhanced antibody response when exposed to another mycobacteria (in this case *M.tuberculosis*).

In summary, there appears to be a transient antibody response shortly after vaccination, which may vary with the antigen tested, and that wanes slowly over time but that may persist for several years after vaccination. The serologic response to initial BCG vaccination appears to be enhanced if there is re-exposure to mycobacteria such as BCG or *M.tuberculosis*.

f) Effect of HIV on Serologic Testing

In early HIV infection, when the CD4 count is almost normal, the clinical picture of tuberculosis is similar to that in non-HIV infected individuals. As the individual becomes more immunosuppressed, however, atypical presentations for tuberculosis are more common and patients are more likely to be AFB smear negative.[30] It is in these patients that serologic testing would be particularly valuable. Unfortunately, the serologic response (intensity and percent positive) in HIV positive individuals as compared to HIV negative individuals has consistently been substantially lower, regardless of which antigen is tested.[83, 90, 91, 106, 120, 140] The degree of immunosuppression likely affects the antibody response. A humoral response to PPD was found in 8 of 22 (36%) HIV positive patients with active TB as compared to 1 of 20 (5%) patients with more severe immunosuppression (i.e. other AIDS defining illnesses plus tuberculosis).[165] Serologic testing is therefore of limited clinical utility in TB patients co-infected with HIV.

g) Summary of the Humoral Response to *M. tuberculosis*

The humoral response to *M.tuberculosis* is influenced by many factors. The immunologic response at different stages of disease and bacillary load appears to be antigen specific. The 38kDa, 85B Ag, and 19kDa give the most vigorous response in those with advanced disease, and the 38kDa and liproarabinomannan (LAM) are the most sensitive antigens in extrapulmonary disease. Overall, the sensitivity of serologic testing is approximately 20% less in patients with smear negative pulmonary TB and extrapulmonary TB than in those with smear positive pulmonary TB. An antibody response to ESAT-6 and 14kDa are most commonly positive in latent tuberculosis or inactive TB (tables 6-11, pages 46-49). As disease progress and bacillary load increases the cellular immune response shifts to the TH2 type (predominantly humoral) and is associated with increased intensity and number of serologic responses. It remains to be resolved if advanced disease actually leads to a TH2 cellular response or if it is present as a consequence of uncontrolled mycobacterial infection. Serologic testing is therefore most sensitive in those with advanced disease and is often negative in those with paucibacillary disease.

Purified antigens are consistently more sensitive than recombinant antigens, but with little difference in specificity. Mycobacterium tuberculosis elicits a heterogeneous humoral response suggesting that combining several antigens would increase sensitivity.[107] Studies of the humoral response with combination of antigens have in general resulted in increased sensitivity but at a cost of decreased specificity.[68, 99, 110-113, 141]

Table 15: Summary of Factors Influencing the Humoral Response to *M.tuberculosis*

Disease Stage (Antigen most likely to be positive)
Smear Positive Pulmonary (38kDa, 19kDa, Ag85B)
Smear Negative pulmonary (38kDa, 19kDa)
Extrapulmonary (38kDa, LAM)
Inactive Disease (ESAT6, 14kDa)
Intensity of Antibody Response
Intensity of antibody response and percent positive increase with increasing disease severity and cavitation (except 19kDa)
Intensity of response and percent positive higher in TB endemic countries compared to TB non-endemic countries (? Due to increase disease severity)
Sensitivity of Antibody Response
Increases with increasing disease severity and bacillary load
Lower sensitivity in children than in adults
Lower sensitivity in HIV positive compared to HIV negative (by about 30%)
Sensitivity decreases in HIV positive patients as immunosuppression increases
Effect of Recombinant Antigens
Recombinant antigens have lower sensitivity than purified antigens
Generally combining recombinant antigens increases sensitivity but with a loss of specificity
Effect of Treatment
Increases transiently after treatment

4.6.4. Summary of Background

Tuberculosis is responsible for an enormous global burden of disease that causes significant morbidity and mortality, primarily in economically productive individuals. Despite the fact that TB is preventable and treatable the number of cases continues to rise at a rate of 0.4% per year globally and 6% per year in Sub-Saharan Africa.[2, 3] It is estimated that only 27% of the 8 million new cases of TB that occur each year are detected. This is primarily due to the fact that the rapid diagnostic tests presently available lack sensitivity and are technically cumbersome.[4] The only rapid diagnostic test that is widely available in developing countries is the AFB smear of respiratory secretions. The sensitivity of this test for the detection of pulmonary tuberculosis in the developing world, however, may be as low as 20%. Other diagnostic tests also have limitations and new diagnostic tests are required to improve case detection.

The potentially favorable qualities of serologic testing (rapid, affordable, and technically easy to use in the developing world setting) has fuelled the over 100-year effort to develop such a test. Despite a better understanding of the immunologic response to *M.tuberculosis*, and development of recombinant antigens, clinical utility of serologic testing remains sub-optimal. When single antigens are used, the maximum sensitivity of the humoral response in smear positive active pulmonary TB is 70%. Poor sensitivity is likely due to the stage specific nature of the response to different antigens, and to the heterogeneous nature of the antibody response to *M.tuberculosis.* It was hypothesized that combinations of antigens would resolve this issue. Unfortunately, although this strategy resulted in a minimal increase in sensitivity it was at a cost of reduced specificity. Specificity of single antigens also remains unacceptably low (ranging from 50%-100%) when tested in the most clinically appropriate control populations. This is likely due to the inability of serological testing to distinguish between active disease and latent infection, and due to cross-reactivity of *M. tuberculosis* with NTM and BCG. Since the mid 1990s, new, more restricted antigens have been described that are not present in BCG but are present in most species of NTM, providing hope that serologic tests with these antigens may be equally sensitive but more specific than previous tests.

We performed this serologic study in the Gambia, a country with high rates of tuberculosis, with 7 different mycobacterial antigens (4 shared by most mycobacteria and 3 more restricted) for the diagnosis of active pulmonary tuberculosis. We compared the sensitivity and specificity of the 3 restricted antigens with that of 4 shared mycobacterial antigens to determine if the restricted antigens would be as sensitive as and more specific than the common ones in this setting. Antibodies to these same antigens were tested in household contacts of smear positive pulmonary TB to describe the humoral response in those with potentially recent infection with TB.

5. STUDY METHODOLOGY

5.1 Study Hypothesis

Detection of antibodies to antigens restricted to *M.tuberculosis* will have equal sensitivity and superior specificity for diagnosis of smear positive pulmonary TB as compared to antibody detection to antigens shared by most mycobacteria.

5.2 Study Objectives

1. To assess the sensitivity of detection of antibodies to 7 different mycobacterial antigens for the diagnosis of active smear positive pulmonary tuberculosis in the Gambia.

2. To assess the specificity of these antibodies to 7 mycobacterial antigens in two population groups without active tuberculosis: i) healthy, asymptomatic adults living within three houses of active tuberculosis cases and ii) healthy adults born in countries with a low incidence of tuberculosis and having travelled less than 1 year in countries with high rates of tuberculosis.

3. To compare the sensitivity and specificity of antibodies to antigens more restricted to *M.tuberculosis* (ESAT6, CFP-10 and Rv3871) with 4 antigens shared by most mycobacteria (38kDa, GLU-S, 19kDa and 14kDa), for the diagnosis of active pulmonary tuberculosis.

4. To describe the humoral response to these seven different antigens in household contacts (who potentially have had recent TB infection) of active tuberculosis patients,

5.3 Study Design

In this study, sera from 300 Gambians (100 cases with pulmonary tuberculosis, 100 healthy community controls and 100 household contacts) were tested by an ELISA method for antibodies to 7 mycobacterial antigens. Sera were also obtained from 18 BCG vaccinated and 19 non-vaccinated healthy adults that had been born and raised in countries with a low incidence of tuberculosis (<25/100,000 population).[3] The cut off used to consider an ELISA result positive was calculated from the 18 individuals that had been BCG vaccinated.

The present project was nested within an epidemiologic study conducted by the Medical Research Council Laboratories in the Gambia funded by the European Commission. The objective of this larger (parent) study was to investigate the genetic and environmental factors of susceptibility to tuberculosis (PI: Dr. Christian Lienhardt), and was called the TBGENV study. In this study, adults with active smear positive pulmonary TB, Gambian community controls, and one randomly selected household contact of active cases were recruited and followed prospectively for a minimum of 18 months. Recruitment for the larger study occurred over a three-year period, from 1999-2002.

The serologic study was nested within this larger study and was designed by Dr. Greenaway, the TB research group in the Gambia (Drs. C Lienhardt, R Adegbola and K. McAdam) and Dr. R. Menzies. Dr. Greenaway spent a year at the MRC Laboratories in the Gambia from February 2000 to February 2001 during which time she participated in the fieldwork and day to day running of the TBGENV study and set up, validated, and ran the serologic assays for this study. Sera from patients, their household contact, and the Gambian community control recruited to the TBGENEV study from January 2000 to November 2000 were included in the serologic study. Dr. C. Greenaway recruited the healthy controls from low endemic countries that were used for the cut-off in this study.

5.4 Study Population and Definitions

Cases were adults, >15 years of age, with newly diagnosed smear positive active pulmonary TB, presenting to the National Leprosy and TB Control Program TB clinics in the Greater Banjul area (Banjul Polyclinic, Serrekunda Health Center and the Brikama

Health Center). The diagnosis of tuberculosis was confirmed by 2 different sputa that were smear positive and that subsequently grew *M.tuberculosis*.

Gambian Community Controls were an age matched (± 5 years of the age of the case) individual from a household, chosen randomly, living within three houses of the cases' house. These persons were followed every 3 months for a minimum of 18 months with symptom checks to ensure that they did not develop active TB. Presence of active disease in the controls was ruled out through follow-up of symptoms and chest radiograph, if necessary. All controls with previously treated tuberculosis, presently active tuberculosis, or who developed active tuberculosis during the 18-month study follow-up period were excluded from the serologic study.

Household Contacts- age matched (± 5 years of the age of the case) adults living in the same household as the index cases were selected randomly (the name of all those potentially eligible were placed in a hat and one name was drawn).

Non-Endemic Controls- adults that were born and lived in a country with a low incidence of TB (i.e incidence <25/100,000 population)[3] and had travelled less than 18 months in a TB endemic country. Eighteen of these individuals were BCG vaccinated and 19 were non-vaccinated. For those that were BCG vaccinated the mean age of vaccination was 14 years, and two individuals were vaccinated twice.

5.5 Data Gathering

For all Gambian participants basic demographic information and information pertaining to past medical history and exposures was collected through questionnaires administered by study fieldworkers. The information available for all study participants included age, sex, ethnic origin, religion and country of origin, any known TB contacts, intercurrent diseases (i.e. diabetes, asthma), smoking, alcohol intake and drug use. Height, weight and BCG scars were measured and then recorded. Tuberculin skin tests (2 TU, RT 23, Statens Serum Institut, Denmark) were performed. After 48-72 hours the transverse

diameter of the induration was measured and recorded in millimeters (mm). A width of greater than or equal to 10mm was considered positive.

The following laboratory tests were performed in each study participant; a complete blood count (CBC), eosinophil count, and an HIV test (ELISA). Additional information for TB cases included symptoms such as fever, cough, hemoptysis, and fatigue, prior antituberculous therapy, results of sputum smear including gradation of positivity, mycobacterial culture results and interpretation of the chest radiograph with attention to extent of disease and presence or absence of cavities.

5.6 Measurement of Humoral (Antibody) Response

5.6.1 Antigens

Recombinant mycobacterial antigens (ESAT6, CFP-10, 38kDa, GLU-S, Rv3871, 19kDa and 14kDa) were produced in the laboratory of Dr. Marila Gennaro at the Public Health Research Institute, New York, New York. Seven genes encoding *M.tuberuclosis* culture filtrate protein were cloned and purified in the pQE30 (Quiagen) plasmid vector of *E. coli* as described earlier[166, 167] Recombinant proteins were expressed as NH2-terminally polyhistidine-tagged fusion proteins and purified from *E.coli* cells to near homogeneity by sequential chromatography with metal chelate affinity, size exclusion, and anion exchange columns.[168]

Table 16: Antigens Tested

Shared Antigens	
38kDa	Common to most mycobacteria
19kDa	Common to most mycobacteria
14kDa	Common to most mycobacteria
GLU-S	Common to most mycobacteria
Restricted Antigens	
ESAT-6	RD-1 Region*
CFP-10	RD-1 Region
Rv3871	RD-1 Region

* Antigens absent in all BCG-bovis and present *M.leprae, M. africanum, M.bovis* in many non-tuberculous mycobacteria (M.kansasii, M.szulgai, M.flavescens and M.marinum M.kansasii, M.smegmatis, M.gastri)

5.6.2 ELISA

Polystyrene 96-well microtiter plates were coated overnight with 0.1 ml/well of antigen diluted in carbonate bicarbonate buffer (pH 9.6) in the following concentrations [0.5ug/ml (14kDa), 1 ug/ml (ESAT6 and 19kDa) and 2ug/ml (CFP-10, 38kDa, GLU-S, Rv3871)]. Plates were blocked with phosphate-buffered saline (pH 7.4) containing 1% skim Milk (PBS-1%SM) for 1 hour at room temperature and washed extensively with phosphate-buffered saline with 0.05% Tween (PBS-T). Serum samples were diluted 1:50 in PBS-T 1% SM and 0.1 ml of diluted serum was added to antigen coated wells and incubated for 1 hour at room temperature then washed extensively with PBS-T. 0.1 cc of diluted goat anti-human immunoglobulin G labelled with alkaline phosphatase (Sigma Immunochemicals, St. Louis, MO) was added to each well and incubated for 1 hour at room temperature and then washed extensively with PBS-T. 0.1 ml of alkaline phosphatase substrate (Biorad, Hercules, Calif) was added to each well and incubated for 1 hour at room temperature. 0.1 ml of 1 NH_2SO_4 was then added to stop the reaction and the raw optical density at 450 nm (OD_{450}) were then read with an automatic plate reader. Corrected optical densities for each plate were calculated from an adjustment factor based on the same two high and medium positive controls run on each plate. This method was adapted from other serologic studies done in Dr.Gennaro's lab.[107, 120]

5.7 Data Analysis

5.7.1 Sample Size Calculation

The primary objective of the study was to determine the sensitivity and specificity of seven mycobacterial antigens. The table below outlines the 95% confidence interval around a proportion for different hypothetical sample sizes. Proportions ranging from 60%-90% were chosen as they were felt to be the clinically useful range of sensitivities for a new diagnostic test. There was little difference in the confidence intervals for a sample size of 100, 150 or 200 and given feasibility issues 100 in each group of subjects was chosen.

Table 17a) Sample Size Calculations for 95% Confidence Interval Around a Single Proportion

Proportion (Seropositivity to an Antigens)	Sample Size N=75	Sample Size N=100	Sample Size N=150	Sample Size N=200
60%	60% ± 10.5%	60% ± 9.5%	60% ± 7.5%	60% ± 7.0%
70%	70% ± 10.0%	70% ± 9.0%	70% ± 7.5%	70% ± 6.5%
80%	80% ± 8.5%	80% ± 8.0%	80% ± 6.5%	80% ± 5.5%
90%	90% ± 7.0%	90% ± 5.5%	90% ± 5.0%	90% ± 4.0%

The second objective of the study was to detect a difference between restricted and shared antigens. For proportions ranging from 60% to 80% a 15% difference (α =0.05 and β=0.8) would be detected for a sample size of 100 in each group.

Table 17b) Sample Size Calculations for Comparison of 2 Proportions at (α =0.05, β=0.8)

Seroprevalence	Difference Detected	Sample Size
60%	10%	281
60%	15%	120
70%	10%	231
70%	15%	95
80%	10%	157
80%	15%	60

Based on these calculations and the assumption that the sensitivity obtained in this study would range between 60%-90% we chose a sample size of 100 in each Gambian study group.

5.7.2 Method of Analysis

All analyses were conducted with SAS for personal computers (SAS version 8.2, SAS Institute; Cary, NC). Differences between groups were tested for significance with chi-square for categorical and t tests or analysis of variance for continuous variables and were considered significant at a $p<0.05$. [169] Univariate odd ratios and 95% confidence intervals (CI) were calculated for the relationship of each antigen with age, sex, receipt of BCG, TST and clinical status. Multivariate analysis was used to obtain adjusted odds and 95% confidence intervals.[170]

5.7.3 Definition of Cut-off for a Positive ELISA

The cut-off for each antigen was determined from the BCG vaccinated controls from low TB incidence countries. This was done to control for any potential BCG effect in the Gambian groups. For each antigen the cut-off was calculated from the mean $\pm$ 2 standard deviations of the corrected OD values obtained for the 18 BCG vaccinated controls from low TB incidence countries. This absolute value was then applied to each individual result obtained for all Gambian participants (tuberculosis cases, household contacts and community controls). Corrected OD values above this cut-off were considered positive and corrected OD values below this cut-off were considered to be negative.

5.7.4 Analysis

The primary analysis in this study was the calculated sensitivity and specificity of antibody levels for each antigen for each of the three Gambian groups (smear positive pulmonary TB, community controls and household contacts). Sensitivity and specificity were calculated with standard formulas.[171] The clinical and demographic characteristics (age, sex, history of receipt of BCG, tuberculin skin test result) for each antigen by clinical status (active TB, household contact, Gambian community control) were described and the adjusted odds for all of these characteristics were placed in a multivariate model using Gambian community controls as the reference group.

Secondary analyses included the sensitivity and specificity of each antigen and clinical and demographic characteristics for each of the following groups; HIV positive vs.

HIV negative, tuberculosis patients with cavitary TB vs. those without cavitary TB, and tuberculosis by smear grade (1+, 2+ and 3+). Multivariate analyses were performed comparing active tuberculosis cases with community control (household contacts removed from the dataset) and adjusting for demographic, clinical characteristics and serologic response for each of the antigens. A similar analysis was performed comparing household contacts with community controls (active tuberculosis patients removed from the dataset).

5. RESULTS

Sera from 300 Gambians (100 with active pulmonary TB, 100 household contacts and 100 Gambian community controls) and 38 controls from low TB incidence countries underwent serologic testing (table 18). The mean age of active TB cases was 31.7 years reflecting the fact that the Gambia is a country with high rates of TB with ongoing community transmission. The predominance of male sex (68%) is in keeping with previously described sex differences in tuberculosis. The ethnic, religious and socioeconomic profile of the patient groups did not differ between the study groups and was similar to that expected for the general population. Patients with active TB had significantly lower body weight, lower haemoglobin, and lower mean corpuscular volume (MCV), than community controls (table 19). The significantly higher total white blood cell count and higher platelet count in TB cases, in comparison to community controls, was not clinically important as values from both groups fell within the normal reference ranges. The mean size of tuberculin reactions were significantly different between groups with a mean of 17 mm in those with active tuberculosis, 15 mm in Household Contacts and 13 mm in Gambian community controls (Table 19, page 72 and figure 4, page 86

This was also not thought to be clinically significant as previous epidemiologic data suggests that all individuals with a TST $\geq$ 10mm are at increased risk of developing disease.[59] Patients with active TB were more likely to be HIV positive than community controls (9% vs 1%). These seroprevalence rates are similar to those reported in the Gambia for active TB cases and the general population in 1999.[24] The higher seroprevalence of HIV in household contacts (7%) is not unexpected as many of the household contacts were the spouse of the active TB cases.

Sixty percent of the active TB cases were 3+ smear positive and 62% had cavitary disease on chest radiograph. These patients had higher rates of treatment completion and cure (85%) in comparison to that reported for smear positive TB patients in the Gambia in 1998 (73%).[23] In the study population there was a lower rate of defaulting (5% vs 15%) and a lower mortality rate (2% vs 5%), in comparison to rates for the country, likely reflecting the benefit of close follow up provided by the study personnel. (Table 1, page 16 and table 20, page 73).

A positive test for each antigen was calculated from 18 BCG vaccinated controls from low TB incidence countries (table 21) To assess the effect of BCG, a second cut off was also calculated from 19 non-vaccinated controls from low TB incidence countries (results are presented in table 22a and 22b). For all of the antigens except GLU-S the cut off calculated from BCG vaccinated controls was higher than from unvaccinated controls; these differences were not statistically significant but the trend suggests a BCG effect.

The sensitivity of all seven antigens was poor and ranged from 24% to 75%. Specificity was very poor ranging from 26% to 71% (table 24b, page 77). For all four antigens shared by most mycobacteria (38kDa, 19kDa, 14kDa and GLU-S) and for the restricted antigen Rv3871 there was no significant difference between intensity of antibody response or percent positive between active TB cases, household contacts and community controls (table 24 and 23a, pages 76 & 77). For the restricted antigens ESAT-6 and CFP-10, the intensity of the antibody response and the percent positive was higher in active TB cases in comparison to community controls (Tables 23 and 23a and pages 76 & 77).

In the full dataset, the only antigens associated with active TB in multivariate analysis after adjusting for all clinical and demographic factors were ESAT-6 and CFP-10 (table 25). CFP-10 was associated with household contact status in multivariate analysis (table 25). In a reduced dataset comparing active TB with community controls (household controls removed), using multivariate analysis to adjust for potential confounders, both ESAT-6 and CRP-10 showed a trend towards association with active TB but not did reach significance (table 26). In a similar analysis comparing household contacts with community controls, CRP-10 showed a trend towards an association with household contact status (table 26).

HIV seropositive patients generally had lower mean antibody intensities, but this reached significant levels only for ESAT6 (0.27 vs 0.41, $p<0.01$). HIV seropositive patients also had overall lower percent positive serologic responses than HIV seronegative individuals (table 27) but this was only significant for the 14kDa (12% vs 36%, $p=0.04$). Patients with active cavitary pulmonary TB were significantly more likely to have positive serology and have higher mean antibody levels for all antigens except the 19kDa (table 28). There was no significant association between smear positive status and humoral response (table 29).

There was no association between the size of the tuberculin reaction and the presence or intensity of the antibody response in any of the study groups (Table 30). The frequency distribution of tuberculin skin test reactions was different in the three groups with higher mean and median values in active TB cases as compared to household contacts and community controls. Twenty-five percent of community controls had small TST reactions ranging from 5-9mm (figure 4).

Table 18- Demographic Characteristics of Active TB Cases, Household Contacts, Gambian Community Controls and Low TB Incidence Controls

	Active TB Cases N=100	Household Contacts N=100	Gambian Community Controls N=100	Low TB incidence Controls N=38
Age (years) Range	31.7 ± 12.1 (16-70)	29.7 ± 10.3 (15-61)	32.3 ± 10.2	30.0 ± 9.5 (19-52)
Male Sex (N,%)	68	49	59	16 (42%)
BCG Vaccinated (N,%)	53	54	65	18 (47%)
Religion (N,%) Muslim Other	 91 9	 91 9	 88 12	
Ethnic Origin (N,%) Mandinka Jola Fula Wolof Other	 46 16 15 10 13	 38 15 17 11 19	 31 25 10 11 23	
Marital Status (N,%) Married Single Other	 56 38 6	 57 41 2	 60 36 4	
Income (Dalasis/mo) †	429 ± 506	390 ± 437	478 ± 571	

† 22.8 Dalasi (Gambian currency) = $1 US

Significance testing was done on all variables in the table and none were found to be significant

Table 19: Physical and Laboratory Characteristics of Active Cases, Household Contacts, and Gambian Community Controls

		Active TB Cases N=100	Household Contacts N=100	Gambian Community Controls N=100
Height (cm)		170 ± 11	166 ± 10	168 ± 9
Weight (kg)		50.9 ± 8.8* (34.5-70)	60.5 ± 11 (36-103)	62.3 ± 11.3 (39-107)
BMI (kg/m^2)		17.6*	22	22.1
BCG Scar Present		50	52	67
BCG Scar Width (mm)		7 ± 3 (2-15)	8 ± 3 (3-20)	8 ± 4 (1-15)
BCG Scar Length (mm)		7 ± 3 (3-13)	8 ± 4 (3-21)	8 ± 3 (2-15)
TST Positive (≥10mm)		79/85 (93%)*	70/100 (70%)*	30/99 (30%)
TST Width (mm) (with TST <10mm removed)		17 ± 5* (10-40)	15 ± 4* (10-30)	13± 2 (10-20)
TST=0mm		5*	16*	39
HIV Positive		9*	7*	1
HIV1		6	4	1
HIV2		2	1	
HIV 1 + 2		1	2	
Hemoglobin (Hb)	Mean + SD	10.6± 2.6*	12.7 ± 2.5	12.5 ± 1.96
	Range	(5.4-17.2)	(6-18.6)	(9-18.2)
MCV†	Mean + SD	74± 7*	82 ± 7	83 ± 8
	Range	(57-93)	(56-102)	(49-99)
WBC‡	Mean + SD	7.6± 3.8*	5.6 ± 1.9	6.2 + 2.1
	Range	(1.6-20.3)	(2.4-13)	(2.8-17)
Eosinophils	Mean + SD	2.9± 2.6	3.9 ± 4.1	3.9 + 4.1
	Range	(0-20)	(0-20)	(0-21)
Platelets	Mean + SD	309± 146*	232 ± 190	211 ± 67
	Range	(88-766)	(120-490)	(74-386)

Significance testing compared active TB cases with community controls or household controls with community controls

All (*) values were significant at least at $p < 0.01$

† Mean Corpuscular Volume (MCV), ‡ White Blood Cell Count (WBC)

Table 20: Clinical Characteristics of Active TB Cases

	N=100
Previous TB Treatment	8
Smear (N=100)	
3+	59
2+	27
1+	13
Chest X-Ray	
Miliary	3
Pleural Effusion	5
Cavitation	62
Mean # cavities	2.7 (1-9)
Hilar Enlargement	35
Outcome	
Cured[1]	72
Completed Treatment[2]	13
Defaulted	5
Treatment Failure[3]	2
Transferred Out	4
Death	2
Unknown	2

1. Cured: Completed 6 months of treatment and Acid Fast Stain of Sputum Negative at 5-6 months of treatment.
2. Completed Treatment: Completed 6 months of treatment and no Acid Fast Stain of Sputum at the end of treatment
3. Treatment Failure: Acid Fast Stain of Sputum still positive after 5 months of therapy

(Definitions based on the WHO classification from the Global Report on Tuberculosis Control 2003)

Table 21: Characteristics of Controls from Low TB Incidence Countries

	BCG Vaccinated N=18	**BCG Non Vaccinated N=19**
Age (Mean years) (Range) **Mean age vaccinated** (years)	26 ± 5 (19-36) 14	31 + 12 (19-52)
Female	7 (39%)	14 (74%)
Mean number of months travelled in TB endemic country	4.5 ± 4.0 (1-15)	2.8 + 3.0 (0-14)
Nationality	United Kingdom- 13 Belgian-2 France- 2 New Zealand-1	USA-11 Holland- 7 Canadian-1
Occupation	Medical Student- 7 Medical Doctor- 1 Administrator- 2 Teacher- 2 Tourist- 2 Historian- 1 Economist- 1 Student-1 PhD-1	Medical Student- 5 Peace Corp Worker- 8 Nurse-1 Tourist- 4 Student-1

Table 22a: Percent Positive for Antibodies for Different Mycobacterial Antigens in Active TB Cases, Household Contacts , Gambian Community Controls and Low TB Incidence Controls Calculated with Two Different Cut offs

	N=100	Active TB Cases	Household Contacts	Gambian Community Controls	Low TB Incidence Control
			Shared Antigens		
38kDa	Cut-off 1	49%	48%	50%	3/18 (11%)
	Cut-off 2	60%	55%	61%	1/19 (5%)
19kDa	Cut-off 1	24%	26%	25%	2/18(11%)
	Cut-off 2	27%	29%	27%	0/19(0%)
14kDa	Cut-off 1	36%	32%	35%	1/18(6%)
	Cut-off 2	36%	32%	35%	3/19(16%)
GLU-S	Cut-off 1	75%	82%	74%	1/18(6%)
	Cut-off 2	66%	69%	54%	2/19 (10%)
			Restricted Antigens		
ESAT-6	Cut-off 1	67%	49%	49%	1/18 (6%)
	Cut-off 2	68%	51%	51%	1/19 (5%)
CFP-10	Cut-off 1	63%	54%	45%	1/18 (6%)
	Cut-off 2	72%	57%	54%	1/19 (5%)
RV3871	Cut-off 1	34%	28%	29%	1/18 (6%)
	Cut-off 2	43%	35%	35%	1/19 (5%)

Cut off 1: Calculated from BCG vaccinated controls from low TB incidence countries (n=18)
Cut off 2: Calculated from non-BCG vaccinated controls from low TB incidence countries (n=19)

Table 22b: Mean OD for Different Mycobacterial Antigens in Active TB Cases, Household Contacts , Gambian Community Controls and Low TB Incidence Controls Calculated With Two Different Cut offs

	Cut off 1	Cut off 2
	Shared Antigens	
38kDa	0.65	0.51
19kDa	0.47	0.42
14kDa	0.25	0.25
	Restricted Antigens	
GLU-S	0.34	0.45
ESAT-6	0.20	0.19
CFP-10	0.54	0.42
RV3871	0.52	0.45

Cut off 1: Calculated from BCG vaccinated controls from low TB incidence countries (n=18)
Cut off 2: Calculated from non-BCG vaccinated controls from low TB incidence countries (n=19)

There was no statistical difference between the cut off 1 and cut off 2 for any of the antigens

Table 23: Mean and Median Optical Densities[1] for Different Mycobacterial Antigens

		Active TB Cases	Household Contacts	Gambian Community Controls	Low TB[2] Incidence Controls
Shared Antigens					
38kDa	Mean + SD	1.04 ± 0.88	0.9 ± 0.8	0.92 ± 0.80	0.19 ± 0.19
	Range	(0.06-2.84)	(0.04-2.84)	(0.05-2.84)	(0.04-0.81)
	Median	0.64	0.54	0.65	0.10
19 kDa	Mean + SD	0.39 ± 0.27	0.40 ± 0.40	0.36 ± 0.29	0.22± 0.12
	Range	(0.07-1.52)	(0.06-2.91)	(0.01-1.59)	(0.05-0.62)
	Median	0.39	0.28	0.36	0.19
14 kDa	Mean + SD	0.36 ± 0.44	0.34 ± 0.45	0.30 ± 0.38	0.14 ± 0.09
	Range	(0.04-2.44)	(0.04-2.5)	(0.03-3.05)	(0.04-0.44)
	Median	0.36	0.17	0.15	0.11
GLU-S	Mean + SD	0.89 ± 0.67	1.05 ± 0.78*	0.80 ± 0.70*	0.21 ± 0.16
	Range	(0.05-2.87)	(0.11-2.87)	(0.08-2.87)	(0.06-0.54)
	Median	0.71	0.84	0.50	0.18
Restricted Antigens					
ESAT-6	Mean + SD	0.53 ± 0.60*	0.35 ± 0.36	0.33 ± 0.35*	0.10 ± 0.05
	Range	(0.04-2.56)	(0.04-1.69)	(0.05-1.98)	(0.04-0.25)
	Median	0.32	0.20	0.20	0.09
CFP-10	Mean + SD	1.11 ± 0.87*	0.94 ± 0.84	0.82 ± 0.80*	0.19 ± 0.25
	Range	(0.06-2.85)	(0.06-2.87)	(0.04-2.87)	(0.01-1.3)
	Median	0.79	0.63	0.49	0.12
Rv3871	Mean + SD	0.61± 0.60	0.57 ± 0.65	0.57 ± 0.64	0.21 ± 0.16
	Range	(0.01-3.02)	(0.07-3.02)	(0.05-2.77)	(0.05-0.73)
	Median	0.38	0.57	0.34	0.18

1. All numbers are corrected optical densities (ODs), the calculation for which is described in section 5.6.2 ELISA, page 64.
2. Includes the 18 BCG vaccinated controls raised in low TB incidence countries from which the cut-off was calculated as described in section 5.7.3 Definition of Cut-off, page 66.
3. Significance testing compared active TB cases with community controls or household controls with community controls
4. All highlight values marked with * were significant at least at $p < 0.01$

Table 24a- Percent Positive for Antibodies for Each Mycobacterial Antigen Tested

	Active TB Cases	Household Contacts	Gambian Community Controls
		Shared Antigens	
38kDa	49%	48%	50%
19kDa	24%	26%	25%
14kDa	36%	32%	35%
GLU-S	75% *	82%	74%
		Restricted Antigens	
ESAT-6	67% *	49%	49%
CFP-10	63%	54%	45%
Rv3871	34%	28%	29%

1. Cutoff calculated from BCG vaccinated healthy individuals from low incidence countries.
2. Significance testing compared active TB cases with community controls or household contacts with community controls
3. All highlighted values marked with an * were significant at least at $p < 0.01$

Table 24b- Sensitivity of Humoral Response to Detected Active Pulmonary TB and Specificity from Community controls for Mycobacterial Antigens Tested

	Sensitivity	Specificity
	Shared Antigens	
38kDa	49%	50%
19kDa	24%	75%
14kDa	36%	65%
GLU-S	75%	26%
	Restricted Antigens	
ESAT-6	67%	51%
CFP-10	63%	55%
Rv3871	34%	71%

Figure 3a

Optical Densities for Different Mycobacterial Antigens for Active TB (I), Household Contacts (H), Community Controls (X), BCG Vaccinated Controls from Low TB Incidence Countries (C)

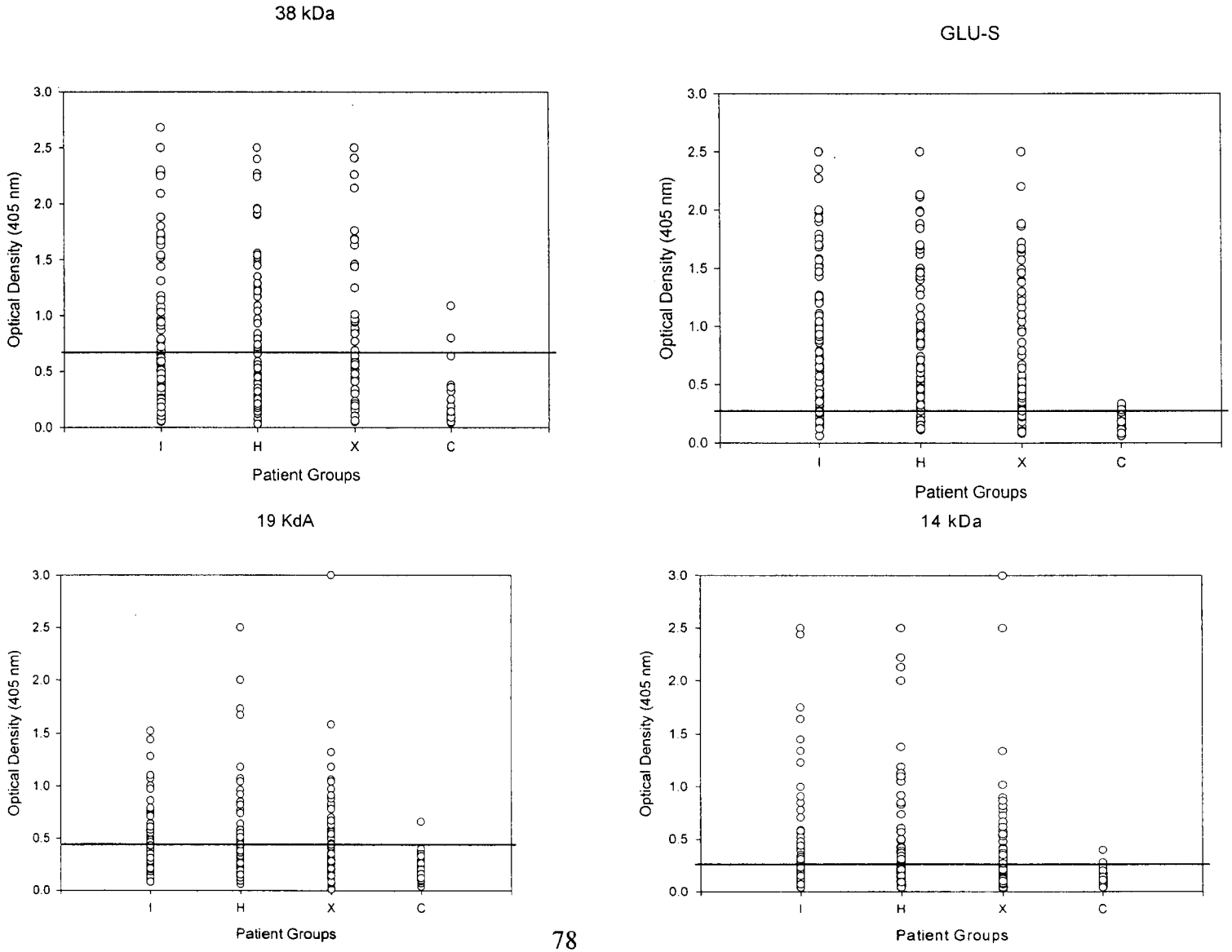


Figure 3b

Optical Densities for Different Mycobacterial Antigens for Active TB (I), Household Contacts (H), Community Controls (X), BCG Vaccinated Controls from Low TB Incidence Countries (C)

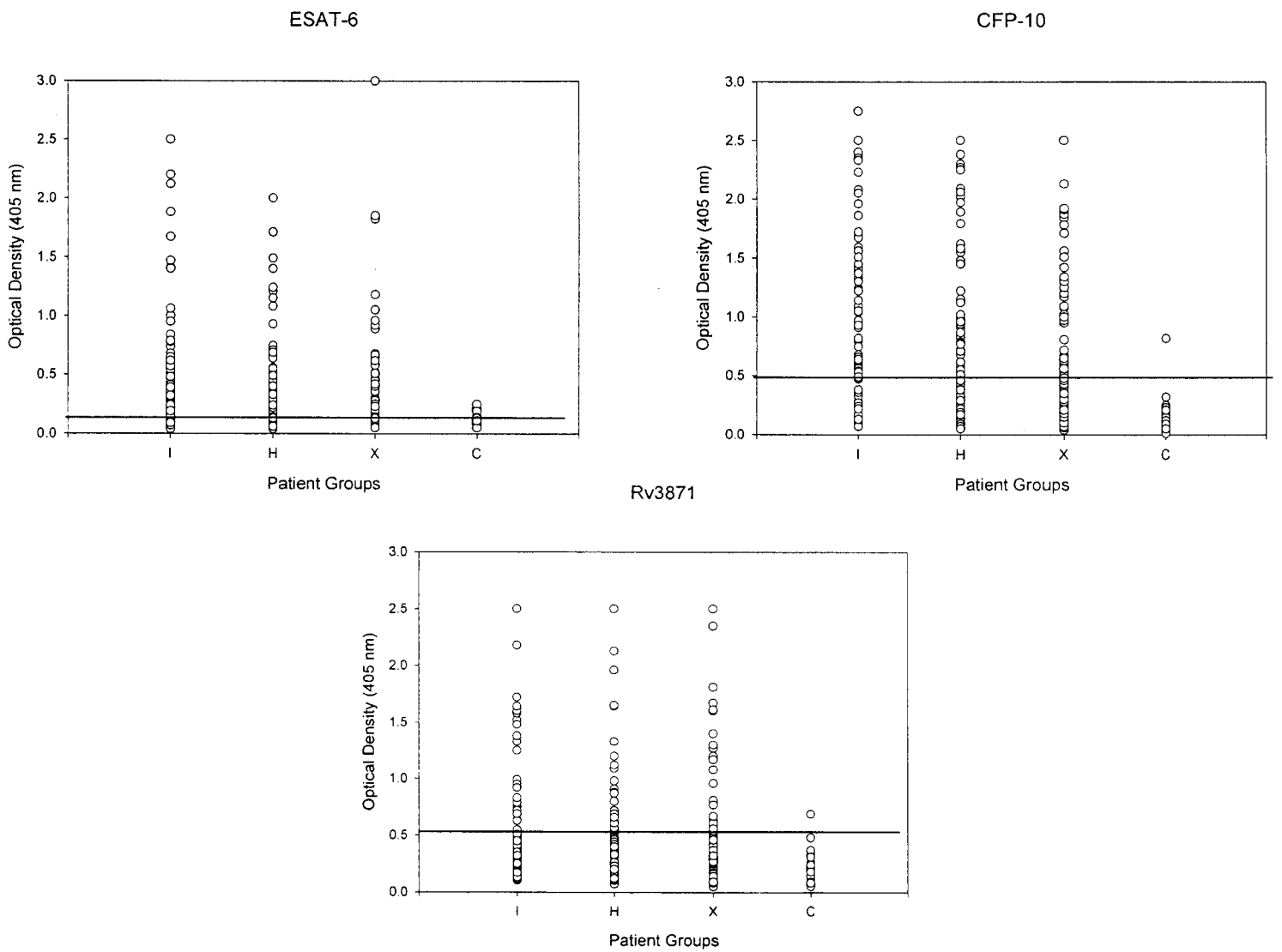


Table 25: Adjusted Odds of Factors Associated with Positive Serologic Response to Each of the Antigens for All Patient Groups.

	38Kd	19Kd	14Kd	GLUS	ESAT6[1]	CFP-10	Rv3871
Active TB	0.4 (0.2-0.97)	0.96 (0.4-2.4)	1.07 (.5-2.5)	0.8 (.32-1.9)	2.3 (1.02-5.1)	3.3 (1.5-7.3)	1.3 (0.5-3.1)
Household Control	0.6 (0.3-1.1)	1.3 (0.6-2.9)	0.86 (0.4-1.8)	1.7 (0.8-3.8)	1.07 (.5-2.2)	2.09 (1.03-4.2)	1.1 (0.5-2.4)
Gambian Controls	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Age	0.99 (.96-1.01)	1.02 (1.0-1.1)	0.99 (.97-1.0)	1.02 (.99-1.0)	1.01 (.99-1.0)	1.01 (.99-1.0)	1.00 (.99-1.0)
Male Sex	0.8 (0.5-1.4)	0.8 (.5-1.6)	1.1 (0.6-2.1)	1.2 (0.7-2.3)	1.05 (0.6-1.8)	0.9 (0.5-1.7)	1.8 (.97-3.3)
BCG	1.03 (0.6-1.8)	.8 (0.4-1.4)	.98 (0.5-1.8	1.3 (0.7-2.5)	0.73 (0.4-1.3)	1.2 (0.7-2.1)	1.09 (0.6-1.9)
TST ≥ 10mm	1.7 (.9-3.4)	.59 (.29-1.2)	.79 (.39-1.6	1.25 (.56-2.7	.75 (.38-1.5)	.61 (.31-1.2)	.92 (.45-1.9)
38Kd		0.99 (0.5-1.9)	1.7 (.93-3.2	2.5 (1.2-5.0)	1.4 (.8-2.4)	2.7 (1.6-4.7)	2.9 (1.6-5.4)
19kDa	1.0 (0.5-1.9)		2.5 (1.3-4.7)	.95 (0.4-2.2)	1.5 (.7-2.8)	1.4 (0.7-2.6)	2.3 (1.2-4.4)
14kDa	1.8 (.96-3.3)	2.5 (1.3-4.8)		2.1 (.95-4.8)	3.02 (1.6-5.5)	2.2 (1.2-4.0)	1.2 (.6-2.2)
GLUS	2.5 (1.2-5.0)	0.9 (0.4-2.2)	1.0 (0.4-2.8)		2.5 (1.3 -4.8)	1.5 (0.8-2.9)	1.7 (0.7-3.9)
ESAT-6	1.4 (0.8-2.4)	1.5 (.77-2.9)	2.9 (1.6-5.5)	2.3 (1.2-4.7)		1.3 (.8-2.4)	1.8 (0.9-3.4)
CFP-10	2.7 (1.6-4.8)	1.4 (0.7-2.6)	2.2 (1.2-4.0)	1.5 (0.8-2.9)	1.3 (.8-2.3)		1.3 (0.7-2.34
Rv3871	2.9 (1.6-5.4)	2.4 (1.2-4.5)	1.3 (.68-2.3)	1.9 (0.8-4.6)	1.8 (.9-3.3)	1.3 (0.7-2.4)	

1. Example Interpretation: Patients with a positive ESAT6 result, were 2.3 times more likely to have active tuberculosis, 3.0 times more likely to have positive result to 14 kDa and 2.5 times more likely to have a positive result to GLU-S after adjusting for age, sex, BCG status, TST status and the other 6 antigens.

2. Highlighted values are significant at a 95% confidence interval.

Table 26: Adjusted Odds (with 95% CI) of Factors Associated with Active TB or Household Contact Status

	Active TB[1]	Household[2] Contact
Age	0.98 (.95-1.01)	.96 (.92-.99)
Sex	0.7 (.3-1.7)	.4 (.2-.8)
BCG	.5 (.2-1.1)	.5 (.3-.99)
TST	43 (15-125)	8.4 (4.1-17)
38Kd	.5 (.2-1.3)	.6 (0.3-1.3)
GLUS	.6 (.2-1.7)	1.7 (0.7-4.0)
19kDa	.7 (.2-1.8)	1.5 (.6-3.3)
14kDa	1.3 (.5-3.5)	0.8 (0.4-1.8)
CFP-10	2.0 (.85-4.9)	2.02 (0.99-4.1)
Rv3871	2.2 (.8-5.9)	.9 (.4-2.0)
ESAT-6	2.5 (0.96-6.4)	1.01 (.5-2.0)

1. Data set with Active TB cases removed and reference group are Gambian Community Controls
2. Data set with Household contact removed and reference group are Gambian Community Controls
3. Highlighted values are significant at a 95% confidence interval.
4. Example Interpretation: In comparison to Gambian Community Controls patients with active TB had a 43 tmes greatere likelihood of having a positive TST, a 2.5 greater likelihood of having a positive serologic test for ESAT-6 after adjusting for age, sex, TST status, BCG status and the other 6 antigens tested.

Table 27: Serologic Response to Seven Antigens by HIV Status

		HIV+		HIV-	
		N=17	% positive	N=283	% positive
Shared Antigens					
38kDa	Mean + SD Range Median	0.78 ± 0.80 (0.12-2.7) 0.48	5/17 (29%)	0.97 ± 0.83 (0.04-2.84) 0.66	140/283 (50%)
19 kDa	Mean + SD Range Median	0.39 ± (0.09-1.7) 0.17	4/17 (23%)	0.38 ± 0.32 (0.01-2.9) 0.39	71/283 (25%)
14 kDa	Mean + SD Range Median	0.25 ± 0.39 (0.04-1.7) 0.17	2/17[1] (12%)	0.34 ± 0.42 (0.03-3.1) 0.18	101/283 (36%)
GLU-S	Mean + SD Range Median	0.84 ± 0.69 (0.23-2.6) 0.61	13/17 (76%)	0.92 ± 0.72 (0.05-2.9) 0.66	218/283 (77%)
Restricted Antigens					
ESAT-6	Mean + SD Range Median	0.27 ± 0.16[2] (0.07-0.55) 0.22	9/17 (52%)	0.41 ± 0.47 (0.04-2.6) 0.26	156/283 (55%)
CFP-10	Mean + SD Range Median	0.87 ± 0.77 (0.07-2.4) 0.51	7/17 (41%)	0.96 ± 0.84 (0.04-2.9) 0.62	155/283 (55%)
Rv3871	Mean + SD Range Median	0.59± 0.61 (0.10-2.3) 0.30	6/17 (35%)	0.59± 0.63 (0.05-3.0) 0.35	85/283 (30%)

The only significant differences for the percent positive tests or intensity of antibody response between HIV+ and HIV- patients are those listed below:

1. For the 14kDa, HIV+ patients were less likely to have a positive test than HIV- patients (12% vs 36%, p=0.04)
2. For ESAT6, HIV+ patients had a lower mean optical density than than HIV- patients (0.27 vs 0.41%, p=0.04)

Table 28: Association of Cavitation with Humoral Response

		Cavitary N=62		Non-Cavitary N=38	
			% positive		% positive
Shared Antigens					
38kDa	Mean + SD Range Median	1.18 ± 0.90 (0.06-2.8) 0.83	36/62 (58%)	0.8 ± 0.80 (0.06-2.8) 0.47	13/38 (34%)
19 kDa	Mean + SD Range Median	0.39 ± 0.28 (0.07-1.5) 0.32	13/62 (21%)	0.37 ± 0.27 (0.1-1.4) 0.30	7/38 (18%)
14 kDa	Mean + SD Range Median	0.42 ± 0.46 (0.04-2.2) 0.25	13/62 (21%)	0.25 ± 0.39 (0.04-2.4) 0.36	2/38 (5%)
GLU-S	Mean + SD Range Median	1.06 ± 0.68 (0.14-2.9) 0.92	52/62 (84%)	0.61 ± 0.54 (0.05-2.4) 0.47	23/38 (61%)
Restricted Antigens					
ESAT-6	Mean + SD Range Median	0.67 ± 0.66 (0.08-2.6) 0.43	47/62 (76%)	0.31 ± 0.40 (0.04-2.4) 0.26	20/38 (52%)
CFP-10	Mean + SD Range Median	1.3 ± 0.86 (0.08-2.7) 1.2	43/62 (69%)	0.79 ± 0.79 (0.06-2.9) 0.5	13/38 (34%)
Rv3871	Mean + SD Range Median	0.67± 0.60 (0.01-2.5) 0.47	24/62 (38%)	0.50± 0.60 (0.01-3.0) 0.28	7/38 (18%)

1. For all antigens except the 19kDa and Rv3871 the mean intensity of the antibody response was significantly higher ($p<0.01$) in those with cavitary disease than those with non-cavitary disease.
2. For all antigens except the 19kDa the percent positive tests was significantly higher ($p<0.01$) higher in those with cavitary disease than those with non-cavitary disease.

Table 29: Association of Bacillary Load seen on AFB Smear with Humoral Response

		Smear 1+		Smear 2+		Smear 3+	
		N=13	% positive	N=27	% positive	N=60	% positive
38kDa	Mean + SD Range Median	0.79 ± 0.76 (0.06-2.8) 0.48	5/13 (38%)	1.3 ± 1.0 (0.1-2.7) 0.72	15/27 (56%)	0.94 ± 0.79 (0.06-2.84) 0.61	28/59 (47%)
19 kDa	Mean + SD Range Median	0.34 ± 0.21 (0.1-0.86) 0.28	2/13 (15%)	0.37 ± 0.23 (0.11-0.93) 0.30	7/27 (26%)	0.40 ± 0.31 (0.07-1.5) 0.32	11/59 (17%)
14 kDa	Mean + SD Range Median	0.24 ± 0.19 (0.08-0.8) 0.19	1/13 (8%)	0.23 ± 0.18 (0.04-0.87) 0.23	2/27 (7%)	0.44 ± 0.53 (0.04-2.54) 0.24	12/59 (20%)
GLU-S	Mean + SD Range Median	0.63 ± 0.61 (0.13-2.4) 0.71	10/13 (76%)	0.86 ± 0.72 (0.05-2.9) 0.69	17/27 (63%)	0.93 ± 0.64 (0.14-2.6) 0.81	47/59 (80%)
ESAT-6	Mean + SD Range Median	0.24 ± 0.17 (0.07-0.72) 0.32	17/13 (54%)	0.36 ± 0.29 (0.04-1.4) 0.34	15/27 (56%)	0.64 ± 0.67 (0.06-2.6) 0.37	44/59 (76%)
CFP-10	Mean + SD Range Median	0.78 ± 0.73 (0.06-2.4) 0.59	5/13 (38%)	1.1 ± 0.86 (0.06-2.6) 0.80	14/27 (52%)	1.17 ± 0.89 (0.08-2.9) 0.81	36/59 (61%)
Rv3871	Mean + SD Range Median	0.51± 0.54 (0.01-1.7) 0.25	3/13 (23%)	0.53± 0.46 (0.11-1.7) 0.35	9/27 (33%)	0.67± 0.67 (0.01-3.0) 0.41	19/59 (32%)

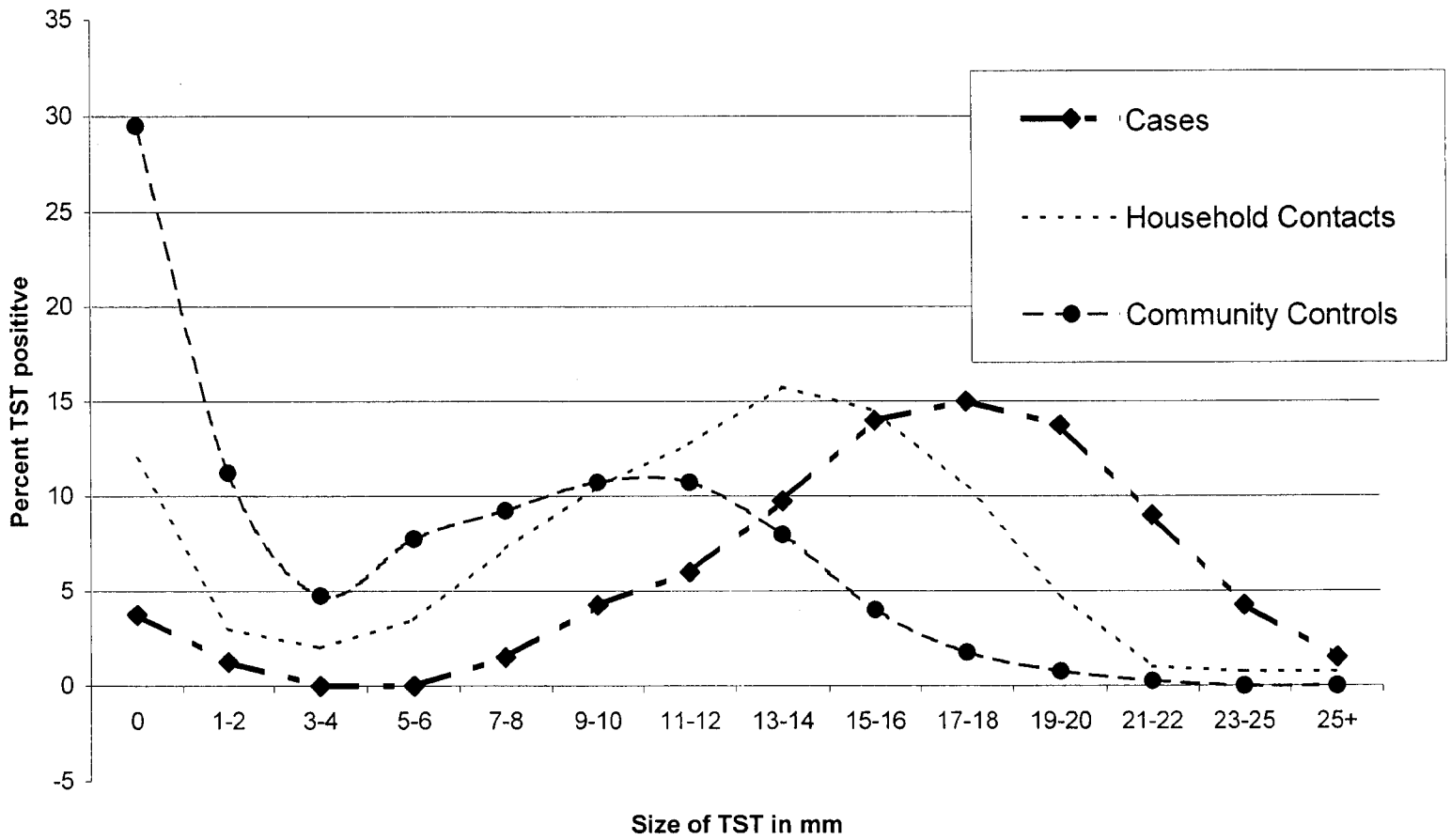
There were no significant differences for the percent positive tests or intensity of antibody response between any of the patient groups

Table 30 : Association of Humoral Response to Each Antigen with Size of TST Reaction in Gambian Community Controls

Antigen	TST=0 (N=39)	TST 1-9 mm (N=30)	TST >10mm (N=30)
Shared Antigens			
38kDa	54%	50%	47%
19kDa	21%	33%	23%
14kDa	38%	40%	27%
GLU-S	69%	77%	80%
Restricted Antigens			
ESAT-6	56%	47%	43%
CFP-10	41%	53%	43%
Rv3871	31%	37%	20%

There were no significant differences in humoral response by TST for any of the antigens

Figure 4 **Percent TST positive for Index Cases , Houshold Contacts and Community Controls**



7. DISCUSSION

7.1 Summary of Results

This study demonstrated that the humoral response in Gambians to seven different mycobacterial antigens showed poor sensitivity and very poor specificity. Only the responses to ESAT-6 and CFP-10, restricted antigens absent from BCG and some non-tuberculous mycobacteria, were able to distinguish those with active TB from community controls. Only CFP-10 distinguished household contacts from community controls. Sensitivity and intensity of antibody response increased with cavitation, suggesting that the humoral response was positively correlated with increasing disease severity.

7.2 Strengths and Weaknesses of the Study

There were many strengths of this study. The most important strength of this study was that the three study groups were well characterized. Cases of active tuberculosis were only included in the study if two sputum specimens were smear positive and were confirmed by culture. The household contacts and community controls were prospectively followed for 18 months to verify that they did not develop active tuberculosis. Individuals that developed symptoms suggestive of pulmonary tuberculosis underwent appropriate investigations. Extensive descriptive demographic and clinical information, measurement of BCG scar, tuberculin skin test results, and HIV serostatus were available for study participants. Patients with active tuberculosis had a detailed description of the chest radiograph findings and the smear positivity was graded. The effect of BCG on the humoral response could be estimated in this study because half of the controls from low TB incidence countries were BCG vaccinated and the other half were not (tables 21, 22a & 22b). The potential effect of non-tuberculous mycobacteria on the humoral response could be inferred due to the availability of a recent Gambian-wide skin test survey of several non-tuberculous mycobacteria.[151] The use of asymptomatic community controls, while not as clinically appropriate as patients presenting with respiratory symptoms, was more appropriate than healthy young volunteers from non-endemic countries used in many other studies.[15, 104, 108, 143]

There were certain limitations to this study. The cross-sectional nature of the design meant that only associations rather than causality could be inferred. Power was limited in some subgroups such as those that were HIV seropositive or those with only 1+ smear positive status. Multiple testing was done and was not corrected for primarily, because even prior to doing so few significant differences between groups were found.

7.3 Sensitivity

Sensitivity of the humoral response is influenced by many factors as outlined in table 15 (page 57). The factors that may have affected results in this study were inclusion of HIV seropositive individuals (reduced sensitivity), the use of recombinant antigens (reduced sensitivity, table 11, page 49), and cavitation on chest radiograph (62% of the study population had significantly greater intensity and percent positive antibody results than the other 38% (table 28, page 83).[95-98, 100] The sensitivity of the humoral response is also influenced by the antigen used and the stage of disease, so these factors need to considered when interpreting our results (table 11, page 49).

The sensitivity for most of the antigens reported in our study was similar to what has been described previously for recombinant antigens in smear positive pulmonary TB, but still was disappointingly low. The sensitivity estimated in this study and the average of previous reported values in the literature are as follow; for 38kDa (49% vs 52%)[17, 97, 98, 107-110], for 19kD (20% vs 25%)[107, 108], and for 14kDa (15% vs 28%)[107, 108]. In contrast, the sensitivity of ESAT-6 estimated in our study was 67% vs 18% reported in the literature[107-109]. The higher than previously described sensitivity to ESAT-6 will be discussed in more detail in the section on specificity, but may be due to high rates of exposure to non-tuberculous mycobacteria in the environment such as *M.marinum* or *M. kansasii* or high rates of latent tuberculosis in the community, as 49% of the Gambian community controls also had positive serologic responses to ESAT-6.

The method of preparation of the antigen has a great influence on the sensitivity of detection of mycobacterial antibodies. Recombinant antigens are less time consuming to produce than are purified antigens and are theoretically more specific.[12] Studies from different countries on recombinant 38kDa[12, 15, 17, 82, 97-100, 104-110], 14kDa[12, 15, 82, 104, 107, 108], 19kDa[12, 15, 82, 100, 104, 107, 108, 114] and Antigen 85B[12, 107, 108, 117] tested at different stages of

disease, but particularly with smear positive pulmonary disease, have been 20% to 40% less sensitive than purified antigens. This was first noted in a study by Verbon et al where the sensitivity of a truncated recombinant 38kDa antigen that lacked the first 42 residues from the amino-terminus and the last four from the carboxy-terminus end, had a sensitivity of 50%, much lower than had previously been described.[17, 113] Different hypotheses have been suggested (but not systematically studied) to explain the fact that recombinant antigens have a lower sensitivity than purified proteins. One plausible explanation could be that post-translational events result in a "poorer fitting antigen". This means that proteins produced in an *E.coli* clone may have additional sugars or other molecules, or that the protein produced may not be folded in the same manner as a protein produced in a mycobacterium. This results in antigens that have less affinity to the antibody, resulting in reduced sensitivity.

One strategy to improve the performance of serologic testing has been to combine several antigens (due to the heterogeneous nature of the antigen response to tuberculosis).[107] Two commercial multi-antigen tests that each contain 5 recombinant antigens or peptides [the ICT Tuberculosis (AMRAD-ICT, Sydney, Australia), and Detect-TB (BioChem ImmunoSystems, Montreal, Canada)] have been tested in different populations. These studies demonstrated moderate sensitivity (mean 63%, range 31%-87%) for smear positive pulmonary TB but at a cost of unacceptably poor specificity (mean 84%, range 79%-88%) in hospitalized patients or those presenting with pulmonary symptoms.[110-113, 141]

The sensitivity of mycobacterial antibodies to detect active TB in HIV positive patients has been shown to be lower than in HIV negative patients in several studies.[83 90, 91, 106, 140] Only 9% of patients with active TB, 7% of household contacts and 1% of community controls were HIV positive in this study (table 19, page 72). The small number of HIV positive patients would not have affected the overall sensitivity of the serologic responses in this study (table 27, page 82).

7.4 Specificity

The specificity for the 7 different antigens in our study ranged from 26%-85%. This is much poorer than in any previously reported study. Specificity in healthy controls

from low TB incidence countries is generally >95%[15, 104, 108] but decreases to 85%-90% when more appropriate controls are tested, such as patients with pulmonary symptoms or hospitalized patients with other illnesses.[89, 109, 110] Specificity was only 75%-90% for those with non-tuberculous mycobacterial disease [86, 100, 109, 112] and 79%-88% when multiple antigens were tested[68, 111-113, 117] Interestingly, the only other study to have reported specificities as poor as those found in our study was also conducted in an African setting.[106] In a Tanzanian study, when tested with the 38kDa, the specificity in HIV negative hospital controls was 70% and was 50% in HIV positive hospital controls. When both the 38kDa and the 14kDa were tested, specificity decreased to 54% in HIV negative hospital controls and remained 50% in HIV positive hospital controls. [106]

Several factors may have contributed to the poor specificity seen in our study. These include the effect of BCG, cross-reactivity with other mycobacteria in the environment or other microorganisms, and the inability to distinguish active from latent TB. Each of these factors will be discussed separately below.

7.4.1. Effect of BCG on Specificity

BCG can theoretically cause false positive serologic reactions to the 4 shared antigens (38kDa, 19kDa, 14kDa and GLU-S) tested. BCG should not, however, cause false positive reactions to the more restricted antigens (ESAT-6, CFP-10 and Rv3871) since they are encoded in the RD-1 region of *M.tuberculosis* and are not found in BCG. In this study, we had an opportunity to estimate the effect of BCG from the results for each of the antigens in 37 controls from countries with low TB incidence, 18 of whom were BCG vaccinated and 19 who were not. The effect of BCG on the humoral response was estimated from these two control groups, as shown in table 22a and 22b, page 75. The differences are described below suggest a trend towards a BCG effect, but none of the differences were significantly different.

The mean serologic responses to the 38kDa and the 19kDa were higher in the BCG vaccinated group as compared to the unvaccinated group. There were no differences between these two groups when tested with the 14kDa. For reasons that are unclear, the mean serologic response to GLU-S was higher in the unvaccinated group than in the BCG vaccinated group. As would be expected, the mean optical densities for ESAT-6 (an

antigen not found in BCG) in both the BCG vaccinated and the unvaccinated groups were similar. Humoral response to the Rv3871 and CFP-10 antigens (from the RD1 region and absent from BCG) however, was greater in BCG vaccinated than unvaccinated suggesting that these antigens must have been contaminated with some other antigen(s).

None of the differences between the BCG vaccinated group and the unvaccinated group were statistically significant, but the trend overall is suggestive of a BCG effect. The fact that BCG affected the serologic response in the BCG vaccinated controls from low endemic countries is likely due to the fact that most were relatively "recently" vaccinated as they were vaccinated at a mean age of 14 and had an average age of 26.

Most of the participants in this study had been BCG vaccinated given the fact that universal BCG vaccination at birth has been instituted in the Gambia since the late 1970s with excellent average country wide coverage (85% in 1980, 98% in 1990 and 99% in 2000).[172] To account for the possibility that BCG affected the serologic response in study subjects, the cut-off used to determine positive results was calculated using results from the BCG vaccinated controls from low TB incidence countries. The poor specificity reported in this study therefore, should not been due to BCG.

7.4.2 Effect of Non-tuberculous Mycobacteria on Specificity

Members of the genus Mycobacterium share many antigens in common and cross-reactivity of immune response increases with greater phylogenetic closeness to *M.tuberculosis*. The non-tuberculous mycobacteria most closely related to *M.tuberculosis* are *M.marinum* and *M. kansasii*, followed by *M.avium, M. scrofulaceum*, and *M. intracellulare*. In a large population based study in Malawi the greatest cross-reacting effect on tuberculin skin testing was due to *M.marinum* and *M. kansasii*. This effect decreased with decreasing genetic relatedness of the mycobacterial species tested.[150] In tropical countries, including the Gambia, non-tuberculous mycobacteria are ubiquitous in the environment, resulting in a high prevalence of skin test reactivity, ranging from 20%-80% for the most common species (table 13, page 53 and table 14, page 54).

Non-tuberculous mycobacteria appear to stimulate some cell mediated immunity to *M.tuberculosis* as demonstrated by small tuberculin skin test reactions (usually <10mm).[150] Non-tuberculous mycobacteria also stimulate the humoral immune system and have been

known to cause false positive serologic responses to *M.tuberculosis* antigens.[109] In patients with non-tuberculous mycobacterial disease (predominantly *Mycobacterium avium intracellulare*) 10-15% had antibodies to the 38kDa [100], 25% to Ag60 [86], 11%-26% to the 14kDa [100, 109] , 15% to ESAT-6 [109] and 26% with the DETECT TB (ITC, Melboure, Australia). In areas with a high prevalence of non-tuberculous mycobacteria, such as the Gambia, false positive serologic results to TB may be more common.

The finding of high background rates of humoral response to ESAT-6 was corroborated by the study by Vekeman et al where 30% of community controls from the same Gambian study population also had cellular immune responses to ESAT-6.[125] The *esat6* gene (and therefore, very likely, the gene for CFP10, which is within the same operon as *esat6*) has recently been shown to be present in many NTM.[80, 81] Interestingly, in the region of residence of study participants, the skin test prevalence to *M.marinum* was very high (43%) and to *M. kansasii* was moderate (11%).[151] The distribution of TST reactions in the Gambian community controls (with 25% having small reactions in the 5-9 mm range) is typical of that seen in populations with high background rates of non-tuberculous mycobacterial exposure and sensitization (figure 4, page 86). These facts support the possibility that non-tuberculous mycobacteria contributed to the low specificity of the serologic response in the study population.

7.4.3 Effect of Latent Tuberculosis on Specificity

The only test that distinguishes between active tuberculosis and latent tuberculosis infection is culture of the mycobacteria from an individual. In a study in Bombay by Lalvani et al, 56% of community controls had cellular responses to ESAT-6 and 76% had responses to CFP10 in comparison to none of the UK controls.[130] This data and the 30% cellular response to ESAT-6 in Gambian Community controls is likely due to the high proportion of individuals with latent TB infection in these countries.[125] One study also suggested that serologic responses to ESAT-6 may be a marker of "inactive TB" (latent tuberculosis with fibronodular changes on chest radiograph). In the study by Silva et al 57% of patients with inactive tuberculosis (stable fibrosis on chest radiograph for >2 months, TST >5mm, and smear and culture negative) had a positive serologic test to ESAT-6 compared to 9% of those with latent tuberculosis (normal chest radiograph and

TST ≥10mm).[109] Based on these data it is plausible that some of the non-specificity to ESAT-6, as well as the other antigens in this study, could be due to latent TB infection or even inactive tuberculosis, as controls did not have a chest radiograph but were followed only with symptoms.

7.4.4 Effect of Cross-reactivity with Other Organisms on Specificity

There is cross reactivity between mycobacterial and fungal antigens. Wheat at al described false positive histoplasmosis serology in patients with active tuberculosis and Sada et al described false positive serology to lipoarabinomannan (LAM) in patients with active histoplasmosis.[91, 92] The extent to which histoplasmosis may have caused false positive serologic reactions in our study is unknown, as the prevalence of histoplasmosis in the Gambia is unknown.

7.5 Relationship Between Disease Stage and Humoral Response

Antibodies are not thought to play an important role in the protective immunity to *M.tuberculosis.* Antibodies to different antigens are found, however, in various stages of disease. The predominant immune response early in infection and with minimal disease is a cell mediated TH1 response, whereas in more advanced disease a humoral and TH2 cellular response predominates. Greater intensity of antibody response has been documented with increasing disease severity (smear positive vs smear negative)[14, 97], with increased length of symptoms (>1 month and >4 months),[98, 99] and with very advanced vs moderately advanced disease.[14, 95, 96] In a study of patients with culture positive TB, 88% of those that had been symptomatic for either < 4 months or > 4 months had serum antibodies. Seventy-five percent of those symptomatic for > 4 months had a strong antibody response, whereas only 28% of those that had been symptomatic for < 4 months showed a strong antibody response.[98] In our study, humoral response to all antigens except the 19kDa was more frequent and of greater intensity with increasing disease severity (measured by cavitation on chest radiograph). One study noted a negative correlation of the 19kDa with cavitation on chest radiography, a finding that is intriguing and unexplained.[14] One possible explanation would be that different mycobacterial antigens elicit a humoral

response at different stages of disease (which occurs with other diseases such as hepatitis B and Epstein Barr Virus infections).

The TH2 shift in patients with active TB in our patient population is supported by data from the larger study in which this serologic study was nested. Lienhardt et al looked at the cellular response to *M.tuberculosis* in cases of active tuberculosis, household contacts, and community controls from the Gambia and Guinea. They reported on 414 groups (cases of active smear positive pulmonary disease each matched to one household contact and one community control), 285 of whom were from the Gambia. They demonstrated that in active tuberculosis cases there was predominantly a TH2 response, in household contacts there was predominantly a TH1 response, and in community controls there was a mixed response.[173] Several other groups have also shown an increase in Th2-type cytokines in tuberculosis patients with more severe disease, particularly cavitary disease.[39-43] The predominant TH1 cellular response (low humoral response) in patients with early disease (contacts) or pauci-bacillary disease suggests that serologic testing will not be a useful method of detecting active tuberculosis in these patient groups.

7.6 Conclusions

Over the past 100 years an enormous amount of effort has been expended to develop a serologic test for the diagnosis of active tuberculosis. During this time, the understanding of the biology and immune response of *M.tuberculosis* has increased dramatically, and new restricted, immunogenic, recombinant antigens have been produced. Despite this, the accuracy of serologic testing has not substantially improved.

The inability to improve the sensitivity and specificity of serologic testing to diagnose active tuberculosis may be attributable to the characteristics of the humoral immune response to *M.tuberculosis.* This is a complex process influenced by many factors. Sensitivity is limited by the heterogeneous nature of the humoral response and is antigen, disease stage, and host specific. No single antigen has a sensitivity of greater than 70% even in smear positive pulmonary disease, and sensitivity is poor for all antigens in pauci-bacillary disease (including children, and HIV infected individuals). New recombinant antigens have much lower sensitivity than purified antigens. The utility of serologic testing is further decreased by poor specificity of the humoral response. Serologic testing cannot distinguish patients with active disease from those with latent TB infection, and there is considerable cross-reactivity with non-tuberculous mycobacteria and some cross-reactivity with BCG vaccine. Combinations of recombinant antigens have improved sensitivity but at the cost of decreased specificity. The results of our study show that the new restricted antigens have unacceptably low sensitivity and specificity. Based on these results, clinical utility of serologic testing in countries with low or high incidence of TB remains limited. It is unlikely that, in the future, serologic testing will become an important diagnostic tool to detect active tuberculosis.

9. REFERENCES

1. Dye C, Scheele S, Pathania V, Raviglione M, The WHO Global Surveillance and Monitoring Project. Global Burden of Tuberculosis, Estimated Incidence, Prevalence, and Mortality by Country. JAMA 1999; 282:677-686.

2. Corbett EL, Watt CJ, Walder N, et al. The Growing Burden of Tuberculosis. Global Trends and Interactions with the HIV Epidemic. Arch Intern Med. 2003; 163:1009-1021.

3. WHO Report 2003. Global Tuberculosis Control, Surveillance, Planning, Financing. Geneva: World Health Organization,, 2003.

4. Kindhauser MK. Communicable Diseases 2003. Global defence against the infectious disease threat. Geneva: World Health Organization, 2003.

5. Raviglione MC, Snider DE, Kochi A. Global Epidemiology of tuberculosis. JAMA 1995; 223:220-226.

6. Foulds J, O'Brien R. New Tools for the Diagnosis of Tuberculosis: the perspective of developing countries. Int J Tuberc Lung Dis 1998; 2(10):778-783.

7. Harries AD, Maher D, Nunn P. An approach to the problems of diagnosing and treating adult smear-negative pulmonary tuberculosis in high-HIV-prevalence settings in sub-Saharan Africa. Bull of the World Health Organization 1998; 76:651-662.

8. Hawken MP, Muhindi DW, Chakaya JM, Bhatt SM, Ng'ang'a LW, Porter JDH. Underdiagnosis of smear-positive pulmonary tuberculosis in Nairobi, Kenya. Int J Tuberc Lung Dis 2000; 5:360-363.

9. Barnes P, Verdegem T, Vachon L, Leedom J, Overturf G. Chest roentgenogram in pulmonary tuberculosis. Chest 1988; 94:316-320.

10. Markey AC, Forster SM, Mitchell R, Larson E, Smith H. Suspected cases of pulmonary tuberculosis referred from port of entry into Great Britain, 1980-3. British Medical Journal 1986; 292:378.

11. Laszlo A. Tuberculosis Bacteriology Laboratory Services and Incremental Protocols for Developing Countries. Clinics in Laboratory Medicine 1996; 16:697-716.

12. Bothamley GH. Serological diagnosis of tuberculosis. Eur Respr J. 1995; 8:676S-688S.

13. Kox LFF. Tests for detection and identification of mycobacteria. How should they be used? Respiratory Medicine 1995; 89:399-408.

14. Bothamley GH, Rudd R, Festenstein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax 1992; 47:270-275.

15. Jackett PS, Bothamley GH, Harsh VB, Mistry A, Young DB, Ivanyi J. Specificity of antibodies to immunodominant mycobacterial antigens in pulmonary tuberculosis. J Clin Microbiol 1988; 26:2313-2318.

16. Kaplan MH, Chase MW. Antibodies to mycobacteria in human tuberculosis. I. Development of antibodies before and after antimicrobial therapy. J of Infectious Diseases 1980; 142:825-834.

17. Verbon A, Weverling GJ, Kuijper S, Speelman P, Jansen HM, Kox LFF. Evaluation of different tests for the serodiagnosis of tuberculosis and the use of likelihood ratios in serology. Amer Rev Resp Dis 1993; 148:378-384.

18. Wobester WR, Krajden M, Conly J, Simpson H, Yim B, D'Costa M. Evaluation of Roche amplicor PCR assay for *Mycobacterium tuberculosis*. J Clin Microbiol 1996; 34:134-139.

19. Arikan S, Tuncer S, Us D, Unal S, Ustacelebi S. Anti-Kp 90 IgA Antibodies in the Diagnosis of Active Tuberculosis. Chest 1998; 114:1253-1257.

20. Swaminathan S, Umadevi P, Shantha S, Radhakrishnan A, Datta M. Sero diagnosis of tuberculosis in children using two ELISA kits. Indian Journal of Pediatrics 1999; 66.

21. Raviglione M, Snider DE, Kochi A. Global epidemiology of tuberculosis: morbidity and mortaltiy of a worldwide epidemic. JAMA 1995; 273:220-226.

22. World Health Organization. Global Tuberculosis Control. WHO Report. Geneva, 1998.

23. Année-van Bavel JACM. The National Leprosy and Tuberculosis Control Programme of the Gambia. Progess report #7 of the advisory visit, October 2-17, 1999.

24. O'Donovan D, Ariyoshi K, Milligan P, et al. Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates of HIV-1 and HIV-2 in the Gambia. AIDS 2000; 14:441-448.

25. UNICEF. The State of the World's Children, 2003.

26. Adegbola RA, Hill P, Baldeh I, et al. Surveillance of drug-resistant *Mycobacterium tuberculosis* in The Gambia. Int J Tuberc Lung Dis 2003; 7:390-393.

27. Organization WH. Global Tuberuclosis Control. WHO Report. Geneva, 1998.

28. Tomkinson M. Gambia. Oxford: Michael Tomkinson Publishing, 2000.

29. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. Color Atlas and Textbook of Diagnostic Microbiology. Philadelphia: Lippincott-Raven, 1997.

30. Iseman MD. A Clinician's Guide to Tuberculosis. Philadelphia: Lippincott Williams & Wilkins, 2000.

31. Brennan PJ. Structure of mycobacteria: Recent developments in defining cell wall carbohydrates and proteins. Reviews of Infectious Diseases 1989; 11:S420-S430.

32. Andersen P. Host Responses and Antigens Involved in Protective Immunity to Mycobacterium Tuberculosis. Scand.J.Immunol. 1997; 45:115-131.

33. van Crevel R, Ottenhoff THM, van der Meer JWM. Innate immunity to *Mycobacterium tuberculosis*. Clin Micro Reviews 2002; 15:294-309.

34. Taha RA, Olivenstein R, Hamid QA. Immunopathology of Pulmonary Tuberculosis: Role of Cytokines. Canadian Journal of Allergy & Clinical Immunology 2001; 6:16-24.

35. Taha RA, Kotsimbos TC, Song YL, Menzies D, Hamid Q. IFN-gamma and IL-12 are increased in active compared with inactive tuberculosis. Am J Respir Crit Care Med 1997; 155:1135-1139.

36. Cooper AM, Dalton DK, Stewart TA. Disseminated tuberculosis in interferon-gamma gene-disrupted mice. J Exp Med 1993; 178:2243-2248.

37. Flynn JL, Chan J, Tribold KJ. An essential role for interferon-gamma in resistance to *M. tuberculosis* infection. J Exp Med 1993; 178:2249-2254.

38. Dahl KE, Shiratsuchi H, Hamilton BD, Ellner JJ, Toossi Z. Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. Infect. Immun 1996; 64:399-405.

39. Bhattacharyya S, Singla R, Dey AB, Prasad HK. Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis. Infect Immun 1999; 67:5597-5603.

40. Dlugovitzky D, Bay ML, Rateni L, et al. *In vitro* synthesis of interferon gamma, interleukin-4, transforming growth factor-beta and interleukin-1 beta by peripheral blood mononuclear cells from tuberculosis patients: Relationship with the severity of pulmonary involvement. Scand J Immunol 1999; 49:210-217.

41. Seah GT, Scott GM, Rook GAW. Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis. J Infect Dis 2000; 181:385-389.

42. Surcel HM, Troye-Blomberg M, Paulie S, et al. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 1994; 81:171-176.

43. van Crevel R, Karyadi E, Preyers F, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. J Infect Dis 2000; 181:1194-1197.

44. Barnes PF, Lu S, Abrams Js, Wang E, Yamamura M, Modlin RL. Cytokine production at the site of diease in human tuberculosis. Infect Immun 1993; 61:3482-3489.

45. Lin Y, Zhang M, Hofman FM, Gong J, Barnes PF. Absence of a prominant Th2 cytokine response in human tuberculosis. Infection and Immunity 1996; 64:1351-1356.

46. Hernandez-Pando R, Rook GAW. The role of TNF-alpha in T-cell mediated inflammation depends on the TH1/Th2 cytokine balance. Immunology 1994; 82:591-595.

47. Lai CKW, Ho S, Chan CHS, et al. Cytokine Gene Expression Profile of Circulating CD4+ T Cells in Active Pulmonary Tuberculosis. Chest 1997; 111:606-611.

48. WHO Tuberculosis Diagnostics Workshop: Product Development Guidelines. Cleveland, Ohio: World Health Organization, Global Tuberculosis Program, 1997.

49. Metchock BG, Nolte FS, Wallace RJ. Mycobacterium. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolden RH, eds. Manual of Clinical Microbiology. Washington D.C.: American Society of Microbiology, 1999.

50. Toman K. Tuberculosis: Case-finding and chemotherpy: Questions and answers. Geneva: World Health Organization, 1979.

51. Kanaya AM, Glidden DV, Chambers HF. Identifying Pulmonary Tuberculosis in Patients with Negative Sputum Smear Results. Chest 2001; 120:349-355.

52. Long R, Scalcini M, Manfreda J, Jean-Baptiste M, Hershfield E. The impact of HIV on the usefulness of sputum smears for the diagnosis of tuberculosis. Am J Public Health 1991; 81:1326-1328.

53. Long R. Smear-negative pulmonary tuberculosis in industrialized countries. Chest 2001; 120:330-334.

54. Githui W, Nunn P, Juma E, al e. Cohort study of HIV-positive and HIV-negative tuberculosis, Nairobi, Kenya: comparison of bacteriological results. Tubercle Lung Dis 1992; 73:203-209.

55. Treatment of Tuberculosis: Guidelines for National Programmes. Geneva: World Health Organization, 2003.

56. Control of tuberculosis transmission in health care setting: A joint statement of the International Union Against Tuberculosis and Lung Disease (IUATLD) and the Tuberculosis Programme of the World Health Organisation. Paris, France: IUATLD, 1993.

57. Burman W, Stone B, Reves R, Wilson A, Yang Z, El-Hajj H. The incidence of false-positive cultures for mycobacterium tuberculosis. Am J Respir Crit Care Med 1997; 155:321-326.

58. Menzies D. Interpretation of repeated tuberculin tests, boosting, conversion, and reversion. Am J Respir Crit Care Med 1999; 159:15-21.

59. Al Zahrani K, Jahdali HA, Menzies D. Does size matter? Utility of the size of tuberculin reactions for the diagnosis of mycobacterial disease. Am J Respir Crit Care Med 2000; 162:1419-1422.

60. Rooney JJ, Crocco JA, Kramer S, Lyons HA. Further observations on tuberculin reactions in active tuberculosis. Am J Med 1976; 60:517-521.

61. Holden M, Dubin MR, Diamond PH. Frequency of negative intermediate-strength tuberculin sensitivity in patients with active tuberculosis. New Engl J Med 1971; 285:1506-1509.

62. Harrison BDW, Tugwell P, Fawcett IW. Tuberculin reaction in adult Nigerians with sputum-positive pulmonary tuberculosis. Lancet 1975.

63. Graham NMH, Nelson KE, Solomon L, Bonds M, Rizzo RT, Scavotto J. Prevalence of tubeculin positivity and skin test anergy in HIV-1 seropositive and HIV-seronegative person. JAMA 1992; 267:369-373.

64. Markowitz N, Hansen NI, Wilcosky TC, Hopewell PC, Glassroth J, Kvale PA. Tuberculin and anergy testing in HIV-seropositive and HIV-seronegative persons. Ann Intern Med. 1993; 119:185-193.

65. World Health Organization. Further studies of geographic variation in naturally acquired tuberculin sensitivity. Bull World Health Organization 1955; 22:63-83.

66. Roberts GD, Hall L, Wolk DM. Mycobacteria. In: Truant AL, ed. Manual of Commerical Methods in Clinical Microbiology. Washington, D.C.: American Society for Microbiology, 2002.

67. Clarridge JE, Shawar RM, Shinnick TM, Plikaytis BB. Large-scale use of polymerase chain reaction for detection of mycobacterium tuberculosis in a routine mycobacteriology laboratory. J Clin Microbiol 1993; 31:2049-2056.

68. Al Zahrani K, Jahdali HA, Poirier L, Rene P, Gennaro ML, Menzies D. Accuracy and utility of commercially available amplification and serologic tests for the diagnosis of minimal pulmonary tuberculosis. Am J Respir Crit Care Med 2000; 162:1323-1329.

69. Catanzaro A, Perry S, Clarridge JE, Dunbar S, Goodnight-White S, LoBue PA. The role of clinical suspicion in evaluating a new diagnostic test for active tuberculosis. Results of a multicenter prospective trial. JAMA 2000; 283:639-645.

70. Abe C, Hirano K, Wada M, Kazumi Y, Takahashi M, Fukasawa Y. Detection of mycobacterium tuberculosis in clinical specimens by polymerase chain reaction and the gen-probe amplified *Mycobacterium tuberculosis* direct test. J Clin Microbiol 1993; 31:3270-3274.

71. Daniel TM, Debanne SM. The serodiagnosis of tuberculosis and other mycobacterial disease by enzyme-linked immunosorbent assay. Amer Rev Resp Dis 1987; 135:1137-1151.

72. Orme IM. Induction of nonspecific acquired resistance and delayed-type hypersensitivity, but not specific acquired resistance in mice inoculated with killed mycobacterial vaccines. Infect Immun 1988; 56:3310-3312.

73. Romain F, Augier J, Pescher P, Marchal G. Isolation of a proline-rich mycobacterial protein eliciting delayed-type hypersenstivity reactions only in guinea pigs immunized with living mycobacteria. Proc.Natl.Acad.Sci.U.S.A. 1993; 90:5322-5326.

74. Andersen P, Askgaard D, Ljungqvist L, Bennedsen J, Heron I. Proteins released from *Mycobacterium tuberculosis* in growth. Infection and Immunity 1991; 59:1905-1910.

75. Horwitz MA, Lee BWE, Dillon BJ, Harth G. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc.Natl.Acad.Sci.U.S.A. 1995; 92:1530-1534.

76. Behr MA, Wilson MA, Gill WP, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999; 284:1520-1523.

77. Sorensen A, Nagai S, Houen G, Andersen P, Andersen AB. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infection and Immunity 1995; 63:1710-1707.

78. Berthet FX, Rasmussen PB, Rosenkrands.I., Andersen P, Gicquel B. Mycobacterium tuberculosis operon encoding ESAT-6 and a novel low-molecular-mass culture filtrate protein (CFP-10). Microbiology 1998; 144(Pt11):3195-3203.

79. Vinther Skjot RL, Oettinger T, Rosenkrands.I., et al. Comparative evaluation of low-molecular-mass proteins from *Mycobacterium tuberculosis* identifies members

of the ESAT-6 family as immunodominant T-cell antigens. Infection and Immunity 2000; 68(1):214-220.

80. Gey van Pittius NC, Gamieldien J, Hide W, Brown GD, Siezen RJ, Beyers AD. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G + C gram-positive bacteria. Genome Biol 2001; 2 (10):Epub 2001 Sept 19.

81. Gey van Pittius NC, Warren RM, van Helden PD. ESAT-6 and CRP-10 : What is the Diagnosis? Infection and Immunity 2002; 70:6509-6510.

82. Wilkins EGL. The serodiagnosis of tuberculosis. In: Davies PDO, ed. Clinical Tuberculosis. Vol. 367-380. London: Chapman & Hall, 1994.

83. Chan ED, Heifets L, Iseman MD. Immunologic diagnosis of tuberculosis: a review. Tuberc Lung Dis 2000; 80:131-140.

84. Cocito CG. Properties of the mycobacterial antigen complex A60 and its applications to the diagnosis and prognosis of tuberculosis. Chest 1991; 100:1687-1693.

85. Charpin D, Herbault H, Gevaudan MJ, et al. Value of ELISA using A60 Antigen in the diagnosis of active pulmonary tuberculosis. Amer Rev Resp Dis 1990; 142:380-384.

86. Gevaudan MJ, Bollet C, Charpin D, Mallet MN, Micco PD. Serological response of tuberculosis patients to antigen 60 of BCG. Eur J Epidemiol 1992; 8:666-676.

87. Gupta S, Kumari S, Banwalikar JN, Gupta SK. Diagnostic utility of the estimation of mycobacterial Antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis. Tuberc Lung Dis 1995; 76:418-424.

88. Alifano M, Sofia M, Mormile M, et al. IgA Immune Response against the Mycobacterial Antigen A60 in Patients with Active Pulmonary Tubeculosis. Respiration 1996; 63:292-297.

89. Alifano M, Pascalis RD, Sofia M, Faraone S, Pezzo MD, Covelli I. Evaluation of IgA-mediated humoral immune response against the mycobacterial antigen P-90 in diagnosis of pulmonary tuberculosis. Chest 1997; 111:601-605.

90. Somi GR, O'Brien RJ, Mfinanga GS, Ipuge YA. Evaluation of the MycoDot test in patients with suspected tuberculosis in a field setting in Tanzania. Int J Tuberc Lung Dis 1999; 3:231-238.

91. Sada E, Brennan PJ, Herrera T, Torres M. Evaluation of Liparabinomannan for the Serological Diganosis of Tuberculosis. J Infect Dis 1990; 28:2587-2590.

92. Wheat J, French MLV, Kamel S, Tewari RP. Evaluation of cross-reactions in *Histoplasma capsulatum* serologic tests. J Clin Microbiol 1986; 23:496-499.

93. Julian E, Matas L, Perez A, Alcaide J, Laneelle M-A, Luquin M. Serodiagnosis of tuberculosis: Comparison of immunoglobulin A (IgA) response to sulfolipid I with IgG and IgM responses to 2,3-diacytrehalose, 2,3,6-triacyltrehalose, and cord factor antigens. J Clin Microbiol 2002; 40:3782-3788.

94. Young D, Kent L, Rees A, Lamb J, Ivanyi J. Immunological activity of 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect. Immun. 1986; 54:177-183.

95. Daniel TM. Enzyme-Linked Immunosorbent Assay Using Mycobacterium tuberculosis Antigen 5 and PPD for the Serodiagnosis of Tuberculosis. Chest 1985; 88:388-392.

96. Ma Y, Wang Y, Daniel TM. Enzyme-linked immunosorbent assay using *Mycobacterium tuberculosis* antigen 5 for the diagnosis of pulmonary tuberculosis in China. Am Rev Respir Dis 1986; 134:1273-1275.

97. Cole RA, Lu HM, Shi YZ, Wang J, De-Hua T, Shou AT. Clinical evaluation of a rapid immunochromatographic assay based on the 38kDa antigen of *Mycobacterium tuberculosis* on patients with pulmonary tuberculosis in China. Tubercle and Lung Disease 1996; 77:363-368.

98. Zhou AT, Ma WL, Ahang PY, Cole RA. Detection of pulmonary and extrapulmonary tuberculosis patients with the 38-kilodalton antigen from Mycobacterium tuberculosis in a rapid membrane-based assay. Clinical & Diagnostic Laboratory Immunology 1996; 3:337-341.

99. Mathur ML, LoBue PA, Catanzaro A. Evaluation of a serologic test for the diagnosis of tuberculosis. Int J Tuberc Lung Dis 1999; 3(8):732-735.

100. Wilkins EGL, Ivanyi J. Potential value of serology for the diagnosis of extrapulmonary tuberculosis. Lancet 1990; 336:641-644.

101. Daniel TM, DeMurillo GL, Sawyer JA, et al. Field Evaluation of Enzyme-Linked Immunosorbent Assay for the Serodiagnosis of Tuberculosis. Amer Rev Resp Dis 1986; 134:662-665.

102. Balestrino EA, Daniel TM, DeLatini MDS, Latini OA, Ma Y, Scocozza JB. Serodiagnosis of pulmonary tuberculosis in Argentina by enzyme-linked immunosorbent assay (ELISA) of IgG antibody to Mycobacterium tuberulosis antigen 5 and tuberculin purified protein derivative. Bull of the World Health Organization 1984; 62(5):755-761.

103. Ivanyi J, Krambovitis E, Keen M. Evaluation of a monoclonal antibody (TB72) based serological tet for tuberculosis. Clin Exp Immunol 1983; 54:337-345.

104. Bothamley GH, Udani P, Rudd R, Festenstien F, Ivanyi J. Humoral response to defined epitopes of tubercle bacilli in adult pulmonary and child tuberculosis. Eur J Clin Microbiol Infect Dis 1988; 7:639-645.

105. Wilkins EGL, Bothamley GH, Jackett PS. A rapid, simple ELISA to measure antibody to individual epitopes in the serodiagnosis of tuberculosis. Eur J Clin Microbiol Infect Dis 1991; 10:559-563.

106. Thybo S, Richter C, Wachmann H, et al. Humoral resonse to *Mycobacterium tuberculosis* specific antigens in African tuberculosis patients with high prevalence of human immunodeficiency virus infection. Tuberc Lung Dis 1995; 76:149-155.

107. Lyashchenko K, Colangeli R, Houde M, Jaldali HA, Menzies R, Gennaro ML. Heterogeneous antibody responses in tuberculosis. Infection and Immunity 1998; 66:3936-3940.

108. Lyashchenko KP, Singh M, Colangeli R, Gennaro ML. A multi-antigen print immunoassay for the development of serolgical diagnosis of infectious diseases. Journal of Immunological Methods 2000; 242:91-100.

109. Silva VMC, Kanaujia G, Gennaro ML, Menzies D. Factors associated with humoral response to ESAT-6, 38kDa and 14kDa in patients with a spectrum of tuberculosis. Int J Tuberc Lung Dis 2003; 7:478-484.

110. Pottumarthy S, Wells VC, Morris AJ. A comparison of seven tests for serological diagnosis of tuberculosis. J Clin Microbiol 2000; 38:2227-2231.

111. Rasolofo V, Rasolonavalona T, Ramarokoto H, Chanteau S. Predictive values of the ICT Tuberculosis test for the routine diagnosis of tuberculosis in Madagascar. Int J Tuberc Lung Dis 2000; 4:184-185.

112. McConkey SJ, Youssef FG, Azem E, Frenck RW, Weil GJ. Evaluation of a rapid-format antibody test and the tuberculin skin test for diagnosis of tuberculosis in two contrasting endemic settings. Int J Tuberc Lung Dis 2002; 6:246-252.

113. Perkins MD, Conde MB, Martins M, Krtski AL. Serologic diagnosis of tuberculosis using a simple commercial multiantigen assay. Chest 2003; 123:107-112.

114. Bothamley GH, Batra HV, Ramesh V, Chandramuki A, Ivanyi J. Serodiagnostic value of the 19 kilodalton antigen of Mycobacterium tuberulosis in Indian patients. Eur J Clin Microbiol Infect Dis 1992; 11:6-10.

115. Bothamley G, Rudd R, Festenstein F, Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax 1992; 47:270-275.

116. Sada E, Ferguson LE, Daniel TM. An ELISA for the serodiagnosis of tuberculosis using a 30,000-Da native antigen of *Mycobacterium tuberculosis*. J Infect Dis 1990; 162:928-931.

117. Landowski CP, Godfrey HP, Bentley-Hibbert SI, et al. Combinatorial use of antibodies to secreted mycobacterial proteins in a host immune system-independent test for tuberculosis. J Clin Microbiol 2001; 39:2418-2424.

118. Matsumoto S, Matsuo T, Ohara N. Cloning and Sequencing of a unique antigen MPT70 from Mycobacterium tuberculosis H37Rv and expression in BCG using E.coli-mycobacteria shuttle vector. Scand.J.Immunol. 1995; 41:281-287.

119. Matsuo T, Matsumoto S, Ohara N, Kitaura H, Mizuno A, Yamada T. Differential transciption of the MPB70 genes in two major groups of *Mycobacterium bovis* BCG substrains. Microbiology 1995; 141(Pt7):1601-1607.

120. Colangeli R, Antinori A, Cingolani A, et al. Humoral immune responses to multiple antigens of *Myobacterium tuberculosis* in tuberculosis patients co-infected with the human immunodeficiency virus. Int J Tuberc Lung Dis 1999; 3(12):1127-1131.

121. Bothamley GH, Swanson Beck J, Potts RC, Grange JM, Kardjito T, Ivanyi J. Specificity of antibodies and tuberculin response after occupational exposure to tuberculosis. JID 1992; 166:182-186.

122. Daniel TM, Ellner JJ. Immunology of Tuberculosis. In: Richman LB, Hershfield ES, eds. Tuberculosis: A Comprehensive International Approach. New York: Marcel Dekker Inc, 1993:75-101.

123. Harth G, Clemens DL, Horwitz MA. Glutamine synthetase of *Mycobacterium tuberculosis*: extracellular release and characterization of its enzymatic activity. Proc.Natl.Acad.Sci.U.S.A. 1994; 91:9342-9346.

124. Raynaud C, Etienne G, Peyron P, Laneelle M-A, Daffe M. Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis.* Microbiology 1998; 144:577-587.

125. Vekemans J, Lienhardt C, Sillah J, et al. Tuberculosis contacts but not patients have higher gamma interferon responses to ESAT-6 than do community controls in the Gambia. Infection and Immunity 2001; 69:6554-6557.

126. Doherty TM, Demissie A, Olobo J, et al. Immune responses to the *Mycobacterium tuberculosis* specific antigen ESAT-6 signal subclincal infection among contacts of tuberculosis patients. J Clin Microbiol 2002; 40:704-706.

127. Pathan AA, Wilkinson KA, Klenerman P, et al. Direct *ex vivo* analysis of antigen-specific IFN gamma secreting CD4 cells in *Mycobacterium tuberculosis* infected individuals: Associations with disease state and effect of treatment. The Journal of Immunology 2001; 167:5217-5225.

128. Munk ME, Arend SM, Brock I, Ottenhoff THM, Andersen P. Use of ESAT-6 and CFP-10 antigens for diagnosis of extrapulmonary tuberculosis. J Infect Dis 2001; 183:175-176.

129. van Pinxteren LAH, Ravn P, Agger EM, Pollock J, Andersen P. Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP-10. Clinical and Diagnositic Laboratory Immunology 2000; 7:155-160.

130. Lalvani A, Nagvenkar P, Udwadia Z, et al. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium tuberculosis* infection in healthy urban Indians. J of Infectious Diseases 2001; 183:469-477.

131. Colangeli R, Spencer JS, Bifani P, et al. MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis*, elicits tuberculosis-specific, delayed-type hypersensitivity in guinea pigs. Infect.Immun. 2000; 68(2):990-993.

132. Arend SM, Andersen P, van Meijgaarden K, et al. Detection of active tuberculosis infection by T cell responses to early-secreted antigenic target 6-kDa protein and culture filtrate protein 10. J Infect Dis 2000; 181:1850-1854.

133. Brusasca PN, Colangeli R, Lyashchenko KP, et al. Immunological Characterization of Antigens Encoded by the RD1 Region of the *Mycobacterium tuberculosis* Genome. Scand. J. Immunol. 2001; 54:448-452.

134. Yamaguchi R, Matsuo K, Yamazaki A, et al. Cloning and characterization of the gene for immunogenic protein MPB64 of *Mycobacterium bovis* BCG. Infect.Immun. 1989; 57:283-288.

135. Oettinger T, Andersen AB. Cloning and B-cell-epitope mapping of MPT64 from *Mycobacterium tuberculosis* H37Rv. Infect.Immun. 1994; 62:2058-2064.

136. Wilcke JTR, Jensen BN, Ravn P, Andersen AB, Haslov K. Clincial evaluation of MPT-64 and MPT-59, two proteins secreted from *Mycobacterium tuberculosis*, for skin test reagents. Tubercle and Lung Disease 1996; 77:250-256.

137. Elhay MJ, Oettinger T, Andersen P. Delayed-type hypersensitivity responses to ESAT-6 and MPT-64 from *Mycobacterium tuberculosis* in the guinea pig. Infection and Immunity 1998; 66(7):3454-3456.

138. Grange JM. The humoral immune response in tuberculosis: Its nature, biological role and diagnostic usefulness. Adv. Tuberc. Res 1984; 21:1-78.

139. Turneer M, Vooren J-Pv, Bruyn JD, Serruys E, Dierckx P, Yernault J-C. Humoral immune response in human tuberculosis: immunoglobulins G, A and M directed against the purified P32 protein antigen of *Mycobacterum bovis* bacillus. J Clin Microbiol 1988; 26:1714-1719.

140. Daniel TM, Sippola AA, Okwera A, Kabengera S, Hatanga E, Aisu T. Reduced sensitivity of tuberculosis serodiagnosis in patients with AIDS in Uganda. Tuberc Lung Dis 1994; 75:33-37.

141. Amicosante M, Houde M, Guaraldi G, Saltini C. Sensitivity and specificity of a multi-antigen ELISA test for the serological diagnosis of tuberculosis. Int J Tuberc Lung Dis 1999; 3:736-740.

142. Delacourt C, Gobin J, Gaillard J, Blic Jd, Veron M, Scheinmann P. Value of ELISA using antigen 60 for the diagnosis of tubeculosis in children. Chest 1993; 104:393-398.

143. Alifano M, Pascalis RD, Sofia M, Farone S, Pezzo MD, Covelli I. Detection of IgG and IgA against the mycobacterial antigen A60 in patients with extrapulmonary tuberculosis. Thorax 1998; 53:377-380.

144. Chiang I-H, Suo J, Bai K-J, et al. Serodiagnosis of tuberculosis. A study comparing three specific mycobacterial antigens. Amer Rev Resp Dis 1997; 156:906-911.

145. Torres M, Mendez-Sampeiro P, Jimenez-Samudio L. Comparison of the immune response against *Mycobacterium tuberculosis* antigens between a group of patients with active pulmonary tuberculosis and healthy household contacts. Clin Exp Immunol 1994; 96:75-78.

146. Wilsher ML, Hagan C, Prestidge R, Wells AU, Murison G. Human in vitro immune responses to *Mycobacterium tuberculosis*. Tuberc Lung Dis 1999; 79:371-377.

147. Paul RC, Stanford JL. Multiple skin testing of Kenyan schoolchildren with a series of new tuberculins. J Hyg Camb 1975; 75:303-313.

148. Kardjito T, Beck JS, Grange JM, Standford SL. A comparison of the responsiveness to four new tuberculins among Indonesian patients with pulmonary tuberculosis and healthy subjects. Eur J Respir Dis 1986; 69:142-145.

149. von Reyn F. Evidence of Previous Infection with *Mycobacterium avium-Mycobacterium intracellulare* Complex among Healthy Subjects: An International Study of Dominant Mycobacterial Skin Test Reactions. J Infect Dis 1993; 168:1553-1558.

150. Fine PEM, Floyd S, Stanford JL, et al. Environmental mycobacteria in northern Malawi: implications for the epidemiology of tuberculsis and leprosy. Epidemiol.Infect. 2001; 126:379-387.

151. Corrah PT. Studies of Tuberculosis in the Gambia. Medical Research Council Laboratories, Fajara, the Gambia: University College, London, 1994.

152. Edwards LB, Acquaviva FA, Livesay VT, Cross FW, Palmer CE. An Atlas of Sensitivity to tuberculin, PPD-B, and Histoplasmin in the United States. Am Rev Respir Dis 1969; 99:1-132.

153. Jeanes CWL, Davies JW, McKinnon NE. Sensitivity to "Atypical" Acid-Fast Mycobacteria in Canada. Can Med Assoc J 1969; 100:888-895.

154. Brickman HF. Prevalence of mycobacterial sensitivity in Montreal children. Can Med Assoc J 1974; 110:640-644.

155. Frappier-Davignon F, Fortin R, Desy M. Sensitivity to "Atypical" Mycobacteria in High School Children in Two Community Health Departments. Can J Public Health 1989; 80:335-338.

156. Palmer CE, Edwards LB, Hopwood L, Edwards PQ. Experimental and Epidemiologic Basis for the Interpretation of Tuberculin Sensitivity. The Journal of Pediatrics 1959; 55:413-428.

157. WHO Tuberculosis Research Office. Further Studies of Geographic Variation in Naturally Acquired Tuberculin Sensitivity. Bull World Health Organization 1955; 12:63-83.

158. Ly HM, Trach DD, Long HT, Thuy NK, Tuan NA. Skin test responsiveness to a series of new tuberculins of children living in three vietnamese cites. Tubercle 1989; 70:27-36.

159. Amano H, Mizoguchi K, Tsukamura M, Murate T, Hasegawa T, Shimokata K. Enzyme-Linked Immunosorbent Assay for the Differential Diagnosis of Pulmonary Tuberculosis and Pulmonary Diseases due to *Mycobacterium avium-intracellulare Complex.* Jpn J Med 1989; 28:196-201.

160. Wayne LG, Anderson B, Chetty K, Light RW. Antibodies to mycobacterial peptidioglycolipid and to crude protein antigens insera from different categories of human subjects. J Clin Microbiol 1988; 11:2300-2306.

161. Fusillo MH, Weiss DL. Lack of circulating antibodies after BCG immunization as assayed by the globulin titration technique. Am Rev Tuberc 1958; 78:793.

162. Mauch H, Brehmer W. Mycobacterial antibodies after tuberculin testing, BCG-immunotherapy and against cross-reacting antigens in a solid-phase radioimmunoassay. Zentbl. Bakt. Hyg. I. Abt. Orig A 1982; 251:380-388.

163. Lagercrantz R, Peterson IC, Lind I. Futher studies of tuberculin hemagglutination in tuberculous infection, benign and virulent. Acta Paediat 1953; 42:113-125.

164. Kardjito T, Handoyo I, Grange JM. Diagnosis of active tuberculosis by immunological methods. 1. The effect of tuberculin reactivity and previous BCG

vaccination on the antibody levels determined by ELISA. Tubercle 1982; 63:269-274.

165. Barrera L, de Kantor I, Ritacco V. Humoral response to *Mycobacterium tuberculosis* in patients with human immunodeficieny virus infection. Tuberc Lung Dis 1992; 73:187-191.

166. Manca C, Lyashchenko K, Wiker HG, Usai D, Colangeli R, Gennaro ML. Molecular cloning, purification, and serologica characterization of MPT63, a novel antigen secreted by Mycobacterium tuberculosis. Infect.Immun. 1997; 65:16-23.

167. Manca C, Lyashchenko K, Colangeli R, Gennaro ML. MTC28, a novel 28-kilodalton proline -rich secreted antigen specific for the Mycobacterium tuberculosis complex. Infect.Immun. 1997; 65:4951-4957.

168. Colangeli R, Heijbel A, Williams AM, et al. Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of Mycobacterium tuberculosis. J Chromatogr B Biomed Sci Appl 1998; 714:223-35.

169. Dawson-Saunders B, Trapp RG. Basic and clinical biostatistics. Norwalk, CT: Appleton & Lange, 1990.

170. Kleinbaum DG, Kupper LL. Applied regression analysis and other multi-variate methods. North Scituate, MA: Duxbury Press, 1978.

171. Sackett DL, Haynes RB, Tugwell P. Clinical epidemiology. Toronto, Canada: Little, Brown & Co., 1985.

172. World Health Organization. State of the World's Vaccines and Immunization. Geneva: World Health Organization, 2002:96.

173. Lienhardt C, Azzurri A, Amedei A, et al. Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity *in vivo*. Eur J Immunol 2002; 32:1605-1613.

9.0 APPENDICIES

Ethics approval letter from McGill University, July 19, 2000

Certification of Ethical Acceptability for Research Involving Human Subjects, McGill University, July 19, 2000.

Ethics approval letter from McGill University, Sept 25, 2001

Ethics approval letter from McGill University, June 18, 2002

Ethics approval letter from MRC Laboratories, the Gambia, May 4, 2000

Ethics approval letter from MRC Laboratories, the Gambia, December 18, 2000

E-mail permission from Dr. J. van der Meer to publish figure 2, August 22, 2003.

Greenaway Chris

From: vanderMeer J. [J.vanderMeer@AIG.umcn.nl]
Sent: August 22, 2003 12:02
To: Greenaway Chris
Cc: vanCrevel R.
Subject: RE: article on innate immunity to TB in Clinical Micro Reviews April 2002

Of course, we grant you permission.
Good Luck with your thesis defense.
Yours sincerely
Jos WM van der Meer, MD PhD FRCP
Professor of Medicine
> ----------
> From: Greenaway Chris
> Sent: Friday, August 22, 2003 16:03 PM
> To: vanderMeer J.
> Subject: article on innate immunity to TB in Clinical Micro Reviews April 2002
>
> Dear Dr. van der Meer,
>
> I am an infectious disease specialist and microbiologist at the
> Jewish General Hospital, a McGill affiliated hospital in Montreal
> Canada. I am doing my masters in Epidemiology and Biostatistics at
> McGill. For the field work for my thesis for this degree I spent a
> year in the Gambia on a research fellowship studying the humoral
> response to tuberculosis in cases with active TB in comparison to
> community controls in an attempt to develop a diagnostic test for TB
> that could be used in developing countries. We unfortunately found
> that both the sensitivity and specificity of the antigens we tested
> were poor. I am planning to submit my masters thesis next week and in
> the back ground I discuss both the cellular and humoral immune
> response to TB. During my writing I found and read with interest
> your article on > "> Innate Immunity to Mycobacterium tuberculosis> ">
> . I was wondering if I could have permission to add figure 3 in that
> article (inflammatory response of phagocytic cells upon activation of
> M.tuberculosis) in my thesis. I look forward to hearing from you.
>
> Sincerely,
>
> Chris Greenaway
>
>