EFFECTS OF MALARIA ON IMMUNE RESPONSE AGAINST TUBERCULOSIS AMONG CHILDREN IN UASIN GISHU COUNTY, KENYA

BY

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DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been presented for a degree in any other University. No part of this thesis may be reproduced without the prior written permission of the author and/or University of Eldoret.

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This thesis is dedicated to my parents, wife and children.

ABSTRACT

Among tuberculosis (TB) high incidence regions, Sub-Saharan Africa is particularly affected with approximately 1.6 million new cases every year and 1 million people die from malaria every year (most are children 2 - 5 years old). The efficacy of *Bacille Calmette Guerin* (BCG) is low and incidence of TB is high in those areas where malaria is endemic. Besides this dramatic situation, the effect of malaria on immunity to TB and data on the diversity of *Mycobacterium tuberculosis* complex (MTBC) strains causing this epidemic in this area are only sparsely available. The population of MTBC strains circulating and their drug susceptibility trends from malaria and TB co-infected children in Uasin Gishu County was analysed.The effect of malariaon haematological indices, BCG (before and after) vaccination, CD4+ T/CD8+ T cells and cytokine response to *M. tuberculosis* in malaria and TB co-infected patients and in patients with TB alone were also investigated. ZN staining was done for AFB bacilli and sputum specimens cultured in liquid (BACTEC™ MGIT 960) and solid media (LJ), and drug susceptibility tests performed for first-line drugs including (Isoniazid, Rifampicin, Streptomycin and Ethambutol) using both Genotype®MTBDR*Plus* andBACTEC™ MGIT 960. MTBC genotyping was carried out by Hain lifescience Line probes, while microscopy was done on Giemsa stained blood slides to examine for malaria parasites.Among the strains analyzed, the results showed that 20 (5.2%) were resistant to Isoniazid, 4 (1.4%) to rifampicin, and 2 (0.5%) were multidrug resistant (at least resistant to Isoniazid and Rifampicin). The population diversity of circulating MTBC strains was high with four different species: *M. tuberculosis* (91.4%), *M. africanum* (6.5%), *M. bovis* BCG(1.8%) and *M. bovis* (0.3%). Th1 (IL-12p40), Th2 (IL-4 and IL-5), pro-inflammatory (IL-6 and IL-8) and anti-inflammatory (TGF-β and IL-10) cytokines were measured by ELISA in 72 hour old peripheral blood mononuclear cell (PBMC) culture supernatants from 320 co-infected and 64TB patients while Th1 (IFN-γ) was measured by ELISpot. In response to PPD antigen stimulation, significantly increased levels of IL-6, IL-8, IL-10 and TGF-β and decreased IFN-γ and IL-12p40 were seen in malaria and TB co-infected patients compared to those with TB alone. In conclusion, strain classification revealed that the majority of MTBC strains circulating in this region belonged to *M. tuberculosis*. Resistance rates amongst these patients were at an alarming level. The population of MTBC strains circulating amongst the study patients shows an intriguing diversity raising the question of possible consequences for TB epidemic and for the introduction of new diagnostic tests or treatment strategies in malaria endemic regions. The study showed that malarial infection differentially modulates the various cytokine immune responses to *M. tuberculosis* antigens, which may influence the cellular and humoral immune responses to *M. tuberculosis* infection in a susceptible host. The study recommended a search for better diagnostic tools with respect to *M. africanum* and *M. bovis*. It also recommended for a better screening tools with respect to mycobaterial infection in malaria endemic region since TST is a delayed hypersensitivity reaction which is T-cell dependent and it was shown that malarial infections caused a depression in T-cell function which might depress the host's response to tuberculin antigen leading to a false-negative result.

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LIST OF ABBREVIATIONS

AFB	Acid-Fast bacilli
AMPATH	Academic Model Providing Access to Healthcare
APCs	Antigen Presenting Cells
AIDS	Acquired Immunodefiency Syndrome
BCG	Bacille Calmette-Guerin
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
DCs	Dendritic Cells
DNA	Deoxynucleic Acid
DST	Drug Susceptibility Testing
EDTA	Ethylene Diamine Tetramethlene Acetic acid
EMB	Ethambutol
ELISA	Enzyme Linked Immunosorbent Assay
EPI	Expanded Programme of Immunization
FACS	Fluorescence Associated Cell Sorter
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
IGRAs	Interferon Gamma Release Assays
inhA	encyl acyl reductase gene
iNOs	inducible Nitric Oxide synthatase
IUATLD	International Union of Against Tuberculosis and Lung Diseases

IFN	Inteferon
INH	Isoniazid
IL	Interleukin
katG	Catalase peroxidase gene
LAM	Lipoarabinomannan
LM	Lipomannan
MDR-TB	Multi-Drug Resistant-TB
MGIT	Mycobacterium Growth Inhibition Tube
MHC	Major Histocompatibility Complex
MTBC	*Mycobacterium tuberculosis* Complex
MTRH	Moi Teaching and Referral Hospital
MUSOM	Moi University School of Medicine
NALC-NaOH	N-acetyl-L-Cysteine – Sodium hydroxide
NK	Natural Killer cells
NO	Nitric Oxide
NTM	Non-tuberculous mycobacterium
LJ	Lowenstein-Jensen
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PfEMP-1	*Plasmodium falciparum* Erythrocyte Membrane atigen-1
PILAM	Phosphoinositol Lipoarabinomannan
PIM	Phosphadylinositol Mannosides

PPD	Purified Protein Derivative
PV	Parasitophorous Vacuole
RIF	Rifampicin
RNA	Ribonucleic Acid
RNs	Reactive Nitrogen species
rpm	Revolution per minute
rpoβ	RNA polymerase oligomer β subunit
SFU	Spot Forming Units
STR	Streptomycin
TB	Tuberculosis
TGF	Tumor growth factor
TREM	Triggering Receptor Expressed on Myeloid cells
Tregs	T regulatory cells
TST	Tuberculin skin test
WBCs	White blood cells
WHO	World Health Organization
XDR-TB	Extremely drug resistant-TB
ZN	Zeihl Neelsen

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CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Malaria and tuberculosis (TB) are infectious diseases worldwide and affect 365 million and 2 billion people respectively, per year (WHO, 2004b; Hotez *et al.*, 2006). Among TB high incidence regions, Sub-Saharan Africa is particularly affected with approximately 1.6 million new cases every year (Corbett *et al*., 2006; WHO, 2007) and 1 million people die from malaria every year (WHO, 2002) with a high likelihood of individuals being infected with both diseases contemporaneously. This might be related to poverty and suitable environment for these pathogens to thrive and spread.

Co-infections have much influence on the immune competence, vaccination response, treatment efficacy, correct diagnosis and pathologies of the involved diseases especially anaemia (Paolo, 2008). Furthermore, treatment and control of TB is increasingly complicated by the emergence of drug resistant or even multidrug resistant (MDR, resistance to at least isoniazid (INH) and rifampin (RIF) strains (WHO, 2004a). Levels of MDR-TB among new patients have reached 14% in some areas of the world making successful treatment very difficult (WHO, 2004a).

TB epidemic in Africa may be reinforced by the mutual interaction of TB and malaria as malaria may increases the risk of reactivation of latent TB as well as the rapid progression to active TB after infection. Besides this dramatic situation in several Sub Saharan countries, data on the diversity of MTBC strains causing the epidemic in this

area are only sparsely available. However, reports have indicated that the genetic heterogeneity of MTBC strains may be higher than is anticipated and may influence host pathogen interaction, immunogenicity, transmissibility, development of drug resistance, and the performance of diagnostic tests (de Jong *et al.,* 2006; Gagneux*et al.,* 2006a & b; Gagneux and Small, 2007).

The public health importance of TB and malaria is neglected worldwide. In malaria endemic region, immunosuppression due to chronic malaria may reactivate latent TB and as a consequent increase the risk of active TB and anti-TB drugs may become less effective. Malarial infection could be one of the risk factors for persistence of TB in the tropics where both pathogens are co-endemic. Page *et al.,* (2005) showed that TB-induced potentiation of type 1 immune responses is associated with protection against lethal murine malaria. Human populations are rarely exposed to one pathogen only. Malarial and TB co-infection may cause immune perturbation, increased pathogenicity and treatment failure in TB patients. The finding in this study can be a good explanation for the protective effect of TB in malaria coinfection and also gives the new view on the pathogenesis of malaria-TB co-infection.

During the rainy season, malaria burden increases (Dgedge *et al.,* 2001; Hongoro and McPake. 2003; Soumare *et al.,* 2008) in many African countries where TB incidence is also high, peaking morbidity and mortality. Several studies have shown that malarial infection affects severely ill TB patients who are already compromised by malnutrition, deprived immunity or disseminated disease (Anya, 2004; Russell, 2004; Romagosa *et al.,* 2007). Ellner (2010) noted that in pulmonary TB, there is a transient systemic

immunosuppression due to over expression of transforming growth factor beta (TGF-β) and interleukin-10 (IL-10). Scott *et al.*, (2004) demonstrated interactions between TB and malaria *in vitro* and *in vivo* while Hawkes *et al.,* (2010) and in their demonstrations showed that malariacan exacerbate TB infection.

Studies have insuniated that the reasons for this are not completely explored but seem to involve parasite-parasite interaction and host-parasite interaction (Scott *et al*., 2004; Hawkes *et al.,* 2010; Enwere *et al.,* 1999). Malaria causes a further depression in immunity through a qualitative and quantitative defect in T lymphocytes, mainly the CD8+ T cells that are necessary for anti-mycobacterial response, and through a deregulation of the cytokine cascade (Lisse *et al*., 1994). Moreover, Parry *et al* (2004) noted that the respiratory distress that is frequent during acute malaria both inchildren (due to metabolic acidosis) and adults (due to pulmonary edema and Acute Respiratory Distress Syndrome), can worsen the respiratory effort related toTB.

The effects of malaria parasite infection on immunity to TB in a coinfected individual have not been studied in detail and given that there is persistence and high upsurge of TB in the tropics where malaria is coendemic, the question is: could malarial infection be one of the reasons for the persistence of TB in malaria-endemic regions? In order to approach this challenging but scientifically and clinically highly relevant question on how malaria affects host immunity againstTBand cause the disease, the levels of proliferative T cells and cytokine profiles in TB infected, Malaria infected, and in TB and Malaria co-infected patients using their peripheral blood mononuclear cells (PBMCs) were determined.

In areas of seasonal malaria transmission such as Uasin Gishu County, children typically are exposed at a later age, and the disease manifests as cerebral malaria. Conversely, in regions of stable transmission, children are chronically exposed to the malaria parasite at a very young age, and this chronicity of infection leads to severe malarial anemia. Young children and especially newborns are at a high risk when exposed to a contagious TB source (Dye *et al*., 1999). A comprehensive review of the natural history of childhood TB showed that primary infection before 2 years of age frequently progressed to active disease within 12 months (Marais *et al.,* 2004). As such, pediatric TB is a sentinel event reflecting recent TB transmission from an infectious contact in the community. The number of children with TB in a community is an indirect parameter for assessing the effectiveness of a local TB control program (Morcillo, 2007).

Immune response in individuals with patent (or active) malaria infections of all species is a profound inability of $CD4^{+}$T cells to proliferate or produce cytokines associated with a Type-1 (IL-2 and IFN-γ) and Type 2 (IL-5) response following parasite antigen stimulation *in vitro*. This parasite-specific anergy is mediated primarily by IL-10 and, to a lesser extent, by TGF-β (Metenou *et al.,* 2013).

1.2 Statement of the problem

There is high upsurge of incidence of TB in the tropics and sub-Saharan Africa where malaria is endemic. In the same regions, the efficacy of BCG is low.

1.3 Justification of the study

Chronic malarial infection could affect the ability of the host to control TB infections and/or the efficacy of vaccination against TB. At the present time a number of vaccine candidates are entering clinical trials and indulging into such an expensive venture may prove futile without proper consideration of why the existing vaccine needs to be improved.

Immunomodulation by malaria could provide part of the explanation why the incidence of TB is the highest in the tropics and why BCG performs so badly in these regions.

This is a study with practical consequences and this could offer a novel means to reduce the burden of TB in areas where malaria is endemic.

1.4 Research questions

i) Could malarial infection affect the host's immune response against TB infections and hence being one of the reasons for the persistence of TB in malaria endemic regions?

ii) Which strain(s) of MTBC is most common among the TB isolates in malaria infected children and how is the pattern of their drug susceptibility profile?

1.5 Objectives

1.5.1 Broad objectives

To determine the effect of malarial infection on host's immune response against *M. tuberculosis* complex infections; andcharacterize MTBC strains isolated from malaria infected patients and determine their drug susceptibility profiles.

1.5.2 Specific Objectives

i) To determine co-infection and characterize strains of MTBC isolated from malaria infected patients.

ii) To determine the prevalence of anti-TB drug resistance in malaria and TB co-infected patients.

iii) To determine hematologic profiles and parasite levelsin persons with concurrent malaria parasite and TB infection

iv) To determine the effect of malarial infection on CD4+ T cells count among TB coinfected patients.

v) To determine the levels of Th1, Th2, Pro- and anti-inflamatorycytokines in patients with concurrent malaria and TB.

1.5.4 Study limitations

The study limitation was that there was reduction in positive blood films during the study period. A reduction in positive blood films was indeed observedfrom the period August-November 2011 to August-November 2012 and 2013.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malarial infection

Malaria is a vector-borne disease caused by the protozoan, apicomplexan parasite *Plasmodium* that is naturally transmitted by the bite of a female Anopheline mosquito. During the blood meal, infectious sporozoites are deposited under the host skin and subsequently reach the liver via the blood stream. Within hepatocytes they develop and rapidly multiply into liver-stage schizonts. Eventually, mature schizonts rupture and release thousands of pathogenic first-generation merozoites into the blood stream where they initiate a progressive cycle of red cell invasion, replication, red cell rupture, and reinvasion (Aly *et al.*, 2009).

The *Plasmodium* life cycle continues as some merozoites develop into the sexual parasite stages, the male and female gametocytes, which can be taken up by mosquitoes during blood meals. In the mosquito, *Plasmodium* sporozoites develop within oocysts residing in the midgut and eventually migrate to the salivary glands for onward transmission into another host (Aly *et al.*, 2009; Cirimotich *et al.*, 2010). The liver stage of *Plasmodium* parasite is an obligatory step during infection and relevant for anti-plasmodial immunity (Hoffman and Doolan, 2000; Mueller *et al.*, 2005; Mueller *et al.*, 2007).

2.1.1 The host's immune response to malarial infection

The initial response to malarial infection is usually non-specific including splenic removal of both parasitized and uninfected red cells (Haynes and Fauci, 1998). Haynes and Fauci (1998) also showed that when schizonts rupture, parasite products arer eleased into the circulation and these trigger activation of phagocytes, with the releaseof proinflammatory cytokines that cause feverand exert other pathological effects. Langhorne *et al.,* (1989) noted that host's specific response involves both humoral and cell-mediated immunemechanisms. Cohen (1986) noted that malarial infection was associated with greatlyenhanced rates of synthesis of immunoglobulins, especially IgG and IgM. The stimulant for this hypergammaglobulinaemia is thought to be aschizont-surface glycoprote in with a molecular weight of 200 kDa (Kataaha, *et al*., 1984). This was shown to have led to a polyclonal activation of the B cells, although this is a T-cell-dependent phenomenon (Brown and Rice-Ficht, 1994).

Much of the immunoglobulins produced have no demonstrable specificity for the malarialparasites and include heterophile andauto-antibodies (Cohen, 1986), of which some are antibodies against the host's B and T lymphocytes. However, specific antibodies are also produced and theseforms the basis of the serological reactions thatare useful in diagnosis (Boudin *et al*., 1993) and that although some of these antibodies are protective and both species-specfic and stage-specific, their levels show no correlation with clinical immunestatus (Aribot *et al*., 1996).

The strongest evidence for the efficacy of antibodies against malaria is provided by the recovery by recipients of sera from immune donors (Sabchareon *et al*., 1991), and the protection conferred, for a few months, by transplacental antibodies in neonates born to semi-immune mothers (Kumaratilake *et al*., 1994). These anti-malarial activities are associated with both IgM and IgG (White and Breman, 1998). Although *et al*., (1994) and Aribot *et al*., (1996) thought that IgG1 and IgG3 were more protective than other immunoglobulins, Boudin *et al*., (1993) and Chumptitazi *et al*., (1996) thought IgG1 and IgG3 were equally protective like other immunoglobulins. Anti-malarial antibodies act in part by blocking attachment of extracellular merozoites to the red blood cells, but do not inhibit the intracellular development of the parasites (Cohen, 1986).

Phagocytes usually kill the asexual, intraerythrocytic stages of malarial parasites by engulfment of the infected erythrocyte and release of mediators (cytokines and reactive oxygen species and nitrogen intermediates) that induce intracellular destruction of the parasite. Phagocytes are also capable of extracting parasites from the erythrocyte without lysing or ingestion of the red cell (Kumaratilake *et al*., 1994; White and Breman, 1998). Cell-mediated immune mechanisms have aprotective role against the extra and intraerythrocytic parasites (Vreden, 1994; White *et al*., 1996) which arealso the case with TB. However, B-cell-deprived animals can acquire resistance to all the stages in the mammalian host (Cohen, 1986).

Red cells do not express Major Histocompatibility (MHC) antigens on their surface (Miller *et al*., 1994) but these antigens areexpressed by infected parenchymatous cells

and recognition of them causes destruction ofthe infected cells by cytotoxic T cells (White and Breman, 1998). During malaria, parasite products also stimulate T-cell responses, leading to the productionof specific T-cell cytokines (Mendis and Carter, 1995). The reactions of the T-helper cells are of both types 1 and 2 (Th1 and Th2).

Experiments in animal models have shown that the Th1 responses, which occur early in the infection, involve the production of IL-2, IFN-γ and TNF-β, and this aids in parasite clearance (Mendis and Carter, 1995). The Th2 response occurs later in the infection, leading to the production of IL-4, IL-5, IL-6 and IL-10 (Langhorne *et al.*, 1989). Th2 inhibits the production of cytokines by the Th1 cells, and also induces strong differentiation of B cells (Brown & Rice-Ficht, 1994). Another subset of T cells, TcRγδ, though not investigated, has been shown to increase in both relative and absolute numbers during malarial infection (Roussilhon *et al*., 1994). In a group of patients experiencing malaria for the first time, this increase was shown to last for upto 3 to 4 months post-infection.

Some of the immune responses to malarial infections may be deleterious to the host. For instance, such infections lead to both a quantitative and a qualitative decrease in T lymphocytes (Lisse *et al*., 1994). In acute malaria, for example, the number of T lymphocytes, the proportion of T lymphocytes that are T helper cells (CD4+T cells), and the ratio of T helper to T-suppressor cells (CD8+T cells) are all reduced (Ho *et al*., 1986). Lisse *et al*., (1994) suggested that the decrease in the number of T cells may be due to`homing' or sequestration of these cells in the spleen.

The malaria-attributable reduction in peripheral lymphocytes, especially of T helper type 1, runs in parallel with the loss of *in-vitro* control of the T cells, and may be to levels encountered in AIDS (Whittle *et al*., 1984). Subclinical malariamay have effects on the important T-cell-controlling mechanism (Lisse *et al*., 1994). This has significant implications in regions where malaria is hyper-endemic and those with asymptomatic parasitaemia form a significant proportion of the population and may have significant impact in host immunity to TB.

Another important aspect of the immune response in malaria is the predominant T helper type 2 responses later in the infection (Mendis and Carter, 1995).This response is inhibitory to the production of IFN-γ and IL-2, both of which are not only important in the control of malaria (Heinzel, 1994) but are also involved in the control of other infections such as those with*Mycobacterium* (Smith *et al*., 1997; Haynes and Fauci, 1998). The switch to T helper type 2 responses may be parasite-protective, permitting the equilibrium state called asymptomaticparasitemia, while depressing the host's immune system (Erard and le Gros, 1994).

The other piece of evidence which indicates that some immune responses to malaria may be harmful to the host is that the malaria-attributable proliferation of TcRγδ cells continues for several months after the clearance of malarial antigens from the peripheral blood (Roussilhon *et al*., 1994). As these cells maintain immune surveillance and cellular defenses against *Mycobacterium* and other intracellular organisms (Haynes and Fauci, 1998), repeated stimulation of this system might lead to either exhaustion or tolerance to

further antigenic stimulation, with consequent loss of function. Also, the results of *in-vitro* studies indicate that some clones of TcRγδ cells exhibit cytotoxic activity against autologous TcRαβ (CD4and CD8) lymphocytes activated with the same parasite extract (Roussilhon *et al*., 1994). Since TcRγδ responses continue for months after the clearance of malarial parasites and antigens (Roussilhon *et al*., 1994), it is likely that a period or window of relative susceptibility to other infections is created as a result of decreased levels of both CD4+ and CD8+ T cells.

Taken together, the above findings present two possible scenarios. The first is that malaria causes a non coordinated T-cell response, some aspects of which are harmful to the host. This might be related to antigenic variations that are known to occur during the infection (Cohen, 1986), with different antigens possibly triggering off their own specific host responses. The family of functionally similar proteins, known as *Plasmodium falciparum*erythrocyte membrane antigen 1(PfEMP 1) is important in this respect. PfEMP 1 antigen is expressed on the surface of infected red blood cells and is thought to play an important role in acquired immunity to malaria, as a target antigen in antibody mediated agglutination of infected erythrocyte (Wahlgren *et al*., 1994). The second scenario is that, by switching the T cell response to a predominantly T helper type 2, malarial parasites may be manipulating the host's immune system to ensure the host's continued survival, thereby exposing the host to further infections, including infection by intracellular organisms such as *M. tuberculosis*. The true situation is likely to be a combination of these two scenarios (Wahlgren *et al*., 1994).

Immunosuppression in malaria also has humoral aspects. Some of the antibodies produced in malarial infection areanti-lymphocyte which causes both qualitative and quantitative decreases in both B and T-lymphocytic responses to infection. Many of these antibodies are in the IgG subgroup which may cross the placenta when malaria occurs in pregnancy (White & Breman, 1998). Some of these antibodies may thereforeproduce the idiotypic phenomenon (Revillard, 1998), thereby influencing the foetal immune system.

In addition, malaria-specific immune complexes are known to depress the phagocytic activity of white cells, impairing their ability to process and present antigens to immunocompetentcells (Brown and Kreier, 1982). This scenario might explain the finding by Greenwood *et al.*, (1980) that both clinical malaria and asymptomatic parasitaemia diminish response to polysaccharide vaccines and if the foregoing is true, the question is does malaria have any special relevance in an environment with a high prevalence of TB? This is what this study is unvestigating.

2.2 Tuberculosis

MTBC is the main causative agent of human TB and kills nearly 2 million people every year with the highest prevalence in the developing world. An estimated 2 billion people worldwide are latently infected with 9 to 10 million new cases every year (WHO, 2009; Parida and Kaufmann, 2010).

The only vaccine available, *Bacille-Calmette-Guérin* (BCG), does not protect adults against pulmonary TB and only a few discoveries in the field of anti-TB drugs have taken

place in the last 40 years. The most efficient way to control TB would be the use of effective vaccines. The current BCGvaccine, an attenuated strain of *M. bovis*, was developed in the 1920's by two French scientists (Albert Calmette and Camille Guerin). This vaccine provides good protection against childhood TB (Trunz *et al.*, 2006) but fails to show consistent efficacy against adult TB (Ponnighaus *et al.*, 1992) and its place in malarial infection is not known. This coupled with the rising TB epidemic worldwide has compelled WHO to declare TB a global emergency (WHO, 1993). This has prompted global effort to control the disease and to develop a vaccine more efficacious than the one currently in use.

Approaches to develop such a vaccine have included the use of live attenuated bacteria, recombinant bacteria, subunit vaccines and DNA vaccines. Out of these viable recombinant bacteria and subunit vaccines are well developed and have entered clinical trials (Kaufmann, 2002). Control of TB in Sub-Saharan Africa poses a big challenge if the knowledge on the effect of coinfection especially with malaria is lacking.

2.2.1 *Mycobacterium tuberculosis* complex in children

The incidence and prevalence of pediatric TB varies significantly across the globe, driven largely by the burden of the disease in different countries. About 1 million children under 15 years of age develop TB worldwide annually, representing 11% of all TB cases (Corbett *et al.* 2003). The majority of these cases occur in low-income countries (Morcillo, 2007) where the prevalence of malaria is high.

Kenya currently ranks 13th on the list of TB high burdened nations globally (WHO, 2007), and pediatric TB accounts for a substantial proportion of these cases. Almost 2

million people per year die as a result of TB, mostly in developing countries like Kenya, but the mortality in children is often underreported. Despite this, TB is one of the ten leading causes of childhood mortality (Rheka, 2007).

Young children and especially newborns are at a high risk when exposed to a contagious source of TB (Dye *et al.*, 1999). A comprehensive review of the natural history of childhood TB showed that primary infection before 2 years of age frequently progressed to active disease within 12 months (Marais *et al.* 2004). As such, pediatric TB is a sentinel event reflecting recent TB transmission from an infectious contact in the community. The number of children with TB in a community is an indirect parameter for assessing the effectiveness of the local TB control program (Morcillo, 2007).

Given the overwhelming burden of TB among the population as a whole and the well-documented vulnerability of young children to active TB including the severe and fatal forms of the disease (Marais *et al.*, 2004), it is perhaps surprising that TB does not feature among the leading causes of death in children. Protection afforded by BCG vaccination among infants and young children, particularly against disseminated TB and TB meningitis, provides one important explanation for this apparent discrepancy (Trunz *et al*., 2006). However, even in this group the protective efficacy of BCG is suboptimal, and the best available estimates suggest a high global burden of childhood TB despite wide-scale neonatal BCG vaccination within the Expanded Programme of Immunization (EPI) (Nelson and Wells, 2004). To reconcile these data a clear understanding of their limitations and the underlying challenges of TB diagnosis in children is required.

The pathophysiology, clinical presentation, and investigation of TB in children differs from that in adults due to a combination of immunological, anatomical, and epidemiological factors, all of which make diagnosis considerably more challenging (Newton *et al.,* 2008). Young children are at a much higher risk of progression to activeTB following infection (Marais *et al.,* 2004). Larger inocula due to prolonged close contact with adults in the household from whom they frequently acquire their infection might contribute to this higher risk (Guwatudde *et al.,* 2003; Schaaf *et al.,* 2003), but age-related differences in both innate and adaptive immune responses to TB are thought to be the most important determinants of their increased vulnerability (Jones *et al.,* 2011).

Although poorly understood, a less robust immune response is also likely to underlie the paucity of lung cavitations in young children with TB, in stark contrast with the classic picture of adult pulmonary TB in which large numbers of bacilli may "spill" from the cavities into the airways. Whatever the exact mechanisms, important consequences of these differences in host immunity include relatively fewer bacilli, particularly in clinical specimens ("paucibacillary" disease), and an increased propensity for disseminated, extra-pulmonary disease in young children (Newton *et al.,* 2008).This in turn has implications for diagnosis since specimen collection is often more difficult from extra-pulmonary sites, especially in low-resource settings, and low numbers of bacilli greatly reduce the sensitivity of both smear microscopy and mycobacterial culture.

In addition, anatomical differences limit access to appropriate respiratory specimens in childhood pulmonary TB since smaller airways and less tussive force limit a child's ability to expectorate sputum, necessitating more invasive and labour intensive methods of specimen collection such as sputum induction or gastric aspiration (Zar *et al.*, 2005). Immune-based diagnosis using Tuberculin Skin Testing (TST) or newer interferon gamma release assays (IGRAs) is limited by poor sensitivity and specificity (Ling *et al.*, 2011). Furthermore, although they are recommended components of the TB diagnostic workup for children where available, in practice TST, gastric lavage and sputum induction are often not available in high-burden settings due to limited training, a lack of the basic equipment and consumables required (English *et al.*, 2004a & b), very low yield in the absence of mycobacterial culture facilities (Zar *et al.*, 2005), and cost (WHO, 2006; IUATLD, 2010). IGRAs remain far too expensive and complex for use at the point of care in low-resource settings.

The MTBC strain is composed of 5 biochemically and immunologically related species: *M. tuberculosis, M. bovis, M. africanum, M. microti,* and *M. canetti.* Among these, *M. tuberculosis* species is the most important cause of TB in man. *M. tuberculosis* is an acid-fast bacillus 2–4 μm long. The organism is a weakly gram-positive, non-spore-forming, non-motile obligate aerobe that may appear as beaded or clumped aggregates in clinical specimens or culture media. Distinguishing microbiological properties of mycobacteria include acid fastness: mycolic acids in the cell wall form stable complexes with dyes such as crystal violet, carbolfuchsin, auramine, and rhodamine and resist decoloration with ethanol and hydrochloric or other acids. The lipid-rich cell wall also confers resistance to

the cidal effects of antibody and complement and this is the basis of host's immune response.

The similarity between members of the MTBC strongly suggests a common ancestor that over time has been transferred and adapted to different hosts. It was earlier thought that *M. tuberculosis* arose from *M. bovis*, which causes TB in cattle, around the time when man first domesticated cattle roughly 10,000 years ago. However, comparative genome analyses have shown that *M. tuberculosis* is unlikely to have been derived from its bovine counterpart, and the more likely scenario is that TB was actually transferred from man to animal (Smith *et al.,* 2009). *M. tuberculosis* can be further subdivided into several strain lineages that are genetically distinct from each other. In fact, the average genetic distance between two human-adapted strains is equal to the average genetic distance between the animal-adapted strains, even though the latter have adapted to different host species. This diversity within *M. tuberculosis* can be tightly linked to migration events and demographic changes in human history, resulting in the fact that certain strain lineages of *M. tuberculosis* are over represented in specific parts of the world (Hershberg *et al.,* 2008) and phylogeographic distribution of different strains suggest that they are particularly adapted to infect their sympatric human hosts (Fenner *et al.,* 2013).

So far, seven major strain lineages of *M. tuberculosis* have been identified (Firdessa *et al.,* 2013) and they can be roughly divided into ancient lineages and modern lineages, the former of which are genetically closer to a common ancestor than the latter ones. The modern lineages induce weaker inflammatory responses early in the disease, which might

lead to faster progression and transmission, beneficial in modern times, where human populations are denser. The ancient lineages probably benefitted from staying latent until being able to infect a new generation of hosts within small human populations (Portevin *et al.,* 2011) or re-activated by immunosuppressive infections like in the case of malaria.

Finally, a striking difference between *M. tuberculosis* and many other pathogens is that the most evolutionary conserved parts of the genome are in fact epitopes recognized by human T cells, implying that *M. tuberculosis* actually benefits from recognition by the human immune system (Comas *et al.*, 2010). Taken together, the adaption of strains to particular groups of humans and to changes in the density of human populations indicates a close evolutionary relationship between host and pathogen.

M. tuberculosis pathogen is slow growing with a doubling time of up to 48 hours. It has been classified as an acid-fast bacterium, due to its ability to be stained by only certain dyes, and is coated with a thick cell wall. The genome of *M. tuberculosis* has a size of 4.41 Mb and harbours about 4000 protein-coding genes (Cole *et al.,* 1998).

The host's immune status is determined by their innate susceptibility to the disease and their ability to mount an effective, cell-mediated, immune response. Some conditions, such as drug abuse, malignancies, diabetes mellitus, chronic renal failure and infection with a HIV, favour the development ofTB by suppressing cell-mediated responses to the infection (Flynn, 2004).In Africa, HIV infection is by far the most important risk factor for the development of TB (Dheda *et al.,* 2010; Lin and Flynn, 2010). The immunosuppression caused by malarial infection has not been specifically related to TB.

However, malarial infection in an area with a high rate of transmission of TB may affect the control of TB by causing decreased vaccine take, decreased diagnostic accuracy, and a greater risk of developing TB if infected with *M. tuberculosis*.

Malaria could also prime the immune system of infants to respond less to TB infection.The vast majority of *M. tuberculosis* infected individuals will not develop active TB but remain latently infected for the duration of their lives (Figure 2.1). Reactivation only occurs in 5 to 10% of infected persons and can be triggered by immunosuppression due to age, corticosteroids, malnutrition or other factors (Flynn, 2004). The role played by malarial infection in the host immune response against TB is not known, which is the main idea behind this study. A striking feature of TB is its ability to survive under hypoxic and nutrient-poor conditions by shifting to a dormant life stage. This is characterized by alternative energy catabolism and a thickening of the cell wall (Graham and Clark-Curtiss, 1999).

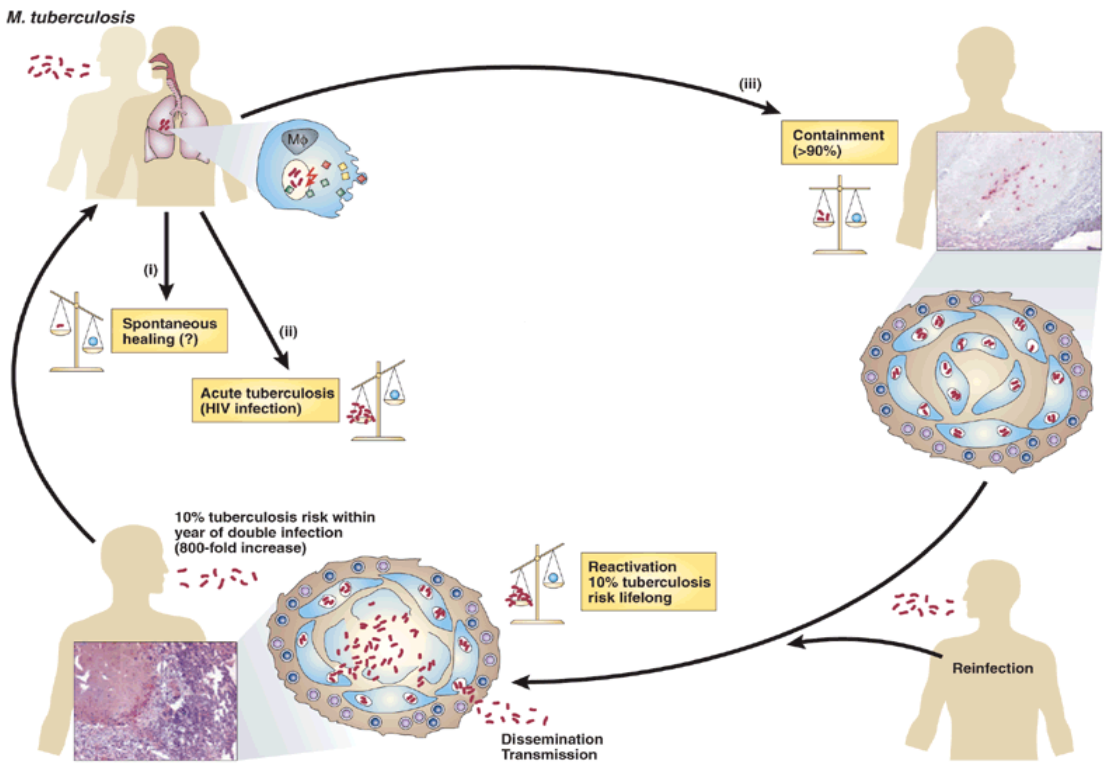


Figure 2.1: Immunosuppression of host's immune response by co-infection leads reactivation of latent *M. tuberculosis*. Modified after

(Source: Kaufmann & McMichael, 2005)

2.3 The host's immune response to TB infection

The host's first response to invading TB involves phagocytosis (Figure 2.2a) of the microorganisms by macrophages (Smith *et al.*, 1997; Hayne and Fauci, 1998). This initial interaction can result in destruction of the organisms or their persistence and replication within the macrophages (Smith *et al.*, 1997). The destruction of the organisms involves the liberation of reactive oxygen species and nitrogen intermediates, acidic lysozomal components and hydrolytic enzymes within the phagolysomes (Chan and Kaufmann,

1994). However, mycobacteria have developed methods of circumventing their destruction by these chemicals (Myrvik *et al*., 1984) and may consequently survive, replicate and disseminate within phagosome (Figure 2.2b).

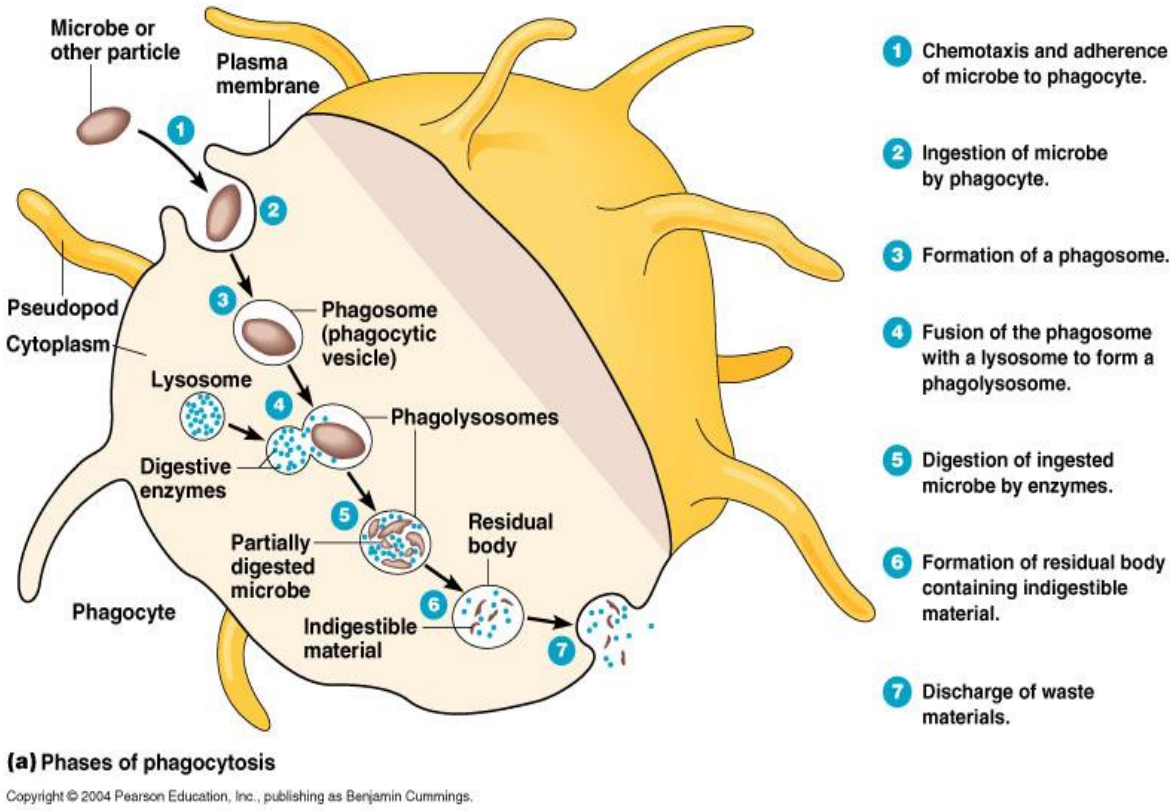


Figure 2.2a: Phagocytosis of *M. tuberculosis* by macrophages

(Source: Pearson Education, Inc., 2004).

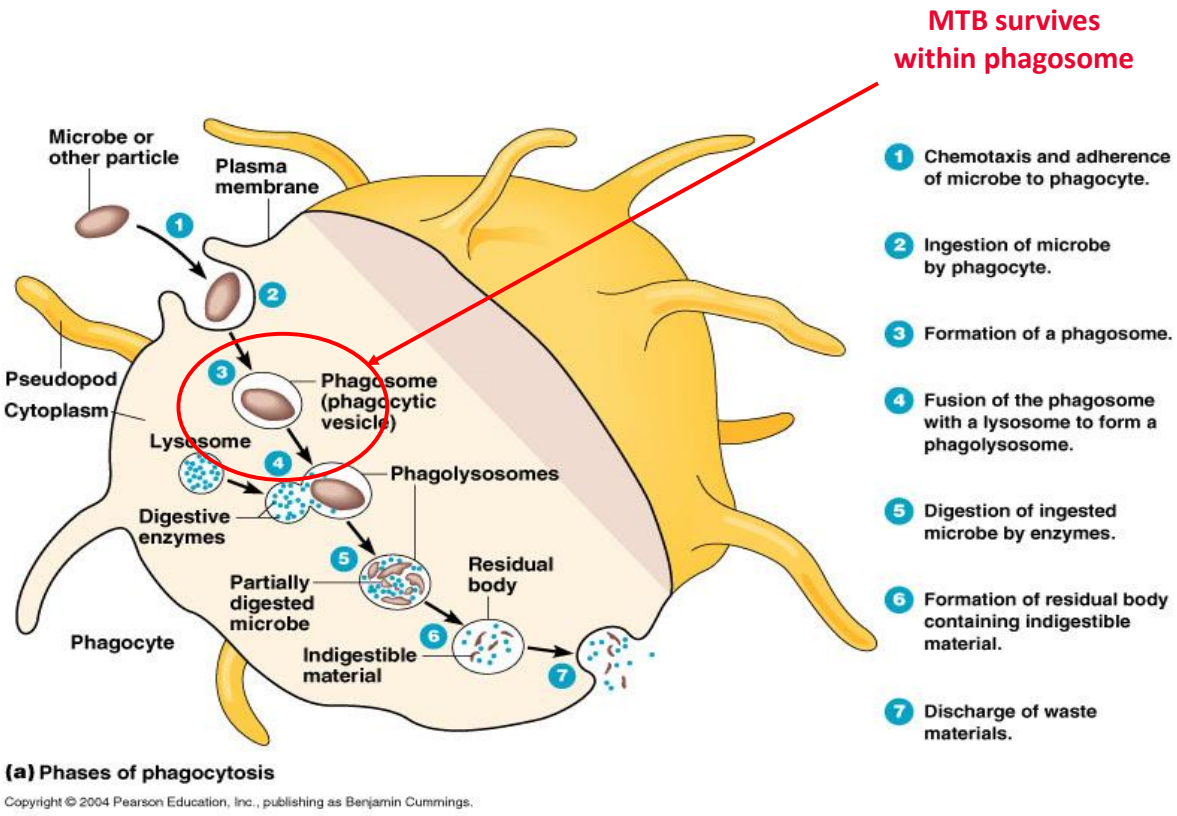


Figure 2.2b: *M. tuberculosis* survival within phagosome

(Source: Pearson Education, Inc., 2004).

2.4 Cytokines

Host defense against all invading pathogens, as well as the subsequent resolution of inflammation, rely on the induction and the effects of a plethora of cytokines. Many of them are produced by the human macrophage and act both as a link to the adaptive immunity and to enhance macrophage effector functions. Many cytokines play a role when mounting an appropriate immune response against *M. tuberculosis.*

Upon antigenic stimulation, CD4+ Th cells differentiate into Th1 cells, which secrete cytokines that are involved in cell-mediated immune response (IFN-γ and IL-12) or Th2 cells that secrete mediators of humoral immunity (IL-4 and IL-5) (Schluger *et al.,* 1998). Other pro-inflammatory (IL-6 and IL-8) and anti-inflammatory cytokines (TGF-β and IL-10) also regulate immune response to TB. IFN-γ, the signature Th1 cytokine, activates infected macrophages to eliminate intracellular pathogens. IL-12, which comprises of p35 and p40 subunits, directs the development of Th1 cells, while IL-4, the principal Th2 cytokine, suppresses the Th1 response (Lammas *et al.,* 2000). IL-10 secreted by alternatively activated macrophages and T cells is known to downregulate IL-12 production (de Waal *et al.,* 1991). IL-8 (CXCL8) is a chemokine secreted by macrophages and T cells that attracts neutrophils and T cells (Zhang *et al*. 1995). TGF-β is implicated in suppression of T cell and antibacterial immune responses in TB (Toossi *et al.,* 1996). IL-6 secreted by T cells and macrophages regulates various cell types (Mangan *et al.,* 2006).

After *M. tuberculosis* is recognized and taken up by the alveolar macrophage, the bacterium interferes with key antimicrobial mechanisms and can start replicating intracellularly. If the infected cell is unable to handle the infection it will produce a range of cytokines and chemokines that will attract additional monocytes, neutrophils and dendritic cells (DCs). Traditionally, this stage of infection is thought to promote bacterial growth, and it is not until adaptive immunity is initiated that bacterial numbers stabilize due to the strong macrophage activation of IFN-γ produced by CD4+ cells arriving at the scene of infection (Russell *et al.,* 2010).

The CD4+ T lymphocytes recognize antigens in association with MHC antigen class II (Revillard, 1998), resulting in a Th1-typeresponse and production of IL-2 and IFN-γ. These in turn induce an exponential increase in the production of TNF, 1 α, 25-dihydroxycholecalciferol and NO by macrophages (Denis, 1991; Howie *et al*., 1994). The overall effect of these immunological responses is inhibition of growth or killing of the mycobacteria. Cytotoxic CD8+ T cells are also stimulated and these are cytolytic to cells infected with mycobacteria (Smith *et al*., 1997). However, several IFN-γ-independent macrophage stimulation pathways and mechanisms have also been shown to induce intracellular killing in human macrophages, suggesting that the battle between host and pathogen can be decided before involvement of adaptive immunity (Gutierrez *et al.,* 2004; Tan *et al.* 2006 a & b).

After arriving at the scene, immune cells of both the innate and adaptive immunity form the classical granuloma formation; a macroscopic structure with heavily infected macrophages surrounded by multinucleate (giant cells) and lipid-rich (foamy) macrophages located in the center. The macrophage-containing center can be further encapsulated by a layer of fibrous tissue with both B and T lymphocytes in the peripheral area. Other immune cells like neutrophils and DCs are also present in the granuloma.

Granulomas are dynamic and heterogeneous structures with immune cells constantly trafficking in and out. Individual granulomas at any given time within a single patient might differ in several aspects including bacterial numbers, cellular composition, oxygen and nutrient levels and fibrosis. However, if bacterial growth is controlled, individuals

can have granulomas present subclinically for decades (latent infection) but upon disease progression increased central necrosis is observed. Subsequent granuloma cavitation leads to spillage of bacteria into the airways of the infected individual, enabling transmission to a new host. The strong proinflammatory response and subsequent granuloma formation has long been considered to be beneficial for the host; a view that has been questioned during the last few years (Flynn and Chan, 2005; Russell *et al.*, 2010).

Interferon-gamma is crucial for the T cell response in mycobacterial disease. In the mouse model this has been demonstrated by the dramatically altered course of infection in IFN-γ knock-out mice while human individuals with defects in the IFN-γor the IFN-γreceptor gene are more susceptible to severe TB (Cooper *et al*., 1993; Ottenhoff *et al.*, 1998). IFN-γ is expressed by both CD4+ and CD8+ T cells in TB, but can also be secreted by infected macrophages in an IL-12-dependent manner (Fenton *et al.*, 1997; Wang *et al.*, 1999). Although IFN-γproduction alone is insufficient to control *M. tuberculosis* infection, it is required for protection against this pathogen. However, IFN-γ is produced by healthy PPD+ individuals as well as those suffering from active TB, suggesting that this cytokine may be an unreliable immune correlate of protection (Flynn and Chan, 2001).

The cytokine IL-2 is also very important since it is required for secondary expansion of memory T cells (Williams *et al.*, 2006). A deficiency in the IL-2 induced T cell proliferation has been observed in patients with active TB, yet the treatment of adults

suffering from the infection with recombinant IL-2 did not lead to an improvement. This underlines the complexity of this cytokine network (Toossi *et al.,* 1986; Schauf *et al.,* 1993; Johnson *et al.,* 2003; Tufariello *et al.,* 2003).

Interleukin-6 is a pleiotropic cytokine affecting a number of tissues and is produced by macrophages infected with *M. tuberculosis*. Mice lacking IL-6 have a higher bacterial burden in their lungs and impaired IFN-γproduction early in the infection, suggesting a beneficial role for IL-6 in TB (Saunders *et al.,* 2000). As opposed to this, IL-6 can also inhibit IFN-γ stimulated gene transcription in macrophages, indicating that *M. tuberculosis* might benefit from the production of IL-6 (Nagabhushanam *et al.,* 2003). Though the importance of IL-6 in TB is still not completely understood, it is not crucial for protection against TB and supposedly plays a more regulatory role.

In contrast to IFN-γ, IL-10 is considered as an anti-inflammatory cytokine produced by T cells during *M. tuberculosis* infection. It leads to downregulation of IL-12, produced by the macrophages, which subsequently decreases the IFN-γ expression by T cells. It directly limits Th1 immunity and exacerbates disease (Ordway *et al.,* 2007).

The role of the Th2 cytokine IL-4 in immune defense remains controversial. Since *M. tuberculosis* is a strong inducer of IL-12, the Th1 response is favoured in infected hosts. Nevertheless, some reports document the existence of Th2 responses in TB, but in PBMC from TB patients, no such phenomenon can be observed. While IFN-γ expression is elevated in human granulomas, only little IL-4 RNA could be detected in lymph nodes (Zhang *et al.,* 1995; Lin *et al.,* 1996; Bhattacharyya *et al.,* 1999).

The production of TNF-α and inflammatory chemokines from the infected macrophages drives the recruitment of neutrophils, NK cells, CD4+ and CD8+ T cells, each of which produce their own set of chemokines and cytokines that lead to the remodelling of the infection site into a structure called granuloma (Algood *et al*., 2003; Ulrichs and Kaufmann, 2006; Russell, 2007). The formation of a stable granuloma is responsible for the immune containment during the latent, or subclinical, period of the infection. The latent infection of mycobacteria can be reactivated in an immunosuppressed host.

In experimental TB, a strong Th1-dominated immune response with IFN-γ-producing CD4+ T cells is necessary for protection. In murine macrophages, IFN-γ has many effects, including upregulation of inducible nitric oxide synthatase (iNOS), which in turn generates nitric oxide (NO) and reactive nitrogen species (RNS) capable of killing *M. tuberculosis* (Flynn and Chan, 2001). The necessity of IFN-γ has also been clearly demonstrated *in vivo,* where mice lacking IFN-γ are extremely sensitive to TB, displaying both increased tissue destruction and bacterial load (Cooper *et al.,* 1993). To counteract this important cytokine, *M. tuberculosis,* through its 19 kDa lipoprotein and certain cell wall components, has the ability to inhibit the effects of IFN-γ on macrophages such asthe induction of specific IFN-γresponsive genes (Fortune *et al.,* 2004). The importance of IFN-γ in humans was early shown by the finding that individuals, who have deficiencies in IFN-γ signaling, have increased susceptibility to mycobacterial infections (Newport *et al*., 1996). However, the ability to induce mycobacterial killing in human macrophages through IFN-γ *in vitro* has been more of a theoretical idea than a well established phenomenon. Recently this has been clarified by studies showing that human

macrophages stimulated with IFN-γ also require the presence of active vitamin D, which leads to increased expression of antimicrobial peptides, increased phagolysosomal fusion and enhanced killing of *M. tuberculosis* (Fabri *et al.*, 2011).

Due to its crucial role in TB, IFN-γ has commonly been used as a marker for effective immunity against TB, but the correlation between the levels of IFN-γ and protection is poor (Goldsack and Kirman, 2007). While CD4+T cells are important source of IFN-γ, other T cell populations were also shown to produce IFN-γ. Such cells include CD8+T cells as well as γδ T cells that are restricted by unconventional presentation molecules (CD1) (Figure 2.3).

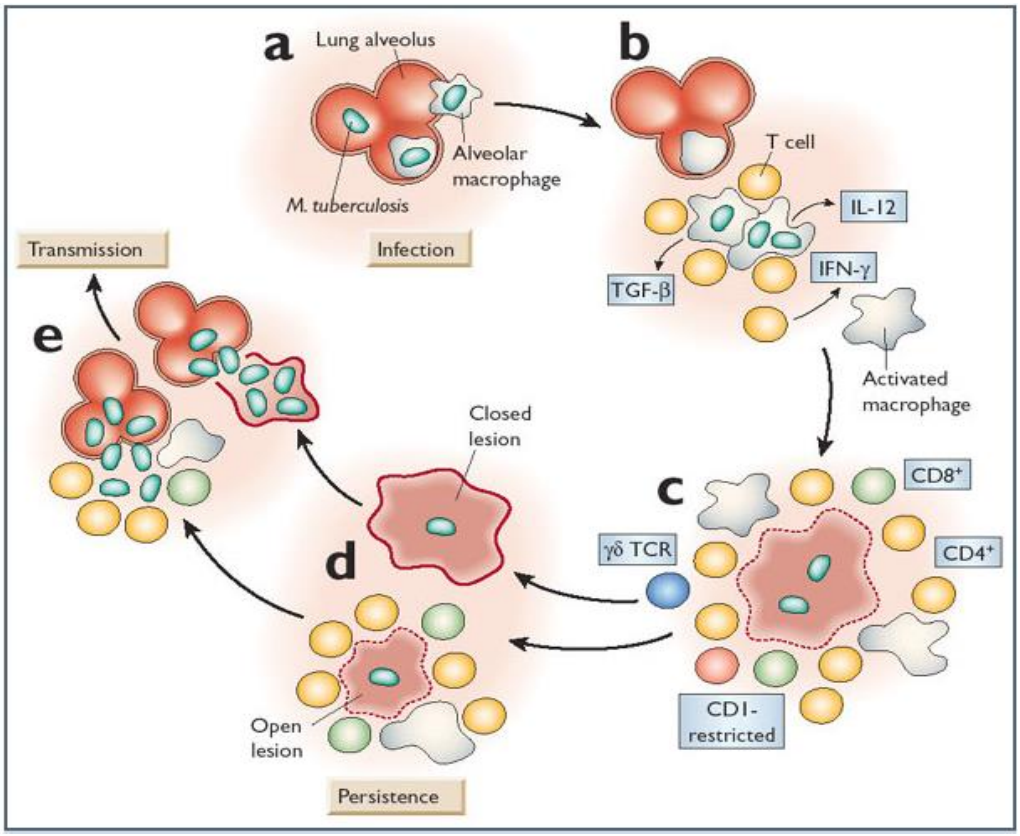


Figure 2.3: Host's immunological response to *M. tuberculosis* infection (Source: www.nature.com)

Cytotoxic (CD8+ T) cells recognize antigenic peptides in the context of MHC class I and are thought to contribute to immune responses against TB via cytotoxic activity (Stenger and Modlin, 1999) and IFN-γ production (Serbina and Flynn, 2001; Shams *et al*., 2001). Gamma delta T (γδ T) cells recognize non-peptide antigens in the context of CD1 molecules and were shown to be involved in *M. tuberculosis* induced immune responses (Hoft *et al.,* 1998). The exact role of γδ T cells in TB immunology is not clearly defined but is thought to contribute by cytokine production in the early phases of infection which is thought to provide help to the antigen presenting cells (APCs) and were also shown to be involved in cytotoxic functions (Dieli *et al*., 2004). Although IFN-γ is crucial for protection against TB, protection is not directly associated with the magnitude of IFN-γ responses (Elias *et al*., 2005; Rook *et al.,* 2005b).

The regulatory T cells are normally associated with down-regulation of the immune response and were initially thought to be solely involved in inhibiting pathogenic and auto reactive T cells (Wood *et al*., 2003; Hori and Sakaguchi, 2004) however their involvement during infections is becoming more and more clear (Belkaidand Rouse, 2005) and evidence is accumulating that the outcome of primary infection with *M. tuberculosis* is influenced by the balance between these two subsets of CD4 T cells (Hori and Sakaguchi, 2004).

2.4.1 CD4+ T cell subsets involved in the immune response to *M. tuberculosis*

Since *M. tuberculosis* resides primarily in vacuoles within macrophages, the presentation of microbial antigens via MHC class II to CD4+ T cells is the main route of immune recognition. Studies in mice without CD4+ T cells have shown the importance of this

subset for the control of infection (Muller *et al.,* 1987; Caruso *et al.,* 1999). In humans the rising number of HIV patients co-infected with *M. tuberculosis* also provides evidence for the importance of CD4+ T cells. HIV patients with latent TB have 8-10% annual risk of developing active TB compared to a 10% lifetime risk in subjects without HIV co-infection (Selwyn *et al.,* 1989). The primary function of CD4+ T cells in the immune defense is believed to be the production of IFN-γ and other cytokines for recruitment and activation of macrophages, which can lead to the control of infection. CD4+ T cells also provide help to CD8+ T cells through the secretion of IL-2.

CD4+ T cells activated by *M. tuberculosis* antigens can also differentiate in cytotoxic T lymphocytes (CTLs) and help the infected macrophages control the intracellular bacterial load (Boom *et al*., 1991; Kaufmann, 1999). Along the lines of cytotoxic effector functions, CD4+ CTLs express granzymes, Fas-L, granulysin and perforin (Canaday *et al*., 2001). Granulysin has been found to express potent bactericidal activity against *M. tuberculosis* (Stenger *et al.,* 1998). Even single peptides derived from granulysin demonstrate bactericidal activity against *M. tuberculosis* (Siano, 2010). It has been noted that a subset of functional T cells produces IL-17 (Th17) and these cells can be observed in the mouse model as well as humans exposed to TB (Khader and Cooper, 2008). After blocking of IL-17 during a high-dose challenge in mice, neutrophils recruitment was hindered and led to an alteration of the early inflammation response (Umemura *et al.,* 2007). Whether these cells are protective or damaging is yet to be investigated.

Regulatory T cells (Tregs) expand in the lung during *M. tuberculosis* infection and their deletion leads to improvement in the immune response (Scott-Browne, 2007). The depletion of Treg also leads to an increased number of IFN-γ producing T cells *in vitro* suggesting a suppressive role for Treg of Th1 response in TB (Garg *et al.*, 2008).

Despite all this knowledge about cellular mechanism counteracting against *M. tuberculosis* that were accumulated over the past centuries, a person is dying of TB every 20 seconds. This reminds us of our responsibility as researcher to better ways to diagnose, prevent and treat this devastating disease (Kaufmann, 2006).

2.5 Effect of malaria induced immune response on the host's defense against *M. tuberculosis*

Malarial infection can affect the host's Tcells, both qualitatively and quantitatively. Since the containment of *M. tuberculosis* infection depends on an intact cellular immunity, the influence on TB of a malaria-induced impairment of the immune response could bedue to effects on T cells which may lead to decrease in number of the CD4+ Tcells which decrease the effective Th1 response necessary for TB control or the switch to a predominantly Th2 response that occurs later in the malarial infection, at the expense of the Th1 response, could impair the host's protective mechanism against *M. tuberculosis.* A Th2 response alsohas been shown to cause a switch of T cells from CD8+ to CD8−, which are ineffective in immune control (Erard and leGros, 1994).

The relative proliferation of the TcRγδ cells, that also have cytolytic effects on CDαβ (CD4+ and CD8+) T cells, will furtherjeopardize effective immunity againstTB in malarial infection. Because the TcRγδ T cells also have immune-protective effects in mycobacterial infection, persistent stimulation of them byrepeated malarial infection could lead to immune exhaustion and unresponsiveness to mycobacterial infection. Studies have shown that, when there is CD4+ T cell ligation, the occurrence of a second, concurrent infection to which the individual is sensitized results in depletion of memory T lymphocytes specific for that infection (Howie *et al*., 1994). Mycobacterial infection of those already infected with *Plasmodium* might therefore lead to depletion of TB specific memory T lymphocytes.

Although Whittle *et al.,* (1984) thought that the malaria-attributable immunosuppression was transient, with complete recovery of T-cell control immediately after the acute episode, Lisse *et al*., (1994) found that this impairment could be demonstrated in those with asymptomatic parasitaemia. By impairing T-cell function, malarial infection may create a window of relative immune ineffectiveness, during which the host may be particularly vulnerable to TB.

2.5.1 Malarial and mycobacteria infections

During the rainy season, malaria burden increases (Dgedge *et al.*, 2001; Hongoro and McPake. 2003; Soumare *et al.*, 2008) in many African countries, peaking morbidity and mortality. Several studies have shown that malarial infection affects severely ill TB patients who are already compromised by malnutrition, deprived immunity or

disseminated disease (Russell, 2004; Anya, 2004; Romagosa *et al.,* 2007). Ellner (2010) noted that in pulmonary TB there is a transient systemic immunosuppression due to over expression of transforming growth factor beta (TGF-β) and interleukin-10 (IL-10). Scott *et al.,* (2004) demonstrated interactions between TB and malaria in both *in vitro* and *in vivo*: *Plasmodium falciparum* modulates *M. tuberculosis* infection and Hawkes *et al.,* (2010) showed that malariacan exacerbate mycobacterial infection. The reasons for this are not completely explored but seem to involve parasite-parasite interaction and host-parasite interaction (Enwere *et. al.,* 1999; Scott *et al*., 2004; Hawkes *et. al.,* 2010).

Malaria causes a further depression in immunity through a qualitative and quantitative defect in T lymphocytes, mainly the CD8+ T cells that are necessary for anti-mycobacterial response, and through a deregulation of the cytokine cascade. Moreover, Parry *et al.,* (2004) noted that the respiratory distress that is frequent during acute malaria both inchildren (due to metabolic acidosis) and adults (due topulmonary edema and Acute Respiratory Distress Syndrome), can worsen the respiratory effort related to TB. Therefore, given the multiple interactions between malaria and TB, new strategies of integrated management should be investigated, especially during malaria peak transmission seasons. The identification of efficacious and low cost methods for reducing malaria in TB patients could largely improve clinical outcome and public health strategies optimizing the use of current limited resources in Africa.

The relationship of host immune responses to the development of TB at different times during malarial infection is not clear. Cytokines play an important role in the process of

host defense against *M. tuberculosis*. The production of IFN-γ appears to be crucial to the control of *M. tuberculosis* infection (Flynn *e.t al.,* 1993).Tumour necrosis factor-alpha (TNF-α) plays an essential role in preventing reactivation of persistent TB, modulates the pulmonary expression of specific immunologic factors and limits the pathological response of the host (Mohan *et. al*., 2001).

The critical role of CD4+ T cells in the control of both malaria and TB is well documented, although their exact function is more controversial. Several studies have elucidated the important roles play by CD8+ T and γδ+ T cells in the immune responses during TB infection (Hernandez-Pando *et al.,* 1996; Chackerian *et al.,* 2001; Flynn and Chan, 2001; Li *et al.,* 2001; Zhang *et al.,* 2001). Mounting evidence shows that theearly stages of malaria are characterized by the production ofIL-12 and IFN-γ by Th1 T cells. Langhorne *et al.,* (2002) noted that to aid in clearance of malaria parasite, the host's immune response shifts to a predominantly Th2 response characterized by the production of IL-4 and other cytokines that initiate the antibody-dependent mechanisms required for complete clearance or resolution of infection.

During infection with TB, a predominantly Th1 cytokine profile has been shown to persist throughout the course of the infection and playsan important role in containment of bacterial growth (Cherise *et al.,* 2004). Different studies conducted by Cooper *et al.,* (1993) and by Tascon *et al* (1998) on CD4- and IFN-γ-null mice, further exemplified the significance of Th1 type immunity and noted that the mice rapidly succumbto infection with TB. The Th1 to Th2 shift observed in malaria infection does not appear to occur in

TB infection (Hernandez-Pando *et al.,* 1996; Arriaga *et al.,* 2002). Conversely, however, IL-4 and IL-10 knockout mice are not better able to defend themselves against TB (North, 1998).

The question of whether BCG vaccination at birth may bias the immune response toward a lasting Type-1 response and consequently decrease susceptibility to mycobacterial infection is still unanswered. The immunogenicity of BCG vaccination has been shown to be impaired in malarial-infected individuals and this is associated with enhanced TGF-β production but not enhanced Th2 responses. On balance, *in-vitro* responses to nonparasitic antigens including the mycobacteria antigens [purified protein derivative (PPD), culture filtrate protein (CFP), and crude *M. tuberculosis* extract] appear to remain largely intact (as measured by lymphocyte proliferation and/or IFN-γ production) in those with malarial infection, and Type-1 responses to mycobacteria antigens are relatively normal.

2.5.2 Malaria modulation of immune responses in mycobacterial infections

The control of TB requires clearly delineated Th1 responses (IL-12, IFN-γ, and TNF-α) and, to a lesser extent, Th17 responses (IL-17a and IL-23), with both T-cell subsets playing important roles in the induction and maintenance of protective immune responses in mouse models of infection (Lockhart *et al.,* 2006; Khader and Cooper, 2008) or for control of infection (as seen in latent TB) (Berrington and Hawn, 2007; Holland, 2007). Since immune mediated protection against *M. tuberculosis* is characterized by strong *Mycobacterium*-specific Th1 responses, it has been postulated that co-infections with

malaria parasites could modulate these immune responses by driving Th2 and regulatory T cells (Tregs) that induce anti-inflammatory responses. Indeed, while latent TB is characterized by the predominant secretion of the Th1-associated and pro-inflammatory cytokines: IL-1β, IL-6, IL-8, IL-12, and IFN-γ, in early filarial infection has been characterized by a mixed (proinflammatory/Th1/Th2) response that has been found to transitions to a more downregulatory cytokine phenotype in chronic infection, dominated by IL-10 (Metenou *et al.*, 2013). Multiple mechanisms could therefore potentially influence how malarial infections affect the immune responses to TB.

This situation suggests that malarial infections can modulate various levels of *M. tuberculosis*-specific responses though whether this mediates predisposition to the development of active TB awaits clarification. Due to chronicity of malaria and the prototypical immune responses (such as Th2, IL-10, TGF-β, Tregs) they engender – clearly should modulate those responses (IL-12, IFN-γ, and IL-17) associated with susceptibility to active TB. Definitive proof, however, of this phenomenon is lacking not only in animal models of malaria and mycobacteria co-infections, but also in population-based human studies.

CHAPTER THREE

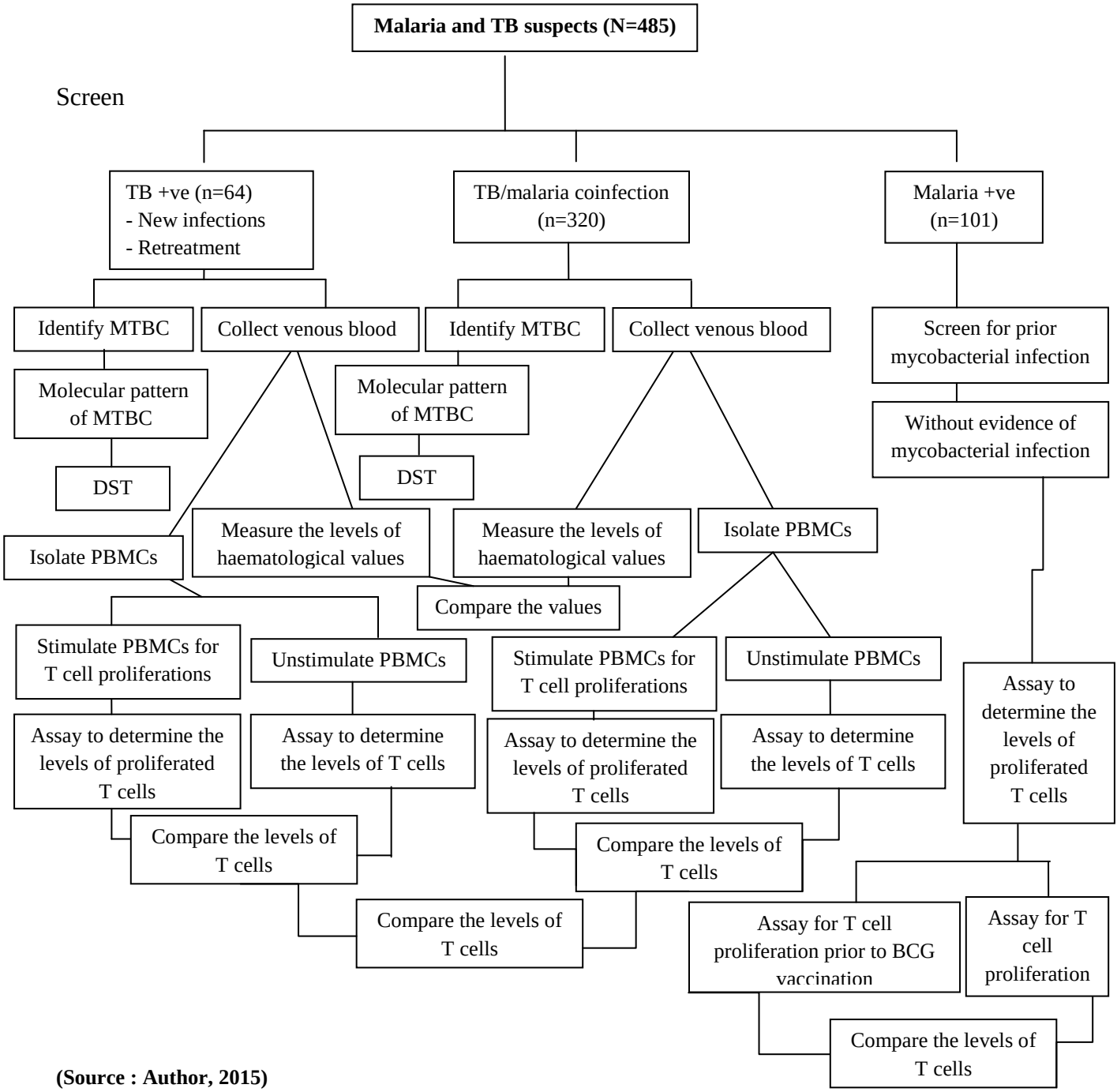
MATERIALS AND METHODS

3.1 Study design

This was a prospective study.All suspected cases with symptoms suggestive of TB and malaria visiting Uasin Gishu and Huruma sub-County Hospitals were enrolled and screened for both infections. Specimen samples were obtained from TB and malaria smear positive patients in the outpatient departments of the two hospitals. Samples were also obtained from TB healthy household contacts (controls). Samples obtained included sputum for staining for acid fast bacilli as well as blood for malarial status, cytokine levels, levels of proliferative T cell response and haematological profiles. Mycobacteria were isolated from smear positive TB sputum samples, MTBC isolates were identified and strain characterization using molecular pattern done. Drug susceptibility testing was also done on the isolated MTBC strains.

Patients who were positive for malaria alone were also screened for prior mycobacterial infection using the Mantoux test and those without evidence of mycobacterial infection were BCG vaccinated and later response (8 weeks) of their peripheral mononuclear cells (PBMCs) to *M. tuberculosis* antigen analyzed and compared.

3.2 Sampling Frame



(Source : Author, 2015)

3.3 Study site

The subjects were drawn from patients who presented themselves between July 2011 and July 2013 at Uasin Gishu and Huruma sub-County hospitals where diagnosis was done. Huruma Hospital serves residents mostly from the Huruma slum and Eldoret West both of which are informal settlements in Eldoret town while Uasin Gishu hospital serves residents mostly from Munyaka and Langas areas which are the biggest informal settlement in the town. The two hospitals also serve the surrounding rural villages. Highest incidence of TB has been shown in the poorest villages and this may appear uninterestinggiven the notion that TB is a disease enhance by poverty, poor nutrition and overcrowding. As the economic town of North Rift, Eldoret town attracts people of all social backgrounds seeking employment, besides business people, tourists and farmers in the region.

The area has rainy and dry seasons, running from mid-March to October and from November to mid-March respectively. The town has several swampy sites during the rainy season, stagnant water pools around houses in unpopular quarters especially during the rainy season, poor waste disposal in a constantly increasing population; all these factors support breeding of female *Anopheles* mosquitoes, the vectors of the malaria parasite.

Malaria is endemic in Eldoret and most parts of Kenya, with high transmission during and just after the rainy season, andis predominantly due to *Plasmodium falciparum*while TB is due to MTBC infection. The two hospitals, therefore, were ideal sites to investigate

the effect of *Plasmodium* infections on the host's immune response to mycobacterial infections in Uasin Gishu County.

3.4 Study population

The study comprised of both male and female aged 2 – 18 years. The distribution of the disease conditions of the subjects was TB positive, Malaria positive, co-infection of malaria and TB, and healthy controls who tested negative for diabetes, helminthes and HIV. Malaria and TB suspects who attended the two Hospitals' chest clinics for routine follow up and/or treatment and/or re-treatment were also enrolled.

Consent was sought from the participants or from the guardians or from the parents. From each of the enrolled patients, sputum samples (supervised spot and unsupervised early morning at home the next day) were collected for direct AFB smear microscopy, culture, MTBC molecular pattern and drug susceptibility testing. Blood samples were then collected for thick and thin smear microscopy, standard full blood count as well as for T cell proliferation.

In addition, household contacts were included based on the information obtained from the TB patient. A household contact was defined as a person who lives together (> 6 months) and spends more than 12 hours per day with a TB patient.

3.5 Inclusion and exclusion criteria

3.5.1 Inclusion criteria

Patients aged 2 – 18 years old who consented (Appendix I).

All TB, malaria, TB and malaria co-infected patients.

Both male and female

Household contacts (Healthy controls) were included based on the information obtained from the TB patient. A household contact was defined as a person who lives together (> 6 months) and spends more than 12 hours per day with a TB patient.

3.5.2 Exclusion criteria

Pregnant mothers

All patients with clinical signs or medical treatment indicating any concomitant chronic or infectious diseases other than TB and malaria

Patients with diabetes, helminthes and HIV

Alcohol consumers and cigarette smokers

3.6 Sample size

Sample size was determined using the formula as used by Mugenda and Mugenda and the prevalence of 45% from the study carried out by Colombatti *et al.*, (2011).

$$N = \frac{Z\alpha^2 P (1-P)}{d^2}$$

N = Number of samples

P = Prevalence of incidence of coinfection of malaria/TB= 45%.

d = allowable error (5% at 95% Confidence interval).

$Z\alpha^2$= Standard normal value corresponding to the desired level of significance at 95% confidence.

d= 0.05

$Z\alpha$ =1.96

P=45%= 0.45

Q= 1-P =1-0.45=0.55, where Q is1-P

$$N = \frac{1.96^2 \times 0.45 (0.55)}{0.05^2} = \mathbf{380}$$

3.7 Summary of the study methods

After due informed consent was obtained, 10ml of venousblood as well as two (supervised spot and unsupervised early morning at home) sputum samples were collected from each of the suspected cases with symptoms suggestive of TB and malaria visiting the facilities. Blood samples were screened at the site laboratory to confirm malaria status of the patients. Sputum samples were screened for AFB to confirm TB status using both ZN and culture methods. The cytokine and $CD4^+$ T cell profiles were estimated from aliquots of each of the blood samples using ELISA and Fluorescence

associated cell sorter (FACS) count respectively whilehaematological and parasitological values were estimated using Coulter counter and microscopical technique respectively.Malaria status was determined by using both thick and thin blood smears stained with 10% Giemsa's stains. HIV status was excluded by use ofHIV rapid screening assay (Determine™HIV-1/2, Abbott®, Tokyo, Japan).

The early morning sputum samples were incubated in both solid Loewenstein Jensen (LJ) medium and in liquid Mycobacterium Growth Indicator Tubes (MGIT) in automated BACTEC MGIT 960 machine (Bacton Dickinson Diagnostic Instrument Systems®). The automated BACTEC MGIT 960 machine monitors growth. Samples that failed to show any growth after 42 days of incubation in BACTEC MGIT 960 machine were removed and classified as negative based on the manufacturer's protocol. Cultures were considered positive for MTBC if they showed a positive growth on the MGIT and showed presence of AFB upon Ziehl-Neelsen (ZN) staining. The culture confirmed MTBC samples were then characterized with genotype MTBC test (Hain Lifescience GmbH, Nehren, Germany®) to further identify the different MTBC species. Cultures with positive MGIT growth but negative for MTBC on genotype MTBC were considered NTM mycobacterium isolates and were excluded from further testing. Identification of MTBC species was performed on DNA extracts from a subset of the MTBC positive specimens at the MUSOM *M. tuberculosis* research laboratory.

3.8 Sputum collection and transport

Sputum samples (both spot and early morning specimens) were collected (in 50 ml Falcon tubes) for direct AFB smear microscopy, culture, polymerase chain reaction (PCR) molecular characterization, and drug susceptibility testing. At the peripheral laboratory, the standard AFB direct smear microscopy using ZN staining was done on the initial sputum to confirm TB diagnosis of suspected patients. A second sputum specimen was then collected before start of treatment and then transported in cold boxes within 48 hours to the MUSOM *M. tuberculosis* research laboratory on the same day for culture.

3.8.1 Sputum smear examination

AFB staining (Appendix II) and examination was done at baseline on morning sputum samples for two consecutive days. The AFB load in sputum smears was measured as previously described by the World Health Organization (WHO, 2010) as: negative (0 AFB/100 oil fields), scanty (1-9 AFB/100 fields), 1+ (10-99 AFB/100 fields), 2++ (1- 10 AFB/ field) and 3 +++ (> 10AFB/field). Sputum conversion was defined as all two consecutive sputum smears turning negative for AFB.

3.8.2Sputum culture on LJ slopes

Sputum was cultured on LJ slopes (Appendix III). From the bacterial growth, a loopful of the colonies was harvested from the LJ slants, suspended in 1ml sterile phosphate buffer (pH 6.8) and the large clumps broken using a vortex. The suspension was adjusted to a standard 0.5 McFarland turbidity by visual comparison. Loewenstein Jensen (LJ) which is an egg based solid culture media, was used for primary culture of *M. tuberculosis*. Sputum specimens were first decontaminated using 4% sodium hydroxide (NALC-

NaOH) (Becton, Dickinson and company Sparks®, USA) to eliminate the associated commensal flora (Appendix III). The decontaminated sputum specimens were then homogenized by vortexing and then centrifuged for 15 minutes at 300 revolutions per minute (rpm). The sediment obtained after centrifuging was inoculated directly to the LJ culture media slopes and incubated at 37^0C. The slopes were examined weekly for any visible growth. A positive culture of *M. tuberculosis* confirmed the diagnosis of active TB disease.

3.8.3 Sputum culture on BACTECTM MGIT 960 machine

The early morning sputum samples were incubated in liquid MGIT in automated BACTEC MGIT 960 machine (Bacton Dickinson Diagnostic Instrument Systems®) which monitors growth (Appendix IV). Samples that failed to show any growth after 42 days of incubation were removed and classified as negatives based on the manufacturer's protocol. Cultures were considered positive for MTBC if they showed a positive growth on the MGIT and showed presence of AFB by ZN stain. The culture confirmed MTBC samples were then characterized with genotype MTBC test (Hain Lifescience GmbH, Nehren, Germany®) to further identify the different MTBC species. Cultures with positive MGIT growth but negative for MTBC on genotype MTBC were considered NTM isolates and were excluded from further testing. Identification of MTBC species was performed on DNA extract from a subset of the MTBC positive specimens at the MUSOM *M. tuberculosis* research laboratory.

3.8.4 Strain cultivation and drug susceptibility testing

Primary isolation and cultivation of mycobacterial isolates were performed at the MUSOM *M. tuberculosis* research laboratory. For all strains isolated, resistance to the key anti-mycobacterial drugs: Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB) and Streptomycin (STR) was determined by using both liquid medium (automated BACTEC MGIT 960 machine [Bacton Dickinson® Diagnostic Instrument Systems]) and line probe Genotype®MTBDR*plus* (Hain Lifescience® GmbH, Nehren, Germany) methods. Both methods were performed according to manufacturer's instructions.

3.8.5 Drug susceptibility testing using MGIT 960 method

Strains of MTBC isolates from LJ slopes were subjected to drug susceptibility testing (DST): INH, RIF, STR, and EMB using the BACTEC™ MGIT 960 automated method.

A loopful of the colonies was harvested from the LJ slants, suspended in 1ml sterile PBS at a pH=6.8 and vortexed to break the large clumps. The suspension was adjusted to a standard 0.5 McFarland turbidity by visual comparison.

The drug containing and drug-free growth control MGIT tubes were inoculated with the standardized 0.5 McFarland inoculums of the *M. tuberculosis* isolate and entered into the MGIT 960 automated machine in a special rack-carrier with a printed barcode; this is read by the machine when entering the tubes to identify the test and apply the adequate algorithm for susceptibility or resistance interpretation. All readings were performed inside the machine and the results were printed as susceptible or resistant for each drug tested.

3.8.6 Detection of Resistance to Isoniazid and Rifampicin using Genotype MTBDRplus

Cultures confirmed as containing MTBCstrainswere assayed for evidence of resistance to INH and RIF using Genotype®MTBDR*plus* (Hain Lifescience GmbH, Nehren, Germany®) method. This line probe assay was performed according to manufacturer's protocol as described by Nikolayerskyy *et al.*, (2009) (Appendix V). The DNA strip used had a total of 27 reaction zones, of which 21 zones probed mutations and the remaining 6 were control probes for verification of the assay procedures. The control probes consisted of a conjugate control, and amplification control, an MTBC-specific control, an *rpoB* amplification control, a *katG* amplification control and an *inhA* amplification control. Rifampicin resistance was marked by the *rpoB* gene, while INH resistance was marked by the *katG* and *inhA*gene. Resistance to RIF was identified by the absence of at least one of the wild-type bands or the presence of bands in the region of the *rpoB* gene (Appendix V). Similarly, the absence of at least one of the wild-type bands or the presence of bands suggestive of mutation in either *katG* and *inhA* genes or both identified resistance to INH. Joint occurrence of characteristic features for resistance to both drugs indicated the presence of MDR-TB. A sample in which all the wild-type probes of a gene were present and there was no band suggestive of mutation within the region examined was considered sensitive to the respective drug. Bands in all the six control zones were required to appear correctly; otherwise, the result was considered invalid.

3.8.7 Characterization of MTBC using Genotyping®MTBC method

A positive culture of MTBCconfirmed the diagnosis of active TB disease. Strains of MTBC were isolated from LJ slopes and MGIT 960 positive cultures; and were identified using GenoType®MTBC reagents (Hain Lifescience® GmbH, Nehren, Germany) (Appendix V). To obtain the isolates, sputum were processed according to national and international guidelines (Kent and Kubica, 1985; Deutsches Institut für Normung. 1986) by using an *N*-acetyl-l-cysteine-NaOH decontamination procedure, inoculated into BACTEC MGIT 960 tubes (Becton Dickinson® and Co., Cockeysville, Md.) and onto solid slant medium (LJ), and incubated at 37°C for up to 6 and 8 weeks, respectively.

BACTEC™ MGIT 960 tubes that had been reported to be positive by the MGIT instrument were checked for the presence of mycobacteria or contamination by (i) performing an acid-fast smear and (ii) subcultivation on Trypticase soy agar with 5% sheep blood. Specimens were chosen for the test if the acid-fast smear was positive or if the acid-fast smear was negative and contamination by other microorganisms could be excluded (no growth on Trypticase soy agar within 24 hours or no other microorganisms visible in the smear). No more than two specimens from one patient were used. Only specimens growing mycobacteria were included in the study.

3.8.8 Sample preparation for genotyping of MTBC

For the genotype assays (Hain Lifescience GmbH, Nehren, Germany®) technique, aliquots of identical liquid media (MGIT 960) were prepared for the genotype MTBC test which was performed according to the manufacturer's instructions. For this, 1 ml was centrifuged (10,000x*g*, 15 min, room temperature), the supernatant was discarded, and

the pellet was suspended in 300 to 500 µl of distilled water. After 15 min of boiling and 15 min of sonication, the samples were used immediately for amplification or stored at −20°C awaiting the bioassays.

3.8.9 Procedure for GenoType® MTBC assay

The GenoType®MTBC assay is based on an MTBC-specific 23S ribosomal DNA fragment, *gyrB* DNA sequence polymorphisms, and the RD1 deletion of *M. bovis* BCG (Niemann *et al.,* 2000). Specific oligonucleotides targeting these sequences are immobilized on membrane strips. Amplicons derived from a multiplex PCR hybridize to these probes. The assay was performed according to the manufacturer's instructions and as described by Richter *et al.,* (2003). Amplification was done with 35 µl of primer nucleotide mix, amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U of HotStarTaq polymerase (QIAGEN®, Hilden, Germany), and 5 µl of DNA in a final volume of 50 µl with the following amplification protocol: denaturation at 95°C for 15 min; 10 cycles of denaturation at 95°C for 30 s and elongation at 58°C for 120 s; an additional 20 cycles of denaturation at 95°C for 25 s, annealing at 53°C for 40 s, and elongation at 70°C for 40 s; and a final extension at 70°C for 8 min.

Hybridization and detection were carried out in a TwinCubator® automated washing and shaking device (Hain Lifescience® GmbH, Nehren, Germany). Twenty microliters (20µl) of the amplification products was mixed with 20 µl of denaturing reagent (provided with the kit) for 5 min in separate troughs of a plastic well. After the addition of 1 ml of prewarmed hybridization buffer, the membrane strips were added to every trough.

Hybridization took place in TwinCubator® at 45°C for 30 min, followed by two washing steps.

For colorimetric detection of hybridized amplicons, streptavidin-conjugated alkaline phosphatase and the appropriate substrate was added. After the final washing step, the strips were air dried and fixed on a data sheet. Species were identified according to the interpretation table provided (Appendix V). The DNA strip used had a total of 13 reaction zones, of which 10 zones probed strain and the remaining 3 were control probes for verification of the assay procedures. The control probes consisted of a conjugate control, an amplification control, and MTBC-specific control, while *M. tuberculosis*strain was marked by the 4, 5, 6, 7 and 8 *genes* band. *M. africanum* (4, 5, 6, 7 and 10 *genes* band), *M. microti* [*M. bovis caprae*] (4, 5, 6, 10 and 11 *genes* band), *M. canettii* (4, 5, 6, 10 and 11 *genes* band), *M. bovis ssp. Bovis* (5, 7, 10 and 12 *genes* band) and *M. bovis* BCG (4, 7, 9, 10 and 13 *genes* band). Bands in all the three control zones were required to appear correctly; otherwise, the result was considered invalid.

3.9 Parasitological, Haematologic and PBMC isolation

Venous blood (10ml) for both parasitological and haematologic examination, and PBMC isolation was drawn into Heparin-EDTA filled tubes to be used for complete blood counts and examination for malaria parasite. Two slides for study purposes were promptly prepared at the field site laboratory, each with a thin and thick blood smear. A third blood smear was prepared, stained and examined immediately in the local clinic

laboratory for the present of malaria parasite and interpreted according to routine clinic procedures for therapeutic purpose.

3.9.1 Examination for haematological parameters

The venous blood sample that was drawn into Heparin-EDTA tubes was used for preparation of the thick and thin smears and automated for determination of Complete Blood Counts (CBCs). Blood counts were performed using Coulter T-890 automated Haematology Analyzer (Beckman-Coulter®, Inc, Fullerton, CA). The Analyzer provided data on WBCs, RBCs, Hb level, platelet counts, MCV, MCH, MCHC, RDW and five part differentials (lymphocyte count, neutrophil count, monocyte count, eosinophil count, and basophil count).

3.9.2 Examination for malaria parasites

Blood slides were prepared and stained with Giemsa. Microscopic abnormality of blood in smear and presence or absence of malaria were determined, the species and the number of asexual parasites. Thick and thin films were prepared from a portion of blood for each participant's blood sample within 30 minutes of collection. After fixing thin smears with absolute methanol, smears were stained using 10% Giemsa for 10 minutes (Appendix IX) and read by microscopy (Cheesbrough, 2006).

One slide from each study participant was examined independently by two experienced microscopists. They determined the presence or absence of *Plasmodia* parasites and the number of asexual parasites per 200 WBCs. If fewer than 10 asexual parasites per 200 WBCs were observed, counting continued to 500 WBCs. Parasite densities were

calculated as parasites per microliter of blood (parasites/WBCs counted x total WBCs in 1 µl of blood), and the results of the two readings were averaged. Concordance between the two blinded Microscopists' interpretations was assessed and cases of species discordance from the two readings, mostly associated with very low parasitemia, was excluded from this analysis. Malaria parasitaemia was measured as parasites per microliter based on the measured WBC.Another portion of blood was used for CD4+ T cells count. CD4+ T cells were enumerated using the Partec CyFlow® automated cell counter (Partec® GmbH, Munster, Germany).

3.10 Effects of malarial infection on immunity against TB and on immunogenicity of BCG vaccination

3. 10.1 Tuberculin skin testing

Purified protein derivatives (PPD) (2 Tuberculin units; Statens Serum Institute, Denmark) was injected intradermally on the ventral aspect of the forearm and the diameter of skin induration was measured 48 hours later following the guidelines specified in the WHO standard tuberculin test technical guide (Sakol, 1975).

3.10.2 BCG vaccination

BCG vaccine was injected intradermally in the left deltoid region to tuberculin negative subjects. A dose of 0.05ml of vaccine injected into the middle of the deltoid muscle of the right arm was chosen as the most appropriate dose and site for vaccination. A short, bevelled 25–26-gauge disposable needle and a 1 ml disposable syringe were used for the injection. After cleaning and stretching the skin with the fingers, the bevel of needle was

placed upwards and the needle inserted 2 mm into the skin parallel to the long axis of the arm. The contents were injected at high pressure to induce a 7 mm bleb. If the needle was accidentally inserted deep into the skin, or if there was failure to induce a bleb, the entire process was started ab initio. The nursing staff and primary care trainers were also trained to recognize adverse events.

3.10.3 Isolation of PBMC from human blood

For isolation of PBMC, 10 ml of blood was collected from the recruited patients using Vacutainer (BD Bioscience) coated with Sodium-Heparin. The blood was diluted 1:1 in phosphate buffered saline (PBS) and layered over 15ml lymphocyte separation medium (Ficoll-hypaque) in a 50 ml tubes. Ficoll-hypaque is a hydrophilic polysaccharide (SG=1.077g/ml) that allows the separation of PBMCs by density gradient centrifugation. After the layering, tubes were centrifuges at 1000xg for 30minutes at room temperature without brake. The supernatant containing plasma was carefully removed and the layer of PBMCs located at the interface of the gradient was recovered and transferred in a new 50ml tube. The tube was filled up with PBS (PH=6.8) and the sample was centrifuged at 800xg for 10 minutes at 4°C. The supernatant was discarded and the washing step was repeated to remove completely cytotoxic lymphocyte separation medium. The pellet was then resuspended in an appropriate amount of buffer and counted using haemocytometer.

3.10.4 PBMC cell culture handling

All cell procedures were performed under sterile condition using a Biosafety cabinet class II. PBMCs were always resuspended in medium and cultured in cell culture plates in a

CO_2 incubator set at a temperature of 37°C and 5% CO_2 tension. Cells were counted using an Inverted laboratory microscope and a counting chamber.

3.10.5 Cell proliferation (Stimulation)

The isolated PBMCs (1 x 10^6 cells/ml) were stimulated with purified protein derivative (PPD) of *M. tuberculosis* at a concentration of 10 µg/ml for 3 days or48 hoursat 37^0 C in CO_2 incubator under humid condition. After 3 days or48 hours of incubation (37^0 C, 5% CO_2 tension with 95% humidity), cell culture supernatants were collected and assayed for T cell proliferation using ELISA (BioLend® Legend Max™ San Diego, CA).The procedure for cell culture was that PBMC isolated from whole blood were washed twice in RPMI 1640 medium and re-suspended in the culture medium at a concentration of 10^6 cells per ml. Then, 0.5 ml (500 µl) of cell suspension was added to wells of a 24-well tissue culture plate. Next, 0.5 ml (500 µl) of PPD antigen at a 2× final concentration in culture medium or 0.5 ml (500 µl) of additional medium (for the cell control) was added to the wells, yielding a final concentration of 5×10^5 cells/ml. Plates were incubated for 3 days (37°C, 95% air, 5% CO_2, 95% humidity).

3.10.6 Cytokine assays

Cell culture supernatants were harvested and analyzed for cytokines by ELISA techniques with commercially available kits (or were frozen for later analysis). IL-4, IL-5, IL-6, IL-8, IL-10,IL-12p40 and TGF-β kits were obtained from BioLend® Legend Max™ San Diego, CA. All cytokine assays were calibrated against the WHO (2010) international standards by the kit manufacturer. The lower detection limits for the individual assays are as follows: IL-4 and IL-6, 7.8 pg/ml; IL-5, 5 pg/ml; IL-10 and IL-12p40, 3.9 pg/ml; and TGF-β, 10 pg/ml.

3.10.7 Analysis of IFN-γ by ELISpot

PBMCs (250,000/well) were plated onto ester-cellulose-bottomed plates (PVDF plates, Mabtech®, Solna, Sweden) coated with a capture mAb specific for human IFN-γ at 15μg/ml in phosphate buffered saline (PBS). Cells were incubated in a humidified incubator at 37°C in 5% CO_2 for 24 hours with PPD-antigen in duplicate using unstimulated and CD3-stimulated cells as negative and positive controls, respectively. Cells were then removed by washing five times with PBS-0.5% fetal calf serum (FCS), and then biotinylated anti-human IFN-γ were added at 1μg/ml in PBS and incubated for 2 hour at room temperature. After washing five times in PBS, streptavidin-alkaline phosphatase (Mabtech) was added and incubatedfor 1 hour at room temperature. After washing with PBS, filtered (0.45μm filter) BCIP/NPT-plus substrate (Mabtech®) was added. Plates were extensively washed using tap water. After 24 hours of incubation, the number of spots for each well was counted with automated ELISpot reader (AID®, Germany), and the results for each stimuli (PPD, negative and positive control) correspond to the median of duplicates. The results are reported as spot forming units (SFU)/250,000 cells.

3.11 Cytokine ELISA

Cytokine levels (IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p40 and TGF-β) in cell culture supernatants were measured employing standard ELISA protocols using commercially available pair of monoclonal antibodies (BioLend® Legend Max™ San Diego, CA) according to manufacturer's instructions (APPENDIX X).

3.12 Ethical issues

The proposal for this study was approved by MUSOM/MTRH IREC (Approval Number: 000657; FAN: IREC 000657) as attached (Appendix XI). Clearance was obtained from respective health authorities. Informed consents and assents were obtained from TB suspects or their guardians or their parents before they were enrolled into the study. The purpose of the study was explained to the suspects in English, Kiswahili or a local language where necessary before consent was sought. Code numbers were used to identify TB suspects in order to maintain confidentiality (Appendix XI).

3.13 Statistical data analysis

Data analysis was performed using STATA version 13 special edition.Normality distribution of continuous data was determined using The Kolmogorov-Smirnov Test. Categorical variables were summarized as frequencies and the corresponding percentages. Continuous variables were summarized as median and the corresponding inter quartile range (IQR).Categorical data were compared using Pearson Chi-Square (χ^2) Test.Association between two continuous data was assessed by Pearson Correlation. Data not conforming to normal distribution were compared by Spearman's rank correlation Test for association between the continuous and the categorical variables was done using the two-sample Wilcoxon rank sum test. Results were presented in form of tables and charts.

CHAPTER FOUR

RESULTS

A total of 485 patients were enrolledin the study for both malarial and mycobacterial infections. Of the enrolled cases, 320 weremalaria and TB coinfected cases, 64 were TB infected while 101were malaria infected cases alone. Those that were found to be smear positive AFB, but culture negative were considered to have non-tuberculosis (NTM) infection and their samples were excluded from the analyses. The enrolled patients had an overall median age of 14(IQR: 11-15) years with a minimum of 5 years and a maximum of 18 yearswith the male and female subjects almost balanced representing 250 (51.5%) and 235 (48.5%), respectively (Table 4.1).

Table 4.1: Characteristics and infection status of the study participants by age and gender

Characteristics	Overall	Malaria+ve/TB+ve	TB +ve	Malaria +ve	*P-value*
No. of subjects	485	320	64	101	N/A*
Age (Years)					
0 – 5	6 (1.3%)	5 (1.6%)	1 (1.6%)	0	0.608
6 – 10	19 (24.5%)	69 (21.6%)	18 (28.1%)	32 (31.7%)	0.608
11 – 15	216 (44.5%)	152 (47.4%)	26 (40.6%)	38 (37.6%)	0.608
16 – 18	144 (29.7%)	94 (29.4%)	19 (29.7%)	31 (30.7%)	0.608
Gender					
Male	250 (51.5%)	168 (52.5%)	33 (51.6%)	49 (49%)	0.891
Female	235 (48.5%)	152 (47.5%)	31 (48.4%)	52 (51%)	0.891

N/A*= Not applicable; TB+ve = tuberculosis positive

There were a total of 384 out of 485 participants who were infected with TB and whose data were included for analysis. A total of 320 (83%) had a co-infection of malaria while the rest were infected with TB only. Participants co-infected with malaria and TB had the same age as those infected with TB only as previously shown in Table 4.1. This was statistically not significant (P=0.970).

Statistically age of the patients who were co-infected with malaria and MTBCstrains compared to those infected with MTBC alone was not significant (P>0.05). The proportion of male patients co-infected with malaria and MTBCas compared to the male patients infected with *M. tuberculosis* alone was not significant (P>0.05) suggesting that co-infection was independent of gender and age.

A total of 384 of the patients had TB and the result (Figure 4.1) showed that majority of these patients, 178 out of 384 (46.4%), were 11 – 15 years of age and among this age group, 152 (47.4%) were co-infected while 26 (40.6%) had single infection of MTBC as earlier indicated in Table 4.1.

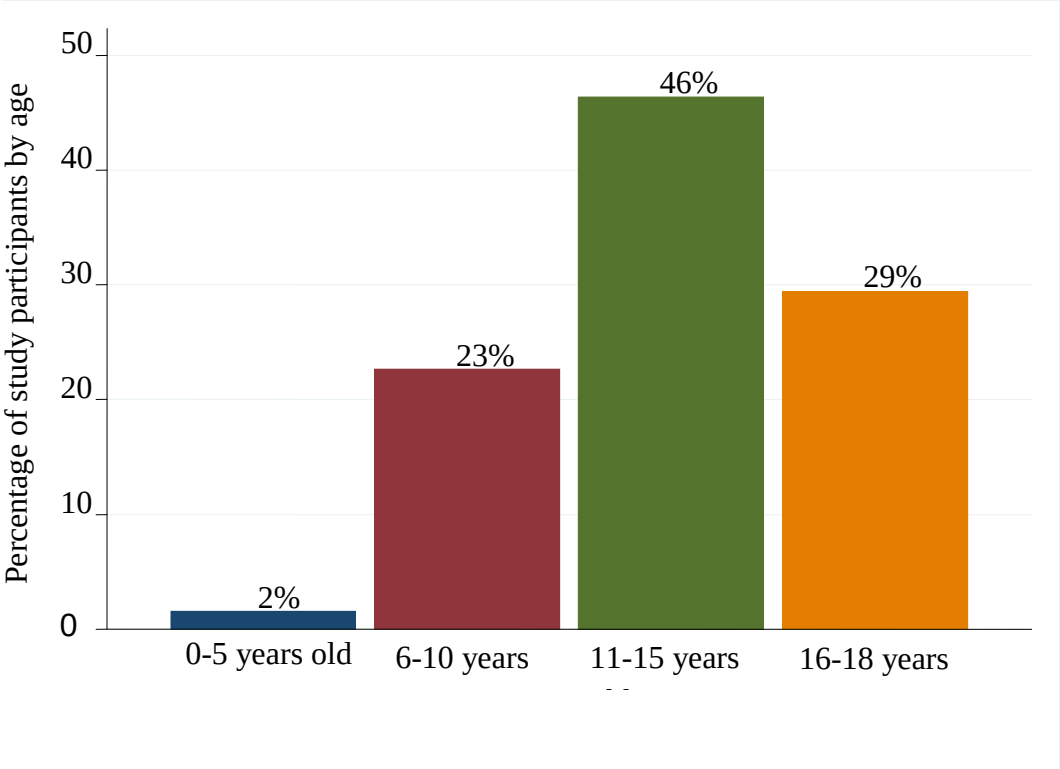


Figure 4.1: Distribution of the participants by age

Among the enrolled patients were newly diagnosed cases, relapsed or retreatment and anti-TB drug interrupters. The results showed that 287/384 (74.73%) of TB infected patients were newly diagnosed cases, 96/384 (25.00%) were relapsed/retreatment cases while one (0.27%) wasanti-TB drug interrupter (Table 4.2).The main reason for registration for re-treatment among the participants was relapse and treatment after interruption.

Table 4.2: Characteristics of the TB patients enrolled in the study

Type of patients	Frequency	Percentage	Cumulative
New patients	287	74.73	73.96
Relapsed (retreatment)	96	25.00	100.00
Defaulter (drug interrupter)	1	0.27	0.27
Total	384	100.00	

Among the MTBC cases identifiedby genotyping, it was found that a total of 351(91.4%) participants were infected with *M. tuberculosis*: representing a majority of the cases; 25 (6.5%) had *M. africanum;* 7 (1.8%) had *M. bovis* BCG while 1 (0.3%) had *M. bovis* infection (Table 4.3).

Out of 351 participants infected with *M. tuberculosis* species, a total of 183(52%) were male and 168(48%) female (Table 4.3). The results also showed that 287(82%) of those infected with *M. tuberculosis* species had a co-infection of malaria. Of the 7 isolates of *M. bovis* BCG, all of them were isolated from malaria and mycobacteria co-infected individuals and same trend was seen in *M. bovis* and *M. africanum* where one (100%) and 25 (100%) of *M. bovis* and *M. africanum* respectively where all the isolates were from co-infected individuals (Table 4.3). The prevalence rate of TB and malaria co-infection in this region was found to be high (320/485 [66%])

Table 4.3: Characteristics of cases with MTBC strain by age and gender

MTBC strains	*M. tuberculosis*	*M. bovis BCG*	*M. bovis*	*M. bovis subsp.*	*M. microti*	*M. africanm*
No. of Subjects	351(91.4%)	7(1.8%)	ND	1(0.3%)	ND	25(6.5%)
Age (years)						
0-5	5(1%)	1(14%)	ND	ND	ND	ND
6-10	77(22%)	1(14%)	ND	ND	ND	9(36%)
11-15	170(48%)	1(14%)	ND	ND	ND	7(28%)
15-18	99(28%)	4(57%)	ND	1(100%)	ND	9(36%)
Gender						
Male	183(52%)	5(71%)	ND	ND	ND	13(52%)
Female	168(48%)	2(29%)	ND	1(100%)	ND	12(48%)
Malaria infection						
Yes	287(82%)	7(100%)	ND	1(100%)	ND	25(100%)
No	64(18%)	ND	ND	ND	ND	ND

ND* = Not detected

To identify associations between the MTBC species and their immunogenicity during malarial infection, ELISpot was used to measure PPD-induced IFN-γ responses in blood samples collected from malaria infected patients who were co-infected with MTBC (n = 384): *M. tuberculosis* (n = 351), *M. africanum* (n = 25), *M. bovis* BCG (n = 7), and *M. bovis* (n = 1).

ELISPot assays were performed as described (Elkhalifa *et al.*, 1991) on a subset of 384 study samples. Quantitative results were expressed as the number of spot-forming units (SFU) that produce IFN-γ in response to *M. tuberculosis* PPD antigen. Positive wells were predefined as containing >10 sfu more than, and at least 2 times as many as, negative control wells. The negative control well was required to have <30 sfu.The risk of infection of *M. tuberculosis* and *M. africanum* among those with a co-infection of malaria was statistically significant (P= 0.0001). These results, therefore, suggest that there is a tendency towards higher risk of infection of *M. tuberculosis* and *M. africanum* among those with a co-infection of malaria (Table 4.4).

On the basis of the assumption that clustering is indicative of recent transmission, PPD ELISpot positivity of clustered strains (Table 4.4) was compared. The data showed that *M. tuberculosis* strains clustered by MTBC genotyping were significantly more likely to produce a positive PPD ELISpot result than are *M. bovis* BCG and *M. bovis* (OR 31.78, 95% CI 9.24–109.28; p = 0.0001) while *M. bovis* and *M. bovis* BCG, a not significant tendency (1.58, 95% CI 0.48 – 5.15; P=0.0.3178 and 0.61, 95% CI 0.21–1.75; P=0.2530 respectively) was shown while for *M. africanum* it was significant at 21.09 (6.09–73.04; p=0.0001).

Table 4.4: ELISpot results for blood samples from patients infected with MTBC strains

Characteristics	Malaria+ve/TB+ve	TB +ve		
No. of subjects (n=384)	320	64	OR	*P-value*
MTBC Strains cluster	ELISpot (+ve)	ELISpot (+ve)		
M. tuberculosis	300	51	31.78 (9.24–109.28)	0.0001
M. africanum	0	7	21.09 (6.09–73.04)	0.0001
M. bovis BCG	0	1	1.58 (0.48–5.15)	0.3178
M. bovis	20	5	0.61 (0.21–1.75)	0.2530

A total of 384 drug sensitivity tests (DST) were performed from isolates of MTBC (Table 4.5). As indicated earlier (Table 4.1), the majority of the patients, 178 out of 384 (46.4%), were 11 – 15 years of age and among this age group, 152 (47.4%) were co-infected while 26 (40.6%) had single infection of MTBC.

The level of drug resistance was found to be high; resistance to any first line drug was found in 33/320 co-infected patients (10.3%) out of which 18 out of 320 (5.6%) were any resistant to INH alone. In total, any resistance to INH was documented in 20 out of 384 MTBCinfected patients (5.2%), 4 were resistant to RIF (1.04%) of which all were from the co-infected patients. MDR-TB was found in a total of 2/384 patients (0.5%). Table 4.5 shows result of the detailed distribution of drug resistance among TB infected subjects. The isolates showed different resistance patterns with monoresistance (resistance to at least one drug) in 27 (7.0%) isolates of which 2 (0.5%) were in single infection of MTBC and 25 (6.5%) in the malaria and MTBC co-infected cases. The double resistance was shown in 5 (1.3%) isolates, and triple resistance in 2 (0.5%). Monoresistance was recorded in all four drugs tested using SIRE kit (Becton Dickinson®). Isolates from MTBC were resistant to the anti-TB drugs tested in the coinfected cases as follows: 14 (3.6%) were resistant to STR; 20 (5.2%) were resistant to INH; 4 (1.04%) were resistant to RIF and 3 (0.8%) were resistant to EMB. The antibiotic resistant pattern among MTBC mono-infected patients was as follows: 1 (0.3%) were resistant to STR; 2 (0.5%) were resistant to INH and 1 (0.3%) were resistant to RIF. Five (1.3%) antibiotics were double drug resistant, two (0.5%) of which were MDR-TB. Two (0.5%) isolates were resistant to INH and RIF, 2 (0.5%) to STR and INH and 1 (0.3%) to

RIF and STR. One MDR-TB isolate was triple resistant with an additional resistance to STR. Of the total MDR-TB isolated, only one (0.3%) was from MTBCco-infected patient.

Resistance to STR, INH, RIF, and EMB were 11 (3%), 14 (4%), 1 (0.3%), and 1 (0.3%) respectively. The difference by whether the participant had a co-infection or not was determined and the results were not significantly ($P > 0.05$) different (Table 4.5) except for for the combination of STR and INH which were significantly different ($P < 0.05$). However, there is a trend towards higher proportions of resistance to individual drugs among those who had a co-infection of malaria.

Multi-drug resistance TB cases weredetermined and it was found out that 2 (0.5%) were resistant to both INH and RIF, 1 (0.3%) were resistant to INH, RIF and STR, 1 (0.3%) were resistant to INH, RIF, and EMB. The risks of multi drug resistance were not different by whether a participant had a co-infection with malaria or not, but there is a tendency towards higher risk among those with a co-infection of malaria (Table 4.4). Other multi drug resistances were investigated and the results were as presented in Table 4.5. The risks of drug resistance were not significantly different by whether a participant had a TB infection only or had a co-infection of malaria.

A total of 37 (10%) were resistant to any of STR, INH, RIF, and EMB. However, a total of 347 (90%) were sensitive to all of the drugs. A higher proportion of sensitivity was

seen among those who had co-infection. However, the difference was not statistically significant (P=0.178).

Table 4.5: Correlates and pattern of resistance to first line anti-tuberculosis drugs

Characteristics	**TB+ve**	**Mal+ve/TB+ve**	**TOTAL**	***P-value***
No. of DST	64	320	384	N/A
Sensitivity to all	60	287	347	
Any resistance	4	33	37	0.178
Mono resistance				
Streptomycin (STR)	1	10	11	0.375
Isoniazid (INH)	1	13	14	0.444
Rifampicin (RIF)	0	1	1	0.229
Ethambutol (EMB)	0	1	1	0.485
Double resistance				
INH + RIF	1	1	2	0.088
INH + STR	0	2	2	**0.027**
RIF + STR	0	1	1	0.670
TOTAL MDR Pattern	1	1	2	0.276
Triple resistance pattern				
INH + RIF + EMB	0	1	1	0.470
INH + RIF + STR	0	1	1	0.600
TOTAL MDR Pattern	0	1	1	0.256

The first line anti-TB drug susceptibility testing of MTBCisolates was perform using GenoType® MTBDRplus 96 reagents (Hain Lifescience GmbH, Nehren, Germany®) and the resultswere pasted in a result sheet (Appendix VIII [a and b), while the susceptibility testing for STR, INH, RIF and EMB was determined using SIRE kit and MGIT 960 automated system the results were shown in a print-out (Appendix VIII).

The median number of malaria parasites was 65 (IQR: 33-90) $x10^3/\mu l$ among those who were co-infected with malaria. The median AFBs was 1 (IQR: 0-2) (10-99 AFBs/100 Fields). The number varied by whether the participant had a co-infection of malaria or

not. The results demonstrated the participants with a co-infection of malaria had a significantly higher median AFB (P=0.002) when compared to those who were infected with TB only (Table 4.6). The overall median Hb was 10.4 (IQR: 8.9-11.7) g/dl. The Hb levels significantly differed between those who were co-infected with malaria and those who were infected with TB only. The results showed that co-infected subjects had significantly (P=0.0001) lower Hb (g/dl) concentration as compared those with TB alone. Overall the median WBC, lymphocytes, monocytes, neutrophils, and eosinophils count were 8.1 (IQR: 6.5-10.7) $x10^3$/µl, 4.5 (IQR: 2.1-7.8) $x10^3$/µl, 0 (IQR: 0-2) $x10^3$/µl, 45 (IQR: 37-62) $x10^3$/µl, and 2 (IQR: 0-7) $x10^3$/µl respectively. As also indicated in Table 4.6, the results showed that there were significant low levels in the median of absolute WBC, lymphocyte and CD4+ T cell counts (P= 0.046, 0.043 and 0.003 respectively) among malaria co-infected patients as compared to those with single infections of malaria and TB alone.

The absolute CD3, CD4, and CD8 were 2.5 (IQR: 1.8-3.3) $x10^3$, 0.3 (IQR: 0.1-1.4) $x10^3$, and 0.7 (IQR: 0.5-1.0) $x10^3$ per millimeter cube respectively. These parameters did not vary by whether the participant had a co-infection or not (Table 4.6). The median ratio of CD4 to CD8 was 0.33 (IQR: 0.2-0.49). This ratio was similar for both the co-infected group and the group with TB only.

Table 4.6: Hematological and parasitological levels of the study participants

Variable	Malaria+ve	TB+ve	Mal+ve/TB+ve	*P-value*
No. of Subjects (n=485)	**101**	**64**	**320**	**N/A***
Hematologic levels (Median [IQR])				
Haemoglobin (g/dl)	10.4(8.9-11.7)	12.3(10.7-13.7)	10.2(8.7-11.4)	0**.0001**
WBC (×10^3/µl)	8.1(6.5-10.7)	9.0(6.3-12.3)	8.0(6.6-10.5)	0.046
Neutrophil (×10^3/µl)	45(37-62)	46(37-62.5)	45(37-62)	0.052
Lymphocyte (×10^3/µl)	4.1(2.1-7.8)	4.5(1.9-7.7)	4.3(2.1-7.8)	0.043
Monocyte (×10^3/µl)	0(0-2)	0(0-1)	0(0-1)	0.412
Eosinophil (×10^3/µl)	2(0-7)	1.5(0-6)	2(1-7)	0.262
Basophil (×10^3/µl)	0.05(0.04–0.06)	0.06(0.04–0.07)	0.08(0.06–0.11)	0.387
Absolute CD3 (x10^3)	2.5(1.8-3.3)	1.36(0.98-1.87)	1.09(0.42-2.08)	**0.001**
Absolute CD4 (x10^3)	0.3(0.1-1.4)	0.82(0.60-1.26)	0.23(0.21-1.41)	**0.003**
Absolute CD8 (x10^3)	0.7(0.5-1.0)	0.61(0.38-0.88)	0.60(0.21-1.48)	0.560
CD4/CD8	0.33(0.2-0.49)	1.4(1.0-2.2)	0.38(0.23-2.10)	**0.010**
Parasitologic and bacteremic density (Median [IQR])				
Parasitemia (x10^3/µl /µl)	56 (35-98)	N/A	65 (33-90)	**0.023**
AFBs (10-99/100Field)	1 (0-2)	1 (0-2)	1 (0-2)	**0.002**

N/A*****= Not applicable; IQR = inter quartile range

In determination of how malarial infection negatively affects immunity against MTBC infection, the Th1 (IFN-γ and IL-12p40), Th2 (IL-4 andIL-5), pro-inflammatory (IL-6 and IL-8) and anti-inflammatory (TGF-β and IL-10) cytokines were measured by use of ELISA in 72 hour old PBMC culturesupernatants from 320 co-infected (test) and 64 TB alone (control) patients.

The cytokine levels in the control patients and the test patients were compared in both unstimulated and stimulated samples and the results were as presented in Table 4.7. As indicated, in response to PPD antigen stimulation, a significant ($P<0.05$) increased levels of IL-4, IL-5, IL-6, IL-8, IL-10 and TGF-β, anda significant ($P<0.05$) decreased IFN-γ and IL-12p40 were seen in test (co-infected) patients compared to control (TB alone) (Table 4.7).

Table 4.7: Comparison of various cytokine levels among the participants infected with TB alone and co-infected with malaria.

	Unstimulated		PPD stimulated		
Variable	**MTB+&M+ve**	**TB+ve**	**MTB+&M+ve**	**TB+ve**	***P-value***
Th1 cytokines (pg/ml) median and the corresponding IQR					
IFN-γ	6.3(6.2-6.3)	10(9.5-10.5)	20(17.5-23.0)	27.5(27.45-27.65)	0.0001
IL-12p40	250(235-275)	505(495-515)	1750(1745-1755)	1895(1890-1900)	0.002
Th2 cytokines (pg/ml) median and the corresponding IQR					
IL-4	11.3(11.25-11.35)	7.5(7.4-7.6)	31.25(31.2-31.3)	30(27.5-35.0)	<0.0001
IL-5	95(90-100)	65(60-70)	115(105-125)	85(75-95)	0.05
Pro-inflammatory cytokines (pg/ml) median and the corresponding IQR					
IL-6	160(155-160)	155(154-155)	180(175-185)	175(170-180)	0.04
IL-8	475(470-475)	425(420-425)	495(490-500)	445(440-450)	0.015
Anti-inflammatory cytokines (pg/ml) median and the corresponding IQR					
TGF-β	1000(995-1150)	750(725-760)	1395(1390-1400)	1145(1140-1150)	0.001
IL-10	1250(1245-1255)	750(745-755)	1290(1285-1295)	770(770-775)	0.0001

IQR = inter quartile range; MTB+&M+ve= *M. tuberculosis* positive and malaria positive; TB+ve = tuberculosis positive

A significant increased in IL-10 and TGF-βresponse in malaria and TB co-infectedcases (Figure 4.2) was observed. TGF-β and IL-10 are inhibitory cytokines. The study suggests that malaria modulate the various cytokine responses to*M. tuberculosis* antigens, which may influence the cellular and humoral immune responses to*M. tuberculosis* infection in a malaria co-infected host.

In an attempt to determine how malarial infection negatively influence the outcome of BCG vaccination against mycobacterial infection, Th1 (IFN-γ and IL-12p40), Th2 (IL-4 and IL-5), pro-inflammatory (IL-6 and IL-8) and anti-inflammatory (TGF-β and IL-10) T cell responses following BCG vaccination were examined in malarial infected patients and compared the results to those of the controls. One hundred and one (101) tuberculin skin test negative individuals in both singly infected malaria cases and control subjects were given BCG vaccination andPPD antigen specific responses [Th1 (IFN-γ and IL-12p40), Th2 (IL-4 and IL-5), pro-inflammatory (IL-6 and IL-8) and anti-inflammatory (TGF-β and IL-10)] were measured by ELISA in 72 hour old PBMC culture supernatants. The PPD specific responses of the various cytokines were measured prior to and 8 weeksafter vaccination. An increase in IL-6, IL-8, IL-10, TGF-β level and a decrease in IFN-γ and IL-12p40 level in response to PPD antigen cell stimulation were observed in malaria positive patients whencompared to controls. In malaria positive patients and controls, a significantincrease in the cytokine level of IL-6, IL-8, IL-10, IFN-γ,IL-12p40, IL-5 ($p \leq 0.05$) was observed in PPD antigen stimulated cultures compared to unstimulated PBMC cultures (controls).

Levels of IFN-γ produced in the culture supernatants of PBMC from malaria infected and control group were measured and it was shown that there were significantly reduced amounts of IFN-γ levels in the malaria infected group in response to PPD antigen stimulation compared with their controls (Figure 4.2, P = 0·04 and 0·03, respectively). In figure 4.2 vaccinees in the control and malaria infected groups were compared with regard to the change in the levels of IFN-γ secretion in response to PPD after BCG vaccination with reference to prevaccination levels. The control group produced a significantly greater amount of IFN-γ after BCG vaccination compared with prevaccination levels (P = 0·03), in the malaria infected group the differences were also significant.

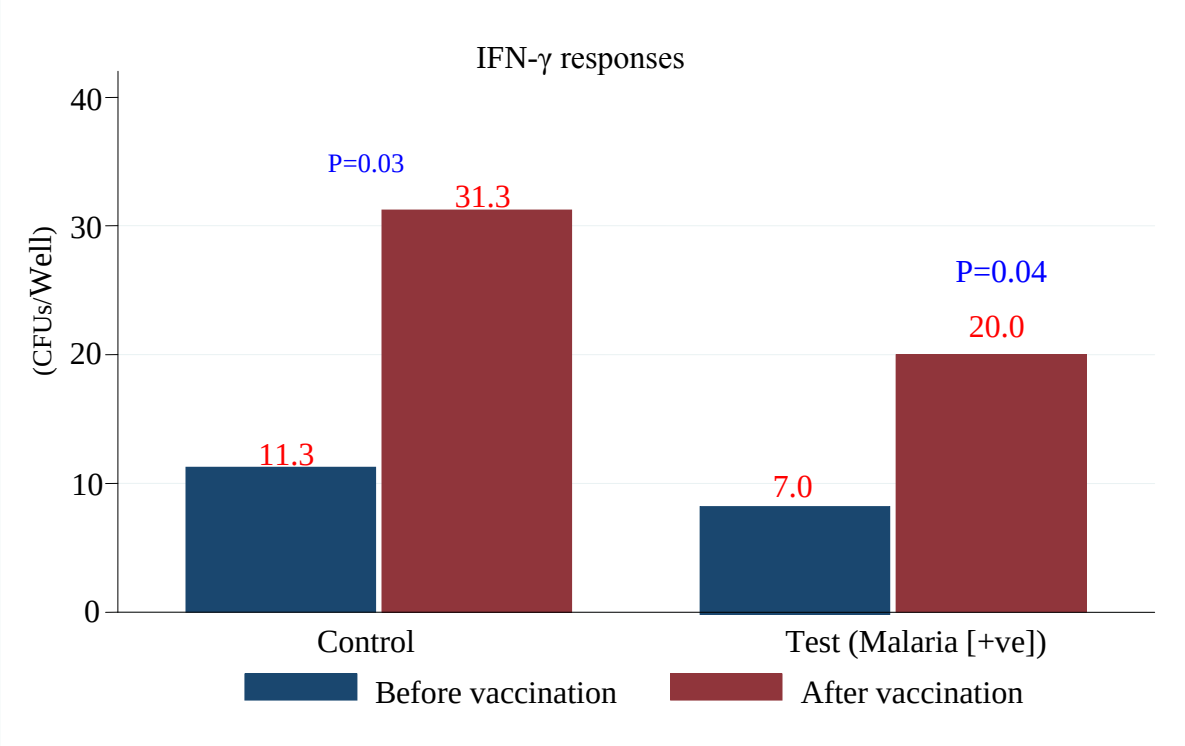


Figure 4.2: *In vitro* IFN-γ responses in malaria infected individuals vaccinated.

Levels of IL-12p40 produced in the culture supernatants of PBMC from malaria infected and control group were measured and it was shown that there were significantly reduced amounts of IL-12p40 levels in the malaria infected group in response to PPD antigen stimulation compared with their controls (Figure 4.3, P = 0·02 and 0·01, respectively). Further analysis of IL-12p40 showed that the malarial infected group had significantly lower PPD-induced IL-12p40 proliferative responses compared with the levels or the responses of control group (Figure 4.3, P=0·02) showing significant differences in the pre- and post-vaccination proliferative responses of PBMC from the malaria infected group.

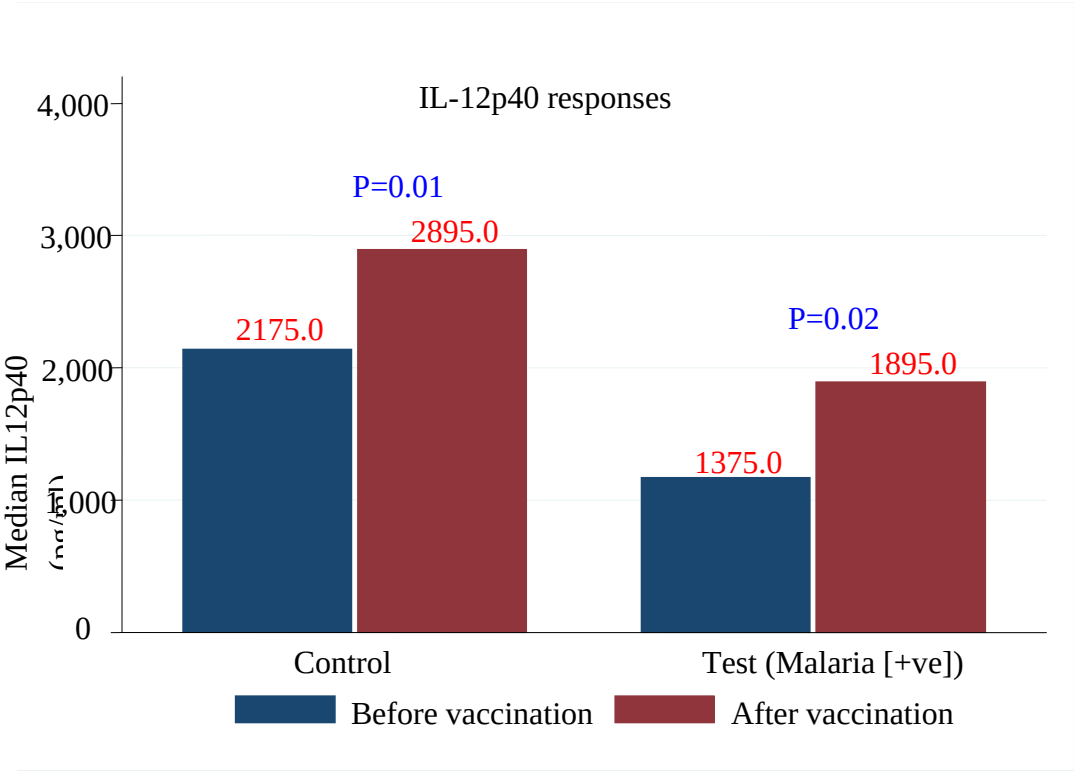


Figure 4.3: *In vitro* IL-12p40 responses in malaria infected individuals vaccinated.

When IL-4 was analysed, it was found that the BCG vaccinated malaria infected group had significantly higher levels of PPD-induced IL-4 responses compared to the levels in the controls (prevaccination levels or/and the responses of control group post-vaccination) (Figure 4.4, P = 0·001 and 0·02, respectively). The same trend was seen in the case of IL-5 proliferative responses (Figure 4.5).

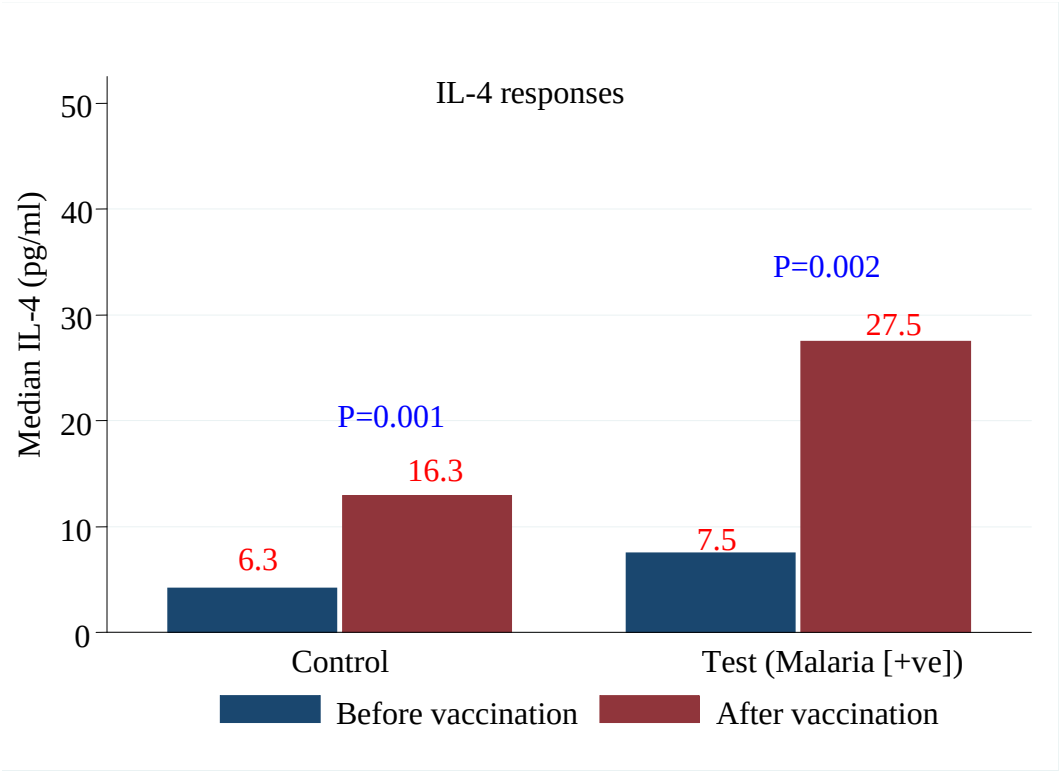


Figure 4.4: *In vitro* IL-4 responses in malaria infected individuals vaccinated.

IL-5 proliferative cytokineswere measured prior to and 8 weeks after BCG vaccination. These were measured by ELISA in 72hour old PPD stimulated and unstimulated peripheral blood mononuclear cell culturesupernatants from 101malaria positive and control subjects and the results showed (Figure 4.5) that PPD specific IL-5 levels were increasedafter BCG vaccination in individuals infected with malaria compared to the control groups.

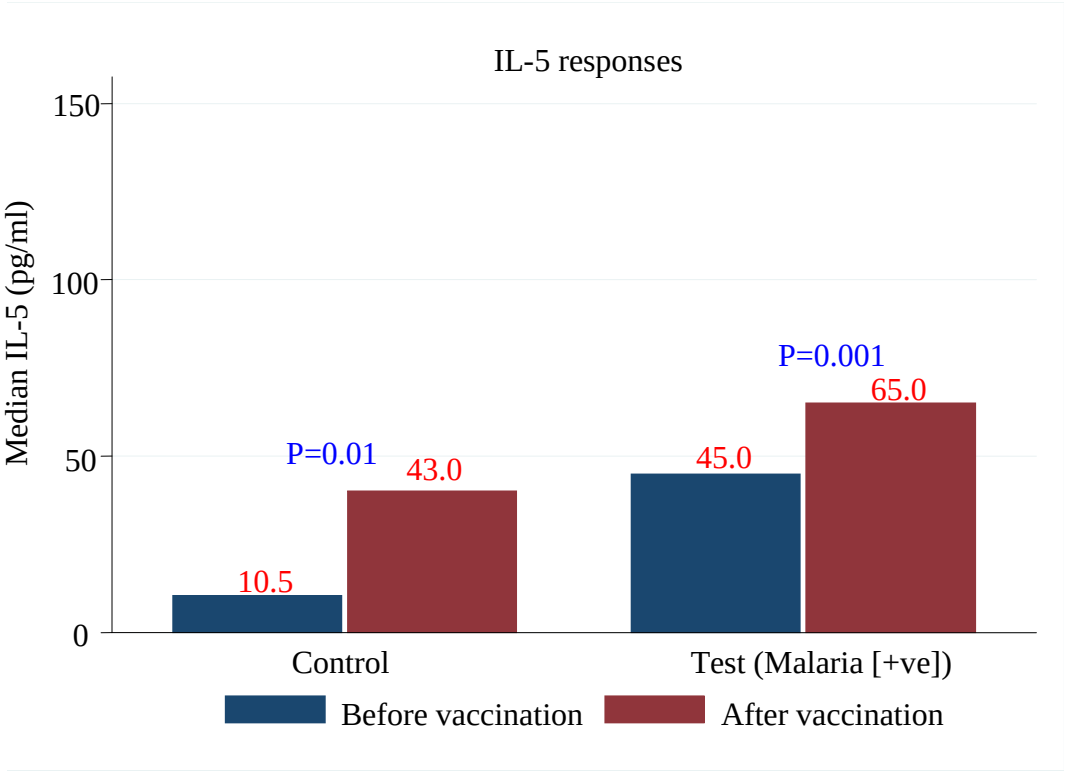


Figure 4.5: *In vitro* IL-5 responses in malaria infected individuals vaccinated.

IL-6 cellular responses to PPD after BCG vaccination showed (Fig. 4.6) that the proliferative response to PPDantigen was significantly decreased in the malaria infected group when compared to the control group (*P*< 0·001).The results, therefore,shows that the median IL-6 cytokines levels measured by ELISA in 72 hour old PBMC culture supernatants after BCG vaccination was significantly high in PBMC obtained before vaccination from malaria infected group as compared to those obtained after BCG vaccination. This trend was seen in both PBMC obtained from the participants infected with malaria and in the control groups.

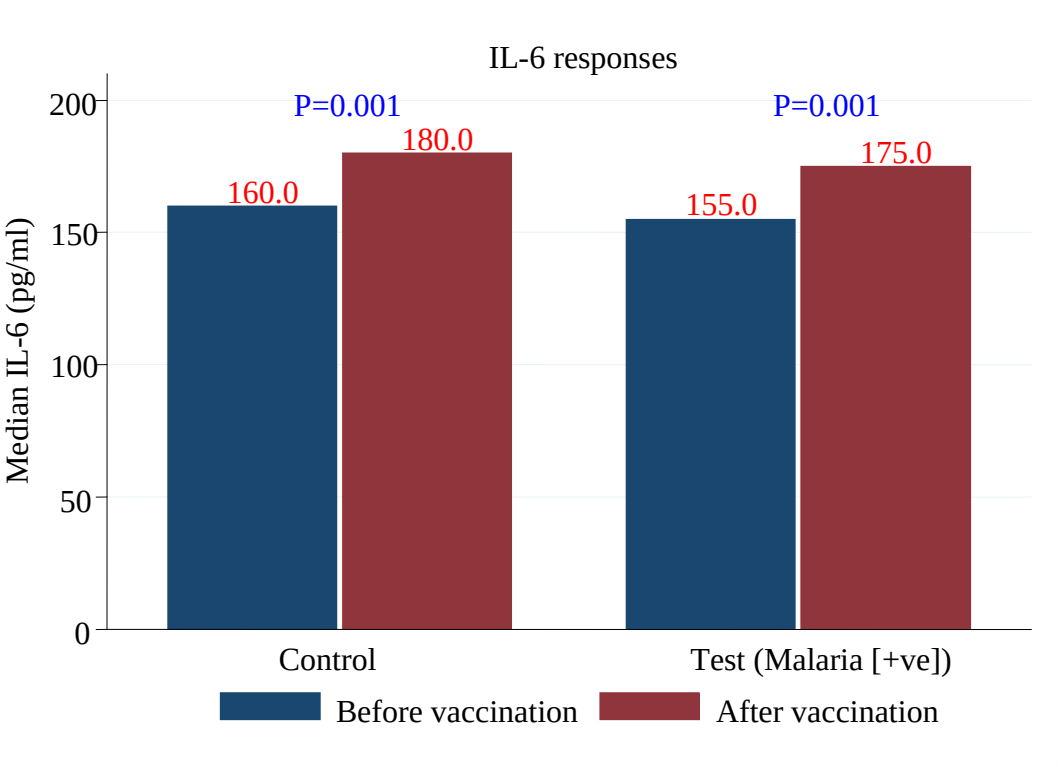


Figure 4.6: *In vitro* IL-6 responses in malaria infected individuals vaccinated.

Levels of IL-8 produced in the culture supernatants of PBMC from malaria infected and control group were measured. After BCG vaccination, significantly higher amounts of IL-8 were measured in the control group in response to PPD antigen stimulation compared with their malaria infected counterparts (Figure 4.7, P = 0·05 and 0·05, respectively). There were decreased levels of IL-8 produced in the culture supernatants of PBMC from BCG vaccinated malaria infected group compared to those of the control group. Vaccinees in the control and malaria infected groups were compared with regard to the change in the levels of IL-8 secretion in response to PPD after BCG vaccination with reference to prevaccination levels (Figure 4.7). The control group produced a significantly greater amount of IL-8 after BCG vaccination compared with prevaccination levels (P< 0·0001), in the malaria infected group the differences were also significant.

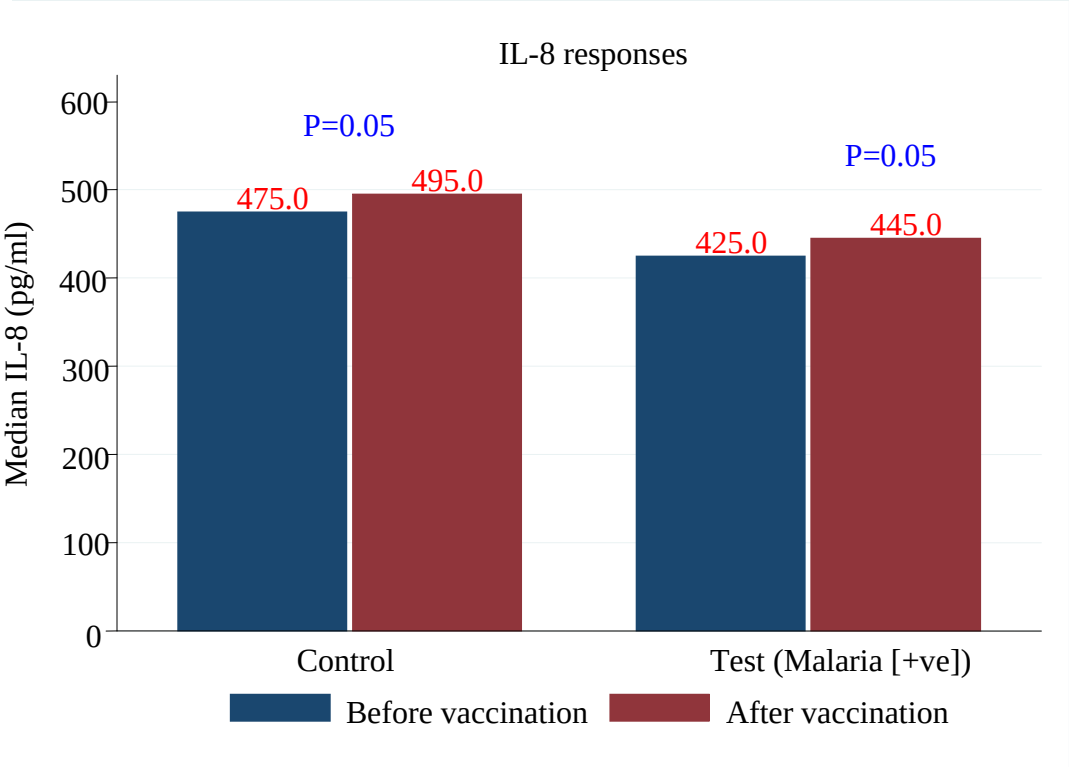


Figure 4.7: ***In vitro*** **IL-8 responses in malaria infected individuals vaccinated.**

A decrease in IL-10 and TGF-β level in response to PPD antigen stimulation observed in BCG vaccinated malaria infected patients whencompared to normal controls (Figure 4.8 and Figure 4.9) shows that malaria do affect the immunogenicity of BCG vaccination. This was also observed by the fact that there was low TB antigen specific Th1 type responses in malaria infected patients compared to controls.

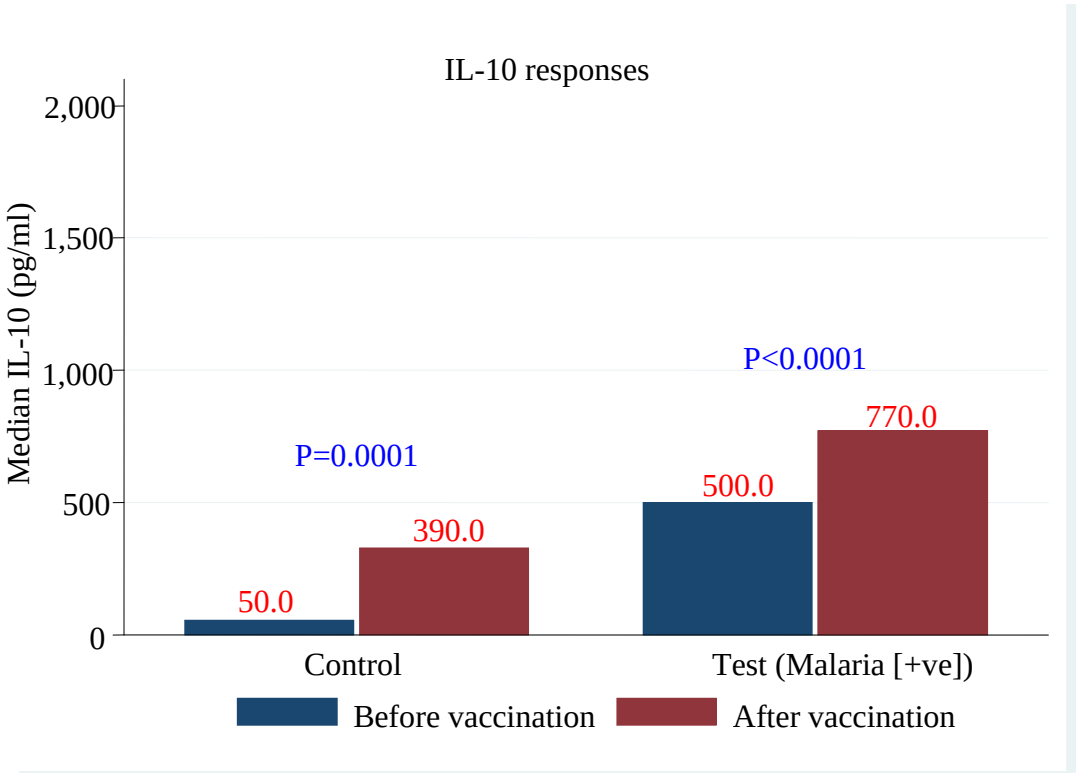


Figure 4.8: *In vitro* IL-10 responses in malaria infected individuals vaccinated.

Levels of TGF-β produced in the culture supernatants of PBMC from malaria infected and control group were measured and compared. After BCG vaccination, significantly higher amounts of TGF-β were measured in the malaria infected group in response to PPD antigen stimulation compared with their control counterparts (Figure 4.9, P = 0·001 and 0·02, respectively). There were decreased levels of TGF-β produced in the culture supernatants of PBMC from BCG vaccinated malaria infected group compared to those of the control group. Vaccinees in the control and malaria infected groups were compared with regard to the change in the levels of TGF-β secretion in response to PPD after BCG vaccination with reference to prevaccination levels (Fig. 4.9). The control group produced a significantly lower amount of TGF-β after BCG vaccination compared with

prevaccination levels (P = 0·02), in the malaria infected group the differences were also significant.

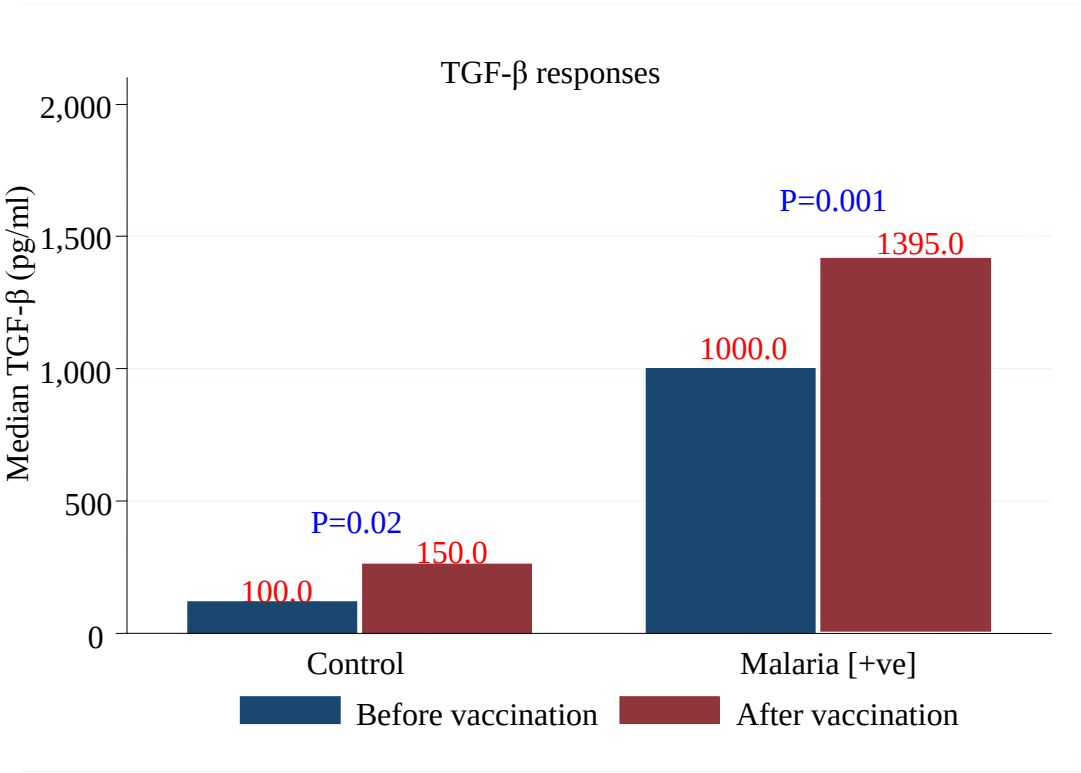


Figure 4.9: *In vitro* TGF-β responses in malaria infected individuals vaccinated.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSIONS

A total of four (4) different genotypes were identified among this small sample size. The most prominent genotype was *M. tuberculosis* (91.4%) followed by *M. africanum* (6.5%) and *M. bovis* BCG (1.8%). If compared with other studies, it is striking that the *M. bovis,* which represents not more than 2% of TB cases in Kenya (Koech, 2001) and 1.4% in world-wide (Cosivi *et al*., 1998; Dankner and Davis, 2000; de Kantor *et al*., 2010; Michel *et al*., 2010; Thoen *et al*., 2010), was not prevalent in Uasin Gishu where it represented only 0.3% of all the strains investigated. This indicates a geographical influence on the distribution of MTBC strains in Western Kenya. Possible reasons, including differences in host-pathogen interaction (Gagneux *et al.,* 2006b), are not yet clear and represent an interesting field for future research. The population of circulating MTBC strains from this region was found to be surprisingly diverse with a total of 4 different genotypes. With 6.5% of all strains investigated, *M. africanum* represent a significant number of cases which appears to be not lower compared to those reported in West Africa (WHO, 2011). The results of MTBC characterization showed that the majority of strains identified belonged to the *M. tuberculosis*. The consequences of this intriguing high MTBC population diversity for the TB epidemic in this high incidence setting as well as on the application of new diagnostic tests or treatment strategies are not yet clear and need to be urgently addressed in further studies. Due to the limited amount of available patient information and the small study sample, we could not perform a deeper analysis of

possible associations between disease characteristics and infection with certain genotypes. However, a striking observation made is that out of 384 MTBC strains two were MDR-TB. Among malaria positive patients, 4 patterns were observed among 320 isolates. The *M. tuberculosis* (91.4%), *M. africanum* (6.5%), *M. bovis* BCG (1.8%) and *M. bovis* (0.3%) were the most common among malaria positive individuals.

With a total of 5.6% of the co-infected patients being resistant to INH alone, the level of drug resistance was found to be high. Resistance to RIF was detected in 4 (1.4%) co-infected patients and MDR-TB was found in 2 patients (0.5%) which are comparable to those of study done in Nairobi Kenya (Ndung'u *et al.,* 2012)which isolated 0.7% MDR-TB resistant. In our study, one MDR-TB isolate was triple resistant with an additional resistance to STR. Of the total MDR isolated, only one (0.3%) was from MTBCcoinfected patient. The data demonstrated that drug resistance rates among the co-infected case in this region is at a level that is comparable with that of other resistance "hot spots" such as those located in Eastern Europe (WHO, 2004a; Cox*et al.,* 2006). These findings argue strongly for a systematic surveillance of drug resistance rates in new as well as previously treated cases to get a clear picture on the actual resistance situation in the region. Systematic susceptibility testing to rapidly detect drug resistance should be implemented in combination with effective treatment adapted to individual resistance profiles. This is essential to avoid an ongoing spread of resistant and MDR-TB strains especially in a particularly vulnerable population with a high malaria rate. The potential danger posed by MDR-TB strains has recently been shown by the longitudinal spread of a highly transmissible MDR-TB strain in the KwaZulu-Natal region which has

finally resulted in the development of extensively drug resistant variants (Pillay and Sturm, 2007).

Hemoglobin (Hb) levels, absolute WBC, lymphocyte, neutrophil and CD4+ T cell counts were significantly ($p<0.001$, 0.046, 0.043, 0.052 and 0.003 respectively) lower in malaria co-infected patients as compared to those with single infections of malaria and TB. Troye-Blomberg *et al.*, (1983) and Whittle *et al*. (1984) showed that malaria- reduction in peripheral lymphocytes, especially of Thelper cells, runs in parallel with the attributable loss of *in-vitro* control of the T cells, and may be to levels encountered in acquired immune deficiency syndrome and that normal control is regained rapidly following cure. Even subclinical malaria, however, may have effects on the important T cell controlling mechanism (Lisse *et al*., 1994) and that this has significant implications in regions where malaria is hyper-endemic and those with asymptomatic parasitaemia form a significant proportion of the population. Our study noted similar results as shown by specific haematological indices which were statistically significant: absolute WBC counts, $p=0.046$; absolute lymphocytes counts, $P=0.043$; absolute neutrophils counts, $P=0.052$, while absolute monocytes counts, $P=0.412$; absolute eosinophils counts, $P=0.262$; and absolute basophils counts, $P=0.387$ we statistically not significant ($P> 0.5$). Malarial infection clearly affects the host's T cells, both qualitatively and quantitatively. Since the containment of *M. tuberculosis* infection depends on an intact cellular immunity, the influence on TB of a malaria-induced impairment of the immune response could be summarised as follows: the decrease in number of the CD4 + T cells (Whittle *et al*., 1984) will decrease the effective Th1 response necessary for TB control. Although Whittle *et al.*, (1984) thought that the malaria-attributable immunosuppression was

transient, with complete recovery of T cell control immediately after the acute episode; Lisse *et al.,* (1994) found that this impairment could be demonstrated in those with asymptomatic parasitaemia. Roussilhon *et al*., (1994) found that some of the responses triggered by malarial infection last for up to 3 months after the disease. By impairing T-cell function, malarial infection may create a window of relative immune ineffectiveness, during which the host may be particularly vulnerable to TB (and other infectious diseases).

The study found out that in malaria and TB co-infected children, there was statistically significant (P=0.003) reduced levels of CD4+ T cells leading to significantly (P=0.01) low CD4/CD8 cell ratios, therefore, some of the features of immune impairment caused by malaria appear to be the same as those seen in AIDS: low CD4/CD8 cell ratios.

Analysis of Th2 cytokine responses (IL-4 and IL-5) in malarial and TB co-infected patients shows that there were high Th2 cytokine levels in the co-infected cases as compared to their controls which hardly made Th2 responses. The study also found out that there was a decreased level of Th1 (IFN-γ and IL-12p40) in co-infected cases as compared to their controls. This indicates that poor resistance against mycobacterial infection is the result of poor Th1 response (Figure 4.2 and 4.3) in malarial infected individuals. Studies elsewhere have shown that one way by which mycobacteria succeed in causing disease is by inducing IL-4 responses in the host which impairs antimycobacterial effector responses and enhances toxicity of TNF-α (Rook *et al.,* 2005a). Consistent with these reports, therefore, the results demonstrated that Th2

cytokine response inducing malarial infection was associated with enhanced increased levels of IL-4 as compared to IL-4 level of controls. The Th2 cytokine IL-4 has been shown to deactivate macrophages, switches of signaling via TLR-2 and potently down regulate iNOS (Bogdan*et al.* 1994) which are crucial for host defense (Rook *et al.*, 2005a). Moreover it has been shown in the mouse model as well as in the humans that IL-4 plays a role in tissue damage (van Crevel *et al.,* 2000).

As was shown by the study, by impairing T-cell function, malarial infection may create a window of relative immune ineffectiveness, during which the host may be particularly vulnerable to TB (and other infectious diseases). There was a switch of T-cell response from Th1 to Th2; and the increase in IL-4 and IL-10 production, concomitant with decrease of IL-12p40 and IFN-γ. The study suggested that the immune impairment caused by malaria led to low CD4/CD8 cell ratios; a switch of T-cell response from Th1 to Th2; and the increase in IL-4 and IL-10 production, concomitant with loss of IFN-γ. If these changes are maintained for a reasonable time, any incompletely controlled TB lesions in the host might be re-activated. The TST is a delayed hypersensitivity reaction which is T-cell dependent (Udani, 1997*b*; Hayne and Fauci, 1998). As malarial co-infected TB patients and the associated anaemia are known to cause a depression in T-cell function, they might depress the host's response to tuberculin antigen. Proliferation of lymphocytes to PPD is decreased by anaemia and during the malaria season. A false-negative result for the Mantoux test may therefore be more likely in those with malarial infection than those without.

The BCG skin test is another test that is of increasing usage in the diagnosis of TB (WHO, 1963). The basis for this test is the accelerated response by the T cells to bacilli, upon presentation by macrophages. These T cells also stimulate naive and memory Th cells to recognize proteins which can only be secreted by live bacilli, leading to a positive result. The study showed that malarial infection affects the quality and quantity of Thelper cells (Th1 and Th2), and it may also leads to loss of memory T-cells, hence leading to sub-optimal response to the BCG test.

The Mantoux and BCG skin tests are important ancillary tools in the identification of people with mycobacterial infection. There are two stages in the maturation of developing T- and B-lymphocytes (Male *et al.* 2006). The first is independent of antigenic stimulation. The second, which takes place in the peripheral lymphoid organs, is dependent on an activation step, often involving lymphocyte specific receptors and antigenic or anti-idiotypic stimulation (Male *et al.* 2006). Through clonal expansion of the stimulated cells, the immune response is able to adapt to environmental stimuli. Priming of foetal cells by antimalarial antibodies transferred from the mother may therefore affect the response of the child to other antigens, such as those of *M. tuberculosis*. Some of the antilymphocyte antibodies resulting from maternal malaria and transferred to the foetus via the placenta may also depress the response of the newborn to BCG immunisation, which is usually given soon after birth.

5.2 CONCLUSIONS

The population of MTBC strains circulating in this region was found to be surprisingly diverse with a total of 4 different genotypes. *M. africanum* is a common cause of TB in West Africa (de Jong *et al.,* 2010). In Uasin Gishu County, *M. tuberculosis* strains co-circulate with *M. africanum* in greater percentage. Identification of *M. africanum* in patients was more likely to be incoinfected and the mere fact it is present causes a reason to worry given the fact it has similarities with *M. bovis*. With 6.5% of all strains investigated, *M. africanum* represence a significant number of cases which appears to be not lower compared to those reported in West Africa (WHO, 2011). However, the majority of strains belonged to the *M. tuberculosis* which was comparable to the results obtains by a study conducted by Gagneux *et al.,*(2006). The consequences of this intriguingly high MTBC population diversity for the TB epidemic in this high incidence setting as well as for the application of new diagnostic tests or treatment strategies are not yet clear and need to be urgently addressed in further studies.

The study demonstrated a high level of drug resistance among malaria and TB coinfected patients in particular resistance to INH in the Uasin Gishu and Huruma district hospitals in Uasin Gishu County. This demonstrated the need for implementing intergrated routine drug susceptibility testing for all culture positive cases and improved public health measures to improve the control of the spread of drug resistant and MDR strains in the region among coinfected patients.

Malaria depresses the levels of Hb concentration, absolute WBC, lymphocytes, Neutrophils and CD4+ T cell counts which were found to be ($p<0.001$, 0.046, 0.043, 0.052 and 0.003 respectively) lower in children co-infected with malaria and TB as compared to those with single infection of malaria and TB. In childrenanaemia, one of the complications of malaria is a known risk factor for progressive TB disease because it causes immuno-incompetence (Udani, 1997*a*, *b*). In conclusion therefore, the Hb finding supports the notion that malaria may be one of the important factors that affect the progression of TB disease. The available literature on malaria and TB also seems to indicate that repeated malarial infection or prolonged asymptomatic parasitaemia (by causing qualitative as well as quantitative depression of host's immune mechanisms-including those related to containment of TB) causes increased susceptibility to, and re-activation and faster progression of TB.

The study found out that in malaria and TB co-infected children, there was a statistically significant reduced level of CD4+ T cells leading to low CD4/CD8 cell ratios.

The study showed that there is incontrovertible evidence showing the effect of malaria on the prevalence of TB. The effects of T cells by malarial infection suggest that BCG immunization during the malaria season could produce minimal protection because of poor response to the vaccine.Therefore part of the problem of TB control in malaria-endemic areas may be that the immunological mechanisms of TB exacerbation occur in malaria, albeit transiently. Since TB is a chronic disease, it is difficult to determine whether or not malarial infection preceded the development of active TB. However, given the observation that malaria modulate immunity by enhancing regulatory T cell

activity and as a consequence increase in Th2 type responses, it may be more plausible to think that it is the malarial infection which predisposes people to getting active TB in malaria endemic region. This possible interaction demands further clarification.

5.3 RECOMMENDATIONS

i) Search for better diagnostic tools with respect to *M. africanum* and *M. bovis*.

M. bovis and *M. africanum* share similarities which often make accurate differentiation harder with conventional methods. This calls for a search for better diagnostic tools with *M. africanum* becoming more relevant for pulmonary TB prevention and control in Kenya.

ii) Search for a better screening tools with respect to mycobaterial infection in malaria endemic region.

The TST is a delayed hypersensitivity reaction which is T-cell dependent. As malaria associated anaemia are known to cause a depression in T-cell function, they might depress the host's response to tuberculin antigen.A false-negative result for the Mantoux test may therefore be more likely in those with malarial infection than those without.

iii) As the world attempts to control malaria and TB, there should be an increased integration of the control of malaria and TB diseases within the same programmes because this will afford a win-win situation in curbing the severity and transmission in co-infected patients.

REFERENCES

Algood, H. M., Chan, J., & Flynn, J. L. (2003).Chemokines and tuberculosis, Cytokine Growth Factor *Rev (Band 14), Nr*. 6, Seite 467-77.

Aly, A.S., Vaughan, A.M., & Kappe, S.H. (2009). Malaria parasite development in the mosquito and infection of the mammalian host, *Annu Rev. Microbiol* 63:195–221.

Anya, S.E. (2004). Seasonal variation in the risk and causes of maternal death in the Gambia: malaria appears to be an important factor. *Am J Trop Med Hyg,* 70: 510-3.

Aribot, G., Rogier, C., Sarthou, J. L., Trape, J. F., Balde, A. T., Druilhe, P., & Roussilhon, C. (1996). Pattern of immunoglobulin isotype response to *Plasmodium falciparum* blood stage antigens in individuals living in a holoendemic area of Senegal. *American Journal of Tropical Medicine and Hygiene*, 54, 449± 457.

Arriaga, A.K., Orozco, E.H., Aguilar, L.D., Rook, G.A., & Hernandez, R. (2002). Immunological and pathological comparative analysis between experimental latent tuberculous infection and progressive pulmonary tuberculosis. *Clin Exp Immunol* 128: 229–237.

Belkaid, Y., & Rouse, B.T. (2005). Natural regulatory T cells in infectious disease. *Nat Immunol* 6: 353-60.

Berrington, W.R., & Hawn, T.R. (2007). *Mycobacterium tuberculosis*, macrophages, and the innate immune response: does common variation matter? *Immunol Rev*. 219:167–186.

Bhattacharyya, S., Singla, R., Dey, A. B., & Prasad, H. K. (1999). Dichotomy of cytokine profiles in patients and high-risk healthy subjects exposed to tuberculosis, *Infect Immun* (Band 67), Nr. 11, Seite 5597-603.

Bogdan, C., Vodovotz, Y., Paik, J., Xie, Q. W., & Nathan, C. (1994). Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J Leukoc Biol* 55:227-33.

Boom, W. H., Wallis, R. S., & Chervenak, K. A. (1991). Human *Mycobacterium tuberculosis*-reactive CD4+ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes, *Infect Immun* 59 (8): 2737-43.

Boudin, C., Chumpitazi, B., DziegieL, M., Peyron, F., Picot, S., Hogh, B., & Ambroise-Thomas, P. (1993). Possible role of specific immunoglobulin M antibodies to *Plasmodium falciparum* antigens in immunoprotection of humans living in a hyperendemic area, Burkina Faso.*Journal of Clinical Microbiology*, 31, 636± 641.

Brown, K. M., & Kreier, J. P. (1982). *Plasmodium berghei* malaria: blockage by immune complexes of macrophages receptors for opsonized plasmodia. *Infection and Immunity*, 37, 1227± 1233.

Brown, W. C., & Rice-Ficht, A. C. (1994). Use of helper T cells to identify potential vaccine antigens of *Babesia bovis. Parasitology Today*, 10, 145± 149.

Canaday, D. H., Wilkinson, R. J., Li, Q., Harding, C. V., Silver, R. F., & Boom, W. H. (2001). CD4(+) and CD8(+) T cells kill intracellular *Mycobacterium tuberculosis* by a perforin and Fas/Fas ligand-independent mechanism, *J Immunol* (Band 167), Nr. 5, Seite 2734-42.

Caruso, A. M., Serbina, N., Klein, E., Triebold, K., Bloom, B. R., & Flynn, J. L. (1999). Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis, *J Immunol* 162 (9): 5407-16.

Chackerian, A.A., Perera, T.V., & Behar, S.M. (2001). Gamma interferon-producing CD4+ T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. *Infect Immun* 69: 2666–2674.

Chan, J., & Kaufmann, S. H. E. (1994). Immune mechanism of protection. In *Tuberculosis: Pathogenesis, Protection and Control*, ed. Bloom, B. R. pp. 392± 399. Washington, DC: ASM Press.

Cheesbrough, M. (2006). District laboratory practice in tropical countries Part 2. 2nd Edition, Cambridge University Press, London 319–321.

Cherise, P.S., Nirbhay, K., Bishai, W.R., & Manabe, Y.C. (2004). Short Report: Modulation of *Mycobacterium tuberculosis* infection by plasmodium in the murine model, *Am. J. Trop. Med. Hyg.*, 70(2), pp. 144–148.

Chumptitazi, B. F., Lepers, J. P., Simon, J., & Deloron, P. (1996). IgG1 and IgG2 antibody responses to *Plasmodium falciparum* exoantigen correlated inversely and positively, respectively to the number of malaria attacks. *FEMS Immunology and Medical Microbiology*, 14, 151± 158.

Cirimotich, C.M., Dong, Y., Garver, L.S., Sim, S., & Dimopoulos, G. (2010). Mosquito immune defenses against Plasmodium infection.*Dev. Compar. Immuno* 34:387–395.

Cohen, S. (1986). Immunology of malaria.*Postgraduate Doctor*, 8, 151± 155.

Cole, S.T., Brosch ,R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier K., Gas S., Barry, C.E. 3rd., Tekaia, F., Badcock, K., Basham, D., Brown, D., Chillingworth, T., Connor, R., Davies, R., Devlin, K., Feltwell, T., Gentles, S., Hamlin, N., Holroyd, S., Hornsby, T., Jagels, K., Krogh, A., McLean, J., Moule, S., Murphy, L., Oliver, K., Osborne, J., Quail, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S., & Barrell, B.G. (1998). Deciphering the

biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393(6685):537-44.

Colombatti, R., Martina, P., Federica, B., Cesaltina, S.V., Antonia, A.L., Fina, V., Simone, T., Carlo, G., & Fabio, R. (2011). Malaria prevention reduces in-hospital mortality among severely ill tuberculosis patients: a three-step intervention in Bissau, Guinea-Bissau. *BMC Infectious Diseases*, 11:57.

Comas, I., Chakravartti, J., Small, P.M., Galagan, J., Niemann, S., Kremer, K., Ernst, J.D., & Gagneux, S. (2010). Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. *Nat Genet* 42(6):498-503.

Cooper, A. M., Dalton, D. K., Stewart, T. A., Griffin, J. P., Russell, D. G., & Orme, I. M. (1993). Disseminated tuberculosis in interferon gamma gene-disrupted mice, *J Exp Med* (Band 178), Nr. 6, Seite 2243-7.

Corbett, E.L., Watt, C.J., Walker, N., Maher, D., Williams, B.G., Raviglione, M.C., & Dye, C. (2003). The growing burden of tuberculosis: global trends and interactions with the HIV epidemic *Arch Intern Med,* 163 (2003), pp. 1009–1021.

Corbett, E.L., Marston, B., Churchyard, G.J., & De Cock, K.M. (2006). Tuberculosis in sub-Saharan Africa: opportunities, challenges, and change in the era of antiretroviral treatment. *Lancet*, 367:926-937.

Cosivi, O., Grange, J.M., Daborn, C.J., Raviglione, M.C., Fujikura, T., Cousins, D., Robinson, R.A., Huchzermeyer, H.F.A.K., de Kantor, I., & Meslin, F.X. (1998). Zoonotic Tuberculosis due to Mycobacterium bovis in Developing Countries. *Emerging Infectious Diseases*, 4(1).

Cox, H.S., Orozco, J.D., Male, R., Ruesch-Gerdes, S., Falzon, D., Small, I., Doshetov, D., Kebede, Y., & Aziz, M. (2004). Multidrug-resistant tuberculosis in central Asia.*Emerg Infect Dis*, 10:865-872.

Dankner, W.M., & Davis, C.E. (2000). *Mycobacterium bovis* as a significant cause of tuberculosis in children residingalong the United States–Mexico border in the Baja California region. *Pediatrics* 105, E79.

Day, N. P., Hien, T. T., Schollardt, T., Loc, P. P., Chuong, L.V., Chau, T. T. H., Mai, N. T. H., Phu, N. H., Sinh, D. X., White, N. J., & Ho, M. (1999). "The prognostic and pathophysiologic role of pro- and anti-inflammatory cytokines in severe malaria." *J Infect Dis* **180**(4): 1288-1297.

de Jong, B.C., Hill, P.C., Brookes, R.H., Gagneux, S., Jeffries, D.J., Out, J.K., Donkor, S.A., Fox, A., McAdam, K.P., Small, P.M., & Adegbola, R.A. (2006). *Mycobacterium africanum* elicits an attenuated T cell response to early secreted antigenic target, 6 kDa, in patients with tuberculosis and their household contacts. *J Infect Dis*, 193:1279-1286.

de Jong, B.C., Ifedayo, A., Brigitte, W., Philip, C. H., Martin, A., Martin, O., & Richard, A. A. (2010). Differences between TB cases infected with *M. africanum*, West-African type 2, relative to Euro-American *M. tuberculosis*-an update *FEMS Immunol Med Microbiol;* 58(1): 102–105.

de Kantor, I. N., LoBue, P. A., & Thoen, C. O. (2010). Human Tuberculosis Caused by *Mycobacterium bovis* in the United States, Latin America, and the Caribean. *International Journal on Tuberculosis and Lung Disease* 14(11): 1369-1373.

de Waal, M. R., Abrahams, J., Bernett, B., Figdor, C. G., &de Vries, J. E. (1991). Inerleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes. An autoregulatory role of IL-10 produced by monocytes.*J Exp Med*, 174: 1209-20

Denis, M. (1991). Killing of *Mycobacterium tuberculosis* within human monocyte: activation by cytokines and calcitrol. *Clinical and Experimental Immunology*, 84, 220± 226.

Dgedge, M., Novoa, A., Macassa, G., Sacarlal, J., Black, J., Michaud, C., & Cliff, J. (2001). The burden of disease in Maputo City, Mozambique: registered and autopsied deaths in 1994. *Bull World Health Organ;* 79: 546-52.

Dheda, K., Schwander, S.K., Zhu, B., van Zyl-Smit, R.N., & Zhang, Y. (2010). The immunology of tuberculosis: from bench to bedside. *Respirology* 15(3):433-50.

Dieli, F., Caccamo, N., Meraviglia, C., & Salerno, A. (2004).Reciprocal stimulation of gamma delta T cells and dendritic cells during the anti-mycobacterial immune immune response. *Eur J Immunol* 34:3227-35.

Dye, C., Scheele, S., Dolin, P., Pathania, V., & Raviglione, M.C. (1999). For the WHO Global Surveillance, Monitoring Project Global burden of tuberculosis: estimated incidence, prevalence and mortality by country *JAMA*, 282 (1999), pp. 677–686.

Elias, D., Akuffo, H., & Britton, S. (2005). PPD induced in vitro interferon gamma production is not a reliable correlate of protection against *Mycobacterium tuberculosis. Trans R Soc Trop Med Hyg* 99:363-8.

Ellner, J. J. (2010). Immunoregulation in TB: observations and implications. *Clin Transl Sci,* 3: 23-8.

Elkhalifa, M.Y., Ghalib, H.W., Dafa'Alla, T., & Williams, J.F. (1991). Suppression of human lymphocyte responses to specific and nonspecific stimuli in human onchocerciasis. *Clin Exp Immunol.* 86:433–439.

English, M., Esamai, F., Wasunna, A.,Were, F., Ogutu, B., Wamae, A., Snow, P.R.W., & Peshu, N. (2004a). "Assessment of inpatient paediatric care in first referral level hospitals in 13 districts in Kenya," *Lancet*, vol. 363, no. 9425, pp. 1948–1953.

English, M., Esamai, F., Wasunna, A., Were, F., Ogutu, B., Wamae, A., Snow, P.R.W., & Peshu, N. (2004b). "Delivery of paediatric care at the first-referral level in Kenya," *Lancet*, vol. 364, no. 9445, pp. 1622–1629.

Enwere, G.C., Ota, M.O., & Obaro, S.K. (1999). The host response in malaria and depression of defence against tuberculosis. *Ann Trop Med Parasitol*., 93:669-78.

Erard, F., & Le Gros, G. (1994). Th2-like CD8 T cells: their role in protection against infectious diseases. *Parasitology Today*, 10, 313± 315.

Fabri, M., Stenger, S., Shin, D.M., Yuk, J.M., Liu, PT., Lee, H.M., Krutzik, S.R., Jo EK., Bloom, BR., & Modlin,RL. (2011). Vitamin D is required for T cell mediated antimicrobial activity of human macrophages. *Experimental Dermatology* 20(2):176-7.

Fenner, L, Egger, M., Bodme, T., Furrer, H., Ballif, M., Battegay, M., Helbling, P., Fehr, J., Gsponer, T., Rieder, H.L., Zwahlen, M., Hoffmann, M., Bernasconi, E., Cavassini, M., Calmy, A., Dolina, M., Frei, R., Janssens, J.P., Borrell, S., Stucki, D., Schrenzel, J., Böttger, E.C., & Gagneux, S. (2013). HIV Infection Disrupts the Sympatric Host-Pathogen Relationship in Human Tuberculosis. *PLoS Genet.* 9(3):e1003318.

Fenton, M. J., Vermeulen, M. W., Kim, S., Burdick, M., Strieter, R. M., & Kornfeld, H. (1997). Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis, Infect Immun* (Band 65), Nr. 12, Seite 5149-56.

Ferrante, A., Staugas, R. E., Rowan-Kelly, B., Bresatz, S., Kumaratilake, L. M., Rzepczyk, C. M., & Adolf, G. R. (1990). "Production of tumor necrosis factors alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites." *Infect Immun* **58**(12): 3996-4003.

Flynn, J.L., Chan, J., Triebold, K.J., Dalton, D.K., Stewart, T.A., & Bloom, B.R. (1993). An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection.*J Exp Med*, 178:2249-54.

Flynn, J.L., & Chan, J. (2001a). Immunology of tuberculosis, *Annu Rev Immunol* 19: 93–129.

Flynn, J. L., & Chan J. (2001b). "Tuberculosis: latency and reactivation." *Infect Immun* 69 (7): 4195-201.

Flynn, J. L. (2004). Immunology of tuberculosis and implications in vaccine development, *Tuberculosis* (Edinb) (Band 84), Nr. 1-2, Seite 93-101.

Flynn, J.L., & Chan, J. (2005). What's good for the host is good for the bug. *Trends Microbiol.* 13(3):98-102.

Firdessa, R., Berg, S., Hailu, E., Schelling, E., Gumi, B., Erenso, G., Gadisa E., Kiros, T., Habtamu, M., & Hussein, J. (2013). Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. *Emerg Infect Dis.* 19(3):460-3.

Fortune, S.M., Solache, A., Jaeger, A., Hill, P.J., Belisle, J.T., Bloom, B.R., Rubin, E.J., & Ernst, J.D. (2004). *Mycobacterium tuberculosis* inhibits macrophage responses to IFN-γ through myeloid differentiation factor 88-dependent and -independent mechanisms. *J Immunol.* 172(10):6272-80.

Gagneux, S., Burgos, M.V., Deriemer, K., Encisco, A., Munoz,S., Hopewell, P.C., Small, P.M., & Pym, A.S. (2006a). Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*.*PLoS Pathog,* 2:e61.

Gagneux, S., Deriemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S., Nicol, M., Niemann, S., Kremer, K., Gutierrez, M.C., Hilty, M., Hopewell, P.C., & Small, P.M. (2006b).Variable host-pathogen compatibility in *Mycobacterium tuberculosis*.*Proc Natl Acad Sci U S A*, 103:2869-2873.

Gagneux, S., & Small, P.M. (2007). Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development.*Lancet Infect Dis*, 7:328-337.

Garg, A., Barnes, P. F., Roy, S., Quiroga, M. F., Wu, S., Garcia, V. E., Krutzik, S. R., Weis, S. E., & Vankayalapati, R. (2008). Mannose-capped lipoarabinomannan- and prostaglandin E2-dependent expansion of regulatory T cells in human *Mycobacterium tuberculosis* infection, *Eur J Immunol* (Band 38), Nr. 2, Seite 459-69.

Goldsack, L., & Kirman, J.R. (2007). Half-truths and selective memory: Interferon gamma, CD4(+) T cells and protective memory against tuberculosis. *Tuberculosis* 87(6):465-73.

Graham, J. E., & Clark-Curtiss,J. E. (1999). Identification of *Mycobacterium tuberculosis* RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS), *Proc Natl Acad Sci* U S A (Band 96), Nr. 20, Seite 11554-9.

Greenwood, B. M., Bradley, A. K., Blakebrough, I. S., Whittle, H. C., Marshall, T. F., & Gilles, H. M. (1980). The immune response to meningococcal vaccine in an African village.*Transactions of the Royal Society of Tropical Medicine and Hygiene*, 74, 340± 346.

Gutierrez, M.G., Master, S.S., Singh, S.B., Taylor, G.A., Colombo, M.I., & Deretic, V. (2004). Autophagy is a defense mechanism inhibiting BCG and *Mycobacterium tuberculosis* survival in infected macrophages. *Cell* 119(6):753-66.

Guwatudde, D., Nakakeeto, M., & Jones-Lopez, E. C. (2003). "Tuberculosis in household contacts of infectious cases in Kampala, Uganda," *American Journal of Epidemiology*, vol. 158, no. 9, pp. 887–898.

Hawkes, M., Li, X., Crockett, M., Diassiti, A., Conrad Liles, W., Liu, J., & Kain, K.C. (2010). Malaria exacerbates experimental mycobacterial infection *in vitro* and *in vivo*. *Microbes Infect*, 12:864-74.

Haynes, B. F., & Fauci, A. S. (1998). Introduction to the immune system. In *Harrison's Principles of Internal Medicine*, 14th Edn, eds Fauci, A. S., Braunwald, E. and Isselbacher, K. J. pp. 1753± 1776. New York: McGraw-Hill.

Heinzel, F. P. (1994). Interleukin 12 and the regulation of CD4+ T cell subset response during murine leishmaniasis. *Parasitology Today*, 10, 190± 192.

Hernandez-Pando, R., Orozcoe, H., Sampieri, A., Pavon, L., Velasquillo, C., Larriva-Sahd, J., Alcocer, J.M., & Madrid, M.V. (1996). Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis, *Immunology* 89: 26–33.

Hershberg, R., Lipatov, M., Small, P.M., Sheffer, H., Niemann, S., Homolka, S., Roach, J.C., Kremer, K., Petrov, D.A.,& Feldman, M.W. (2008). High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. *PLoS Biol*6(12):e311.

Ho, M., Webster, H. K., Looareesuwan, S., Supanaranoud, W., Philipps, R. E., Hantavanitch, P., & Warrell, D. A. (1986). Antigen specific immunosuppression in human malaria due to *Plasmodium falciparum*.*Journal of Infectious Diseases*, 153, 763± 771.

Ho, M., Schollaardt, T., Schollardt, T., Snape, S., Looareesuwan, S., Suntharasamai, P., & White, N. J. (1998). "Endogenous interleukin-10 modulates proinflammatory response in *Plasmodium falciparum* malaria." *J Infect Dis* **178**(2): 520-525.

Hoft, D.F., Brown, R.M., & Roodman, S.T. (1998). *Bacille Calmette-Guerin* vaccination enhances human gamma delta T cell responsiveness to mycobacteria suggestive of a memory-like phenotype. *J Immunol* 161:1045-54.

Hoffman, S.L., & Doolan, D. L. (2000). Malaria vaccines-targeting infected hepatocytes. *Nat. Med.* 6:1218–1219.

Holland, S.M. (2007). Interferon gamma, IL-12, IL-12R and STAT-1 immunodeficiency diseases: disorders of the interface of innate and adaptive immunity. *Immunol Res* 38:342–346.

Hongoro, C., & McPake, B. (2003). Hospital cost of high-burden diseases: malaria and pulmonary tuberculosis in a high HIV prevalence context in Zimbabwe. *Trop Med Int Health*, 8:242-50.

Hori, S., & Sakaguchi, S. (2004). Foxp3: a critical regulator of the development and function of regulatory T cells. *Microbes Infect* 6:745-51.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Ottesen, E., Sachs, S.E., & Sachs, J. D. (2006). Incorporating a Rapid-Impact Package for Neglected Tropical Diseases with Programs for HIV/AIDS, Tuberculosis, and Malaria: A comprehensive pro-poor health policy and strategy for the developing world *PLoS Med.* 2006 May; 3(5): e102.

Howie, S. E. M., Sommerfield, A. J., Gray, E., & Harrison, D. J. (1994). Peripheral T lymphocyte depletion by apoptosis after CD4 ligation *in vivo*: selective loss of CD44 – and activating memory T cells. *Clinical and Experimental Immunology*, 95, 195± 200.

International Union Against Tuberculosis and Lung Disease, (2010). Desk-Guide for Diagnosis and Management of TB in Children, *International Union Against Tuberculosis and Lung Disease*, Paris, France.

Johnson, J. L.,Ssekasanvu E., Okwera, A., Mayanja, H., Hirsch,C. S., Nakibali, J. G., Jankus, D. D., Eisenach, K. D., Boom, W. H., Ellner, J. J., & Mugerwa, R. D. (2003). Randomized trial of adjunctive interleukin-2 in adults with pulmonary tuberculosis, *Am J Respir Crit Care Med* (Band 168), Nr. 2, Seite 185-91.

Jones, C., Whittaker, E., Bamford, A., & Kampmann, B. (2011). "Immunology and pathogenesis of childhood TB," *Paediatric Respiratory Reviews*, vol. 12, no. 1, pp. 3–8.

Kataaha, P. K., Facer, C. A., & Holborrow, E. J. (1984). *Plasmodium falciparum* products enhance human lymphocyte transformation by Epstein± Barr virus. *Clinical and Experimental Immunology*, 56, 567± 572.

Kaufmann, S. H. (1999). Cell-mediated immunity: dealing a direct blow to pathogens, *Curr Biol* (Band 9), Nr. 3, Seite R97-9.

Kaufmann, S.H. (2002). Recent findings in immunology give tuberculosis vaccines a new boost. *Trends Immunol* 26: 660-7.

Kaufmann, S. H., & McMichael, A. J. (2005). Robert Koch, the Nobel Prize, and the ongoing threat of tuberculosis, *N Engl J Med* (Band 353), Nr. 23, Seite 2423-6

Kaufmann, S. H. (2006). Tuberculosis: back on the immunologists' agenda, *Immunity* (Band 24), Nr. 4, Seite 351-7.

Keller, C. C., Davenport, G. C., Dickson, K. R., Hilter, J. B., Kaplan, S. S., Weinberg, J. B., Kremsner, P. G., Perkins, D, J. (2006). "Suppression of prostaglandin E2 by malaria parasite products and antipyretics promotes overproduction of tumor necrosis factor-alpha: association with the pathogenesis of childhood malarial anemia." *J Infect Dis* **193**(10): 1384-1393.

Kent, P. T., & Kubica G. P. (1985). Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services, Centers for Disease Control, Atlanta, Ga.

Khader, S. A., & Cooper, A. M. (2008). IL-23 and IL-17 in tuberculosis, *Cytokine* (Band 41), Nr. 2, Seite 79-83.

Koech, R. K. (2001). Epidemiology of tuberculosis in humans in Narok district with special interest on *Mycobacterium bovis*. MSc thesis, University of Nairobi.88 pp.

Kumaratilake, L. M., Ferrante, A., Kumaratilake, J. S., & Allison, A. C. (1994). Extraction of intraerythrocytic malaria parasites by phagocytic cells.*Parasitology Today*, 10, 193± 196.

Lammas, D.A., Casanova, J.L., & Kumararatne, D.S. (2000). Clinical consequence of defects in the IL-12-dependent interferon-gamma (IFN-gamma) pathway. *Clin Exp Immunol*; 121:417–25.

Langhorne, J., Meding, S. J., Eichmann, K., &Gillard, S. S. (1989). The response of CD4+ T cell to *Plasmodium chabaudi chabaudi*.*Immunological Reviews*, 112, 71± 94.

Langhorne, J., Quin, S.J., & Sanni, L.A. (2002). Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. *Chem Immunol* 80: 204–228.

Lin, Y., Zhang, M., Hofman, F. M., Gong, J., & Barnes, P. F. (1996). Absence of a prominent Th2 cytokine response in human tuberculosis, *Infect Immun* (Band 64), Nr. 4, Seite 1351-6.

Lin, P.L., & Flynn, J.L. (2010). Understanding latent tuberculosis: a moving target.*J Immunol* 185(1):15-22.

Ling, D. I., Zwerling, A. A., Steingart, K. R., & Pai, M.(2011). "Immune-based diagnostics for TB in children: what is the evidence?" *Paediatric Respiratory Reviews*, vol. 12, no. 1, pp. 9–15.

Lisse, I. M., Aaby, P.,Whittle, H.,& Knudsen, K. (1994). A community study of T lymphocyte subsets and malaria parasitaemia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88, 709± 710.

Lockhart, E., Green, A.M., & Flynn, J.L. (2006). IL-17 production is dominated by gamma delta T cells rather than CD4 T cells during *Mycobacterium tuberculosis* infection. *J Immunol*. 177:4662–4669.

Luty, A. J., Mayombo, J., Lekoulou, F. & Mshana, R. (1994). Immunologic responses to soluble exoantigens of *Plasmodium falciparum* in Gabonese children exposed to continuous intense infection. *American Journal of Tropical Medicine and Hygiene*, 51, 720± 729.

Luty, A. J., Perkins, D. J., Lell, B., Schmidt-Ott, R., Lehman, L. G., Luckner, D., Greve, B., Matousek, P., Herbich, K., Schmid, D., Weinberg, J. B., & Kremsner, P. G. (2000). "Low interleukin-12 activity in severe *Plasmodium falciparum* malaria." *Infect Immun* **68**(7): 3909-3915.

Male, D., Jonathan, B., Roth, D. B., & Roitt, I. (2006). Immunology 7th Edition, Internatinal edition, *Mosby Elsevier*.

Mangan, P.R., Harrington, N.E., O'Quinn, D. B., Helms, W.S., Bullard, D. C., Elson, R. D., Wahl, S. M., Schoels, T. R., & Weaver, C. T. (2006). Transforming growth factor-beta (TGF-β) induces development of the Th17 lineage. *Nature* 441, 231-234.

Marais, B. J., Gie R. P., Schaaf H. S., Hesseling A. C., Obihara C. C., Starke J. J., Enarson D. A., Donald P. R., & Beyers N. (2004). The natural history of

childhood intra-thoracic tuberculosis: a critical review of literature from the pre-chemotherapy era, *Int J Tuberc Lung Dis* (Band 8), Nr. 4, Seite 392-402.

Mendis, K. N., & Carter, R. (1995). Clinical diseases and pathogenesis in malaria.*Parasitology Today*, 11, 2± 15.

Metenou, S., Babu, S., & Nutman, T. B. (2013). Impact of filarial infections on coincident intracellular pathogens: *Mycobacterium tuberculosis* and *Plasmodium falciparum. Curr Opin HIV AIDS.* 2012 May; 7(3): 231–238. doi: 10.1097/COH.0b013e3283522c3d

Michel, A., Müller, B., & van Helden P. (2010). *Mycobacterium bovis* at the animal–human interface: A problem, or not? *Veterinary Microbiology* 140: 371-381.

Miller, L. H., Good, M. F., & Milon, G. (1994). Malaria pathogenesis, *Science*, 264: 1878± 1882.

Mohan, V.P., Scanga, C.A., Yu, K., Scott, H.M., Tanaka, K.E., Tsang, E., Tsai, M.M., Flynn, J.L.,& Chan, J. (2001). Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. *Infect Immun*; 69: 1847-55.

Morcillo, N. (2007). Tuberculosis in children. In: Palomino JC, Leao SC, Ritacco V, editors. Tuberculosis 2007—from basic science to patient care. Bernard Sebastian

Kamps and Patricia Bourcillier Fund; 2007, p. 525–58. Available at: http://www.tuberculosistextbook.com (accessed January 2009.).

Muller, I., Cobbold, S. P., Waldmann, H., & Kaufmann, S. H. (1987). Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells, *Infect Immun* (Band 55), Nr. 9, Seite 2037-41.

Mueller, A.K., Labaied, M., Kappe, S.H., & Matuschewski, K. (2005). Genetically modified Plasmodium parasites as a protective experimental malaria vaccine.*Nature.* 433:164–167.

Mueller, A.K., Deckert, M., Heiss, K., Goetz, K., Matuschewski, & Schluter, D. (2007). Genetically attenuated *Plasmodium berghei* liver stages persist and elicit sterile protection primarily via CD8 T cells. *Am. J. Pathol.* 171:107–115.

Myrvik, Q. N., Leake, E. S., & Wright, M. J. (1984). Disruption of phagosomal membrane of normal alveolar macrophages by the H37RV strain of *Mycobacterium tuberculosis*.*American Review of Respiratory Disease*, 129, 322± 328.

Nagabhushanam, V., Solache, A., Ting, L.M., Escaron, C.J., Zhang, J.Y., & Ernst, J.D. (2003). Innate inhibition of adaptive immunity: *Mycobacterium tuberculosis*-

induced IL-6 inhibits macrophage responses to IFN-gamma. *J Immunol* 171(9):4750-7.

Ndung'u, P., Kariuki, S., Nganga, Z., & Revathi, G. (2012). Resistance patterns of *Mycobacterium tuberculosis* isolates from pulmonary patients in Nairobi *J Infect Dev Ctries* 6(1):33-39.

Nelson, L. J., & Wells, C. D. (2004). "Global epidemiology of childhood tuberculosis," *International Journal of Tuberculosis and Lung Disease*, vol. 8, no. 5, pp. 636–647.

Newport, M.J., Huxley, C.M., Huston, S., Hawrylowicz, C.M., Oostra,B. A., Williamson R., & Levin, M. (1996). A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med.* 335(26):1941-9.

Newton, S. M., Brent, A. J., Anderson, S., Whittaker E., & Kampmann, B. (2008). "Paediatric tuberculosis," *The Lancet Infectious Diseases*, vol. 8, no. 8, pp. 498–510.

Nikolayerskyy, V., Balabanova, Y., Simak, T., Malomanova, N., Fedorin, I., & Drobniewski, F. (2009). Performance of the Genotype MTBDRplus assay in the diagnosis of tuberculosis and drug resistance in Samara, Russia Federation. *BMC Clin Pathol*, 9:2.

North, R.J. (1998). Mice incapable of making IL-4 or IL-10 display normal resistance to infection with *Mycobacterium tuberculosis. Clin Exp Immunol* 113: 55–58.

Ordway, D., Henao-Tamayo, M., Harton, M., Palanisamy, G., Troudt, J., Shanley, C., Basaraba, R. J., & Orme, I. M. (2007). The hypervirulent *Mycobacterium tuberculosis* strain HN878 induces a potent TH1 response followed by rapid down-regulation, *J Immunol* (Band 179), Nr. 1, Seite 522-31.

Ottenhoff, T. H., Kumararatne, D., & Casanova, J. L. (1998). Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria, *Immunol Today* (Band 19), Nr. 11, Seite 491-4.

Page, K.R., Jedlicka, A.E., Fakheri, B., Noland, G.S., Kesava, A.K., Scott, A.L., Kumar, N., & Manabe, Y.C. (2005). *Mycobacterium* induced potentiation of type 1 immune responses and protection against lethal malaria are host specific. *Infect Immun*; 8369-80.

Paolo, A. (2008). Pathogenesis of severe malaria anaemia, presentation at workshop, Camerino Italy.

Parida, S. K., & Kaufmann, S. H. (2010). The quest for biomarkers in tuberculosis, *Drug Discov Today* (Band 15), Nr. 3-4, Seite 148-57.

Parry, E., Godfrey, R., Mabey, D., & Gill, G. (2004). Malaria in Principles of Medicine in Africa.Cambridge University Press.

Perkins, D. J., Weinberg, J. B., & Kremsner, P. G. (2000). "Reduced interleukin-12 and transforming growth factor-beta1 in severe childhood malaria: relationship of cytokine balance with disease severity." *J Infect Dis* **182**(3): 988-992.

Pillay, M., & Sturm, A. W. (2007). Evaluation of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in Kwa Zulu-Natal, South Africa.*Clin Infect Dis*, 45: 1409-1414.

Ponnighaus, J.M., Fine, P.E., Sterne, J.A., Wilson, R. J., Msosa, E., Gruer, P. J., Jenkins, P.A., Lucas, S.B., Liomba, N.G., & Bliss L. (1992). Efficacy of BCG vaccine against leprosy and tuberculosis in Northern Malawi. *Lancet*, 339:636-9.

Portevin, D., Gagneux, S., Comas, I., & Young, D. (2011). Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. *PLoS Pathog.* 7(3):e1001307.

Revillard, J. P. (1998). Development of the immune system. *Annales Nestle Â*, 46, 125± 140.

Richter, E., Weizenegger, M., Rüsch-Gerdes, S., & Niemann S. (2003). Evaluation of Genotype MTBC assay for differentiation of clinical *Mycobacterium tuberculosis* complex isolates. *J. Clin. Microbiol.* 41:2672-2675.

Rheka, B., & Swaminathan, S. (2007). Childhood tuberculosis—global epidemiology and the impact of HIV Pediatric Resp Rev, 8, pp. 99–106.

Romagosa, C., Ordi, J., Saute, F., Quinto, L., Machungo, F., Ismail, M.R., Carrilho, C., Osman, N., Alonso, P.L., & Menendez, C. (2007). Seasonal variations in maternal mortality in Maputo, Mozambique: the role of malaria. *Trop Med Int Health*, 12: 62-7.

Rook, G. A., Dheda, K., & Zumla, A. (2005a). Do successful tuberculosis vaccines need to be immunoregulatory rather than merely Th1-boosting? *Vaccine* 23:2115-20.

Rook, G. A., Dheda, K., & Zumla, A. (2005b). Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nat Rev Immunol* 5:661-7.

Roussilhon, C., Agrapart, M., Guglielmi, P., Bensussan, A., Brasseur, P., & Ballet, J. J. (1994). Human TcRγδ+ lymphocyte response on primary exposure to *Plasmodium falciparum.Clinical and Experimental Immunology*, 95, 91± 97.

Russell S., (2004). The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome. *Am J Trop Med Hyg*, 71 (2 suppl): 147-55.

Russell, D. G. (2007). Who puts the tubercle in tuberculosis?, *Nat Rev Microbiol (Band 5)*, Nr. 1, Seite 39-47.

Russell, D.G., Barry, C.E., & Flynn, J. L. (2010). Tuberculosis: what we don't know can, and does, hurt us. *Science.*328(5980):852-6.

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath,P., Bouharoun-Tayoun, H., Chantavanich, P., Foucautt, C., Chongsuphajaisiddhi, T., & Druilhe, P. (1991). Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria.*American Journal of Tropical Medicine and Hygiene*, 45, 297± 308.

Sakol, J.E. (1975). Editorial: Measurement of delayed skin-test responses. *N Engl J Med* 293: 501-2.

Saunders, B.M., Frank, A.A., Orme, I.M., & Cooper, A.M. (2000). Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to *Mycobacterium tuberculosis* infection. *Infect Immun.* 68(6):3322-6.

Schaaf, H. S., Michaelis, I. A., & Richardson, M. (2003). "Adult-to-child transmission of tuberculosis: household or community contact?" *International Journal of Tuberculosis and Lung Disease,* vol. 7, no. 5, pp. 426–431, 2003.

Schauf, V., Rom, W. N., Smith, K. A., Sampaio, E. P., Meyn, P. A., Tramontana, J. M., Cohn, Z. A., & Kaplan, G. (1993). Cytokine gene activation and modified responsiveness to interleukin-2 in the blood of tuberculosis patients, *J Infect Dis* (Band 168), Nr. 4, Seite 1056-9.

Schluger, N.W., & Rom, W.N. (1998). The host immune response to tuberculosis.*Am J Respir Crit Care Med*, 157:679–91.

Scott-Browne, J. P., Shafiani, S., Tucker-Heard, G., Ishida-Tsubota, K., Fontenot, J. D., Rudensky, A. Y., Bevan, M. J., & Urdahl, K. B. (2007). Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis, *J Exp Med* (Band 204), Nr. 9, Seite 2159-69.

Scott, C.P., Kumar, N., Bishai, W.R., & Manabe, Y.C. (2004). Short report: modulation of *Mycobacterium tuberculosis* infection by *Plasmodium* in the murine model. *Am J Trop Med Hyg.,* 70:144-8.

Selwyn, P. A., Hartel, D., Lewis, V. A., Schoenbaum, E. E., Vermund, S. H., Klein, R. S., Walker, A. T., & Friedland, G. H. (1989). A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection, *N Engl J Med* (Band 320), Nr. 9, Seite 545-50.

Serbina, N.V., & Flynn, J.L.(2001). CD8 (+) T cells participate in the memory immune response to *Mycobacterium tuberculosis. Infect Immun*69:4320-8.

Shams, H., Weis, S.E., Samten, B., & Barnes, P.E. (2001). Contribution of CD8 (+) T cells to gamma interferon production in human tuberculosis. *Infect Immun* 69:3497-501.

Siano, A., Tonarelli, G., Imaz, M. S., Perin, J. C., Ruggeri, N., Lopez, M., Santi, M. N., & Zerbini, E. (2010). Bactericidal and hemolytic activities of synthetic peptides derived from granulysin, *Protein Pept Lett* (Band 17), Nr. 4, Seite 517-21.

Smith, S., Jacobs, R. F., & Wilson, C. B. (1997). Immunobiology of childhood tuberculosis: a window on the oncogeny of cellular immunity. *Journal of Pediatrics*, 131, 16± 26.

Soumare, M., Seydi, M., Diop, S.A., Ba, T.A,, Ndour, C.T., Dieng, Y., Diop, B.M., & Sow, P.S. (2008). The place of malaria in an infectious disease department in Dakar Senegal.*Med Trop (Mars)*; 68: 485-90.

Stenger, S., Hanson, D. A., Teitelbaum, R., Dewan, P., Niazi, K. R., Froelich, C. J., Ganz, T.; Thoma-Uszynski, S., Melian, A., Bogdan, C., Porcelli, S. A., Bloom, B. R., Krensky, A. M., & Modlin, R. L. (1998). An antimicrobial activity of cytolytic T cells mediated by granulysin, *Science* (Band 282), Nr. 5386, Seite 121-5.

Stenger, S., & Modlin, R. I. (1999). T cell mediated immunity to *Mycobacterium tuberculosis*. *Curr Opin Microbiol* 2:89-93.

Tan, B.H., Meinken, C., Bastian, M., Bruns, H., Legaspi, A., Ochoa, M.T., Krutzik, S.R., Bloom, B.R., Ganz, T., Modlin, R.L., & Stenger, S. (2006a). Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol* 177(3):1864-71.

Tan, T, Lee, W.L., Alexander, D.C., Grinstein, S., & Liu, J.(2006b). The ESAT-6/CFP-10 secretion system of *Mycobacterium marinum* modulates phagosome maturation. *Cell Microbiol* 8(9):1417-29.

Tascon, R.E., Stavropoulos, E., Lukacs, K.V., & Colston, M.J.(1998). Protection against *Mycobacterium tuberculosis* infection by CD8+T cells requires the production of gamma interferon. *Infect Immun* 66: 830–834.

Telenti, A., Imboden, P., Marchesi, F., Lawurie, D., Cole, S., Colston, M. J., Matter, L., Schopfer, K., & Bodmer, T. (1993). Detection of rifampicin-resistance mutation in *M. tuberculosis. Lancet;* 341: 647-650.

Thoen, C., LoBue, P., & de Kantor, I. (2010). Why has zoonotic tuberculosis not received much attention? *Int. J. Tuberc Lung Dis* 14(9): 1073-1074.

Toossi, Z., Kleinhenz, M. E., & Ellner, J. J. (1986). Defective interleukin 2 production and responsiveness in human pulmonary tuberculosis, *J Exp Med* (Band 163), Nr. 5, Seite 1162-72.

Troye-Blomberg, M., Sjoholm, P. E., Perlmann, H., Patarroyo, M. E., & Perlmann, P. (1983). Regulation of the immune response in *Plasmodium falciparum* malaria: Non specific proliferative response *in vitro* and characterization of lymphocytes. *Clinical and Experimental Immunology*, 53, 335± 344.

Trunz, B. B., Fine, P., & Dye, C.(2006).“Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness,” *Lancet,* vol. 367, no. 9517, pp. 1173–1180.

Tufariello, J. M., Chan, J., & Flynn, J. L. (2003). Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection, *Lancet Infect Dis* (Band 3), Nr. 9, Seite 578-90.

Udani, P. M. (1997a). Diagnosis of tuberculosis in children.*Indian Academy of Paediatrics: Tuberculosis Chapter News Bulletin*, 4, 3± 13.

Udani, P. M. (1997b). TB in children: laboratory investigations, tuberculin and BCG skin tests. *Indian Academy of Paediatrics: Tuberculosis Chapter News Bulletin*, 4, 2.

Ulrichs, T., & Kaufmann, S. H. (2006).New insights into the function of granulomas in human tuberculosis, *J Pathol* (Band 208), Nr. 2, Seite 261-9.

Umemura, M., Yahagi, A., Hamada, S., Begum, M. D., Watanabe, H., Kawakami, K., Suda, T., Sudo, K., Nakae, S., Iwakura, Y., & Matsuzaki, G. (2007). IL-17-mediated regulation of innate and acquired immune response against pulmonary *Mycobacterium bovis bacille Calmette-Guerin* infection, *J Immunol* 178 (6): 3786-96.

Vreden, S. G. S. (1994). The role of Kupffer cells in the clearance of malaria sporozoites from the circulation. *Parasitology Today*, 10, 304± 308.

Wahlgren, M., Fernandez, V., Scholander, C., & Carlson, J, (1994). Rosetting *Parasitology Today*, 10, 73± 79.

Wang, J., Wakeham, J., Harkness, R., & Xing, Z. (1999). Macrophages are a significant source of type 1 cytokines during mycobacterial infection, *J Clin Invest* (Band 103), Nr. 7, Seite 1023-9.

White, K. L., Snyder, H. L., & Krzych, U. (1996). MHC class I dependent presentation of exoerythrocytic antigen to CD81 T lymphocyte is required for protective immunity against *Plasmodium berghes*. *Journal of Immunology*, 156, 3374± 3381.

White, N. J., & Breman, T. G. (1998). Malaria and other diseases caused by red blood cell parasites In *Harrison's Principles of Internal Medicine*, 14th Edn, eds Fauci, A. S., Braunwald, E. & Isselbacher, K. J. pp. 1180± 1189. New York: McGraw-Hill.

Whittle, H. C., Brown, J., Marsh, K., Greenwood, B. M., Seidelin, P., Tighe, H., & Wedderburn, L. (1984). T-cell control of Epstein Barr virus infected B cells is lost during *P. falciparum* malaria. *Nature*, 312, 449± 450.

Williams, M. A., Tyznik, A. J., & Bevan, M. J. (2006). Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells, *Nature* 441 (7095): 890-3.

World Health Organization. (1963). *The WHO Standard Tuberculosis Test*. Document WHO/TB/Technical Guide 3. Geneva: WHO.

World Health Organization. (1993). WHO declares tuberculosis a global emergency. *Soz Praventiv med* 38:251-2.

World Health Organization. (2002). The World Health Organization Report: reducing risks, promoting healthy life. Geneva, World Health Organization.

World Health Organization. (2004a).*Anti-tuberculosis Drug Resistance in the World: Third Global Report*. Geneva, World Health Organization.

World Health Organization. (2004b). *World Health Report 2004: Changing history.* Geneva: World Health Organization; 2004. *http://www.who.int/whr/2004/en*.

World Health Organisation. (2006). Guidance for National Tuberculosis Programmes on The Management of Tuberculosis in Children, WHO, Geneva, Switzerland.

World Health Organization. (2007). Global tuberculosis control: surveillance, planning, financing. WHO Report 2007. WHO/HTM.TB/2007.376. Geneva: World Health Organization; 2007. Available at: *http://www.who.int/tb/publications/global_report/2007/en/index.html*(accessed January 2009).

World Health Organization. (2009). Global Tuberculosis control: Epidemiology, strategy, financing.*[http://www.who.int/tb/publications/global_report/2009/pdf/full_report.pdf]*webcite Last accessed 26 March 2010

World Health Organization. (2011). Global tuberculosis control 2011. Geneva. The Organization, 2011 (cited 2012 Jul 1): *http://whoglobdoc:whoint/publications*/20119789241564380_eng.pdf

Wood, K.J., Ushigome, H., Karim, M., Bushell, A., Hori, S., & Sakaguchi, S. (2003). Regulatory cells in transplantation. *Novartis Found Symp* 252: 177-88; discussion 188-93, 203-10.

Zar, H. J., Hanslo, D., Apolles, P., Swingler, G., & Hussey, G. (2005). "Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study," *Lancet*, vol. 365, no. 9454, pp. 130–134.

Zhang, M., Lin, Y., Iyer, D. V., Gong, J., Abrams, J. S., & Barnes, P. F. (1995). T-cell cytokine responses in human infection with Mycobacterium tuberculosis, *Infect Immun* 63 (8): 3231-4.

Zhang, M., Hisaeda, H., Sakai, T., Li, Y., Ishikawa, H., Hao, Y.P., Nakano, Y., Ito, Y., & Himeno, K. (2001). CD4+ T cells are required for HSP65 expression in host macrophages and for protection ofmice infected with Plasmodium yoelii. *Parasitol Int* 50: 201–209.

APPENDICES

Appendix I: Informed consent form

1.0 Title of the project proposal (FAN: IREC 000657): Genetic diversity among MTBC strains and the immune responses against TB in malaria infected children

Patient's Name……..……

Date of birth……..…Age……Sex……

Tel./Mobile No……..Fax……E-mail……

2.0 Puprpose of the study

To characterize MTBC strains circulating in malaria endemic regions and determine the effects of malarial infection on immunity against TB

You are invited to participate in a research study conducted by investigators from Moi University School of Medicine, to find out the effect of malaria on cellular immune response to TB, the performance of T-cell-based diagnostic test, and sputum smear and sputum cultures in diagnosing active pulmonary TB in malaria infected patients.

3.0 Procedure to be followed

In this study the blood and sputum samples that will be taken from you for routine diagnosis will also be used for TB and malaria co-infection studies and anti-TB drug susceptibility testing. If you accept to participate in the study, you will be requested to remove 5-10mls of blood and at least 3 sputum samples.

4.0 Benefits of Taking Part in the Study

There are no monetary benefits to you for taking part in this study. While there is no monetary benefit, if TB is diagnosed you will be referred for TB treatment and this will ensure that we prevent transmission of TB to your immediate family members and friends. In addition; we will know your TB status before you are advice on where to get anti-TB treatment. Your family members will be asked to come to the hospital for TB screening. You will also be treated for malaria. The overall benefit of this study will be the expected improvement in patient care programs. This will result in benefits to the community of science as a whole, and may have a broader positive effect on health care for TB/malaria co-infected individuals locally and the world at large mostly in sub-Saharan Africa where malaria and TB is endemic.

5.0 Risks

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

6.0 Alternatives to Taking Part in the Study

It is important for you to know that you have freedom to decline to participate in the study and your refusal will not affect relationship between you and those treating and caring for you. Instead of being in the study, you may continue with your usual medical care.

7.0 Confidentiality

Numbers will be used to identify your samples in order to observe confidentiality. Your (Child's) identity will be held in confidence in reports in which the study may be published. Organizations that may inspect and/or copy your research records for quality

assurance and data analysis include groups such as my academic supervisors and my research associates and the Moi Teaching and Referral Hospital (MTRH) / Moi University (MU) Institutional Research Ethics Committee (IREC). No identity of any specific patient in this study will be disclosed in any public reports or publications.

8.0 Costs/Compensation

There will be no cost to you to participate in this study.

9.0 Questions about the Study

You can ask the study staff any questions that you may have about the study. They will be happy to answer any questions at any time during the study. If you have any questions regarding this study or your participation in it or you develop any problems because of your participation in this study, you may contact the clinic or the principal investigator using the following number: 0712-782-580.

10.0 Signature

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

Signature…….Date……

Patients

Signature…….Date……

Patients or legal guardians

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

Signature……Name: **Biegon Richard Kiprono**…….Date……

Investigator

Signature……Name: .....…….Date……

Witness

Address of witness:…….……..……

Appendix II: Ziehl Neelsen (ZN) Staining-Principle, Procedure and Interpretations

Principle

This procedure is used to stain *M. tuberculosis* and *M. leprae*. These bacteria are also called acid fast bacilli. They stain with carbol fuschin, which is a red dye. They retain the dye when treated with acid, which is because of the presence of mycolic acid in their cell wall.

Procedure

1. Fix the smear of the specimen over the glass slide, either by heating or alcohol fixation.
2. Pour carbol fuschin over smear and heat gently until fumes appear. Do not overheat and allow it to stand for 5 minutes, and then wash it off with running tap water.
3. Pour 20% sulphuric acid, wait for one minute and keep on repeating this step until the slide appears light pink in color. Wash off with water.
4. Pour methylene blue, wait for two minutes, again wash with water
5. Allow it to air dry and examine under oil immersion lens.

Result

Acid fast bacilli stain pink, straight or slightly curved rods, at times having beaded appearance. The background appears blue due to methylene blue.

Interpretations

If definite bacilli are seen, report as AFB positive. However, it is better to report the result quantitatively as follows:

- > 10 AFB/high power field –>+++
- 1-10 AFB/high power field –> ++
- 10-100 AFB/100 high power fields –> +
- 1-9 AFB/100 high power fields –> exact number

Appendix III Sputum culture, isolation of mycobacteria and decontamination using NALC-NaOH

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

Procedure for the decontamination of specimen

Note: All specimens had been collected in sterile 500ml Falcon centrifuge tubes marked with patient information.

i) Equal volumes of NALC working solution was added to each specimen. The contents were vortexed for 15 seconds and then mixed. Another vortexing for 15 seconds followed to ensure that the specimen got liquefied. In event that the specimen was still mucoid, some more NALC was added followed by vortexing.

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

iii) The same buffer was added to the other specimen tubes at1 minute intervals.

iv) The tubes were centrifuged (IEC CENTRA® CL3 centrifuge) at 3000g for 20 minutes.

v) The supernatant was decanted into 5% Phenol disinfectant bucket.

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

vii) The tubes were vortexed.

viii) Slides were then prepared from each of the sediments as follows:

- Using a sterile pipette the specimen was spread centrally on the slide (smear size 1 x 2 cm). The slides were then placed on hot plate (thermostat set at 70^0C) under UV light for a minimum of 30 minutes. UV kills aerosols created by the heating of slides.

ix) The slides were stained with the Ziehl-Neelsen stain and examined for AFBs.

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

Inoculation procedures

Note: The LJ medium slopes were always inoculated before the MGIT tubes.

Inoculation of the LJ slopes

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

ii) The slants were then laid out horizontally, with inoculated surface facing upward, for at least 24 – 48 hours, to allow inoculums to absorb into media, after which they were placed upright in the incubator at 37^0C.

iii) The caps were left loosened, for at least the 1^{st} week, to allow for air exchange, and then screwed tight to prevent desiccation.

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

Reporting scheme:

Colony count	Report
0	No growth
1 – 9	Actual number
10 – 100	1+
101 – 200	2+
201 -500 (almost confluent)	3+
500 < (Confluent)	4+

Appendix IV: Sputum culture on BACTEC™ MGIT 960 automated Machine

Inoculation of BACTEC MGIT 960 tubes

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

ii) A volume of 0.8 ml (800µl) of reconstituted MGIT PANTA was added to each MGIT tube that was to be inoculated using a sterile micropipette tip.

iii) Once PANTA had been added the tubes were covered from direct light as they are light sensitive.

iv) Using a sterile micropipette tip, 0.5 ml (500µl) of well mixed concentrated specimen was added to the appropriately labeled MGIT tube. The tube was immediately recapped tightly and contents mixed by inverting the tube several times.

v) The tubes and caps were wiped with 5% Phenol disinfectant and left at room temperature for 30 minutes.

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

Work-up of Positive cultures

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

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

ii) The MGIT tubes were mixed by vortexing. Using sterile Pasteur pipettes, 1 drop of the mixture was placed on to the slide.

iii) The slides were left on a hot plate under UV light for a minimum of 2 hours. The slides were then stained following ZN method and examined for AFBs using 100x oil immersion objective.

Reading of the ZN stain from positive MGIT

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

Pos R	=	Positive roping
Pos NR	=	No roping. ?MOTT
Pos/c	=	Positive mixed with contaminating bacteria
Neg	=	Negative – no organism seen

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

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

Work-up of contaminated Positive cultures

Contaminated positive culture underwent re-decontamination.

i) The entire MGIT broth was transferred into 50 ml centrifuge tube.

ii) An equal volume of 45 sterile NaOH solutions was added, and the contents mixed and left to stand for 15 – 20 minutes.

iii) Phosphate buffer pH 6.8 was added after 15 – 20 minutes up to 40 ml mark on the centrifuge tube. The contents were mixed by inverting the tubes severally.

iv) The tube contents were centrifuge at 3000 x g for 15 – 20 minutes.

v) The supernatant was poured off into a bucket containing Phenol, and the sediment re-suspended in 0.5 ml (500µl) of buffer and mixed well.

vi) A volume of 0.5 ml (500µl) was inoculated into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA®.

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

Decontamination of sputum specimen using Sulphuric Acid

Before inoculating into MGIT tubes, sputum was decontaminated using 6.0% sulphuric acid. For the sulphuric acid (6.0%) decontamination, 2.5 ml of the specimen was put in a 15 ml glass centrifuge tube with a screw cap and an equal volume of sulphuric acid (final concentration of sulphuric acid: 3.0%) is added. After vortexing the specimen is left at room temperature for 15 minutes for decontamination. Then, 5ml sterile normal saline is

added and the mixture is vortex-mixed again. After centrifugation for 15 minutes at 3660 × g, the supernatant is discarded and the sediment is suspended in 10 ml of normal saline. After another centrifugation for 15 minutes at 3660 × g, the supernatant is discarded and the sediment is inoculated in a MGIT.

Procedure

i) Prepare 6.0% sulphuric (H_2SO_4) acid. Put 94 ml of distilled water in a measuring cylinder and then add 6 ml of concentrated H_2SO_4.

NB. Add acid to water.

i) Put 2.5 ml sputum specimen into a 15 ml Falcon tube with a screw cap.

ii) Add 2.5 ml of the prepared 6.0% sulphuric (H_2SO_4)acid[final concentration of sulphuric acid will be 3.0%].

iii) Vortex the mixture and leave at room temperature for 15 minutes for decontamination to take place.

iv) Then add 5ml sterile normal saline and the mixture is vortex-mixed again.

v) Centrifuge for 15 minutes at 3660 × g (≈3000 rpm)

vi) Discard the supernatant and then resuspend the sediment in 10 ml of normal saline.

vii) Centrifuge again for 15 minutes at 3660 × g (≈3000 rpm)

viii) Discard the supernatant and then inoculate the sediment in a MGIT.

Appendix V: Characterization of MTBC species using GenoTyping® method by Hain lifescience

Procedure

The whole procedure is divided into three steps:

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

ii) Multiplex amplification with biotinylated primers.

iii) Reverse hybridization

The hybridization includes the following steps: -

i) Chemical denaturation of the amplification products

ii) Hybridization of the single stranded DNA

iii) Biotin – labeled amplicons to membrane – bound probes

iv) Stringent washing

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

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

Storage and precautions

Store Primer/Nucleotide Mix (PNM) at 2 – 8 ^{0}C upon arrival isolated from potential source of contaminating DNA. If longer storage (more than 4 weeks) is required, store at – 20 ^{0}C. In order to avoid repeated freezing and thawing, aliquote PNM. Store all the kit components at 2 – 8 ^{0}C. Do not use the reagents beyond their expiry date.

DNA Isolation

Bacteria grown either on solid culture (e.g. Lowenstein-Jensen, Middlebrook) or in liquid medium (BACTEC™, MB-Check) will be used, as well as smear-positive direct material (pulmonary samples). The test must not be used to detect mycobacteria directly from smear-negative patient material. The working area must be free from amplified DNA. It is crucial to heat samples to 95-105^{0}C for at least 15 min in order to totally lyse cells and to inactive bacteria. Any DNA isolation procedure producing amplifiable DNA from bacteria can be used. The following quick protocol normally also yields DNA suitable for amplification:

1 (a). When using bacteria grown on solid medium, bacteria will be collected with an inoculation loop and suspend in approximately 300 µl of water (molecular biology grade).

(b). When using bacteria grown on liquid media, 1 ml will be directly applied, when using direct patient material (e.g. sputum), 500 µl of a decontaminated* sample will be applied. Bacteria will be pelleted by spinning for 15 min in a standard table top centrifuge with an aerosol-tight rotor in a class II safety cabinet at approximately 10000 g. Supernatant is then discarded and bacteria is resuspended in 100-300 µl of water (for culture samples), or 100 µl of water (for direct patient material) by vortexing.

2. Incubate bacteria from 1a or 1b for 20 min at 95^{0}C (boiling water bath)

3. Incubate for 15 min in an ultrasonic bath.

4. Spin down for 5 min at full speed and directly use 5 µl of the supernatant for PCR. Incase DNA solution is to be stored for an extended time period, supernatant will be transfer to a new tube.

* Samples must be processed using the NALC/NaOH method according to the DC publication "Public Health Mycobacteriology: A Guide for the Level III Laboratory". Detailed protocols can be obtained from your local distributor or from: www.hain-lifescince.de/pdf/dnaisol_myco.pdf

Amplification

The amplification mix (45 µl) will be prepared in a DNA-free room. The DNA sample will be added in the separated area (Table 5.1).

Per tube mix:

-35 µl PNM

- 5 µl 10x polymerase incubation buffer – not provided

- x µl $MgCl_2$ solution[1)] – not provided

- 1-2 unit(s) thermostable DNA polymerase (refer to manual) – not provided

- y µl water till obtain a volume of 45 µl (not considering volume of enzyme) - not provided

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

[1)] Depending on the enzyme/buffer system used, the optimal $MgCl_2$ concentration may vary between 1.5 and 2.5mM. Please note that some incubation buffers already contain $MgCl_2$.

Determine the number of samples to be amplified (number of samples to be analyzed plus control samples). A negative control sample, for example, contains 5 µl of water instead of DNA solution. Prepare a master mix containing all reagents except for DNA solution and mix well (do not vortex) and aliquot 45 µl in each of the prepared PCR tubes.

Table 5.1: Preparation of master mix for PCR

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

Amplification profile:

	Culture samples	**Direct patient material**
5 min[2)] 95°C	1 cycle	1 cycle
30 sec 95°C 2 min 58°C	10 cycles	10 cycles
25 sec 95°C 40 sec 53°C 40 sec 70°C	20 cycles	30 cycles
8 min 70°C	1 cycle	1 cycle

[2)] When using certain hot start DNA polymerases, this has to be extended (please refer to manual of the enzyme).

Depending on the cycler used the amplification profile might have to be modified. Amplification products can be stored at +4 to –20°C.

For checking the amplification reaction, 5 µl of each sample might be directly applied to a 2% agarose gel without the addition of loading buffer.

The amplicons have a length of approximately 63 bp (Amplification Control). 115bp (MTBC). 166 bp (*rpoB*), 120 bp (*katG*), and 110 bp (*inhA*) respectively.

Hybridization

Preparation (as per the manufacturer's protocol)

Pre-warm shaking water bath/TwinCubator® to 45°C; the maximum tolerated deviation from the target temperature is +/-1°C. Pre-warm solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1. Dispense 20 µl of **Denaturation Solution** (DEN, blue) in a corner of each of the wells used.
2. Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes.

Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.

3. Carefully add to each well 1 ml of pre-warmed **Hydridization Buffer** (HYB, green). Gently shake the tray until the solution has a homogenous color.

   Take care not to spill solution into the neighboring wells.

4. **Place a strip in each well.**

   The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers, turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.

5. **Place tray in shaking water bath/TwinCubator®and incubate for 30 min. at 45^0 C.**

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

6. **Completely aspirate Hybridization Buffer.**

   For example, use a Pasteur pipette connected to a vacuum pump.

7. Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45^0 C in shaking water bath/TwinCubator®.
8. Work at room temperature from this step forward. Completely remove Stringent Wash Solution.

Pour out Wash Solution in a waste container and all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps.

9. Wash each strip once with once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator®(pour out RIN after incubation).
10. Add 1 ml of diluted Conjugate to each strip and incubate for 30 minutes on shaking platform/TwinCubator®.
11. Remove the solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute appropriate 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator®(pour out solution each time).

    Make sure to remove any trace of water after the last wash.

12. Add 1 ml diluted substrate to each strip and incubate protected from light without shaking.

Depending on the test conditions (e.g. room temperature), the substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.

13. Stop reaction by briefly rinsing twice with distilled water.
14. Using tweezers, remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and interpretation of results (as per the manufacturer's protocol)

Strips will be pasted and store protected from light. An evaluation sheet is provided.

When using this evaluation sheet, the developed strips will be pasted in the designated fields by aligning the bands CC and AC with the respective lines on the sheet.

The resistance status will be determined and note down in the respective column; as a help to interpretation.

The template provided serves as an aid for evaluation and must be aligned with the bands CC and AC of the strip as well.

Conjugate Control (CC)

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

Amplification Control (AC)

When the test is performed correctly, a control amplicon generated during amplification will bind to the Amplification set-up on the strip. A missing band therefore indicates mistakes during amplification set-up or the carry-over of amplification inhibitors with the isolated DNA. In case of positive test result, the signal of the Amplification Control zone can be weak. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated.

***M. tuberculosis* complex (TUB)**

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

Locus Controls (*rpo*B, *kat*G, and *inh*A)

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

Appendix VI: Molecular genetic assay for identification of resistance to RIF and/or INH of the *M. tuberculosis* Complex

GenoType® MTBD*plus* method

The *M. tuberculosis* drug susceptibility test was performed using the GenoType® MTBD*plus* test which is based on the DNA-STRIP® technology. This technique permits the molecular genetic identification of *M. tuberculosis* complex and its resistance to RIF and/or INH from cultivated samples or pulmonary smear-positive direct patient material. The identification of RIF resistance is enabled by the detection of the most significant mutations of the *rpoB* gene (coding for the β-sub-unit of the RNA polymerase). For testing of high level INH resistance, the *katG* gene (coding for the catalase peroxidase) is examined and testing of low level INH resistance, the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) is examined. The whole procedure is divided into three steps: DNA extraction from cultured material (culture plates/liquid medium) or direct materials (pulmonary, smear-positive, decontaminated) – using the necessary reagent, a multiplex amplification with biotinylated primers and a reverse hybridization.

The hybridization includes the following steps: chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase conjugate, and an alkaline phosphatase mediated staining reaction. A template ensures the easy and fast interpretation of the banding pattern obtained.

Procedure for *M. tuberculosis* drug susceptibility test

The *M. tuberculosis* drug susceptibility test procedure using the GenoType® MTBD*plus* test which is based on the DNA-STRIP® technology as per manufacturer's protocol was divided into three steps as follows:

i) **DNA extraction** from cultured sputum (culture plate or LJ/ liquid medium),

ii) Multiplex **amplification** with biotinylated primers

iii) Reverse **hybridization**.

i) DNA Extraction

Bacteria grown on LJ or in liquid medium (BACTEC™ MGIT™ 960 tubes) were used, as well as smear positive direct sputum (pulmonary samples). The working area was free from amplified DNA. Samples were heat to 95^0C for at least 20minutes in order to inactivate vegetative bacteria. The following quick protocol which normally yields DNA suitable for amplification was used:

1a. When using bacteria grown on solid medium (LJ), bacteria were collected with an inoculation loop and suspended in 300µl of water (molecular biology grade such as water for injection).

1b. When using bacteria grown in liquid media (BACTEC™ MGIT™960 tubes), 1ml was directly applied and when using direct patient sputum, 500µl of a decontaminated (using NALC-NaOH method according to CDC publication) sample was applied. The bacteria were then pellet by spinning for 15 minutes in a standard bench top centrifuge with an aerosol-tight rotor in a class II biosafety cabinet at approximately 10000 x g. Supernatant

was discarded and bacteria was resuspended in 100-300µl of water (for culture samples), or 100µl of water (for direct patient sputum) by vortexing.

2. Bacteria from 1a or 1b above were incubated for 20 minutes at 95^0 C in a water bath

3. Incubate for 15 minutes in an ultrasonic bath

4. Spin down for 5 minutes at full speed and directly use 5µl of the supernatant for PCR. In case DNA solution is to be stored for an extended time period, the supernatant was transferred to a new tube.

ii) Amplification

The amplification mix (45 µl) was prepared in a DNA-free room. The DNA samples were added in a separate area.

Per tube mix:

- 35 µl PNM

- 5 µl 10x polymerase incubation buffer

- x µl $MgCl_2$ solution

- 1-2 unit(s) thermostable DNA polymerase

- Add 5 µl DNA solution (20-100 ng DNA) leading to a final volume of 50 µl

NB: Depending on the enzyme/buffer system used, the optimal $MgCl_2$ concentration may vary between 1.5 and 2.5 Mm. It was also noted some incubation buffer already contain $MgCl_2$.

Amplification profile:

	Culture samples	**Direct patient samples**
5 min* 95^{0}C	1 cycle	1 cycle
30 sec 95^{0}C	10 cycles	10 cycles
2 min 58^{0}C		
25 sec 95^{0}C		
40 sec 53^{0}C	20 cycles	30 cycles
40 sec 70^{0}C		
8 min 70^{0}C	1 cycle	1 cycle

* When using certain hot start DNA polymerases, this step has to be extended. Refer to manual of the enzyme. Amplification products can be stored at +4 to –20^{0}C.

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

iii) Hybridization

Preparation: Shaking water bath/TwinCubator® was pre-warm to 45^{0}C; the maximum tolerated deviation from the target temperature was +/–1^{0}C. Hybridization buffer (HYB) and stringent (STR) wash solutions were pre-warm to 37–45^{0}C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque). Mix if necessary. Warm the remaining reagents with the exception of CON-C and SUB-C to

room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (**CON-C with CON-D, SUB-C and SUB-D**) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

Hybridization procedure

1. Dispense 20µl of Denturation Solution (DEN, blue) in a corner of each of the wells used.
2. Add to the solution 20µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes.

   Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. Always wear gloves when handling strips.
3. Carefully add to each well 1ml of pre-warmed Hybridization Buffer (HYB, green).

   Gently shake the tray until the solution has a homogeneous color. Take care not to spill solution into the neighboring wells.
4. Place a strip in each well. The strip must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.

5. Place tray in shaking water bath/TwinCubator® and incubate for 30 minutes at 45^0 C. Adjust the shaking frequently of the water bath to achieve a constant and thorough mixing of the solution. To allow adequate heat transfer, the tray must be dipped into the water to at least $^1/_3$ of its height.
6. Completely aspirate Hybridization Buffer. For example, use a Pasteur pipette connected to a vacuum pump
7. Add 1ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45^0C in shaking water bath/TwinCubator®.
8. Work at room temperature from this step forward. Completely remove Stringent Wash Solution.

   Pour out Wash solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps
9. Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/bath/TwinCubator® (pour out RIN after incubation).
10. Add 1ml of diluted Conjugate to each strip and incubate for 30 minutes on shaking platform/bath/TwinCubator®.
11. Remove solution and wash each strip twice for 1 minute with 1ml of Rinse Solution (RIN) and once for 1 minute with approximately 1ml of distilled water (e.g. use wash bottle) on shaking plate/ bath/TwinCubator® (pour out solution each time). Make sure to remove any trace of water after the last wash
12. Add 1ml of diluted substrate to each strip and incubate protected from light without shaking. Depending on the test conditions (e.g. room temperature), the

substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.

13. Stop reaction by briefly rinsing twice with distilled water.
14. Using tweezers remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and interpretation of results

Strips are pasted and store protected from direct light. An evaluation sheet was provided with the reagent kit. When using this evaluation sheet, the developed strips was pasted in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. The resistance status was determined and noted down in the respective column; as a help for interpretation. The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and AC of the strips as well. Each strip has a total of 27 reaction zones (Figure 5.1). Not all bands of a strip have to show the same signal strength.

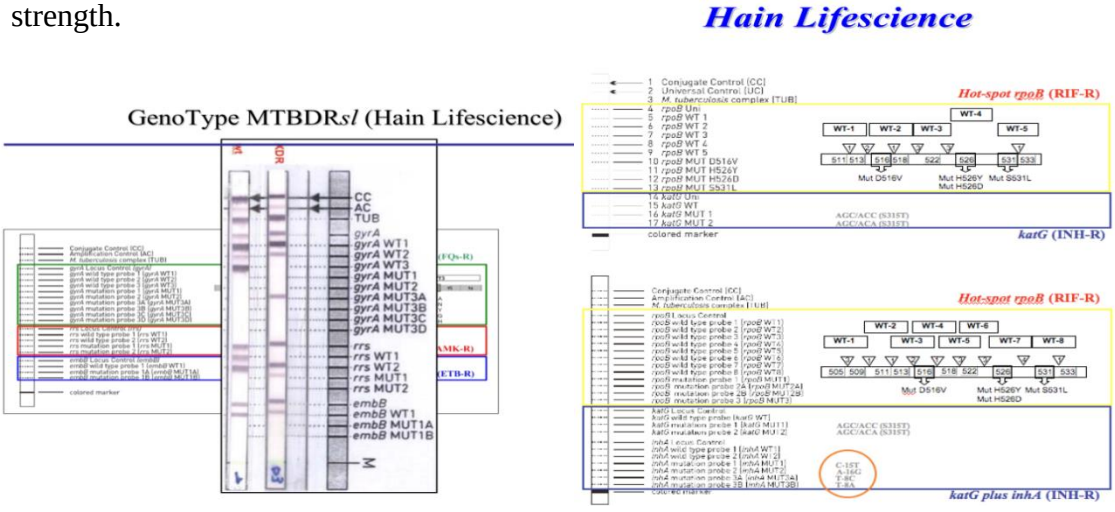


Figure 5.1: MTBDR*plus* DST evaluation template

Conjugate Control (CC)

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

Amplification Control (AC)

When the test is performed correctly, a control amplicon will bind to the Amplification Control zone. If this band is developed, mistakes during extraction and amplification setup and the carry-over of amplification inhibitors can be excluded. In case of a positive test, the signal of the Amplification Control zone can be weak or even vanish totally. This might be due to competition reactions during amplification. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated. A weak or missing AC band in case of a negative test result indicates mistakes during amplification set-up, or carry-over of amplification inhibitors. In this case, the test is not valid and the respective sample has to be repeated.

***M. tuberculosis* complex (TUB)**

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

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

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

Wild type probes

The wild type probes comprise the most important resistance areas of the respective genes. When all wild type probes of a gene stain positive, there is no detectable mutation within the examined regions. Hence the strain tested is sensitive for the respective antibiotic. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a resistance of the tested strain to the respective antibiotic. Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zones are to be considered. Each pattern deviating from the wild type pattern indicates resistance of the tested strain. The banding pattern abtained with *rpoB* probes allows to draw a conclusion about a RIF resistance of the strain tested, the banding pattern obtained with *katG* probes allows to draw a conclusion about a high level INH resistance, the banding pattern obtained with the *inhA* probes allows to draw a conclusion about a low level INH resistance of the strain tested, respectively.

Mutation probes

The mutation probes detect some of the most common resistance mediating mutations (Tables 5.2, 5.3a & b). Compared to the other probes, positive signals of the mutation probes *rpoB* MUT2A and MUT2B may show lower signal strength. Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone are to be considered.

Each pattern deviating from the wild type pattern indicates resistance of the tested strain. The banding pattern obtained with the *rpoB* probes allows to draw a conclusion about a

RIF resistance of the strain tested, the banding pattern obtained with the *katG* probes allows to draw a conclusion about a high level INH resistance, the banding pattern obtained with the *inhA* probes allows to draw a conclusion about a low level INH resistance of the strain tested, respectively.

Note the following special cases:

- There is a possibility that the specimen tested contains a heterogeneous strain. If, at investigation, this strain has developed only a partial resistance, one of the mutation probes as well as the corresponding wild type probe may appear.
- There is a possibility that the tested specimen contains more than one *M. tuberculosis* strain (due to mixed culture or contamination). If at least one of these strains harbors a mutation, one of the mutation probes as well as the corresponding wild type probe may appear.

Table 5.2: Mutations in the gene *rpoB* and the corresponding wild type and mutation probes (modified according to Telenti *et al*. 1993, Lancet, 341: 647-650)

Failing wild type probe(s)	codons analyzed	Mutation probe	Mutation
rpoB WT1	505-509		F505L
			T508A
			S509T
rpoBWT2	510-513		L511P*
rpoBWT2/WT3	510-517		Q513L*
			Q513P
			del514-516
rpoBWT3/WT4	413-519	***rpoB*** MUT1	D516V
			D516Y
			del515
rpoBWT4/WT5	516-522		del518*
			N518I
rpoBWT5/WT6	518-525		S522L
			S522Q
rpoBWT7	526-529	***rpoB*** MUT2A	H526Y
		rpoB MUT2B	H526D
			H526R
			H526P
			H526Q*
			H526N
			H526L
			H526S
			H526C
rpoB WT8	530-533	***rpoB*** MUT3	S531L

S531P

S531Q*

S531W

S533P

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

Table 5.3a: Mutations in the gene *katG* and the corresponding wild type and mutation probes

Failing wild type probe(s)	Codons analyzed	Mutation probe	Mutation
KatG WT	315	*katG* MUT1	S315T1
		katG MUT2	S315T2

Table 5.3b: Mutations in the gene *inhA* and the corresponding wild type and mutation probes

Failing wild type probe(s)	Codons analyzed	Mutation probe	Mutation
inhA WT1	-15	*inhA* MUT1	C15T
	-16	*inhA* MUT2	A16G
*inhA*WT2	-8	*inhA* MUT3A	T8C
		inhA MUT3B	T8A

Evaluation examples

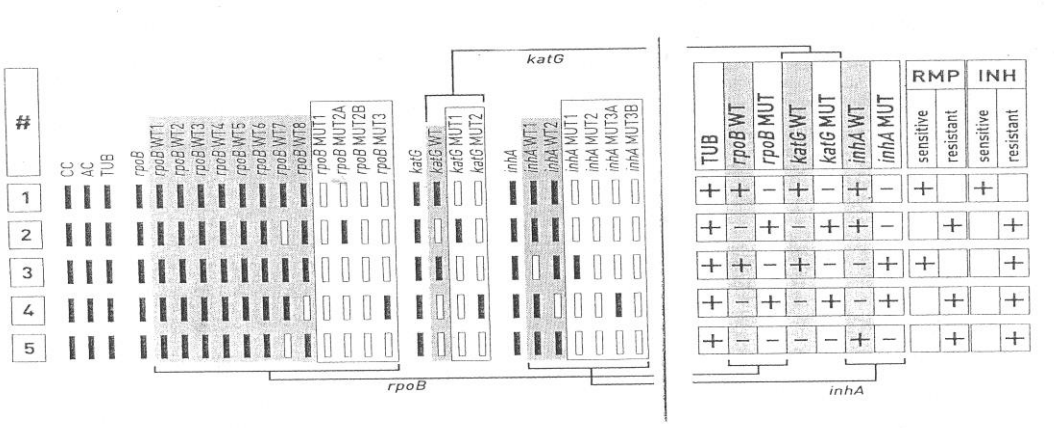


Figure 5.2: Banding patterns and evaluation template with respect to RIFand/or INH resistance

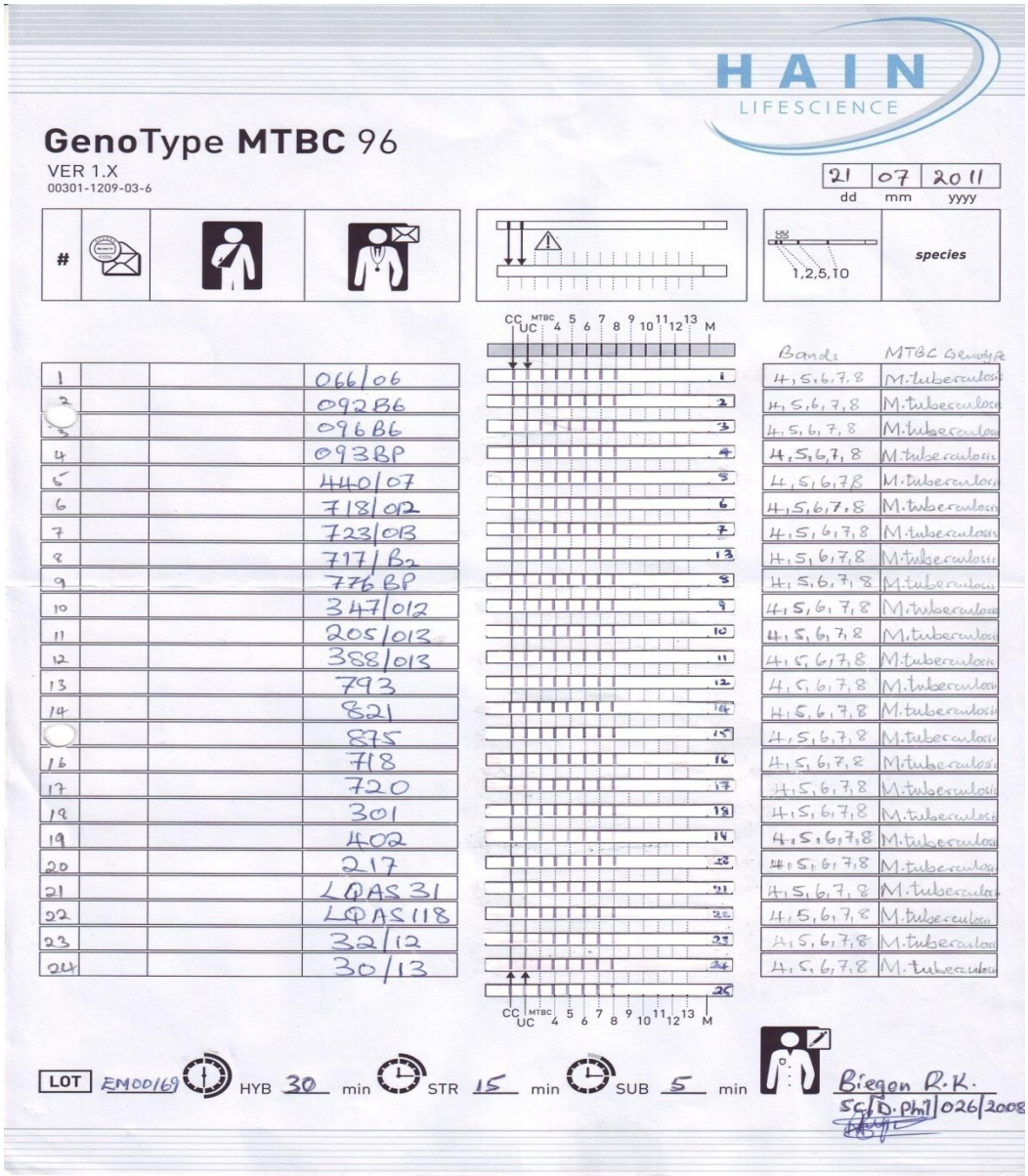
If all wild type bands display a signal, this is classified as positive and marked in the WT column of the respective gene as "+". If at least one of the wild type bands is absent, this is classified as negative and marked in the WT column of the same gene as "–". Negative entries are only made to the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

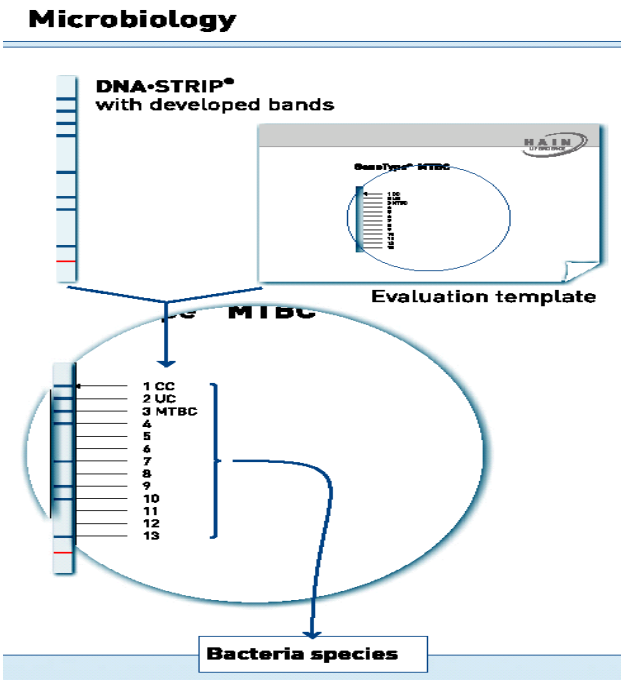
Example 1 (Figure 5.2) shows the wild type banding pattern. All wild type [robes display a signal, but none of the mutations probes; hence, the evaluation chart has a "+" in the three wild type columns and a "–" in the three mutations columns. Accordingly, the boxes fo RIF and INH are marked as sensitive.

Example 5: One of the *rpoB* and the *katG* wild type probes are missing in example 5; hence, the boxes for *rpoB* WT and *katG* WT are marked with a "–". As none of the mutation probes are developed, these boxes are also marked with a "–". The *inhA* promoter region does not deviate from the wild type pattern. The strain is evaluated as RIF and INH resistant. The INH resistance is caused by a mutation in the katG gene and is therefore a high level isoniazid resistance. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a resistance of the tested strain to the respective antibiotic. Only those bands whose intensities are about as strong as or stronger than that of the Amplification Control zone are to be considered.

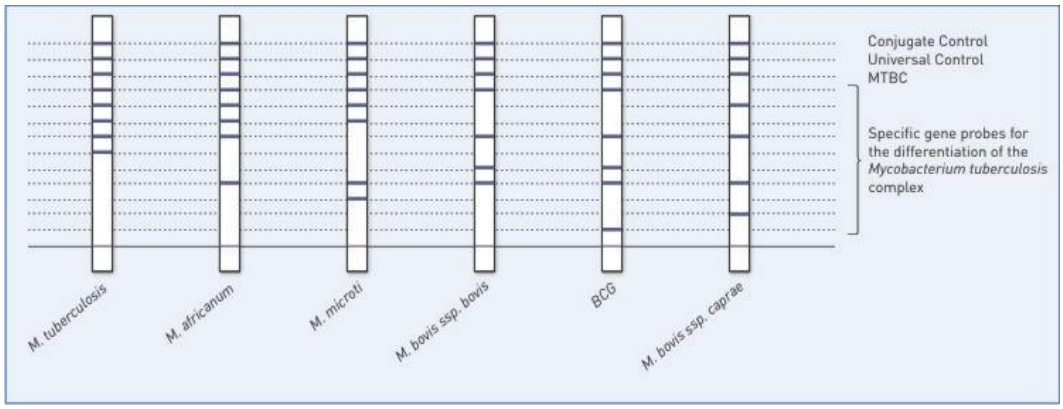
Appendix VII: a) MTBC results obtained pasted on identification sheet, and b) Template for identifying MTBC species

a)




Microbiology
DNA-STRIP®
with developed bands
Evaluation template
1 CC
2 UC
3 MTBC
4
5
6
7
8
9
10
11
12
13
Bacteria species

b)


Conjugate Control
Universal Control
MTBC
Specific gene probes for the differentiation of the Mycobacterium tuberculosis complex
M. tuberculosis
M. africanum
M. microti
M. bovis ssp. bovis
BCG
M. bovis ssp. caprae

Appendix VIII –Results of resistance to INH and RIF pasted on identification sheet

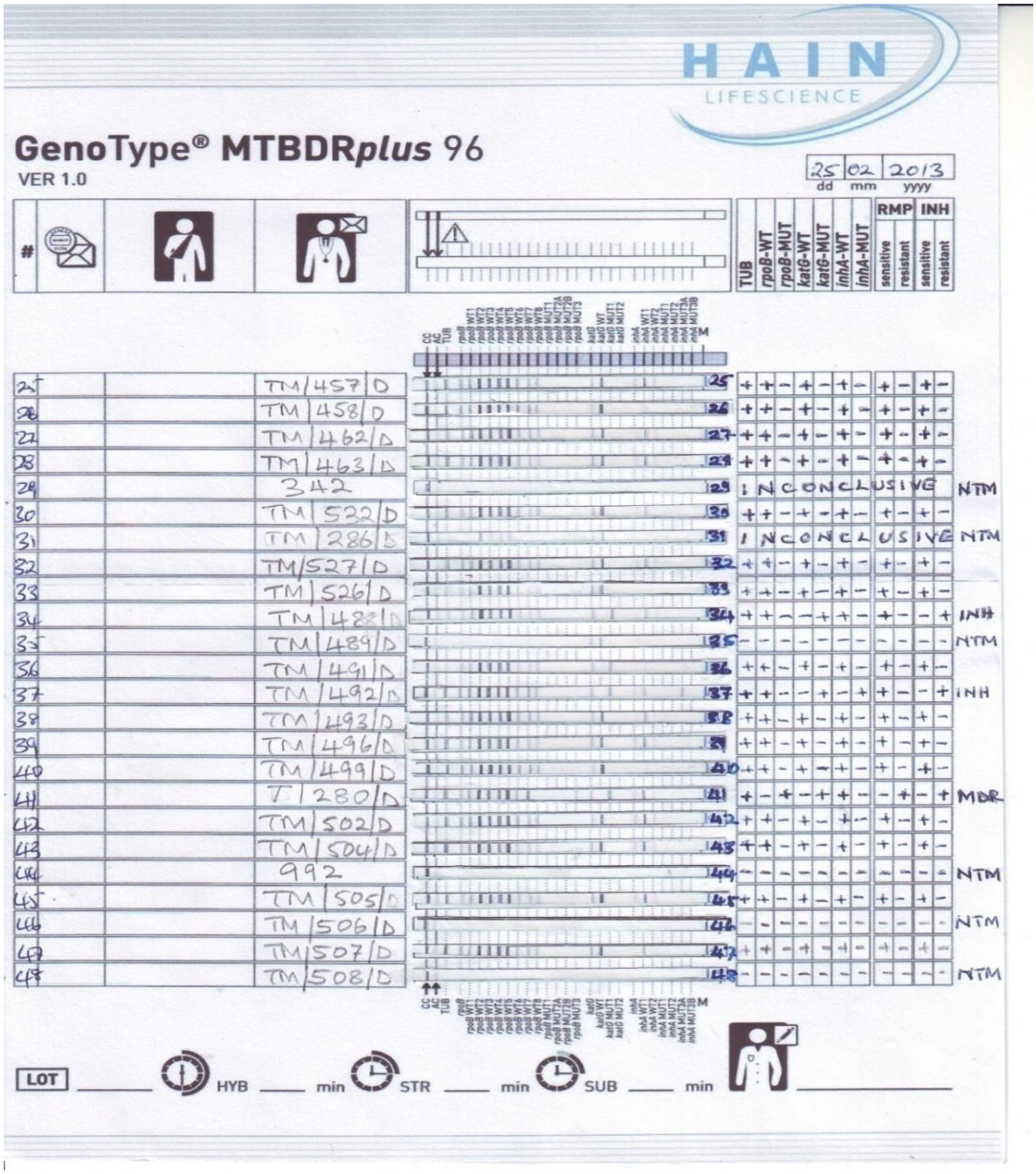


Figure 5.2a: DST on RIF and INH by Hain's GenoType® MTBDRplus 96; Isolates TM/492/D shows resistances to INH; and TM/286/D shows inconclusive results while 992 is MOTT.

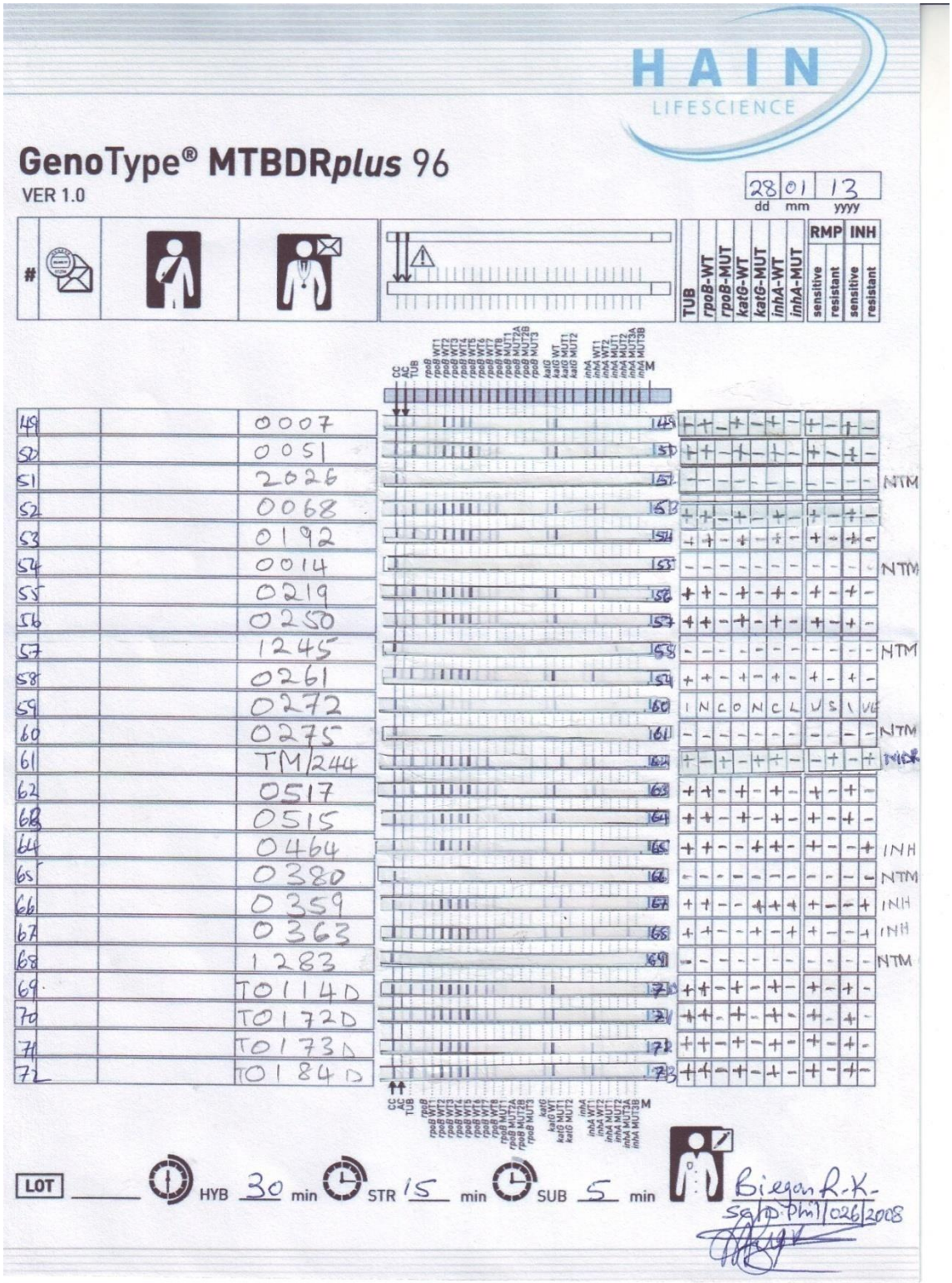
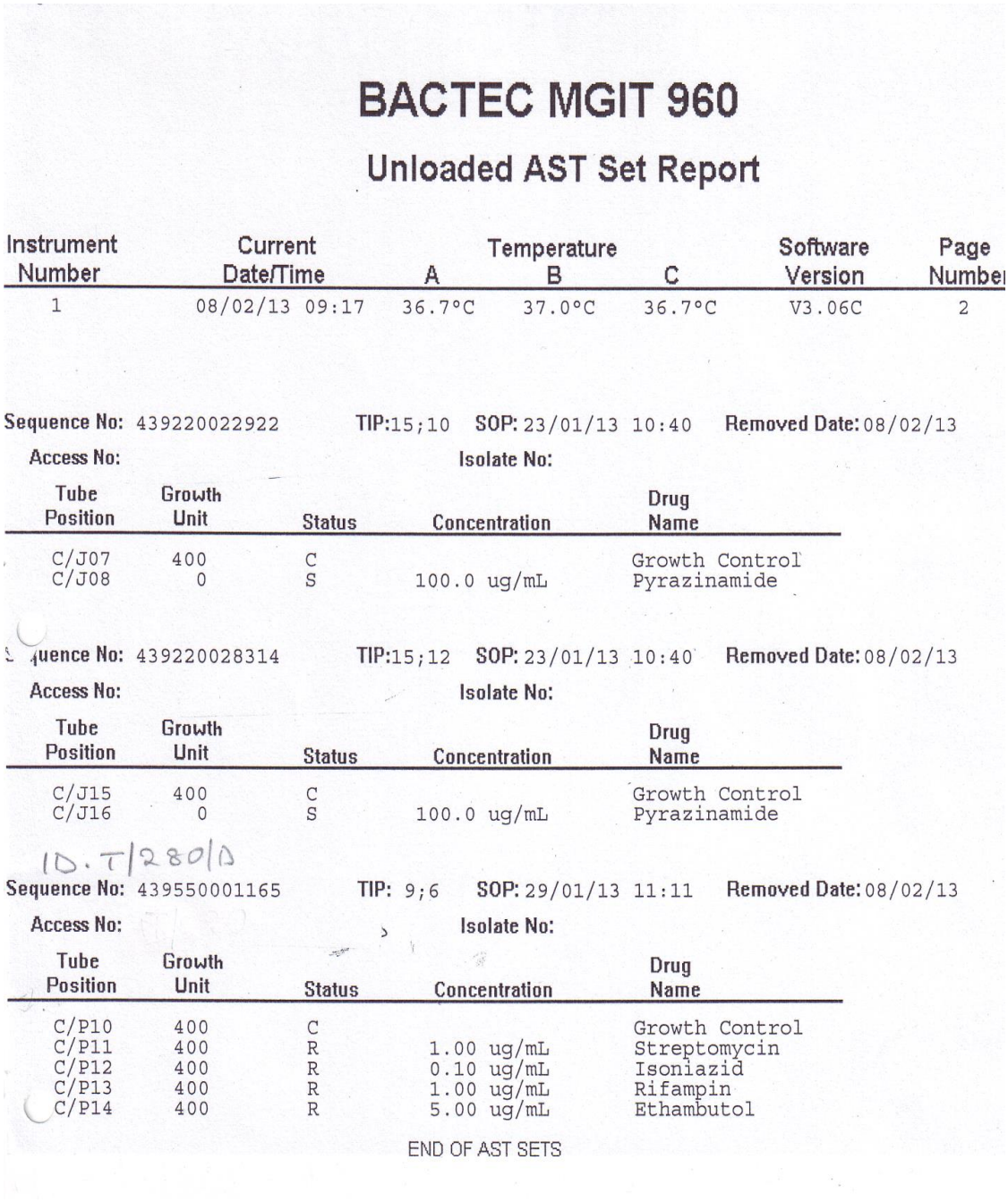


Figure 5.2b: DST on RIF and INH by Hain's GenoType® MTBDRplus 96; Isolates 0464, 0363 and 104 show resistances to INH; and TM/244 shows MDR-TB



BACTEC MGIT 960

Unloaded AST Set Report

Instrument Number	Current Date/Time	Temperature A	B	C	Software Version	Page Number
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Sequence No: 439220022922 **TIP:** 15;10 **SOP:** 23/01/13 10:40 **Removed Date:** 08/02/13

Access No: **Isolate No:**

Tube Position	Growth Unit	Status	Concentration	Drug Name
C/J07	400	C		Growth Control
C/J08	0	S	100.0 ug/mL	Pyrazinamide

Sequence No: 439220028314 **TIP:** 15;12 **SOP:** 23/01/13 10:40 **Removed Date:** 08/02/13

Access No: **Isolate No:**

Tube Position	Growth Unit	Status	Concentration	Drug Name
C/J15	400	C		Growth Control
C/J16	0	S	100.0 ug/mL	Pyrazinamide

ID. T/280/D

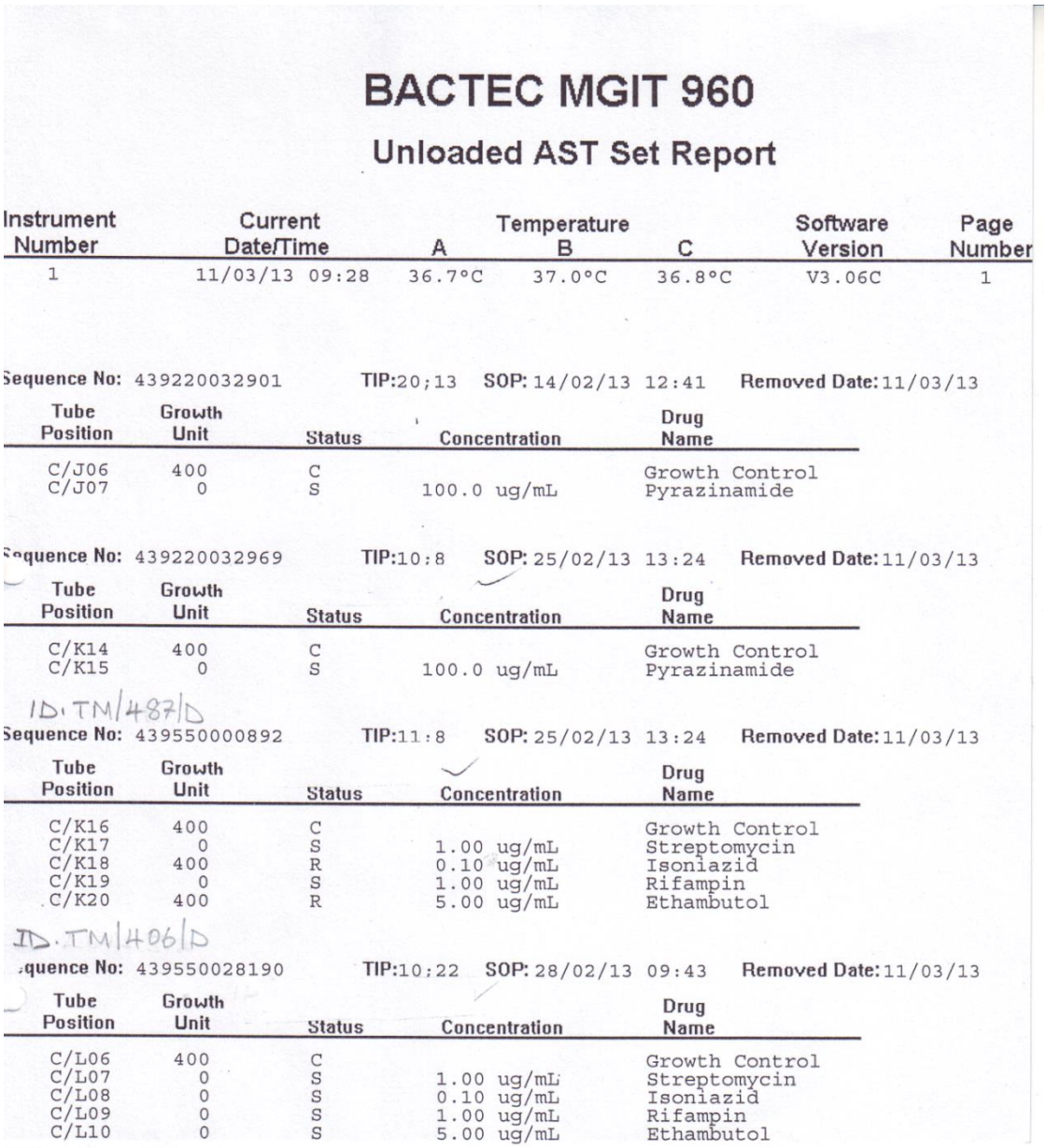
Sequence No: 439550001165 **TIP:** 9;6 **SOP:** 29/01/13 11:11 **Removed Date:** 08/02/13

Access No: **Isolate No:**

Tube Position	Growth Unit	Status	Concentration	Drug Name
C/P10	400	C		Growth Control
C/P11	400	R	1.00 ug/mL	Streptomycin
C/P12	400	R	0.10 ug/mL	Isoniazid
C/P13	400	R	1.00 ug/mL	Rifampin
C/P14	400	R	5.00 ug/mL	Ethambutol

END OF AST SETS

Figure 5.3a: DST on STR, INH, RIF and EMB by BACTEC MGIT 960; Isolates T/280/D shows resistance to all the four drugs (MDR-TB).



BACTEC MGIT 960

Unloaded AST Set Report

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Sequence No: 439220032901 TIP:20;13 SOP: 14/02/13 12:41 Removed Date: 11/03/13

Tube Position	Growth Unit	Status	Concentration	Drug Name
C/J06	400	C		Growth Control
C/J07	0	S	100.0 ug/mL	Pyrazinamide

Sequence No: 439220032969 TIP:10:8 SOP: 25/02/13 13:24 Removed Date: 11/03/13

Tube Position	Growth Unit	Status	Concentration	Drug Name
C/K14	400	C		Growth Control
C/K15	0	S	100.0 ug/mL	Pyrazinamide

ID: TM/487/D

Sequence No: 439550000892 TIP:11:8 SOP: 25/02/13 13:24 Removed Date: 11/03/13

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

ID. TM/406/D

Sequence No: 439550028190 TIP:10;22 SOP: 28/02/13 09:43 Removed Date: 11/03/13

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

Figure 5-3b: DST on STR, INH, RIF and EMB by BACTEC MGIT 960; Isolates TM/406/D shows sensitivity to all the four drugs while TM/487/D shows resistance to both INH and EMB.

Appendix IX: GIEMSA'S staining procedure

Labeling Slides

Glass slides should be clean, grease and scratch free and have smooth edges without any cuts. Label the slides appropriately with a pencil. Write neatly and firmly so that the information can be easily read.

Making Smears

i) For preparation of a thick smear, between one and three drops of blood should be placed in the centre of the slide and spread around evenly with a wooden stick or the corner of another slide to make a circle or square about 1cm.

ii) For preparation of a thin smear, a smaller drop of blood should be placed at the end of the slide. Using another slide, the blood can be spread to create a feathered edge that reaches the other end of the slide.

iii) The smears must be allowed to air dry free from flies and dust. Do not heat the slides as this will damage the parasites.

iv) The thin smear can be fixed by submerging in 100% methanol for 30 seconds and then letting the slide air dry.

v) Since methanol fixation would prevent haemolysis thick smears should not be fixed with methanol. This allows cell lysis necessary for accurate malaria diagnosis, parasite density calculation and identification of gametocytes.

Preparation of Alkaline and Acid buffers

i) Alkaline buffer (one litre)

a) Weigh out 9.5 g of Na_2HPO_4 (dibasic anhydrous)

b) Dissolve in 900 ml of distilled water

c) Fill to a total volume of 1 litre.

ii) Acid buffer (one litre)

a) Weigh out 9.2 g of Na_2HPO_4*H2O (monobasic monohydrate)

b) Dissolve in 900 ml of distilled water

c) Fill to a total volume of 1 litre.

Preparation of Giemsa's Staining Buffer

Mix together the proportions below to achieve a buffer of pH = 6.8:

Giemsa's staining buffer should be prepared every 1-2 weeks as needed.

Staining Thick Smears

1. Prepare 2 % Giemsa's staining solution daily (can be kept for approximately 8 hours).

2. Add Giemsa buffer to stain using the following mixture to achieve 2 % Giemsa:

a) 5 ml of buffer plus 100 µl of Giemsa

b) 10 ml of buffer plus 200 µl of Giemsa

c) 20 ml of buffer plus 400 µl of Giemsa.

3. Stain slides for 30 minutes with 2 % Giemsa staining solution.

4. Rinse slide carefully with distilled water.

5. Allow slide to completely dry (time will vary dependent on ambient temperature, but average is 15 minutes).

Staining Thin Smears

i) After fixing the slide with methanol, allow to dry for 1-2 minutes.

ii) Stain smears with 2 % Giemsa for 30 minutes.

iii) Rinse slide carefully with distilled water.

iv) Allow slide to completely dry (time will vary depending on ambient temperature, but average is 15 minutes).

Appendix X: Cytokines assay procedures

a) Human IL-4 ELISA

IL-4 (Interleukin-4, Ia inducing factor [IaIF], B-cell stimulating factor-1 [BSF-1], Hodgkin's cell growth factor [HCGF], Mast cell growth factor-2 [MCGF-2], macrophage fusion factor [MFF], T cell growth factor-2 [TCGF-2]) is a pleiotropic cytokine that is produced by activated T cells, mast cells and basophils. IL-4 elicits many different biological responses, but has two dominant functions. IL-4 regulates differention of naïve CD4 T cell to Th2 cells. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which tend to favor a humoral immune response while suppressing a cell-mediated immune response controlled by Th1 cells. In addition, IL-4 regulates IgE and IgG1 production by B cells.

Human IL-4 ELISA procedure

Human IL-4 ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is pre-coated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-6 from cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human IL-6 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-6 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-6 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-6 present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the micro well absorbance is read at 450 nm with a micro plate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. Prepare 500 µ1 of the 500pg/ml top standard by diluting 12.5 µl of the standard stock solution in 487.5 µl of assay buffer (provided with the kit). Then six two-fold serial dilutions of the 500 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-6 standard concentrations in the tubes were 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, and 7.8 pg/ml respectively. Assay buffer serves as the zero standard (0 pg/ml)
3. ELISA micro plate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
4. Add 50 µl of assay buffer to each well that will contain either standard dilutions or samples.
5. 50 µl/well of standards or samples were added to appropriate wells.
6. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
7. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
8. After washing, 100 µl of human IL-6 Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
9. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.

10. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.
11. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
12. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-10 were expected to turn blue in color with intensity proportional to its concentration.
13. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
14. The absorbance was then read within 30 minutes at 450 nm

b) Human IL-6 ELISA

IL-6 (also known as B-cell stimulatory factor 2, interferon beta-2, Hybridoma growth factor, and CTL differentiation factor) plays an essential role in the final differentiation of B-cell into Ig-secreting cells. It induces myeloma and plasmacytoma growth, nerve cells differentiation, and acute phase reactants in hepatocytes. IL-6 is expressed by T cells, B cells, monocytes, fibroblasts, hepatocytes, endothelial cells, and keratinocytes. It has been shown that IL-6 plays a critical role in many physiological and pathological conditions, including autoimmune diseases and rheumatoid arthritis.

Human IL-6 ELISA procedure

Human IL-6 ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is pre-coated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-6 from cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human IL-6 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-6 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-6 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-6 present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the micro-well absorbance is read at 450 nm with a micro plate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. Prepare 500 µ1 of the 500pg/ml top standard by diluting 12.5 µl of the standard stock solution in 487.5 µl of assay buffer (provided with the kit). Then six two-fold serial dilutions of the 500 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-6 standard concentrations in the tubes were 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, and 7.8 pg/ml respectively. Assay buffer serves as the zero standard (0 pg/ml)

3. ELISA micro plate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
4. Add 50 µl of assay buffer to each well that will contain either standard dilutions or samples.
5. 50 µl/well of standards or samples were added to appropriate wells.
6. The ELISA micro plate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
7. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
8. After washing, 100 µl of human IL-6 Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
9. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
10. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.
11. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.

12. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-10 were expected to turn blue in color with intensity proportional to its concentration.
13. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
14. The absorbance was then read within 30 minutes at 450 nm

c) Human IL-10 ELISA

Human IL-10 was originally described as Cytokine Synthesis Inhibitory Factor (CSIF) due to its ability to inhibit cytokine production by Th1 clones. IL-10 is expressed in activated CD8+ and CD4+ T cells, activated monocytes, mast cells, and Ly-1 B cells. IL-10 shares over 80% sequence homology with the Epstein-Barr virus protein BCRFI. The functions of IL-10 include inhibition of macrophage-mediated cytokine synthesis and suppression of the delayed type hypersensitivity response.

IL-10 is a powerful type 2 anti-inflammatory cytokine necessary to prevent damage from over-production of pro-inflammatory responses, such as TNF-α, during infection (Ho *et al.*, 1998). Unfortunately, an over-abundant type 2 response is just as detrimental to the resolution of, and recovery from, malaria (Ferrante *et al.*, 1990; Day *et al.*, 1999). It has been shown that IL-10 suppresses IL-12 production using both *in vitro* experiments with PfHz and *in vivo* findings (Keller *et al.*, 2006). Studies conducted in Gabonese children with severe malaria showed low IL-12 levels in the presence of elevated circulating IL-10 and TNF-α concentrations (Luty *et al.*, 2000; Perkins *et al.*, 2000).

Human PBMC Cell proliferation

Human PBMC (1 x 10^6 cells/ml) were stimulated with purified protein derivative (PPD) of *M. tuberculosis* at a concentration of 10 µg/ml for 48 hours at 37^0 C in CO2 incubator. After 48 hours of incubation (37^0 C, 5% CO_2 tension with 95% humidity), cell culture supernatants were collected and assayed for the concentration of human IL-10 using ELISA (BioLend® Legend Max™ San Diego, CA). Centrifuge cell culture supernatant to remove debris prior to analysis. Samples can be stored at ≤ -20^0 C. A void repeated freeze/thaw cycles

Human IL-10 ELISA procedure

Human IL-10 ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA. **Brief procedure:** A human IL-10 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-10 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-10 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-10 present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate ELISA reader.

Assay procedure:

1. Dilute Capture Antibody in 1x coating buffer (provided)
2. Add 100 µl of the Capture antibody solution to all wells of a 96-well plate provided in the set. Seal plate and incubate overnight (16-18 hrs) at 4^0 C.

3. All reagents in the kit were brought to room temperature prior to use
4. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
5. To block non-specific binding and reduce background, add 200 µl 1x Assay diluent per well
6. Seal plate and incubate at RT for 1 hour with shaking at 200 rpm on plate shaker
7. 1,000pg/ml top standard was then prepared at 250 pg/ml from standard stock solution in 1x assay buffer (provided with the kit). The six two-fold serial dilutions of the 250 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-10 standard concentrations in the tubes were 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml,7.8 pg/ml and 3.9 pg/ml respectively. 1x Assay diluent serves as the zero standard (0 pg/ml)
8. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
9. 100 µl/well of standards or samples were added to appropriate wells.
10. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
11. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 8.

12. After washing, 100 µl of human IL-10 Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
13. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 8.
14. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.
15. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
16. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-10 were expected to turn blue in color with intensity proportional to its concentration.
17. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
18. The absorbance was then read within 30 minutes at 450 nm

d) Human IL-12 ELISA

IL-12 is a potent regulator of cell-mediated immune responses that induces IFN-γ production by NK and T cells. It is secreted from DCs, activated monocytes/macropgages, B lymphocytes, and connective tissue type mast cells as a 70 kD heterodimeric glycoprotein comprised of disulfide-bonded 35 and 40 kDa

subunits.IL-12 is secreted in response to bacterial cell wall components, intracellular pathogens, and by CD40 ligation and stimulates production of IFN-γ and TNF-α from T-cells and NK cells, thereby driving type 1 responses. Among its biological activities, IL-12 promotes the growth and activity of activated NK, CD4+, and CD8+ cells and induces the development of IFN-γ producing Th1 cells. Other cytokines and chemokines can either promote IL-12 [e.g., granulocyte macrophage-colony stimulating factor (GM-CSF) and IFN-γ] or hinder its production [e.g., IL-4, IL-10, IL-11, IL-13, monocyte chemotactic protein (MCP)-1/CCL2, and TGF-β]. IL-12 therefore, has myriad of crucial immune functions during infection, but its role as a hematopoietic growth factor is particularly important in malaria infection.

Human IL-12 ELISA procedure

Human IL-12 (p40) ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is precoated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-12 (p40) from cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human IL-12p40 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-12p40 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-12p40 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-12p40 present in the sample. Finally, the stop solution changes the

reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. Prepare 500 µ1 of the 250pg/ml top standard by diluting 6.25 µl of the standard stock solution in 493.8 µl of assay buffer (provided with the kit). Then six two-fold serial dilutions of the 500 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-12 (p40) standard concentrations in the tubes were 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 7.8 pg/ml and 3.9 pg/ml respectively. Assay buffer serves as the zero standard (0 pg/ml)
3. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
4. Add 50 µl of assay buffer to each well that will contain either standard dilutions or samples.
5. 50 µl/well of standards or samples were added to appropriate wells.
6. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
7. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.

8. After washing, 100 µl of human IL-12p40 Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
9. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
10. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.
11. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
12. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-12p40 were expected to turn blue in color with intensity proportional to its concentration.
13. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
14. The absorbance was then read within 30 minutes at 450 nm

e) TCF-β1 ELISA

Transforming growth factor beta 1 (TCF-β1) is a member of the transforming growth factor beta superfamily of cytokines. TCF-β1 precursor contains 390 amino acids withan N-terminal signal prptide of 29 amino acids which is required for secretion from a cell, a 249 amino acid pro-region (latency associated peptide or LAP), and a 112 amino acid C-terminal region that becomes active TCF-β1upon activation. TCF-β1 is an anti-

inflammatory cytokines which regulate immune response to TB. A pathway of T cell activation, initiated by TGF-β and this results in responsiveness to IL-23 result in antigen-specific T cells that produce IL-17 (Th17). Th17 cells have a crucial role in induction of autoimmune tissue injury and are regulated by TGF-β.

Human TGF-β ELISA procedure

Human TGF-β ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is precoated with a capture antibody. This kit is specifically designed for the accurate quantitation of human TGF-β from cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human TGF-βspecific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and TGF-β binds to the immobilized capture antibody. Next, a biotinylated anti-human TGF-β detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by tetramethyl benzidine (TMB) substrate solution, producing a blue color in proportion to the concentration of TGF-β present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. Prepare 500 µ1 of the 500pg/ml top standard by diluting 12.5 µl of the standard stock solution in 487.5 µl of assay buffer (provided with the kit). Then six two-

fold serial dilutions of the 500 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human TGF-β standard concentrations in the tubes were 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml,and 7.8 pg/ml respectively. Assay buffer serves as the zero standard (0 pg/ml)

3. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
4. Add 50 µl of assay buffer to each well that will contain either standard dilutions or samples.
5. 50 µl/well of standards or samples were added to appropriate wells.
6. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
7. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
8. After washing, 100 µl of human TGF-β Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
9. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
10. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.

11. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
12. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human TGF-β were expected to turn blue in color with intensity proportional to its concentration.
13. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
14. The absorbance was then read within 30 minutes at 450 nm

f) Human IL-8 (CXCL8) ELISA

Human IL-8 (CXCL8, neutrophil chemotactic factor, neutrophil activating protein, monocytes derived neutrophil chemotactic factor) is a member of the alpha (C-X-C) subfamily of chemokines. In response to proinflammatory stimuli, IL-8 is produced by monocytes, macrophages, T cells, neutrophils, and fibroblasts. IL-8 promotes neutrophil chemotaxis and degranulation leading to local inflammation in damaged or infected tissues.

Human IL-8 (CXCL8) ELISA procedure

Human IL-8 (CXCL8) ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is precoated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-8 (CXCL8) from

cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human IL-8 (CXCL8) specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-8 (CXCL8) binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-8 (CXCL8) detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-8 (CXCL8) present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. Prepare 500 µ1 of the 1,000pg/ml top standard by diluting 25 µl of the standard stock solution in 475 µl of assay buffer (provided with the kit). Then six two-fold serial dilutions of the 1,000 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-8 (CXCL8) standard concentrations in the tubes were 1,000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml, 31.3 pg/ml and 15.6 pg/ml, respectively. Assay buffer serves as the zero standard (0 pg/ml)
3. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
4. Add 50 µl of assay buffer to each well that will contain either standard dilutions or samples.

5. 50 µl/well of standards or samples were added to appropriate wells.
6. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
7. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
8. After washing, 100 µl of human IL-8 (CXCL8) Detection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
9. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
10. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.
11. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
12. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-8 (CXCL8) were expected to turn blue in color with intensity proportional to its concentration.
13. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
14. The absorbance was then read within 30 minutes at 450 nm

Human IL-5 ELISA

Upon antigenic stimulation, CD4+ Th cells differentiate into Th1 cells, which secrete cytokines that are involved in cell-mediated immune response (IFN-γ and IL-12) or Th2 cells that secrete mediators of humoral immunity (IL-4 and IL-5).

Human IL-5ELISA procedure

Human IL-5 ELISA (BioLend® Legend Max™ San Diego, CA) Kit is a sandwich ELISA with a 96-well strip plate that is precoated with a capture antibody. This kit is specifically designed for the accurate quantitation of human IL-5 from cell culture supernatant, serum, plasma and other biological fluids. **Brief procedure:** A human IL-5 specific monoclonal antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and IL-5 binds to the immobilized capture antibody. Next, a biotinylated anti-human IL-5 detection antibody is added, producing an antibody-antigen-antibody "sandwich". Avidin-horseradish peroxidase is subsequently added, followed by TMB substrate solution, producing a blue color in proportion to the concentration of IL-5 present in the sample. Finally, the stop solution changes the reaction color from blue to yellow, and the microwell absorbance is read at 450 nm with a microplate ELISA reader.

Assay procedure:

1. All reagents in the kit were brought to room temperature prior to use.
2. To block non-specific binding and reduce background, add 200µl assay diluents per well
3. Seal plate and incubate at RT fir 1 hour with shaking at 200rpm on a plate shaker.

4. Prepare 1,000 µ1 of the 250 pg/ml top standard from stock solution in assay diluent. Then perform six two-fold serial dilutions of the 250 pg/ml top standard were performed with 1x assay diluents in separate tubes. Thus, the human IL-5 standard concentrations in the tubes were 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml, 7.8pg/ml and 3.9pg/ml, respectively. Assay buffer serves as the zero standard (0 pg/ml)
5. ELISA microplate was washed 4 times with at least 300 µl of 1x wash buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes were performed similarly.
6. Add 100 µl/well of standard dilutions and samples to the appropriate wells. If needed, samples can be further diluted with assay diluents before adding 100µl/well diluted samples.
7. The ELISA microplate was then sealed with a plate sealer (included in the kit) and incubated at room temperature for 2 hours while shaking at 200 rpm.
8. After 2 hours of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
9. After washing, 100 µl of dilutedDetection Antibody solution was added to each well, sealed the plate and incubated at room temperature for 1 hour while shaking.
10. After 1 hour of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 4 times with 1x wash buffer as in step 3.
11. After washing, 100 µl of Avidin-HRP solution was added to each well, seal the plate and incubated at room temperature for 30 minutes while shaking.

12. After 30 minutes of incubation, the contents of the plate were discarded into the sink, and then the plate was washed 5 times with 1x wash buffer as in step 3. For this final wash, the wells were soaked in 1x wash buffer for 30 seconds to 1 minute for each wash. This was to minimize background.
13. After washing, 100 µl of TMB substrate solution and incubated at room temperature for 30 minutes in the dark. Wells containing human IL-5 were expected to turn blue in color with intensity proportional to its concentration.
14. 100 µl of Stop solution (2N H_2SO_4) was the added to each well to stop the reaction. The solution color should change from blue to yellow.
15. The absorbance was then read within 30 minutes at 450 nm

Appendix XI: IREC Approval letter

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

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P.O. BOX 3
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Reference: IREC/2010/155
Approval Number: 000657

1st July, 2011

Richard K. Biegon
Moi University
School of Medicine
P. O. Box 4606 - 30100
ELDORET- KENYA.

APPROVED
01 JUL 2011

Dear. Mr. Biegon

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:

"Genetic Diversity among Mycobacterium Tuberculosis Complex Strains and Immune Markers Defining Tuberculosis in Malarial – Infected Women and Children in Western Kenya.. "

Your proposal has been granted a Formal Approval Number: **FAN: IREC 000657** on 1st July, 2011. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 2nd July, 2012. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

DR. W. ARUASA
AG. CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc: Director - MTRH
Dean - SOM
Dean - SPH
Dean - SOD