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CLINICAL AND PATHOLOGICAL CHARACTERISTICS OF THE UGANDA GENOTYPE OF *MYCOBACTERIUM TUBERCULOSIS*

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedication

This thesis is dedicated to my mother and all my children.

ABSTRACT

Mycobacterium tuberculosis (Mtb), the aetiological agent of tuberculosis (TB) is the leading infectious cause of death globally. The outcome of Mtb infection is variable and depends on host, bacterial and environmental factors. Mtb has evolved into a number of lineages and sub-lineages exhibiting phylogeographical population structuring and diverse clinical consequences after infection. The Mtb Uganda genotype is the commonest cause of pulmonary TB (PTB) in Kampala, Uganda. Prominent inherited host factors that determine the outcome of TB are human leukocyte antigens (HLA).

To investigate the impact of Mtb genomic diversity and host HLA allelic variability on the clinical outcome of TB infection in Ugandan patients, the clinical and pathological outcome of Mtb Uganda genotype, and the association between HLA II alleles and PTB due to Mtb were studied. The Uganda genotype was found less frequently in extrapulmonary TB (EPTB) than previously reported in PTB in the same setting (Paper I), and tuberculous lymphadenitis patients infected with Mtb Uganda genotype were significantly less prone to have abdominal lymphadenopathy (Paper IV). This may imply that Mtb Uganda genotype has reduced potential to disseminate.

A study of the evolutionary relationships and worldwide distribution of the spoligotypes of Mtb isolates from Ugandan patients with tuberculous lymphadenitis indicated an ongoing evolution of the Uganda genotype, with Uganda at the center of this evolution (Paper II).

HIV negative patients with pulmonary TB and their genetically related healthy household controls were typed for HLA class II alleles (Paper III). The HLA- DQB1*03:03 allele was significantly less frequent in patients compared to healthy controls suggesting that the HLA-DQB1*03:03 allele may be associated with resistance to TB.

To establish the cause and pathology of fatal mycobacterial disease, the mycobacteria and pathology associated with fatal TB were studied (Paper IV). One quarter of fatal mycobacterial disease was associated with non tuberculous mycobacteria (NTM). Pleural effusions were significantly associated with Mtb disease compared to NTM infection (Paper IV).

To explore the potential use of the CD4+ and CD8+ T cell immunoprofile to diagnose tuberculous effusion, CD4+ and CD8+ T cells from pleural effusions were characterized. CD4+ T cells were significantly more abundant in individuals with TB, and the CD4+/CD8+T cell ratios were significantly higher in tuberculous pleural effusion compared to non tuberculous effusion, however this significance was lost after adjusting for age and ethnicity. Analysis of pleural fluid for the quantity of CD4+ and CD8+ T cells may be useful for establishing a diagnosis of TB in suspicious cases (Paper V).

In conclusion, this thesis highlights the genetic diversity of Mtb with Mtb Uganda as the predominant genotype in EPTB patients in Uganda. Both NTM and Mtb are associated with fatal mycobacterial disease and the pathology findings are indistinguishable, though NTM are significantly less likely to cause pleural effusion. Mycobacterial genetic diversity together with host HLA variability may have clinical consequences. This can be exploited in designing TB diagnostic, management and prevention strategies.

LIST OF SCIENTIFIC PAPERS

I. Wamala D, Asiimwe B, Kigozi E, Mboowa G, Joloba M, Källenius G. Clinico-pathological features of tuberculosis due to Mycobacterium tuberculosis Uganda genotype in patients with tuberculous lymphadenitis: a cross sectional study. BMC Clinical Patholology, 2014;14(1):14. doi: 10.1186/1472-6890-14-14.

II. Wamala D, Okee M, Kigozi E, Nalin R , Couvin D, Joloba M, Källenius G. Predominance of Mycobacterium tuberculosis Uganda genotype in Ugandan patients with tuberculous lymphadenitis. BMC Research Notes, 2015, 8:398 doi:10.1186/s13104-015-1362-y,

III. Wamala D, Buteme H, Kirimundu S, Joloba M, Källenius G. Association between Human Leukocyte Antigen Class II and pulmonary tuberculosis due to Mycobacterium tuberculosis in Uganda. BMC Infectious Dis. 2016;16(1):016-1346. 23 doi: 10.1186/s12879-016-1346-0.

IV. Wamala D, Källenius G, Joloba M. Morbid anatomy of patients with mycobacterial infections, a cross sectional autopsy study at a tertiary hospital in a high disease burden setting. Manuscript.

V. Wamala D, Joloba M, Hjerpe A. Potential use of CD4+ and CD8+ T cell immunoprofile from pleural fluid to diagnose tuberculous effusions, Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
ARV	Antiretroviral therapy
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guerin
CD	Cluster of differentiation
CI	Confidence interval
CCR	CC-Chemokine receptor
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	Dendritic cell specific ICAM-3-grabbing non-integrin
DQ	Diff Quick
DTH	Delayed-type hypersensitivity
FNA	Fine needle aspiration
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IRIS	Immune reconstitution inflammatory syndrome
IS	Insertion sequence
MHC	Major Histocompatibility complex
mRNA	Messenger RNA
Mtb	Mycobacterium tuberculosis
NK	Natural killer
NTM	Non tuberculous mycobacteria
OR	Odds ratio
PAMP	Pathogen-associated molecular pattern
Pap	Papanicolaou

PCR	Polymerase chain reaction
PRR	Pathogen recognition receptors
RFLP	Restriction fragment length polymorphism
SNP	Single-nucleotide polymorphism
TB	Tuberculosis
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WHO	World Health Organization
ZN	Ziehl Neelsen

1 INTRODUCTION

1.1 TUBERCULOSIS

1.1.1 Historical overview

The infectious nature of tuberculosis (TB) was first demonstrated in 1865 by a French physician, Jean Antonie Villemin, who demonstrated that rabbits inoculated with TB organisms developed the disease. TB has afflicted man for centuries though its etiology and infectious nature was first described by Robert Koch in 1892 [1]. The earliest evidence was of pulmonary and spinal TB in a 5-year old Egyptian mummy in 3400 BC. In Europe, TB infected between 70 and 90% of the urban population in the late nineteenth century [2]. George Canetti first published the results from early studies on the nature of various types of TB lesions in man during the 1940s. Based on autopsies of 1500 TB patients performed during a 7-year period, Canetti described three different features from a large variety of human TB lesions based on: (i) the histopathologic features of the lesion. (ii) the number of acid-fast bacilli observed by microscopic examination and (iii) the number of culturable bacilli on egg medium. He categorized the three different types of lesions in man further into various subtypes: (i) lesions characterized by marked inflammation including vasodilatation, edema, fibrinous exudate, and an influx of lymphocytes, (ii) lesions characterized by a cellular influx of monocytes transforming into giant cells which are characteristic of a classical granuloma or tubercle and (iii) lesions characterized by necrosis and homogenization. Necrotic lesions may remain solid with very few bacilli, and evolve to sclerosis, or the caseum may soften with accompaniment of enormous bacillary load, which he associated with the onset of clinical TB.

1.1.2 TB Globally

The World Health Organization (WHO) declared TB a global emergency in 1993, with a predicted mortality of 20 million people in a decade [3]. In 2014 TB was ranked alongside HIV as the leading infectious cause of death, accounting for nine million new cases and 1.5 million deaths globally, 400,000 of whom were HIV positive [4]. About one-third of the world's population (1.7 billion) is estimated by WHO to be infected with Mtb. More than half of TB patients live in Asia and Africa [5]. Uganda is one of the countries with the highest TB burden in sub-Saharan Africa, and ranks 15th among the 22 high-burden countries. The

annual TB incidence in Uganda is 559 cases per 100,000 [6]. TB is the leading cause of morbidity and mortality in individuals with HIV in sub-Saharan Africa [7]. Mortality rates of up to 40% per year have been reported in HIV patients co-infected with Mycobacterium tuberculosis (Mtb) on TB treatment [8]. According to WHO, 1.2 million people (13%) of the 9.6 million people who fell ill with TB in 2014 were co-infected with HIV [4]. A significant number of patients with AIDS will have active TB, because the AIDS virus has a profound and detrimental effect on the immune response which normally controls the mycobacterium [9-11].

1.1.3 Pathogenesis of TB

The majority of Mtb infected individuals do not develop active disease for many years. TB re-emergence and spread is determined by i) infectiousness of the infecting person (number of bacilli expelled in air), ii) TB exposure duration, iii) host susceptibility, and iv) virulence of the pathogen. Though Mtb is the predominant cause of TB, other species of the Mtb complex including *M. africanum*, *M. Canetti*, *M. bovis*, *M. bovis* BCG, *M. microti*, *M. caprae* and *M. pinnipedii* [12-14] may also cause disease. After infection with Mtb, the majority of infected people remain asymptomatic (latent TB), the infection being controlled by the immune response and no clinical disease develops [15]. Mycobacterial disease is also caused by various species of non-tuberculous mycobacteria (NTM).

The risk for developing progressive disease is 5% annually (for 5 years) following primary infection [16]. This risk is 1.91% annually (for 5 years) following re-infection [16]. The lifetime risk of developing clinical TB is 10%, this however increases to an annual risk of over 10% in HIV infected individuals [17, 18]. The risk of developing primary TB is lower in children than adults [16], while children have a higher likelihood of developing severe disseminated TB than adults. Young women have an increased likelihood to develop active TB following infection than men of the same age [19, 20].

Tubercle bacilli are transmitted from person to person through aerosols, small droplet nuclei size 5µ, 1-3 bacilli per nuclei generated during coughing, singing, laughing and sneezing. These droplets are expelled and lose moisture by evaporation, leaving a solid nucleus. Coughing is the most efficient method of generating droplet nuclei [21]. Particles less than 5µm filter through the ciliated epithelium and end up in the lung alveoli [22, 23]. In the alveoli, the tubercle bacilli encounter the alveolar macrophages. An inflammatory process is initiated at the site of primary infection whose magnitude depends on the host immune

genetics, extrinsic immune system insults and virulence of the invading tubercle bacilli [24]. Concurrently, there is systemic dissemination of bacilli to other regions of the lung and organs. At this stage, active TB disease may develop if there is sustained proliferation of the bacilli.

1.1.4 Immune system and mycobacteria

TB infection has five stages based on rabbit studies [25]. The first onset stage begins when the bacilli are inhaled into the alveoli and phagocytosed by the non-specifically activated alveolar macrophages with eventual destruction [26]. Bacillary destruction is partially achieved resulting in the second stage called symbiosis [25]. During this stage, the bacilli multiply and reside in the macrophage without causing any progressive disease [27-29], the number of macrophages also increases but do not kill the bacilli. Activation of tissue damaging delayed-type hypersensitivity (DTH) with resultant caseous necrosis terminates the symbiotic stage.

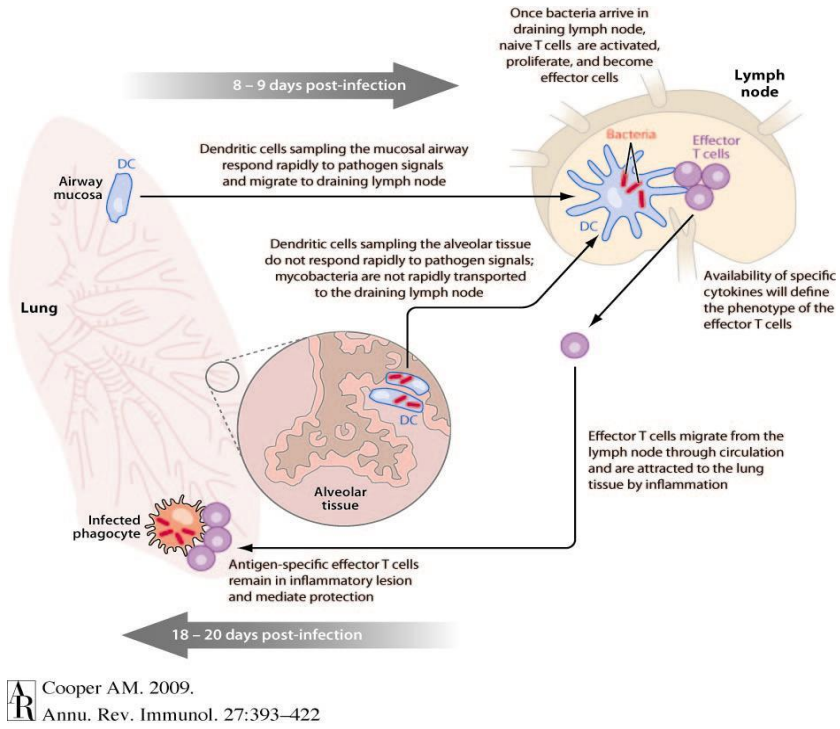


Figure 1. Primary TB: Illustration of the trafficking of Mtb antigen by dendritic cells to regional lymph nodes leading to initiation of adaptive immunity by Cooper AM et al [30]. Permission granted by Cooper AM et al and Elsevier publishers.

The third stage is the caseous stage characterized by bacillary caseation and extensive cellular destruction at the centre of the granuloma or tubercle called caseous necrosis. The fourth stage involves dynamic equilibrium of tissue destruction and immune activation response. The fifth stage is liquefaction and cavity formation characterized by extensive destruction of granuloma tissue due to enzymatic activity. From the alveoli, the bacilli may be released into the environment through coughing or may invade into the blood stream resulting in dissemination. During this innate phase, the granuloma has been shown to be the epitome of pathogenic mycobacterial expansion and systemic dissemination [31].

The bacillary multiplication is usually controlled until the development of adaptive immunity in 3 to 8 weeks after primary infection [32]. Macrophages and dendritic cells provide a link between innate and adaptive immunity through receptor mediated interaction with Mtb. Numerous receptors expressed on phagocytic macrophages bind mycobacteria before their uptake [33]. These include C-type lectin receptors (CLRs), scavenger receptors (SR) and complement receptors [33]. The recognition of pathogen-associated molecular patterns (PAMP) by specific pathogen recognition receptors (PRRs) is pivotal to the initiation and coordination of the host innate immune response [34].

The PRR mediated macrophage phagocytosis includes Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD-) like receptors (NLRs), and C-type lectins. The C-type lectins include mannose receptor (CD207), the dendritic cell-specific intercellular adhesion molecule grabbing nonintegrin (DC-SIGN) and Dectin [35]. Interaction between PRR and PAMP result in intra- cellular signaling culminating in production of immune mediating pro-inflammatory cytokines like IL -1β, TNF-α and IL-6 [35-37].

Dendritic cells and infected macrophages traffic to the regional lymph nodes to present antigen to naïve T cells in association with MHC class I and II or CD1 molecules, priming them against mycobacterial antigens [38].

The T cells are activated, undergo clonal expansion and acquire effector functions [39, 40]. The Mtb antigenic specific T cell subset that directs the cell mediated response against TB in order of decreasing importance are CD4+αβ T lymphocytes, CD8+αβ T lymphocytes, γδ T lymphocytes and CD1 restricted αβ T cells [41]. These T cells can produce cytokines, kill antigen expressing cells in case of cytolytic T lymphocytes (CTL) and mobilize and potentiate other leukocytes (in case of T helper (TH) cells) the effector T cells migrate to the primary infection site (Figure 1). The cytokines functionally potentiate the resident macrophages and induce recruitment of more macrophages to the site of inflammation. The

activated macrophages though more efficient in killing the bacilli, do not completely eliminate them. This primary infection may reactivate later in life, a process called endogenous reactivation. In early phase of TB infection, the bacteria laden macrophages may cross the alveolar barrier to cause systemic spread [22, 42].

The intracellular proliferation of Mtb bacilli, their spread to lymph nodes and systemic dissemination occurs before the development of the adaptive immunity. In the majority of hosts, a cell mediated immune response that develops 2-8 weeks after the infection controls multiplication of the bacilli. The activated T lymphocytes, macrophages, and other immune cells constitute granulomas (Figure 2) that control further replication and spread of the tubercle bacilli [43]. Most Mtb are killed in the caseating granulomas.

Primary TB infection occurs in individuals previously unexposed to Mtb or its antigens [44]. In the majority of patients, the primary TB is inhibited resulting in a standoff between the Mtb bacilli and the host. The primary TB lesion is a ghon focus comprising foci of central necrosis surrounded by epitheloid macrophages and lymphocytes. Although in majority of cases the primary tuberculous caseous lesions heal with fibrosis and scarring, caseous necrosis may enlarge and progress. The dendritic cell traffic the bacilli to regional lymph nodes resulting in caseating granulomatous lymphadenitis, or the bacilli may systematically disseminate to result in disseminated or miliary TB.

A tuberculous lesion will progress into active disease or regress depending on the virulence and burden of the tubercle bacilli and the capacity of the macrophages to inhibit the growth of the bacilli. The main types of tuberculous lesions comprising proliferative and exudative lesions are described [45]. The proliferative lesions are characterized by a cellular influx of lymphocytes, plasma cells, fibroblasts, macrophages and langhan giant cells, morphologically forming a granuloma, while the exudative lesion is characterized by necrosis and homogenization [45].

Liquefied pulmonary caseous lesions or caseating lymph nodes erode into the air passages progressing into a spectrum of disease ranging from small pneumonic foci to lobar or bronchopneumonia. The extent of the resulting disease depends on the quantity of aspirated caseum and bacilli.

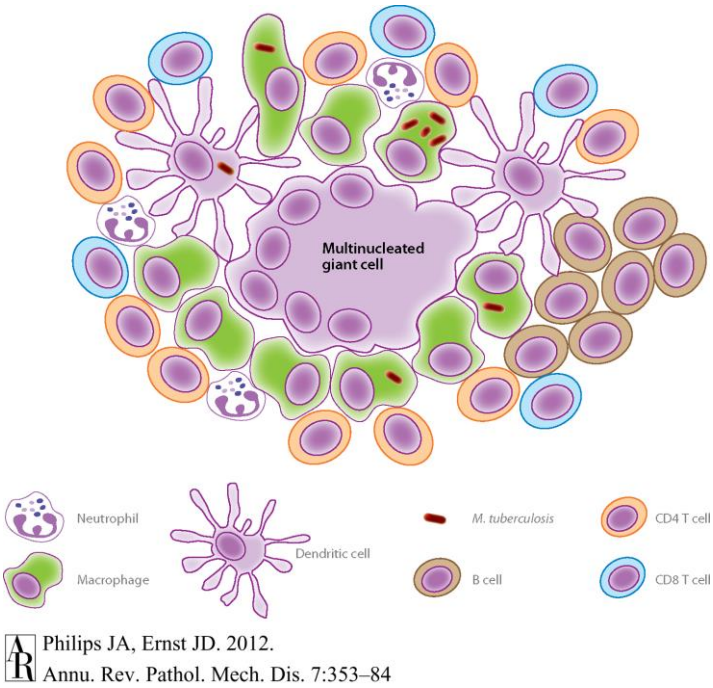


Figure 2. Granuloma structure as depicted by Phillips JA et al [33]. Permission granted by the author and Elsevier Publishers.

1.1.5 Tuberculous pleural effusion

The immunological response during the development and progression of TB pleuritis occurs in three stages. The first stage is mediated by the production of IL-8 and monocyte chemotactic peptide (MCP-1) by activated mesothelial cells which acutely recruit polymorphonuclear neutrophils (PMNs) and monocytes [46]. PMNs and monocytes respond by secreting key cytokines such as TNFα, and IL-6.

The intermediate stage follows in which CD4+ helper cells and CD8+ cytotoxic cells dominate culminating into a CD4+/CD8+ ratio of about 4.3 [47]. Finally, inflammation persists resulting in a sustained CD4+/CD8+ cell response, persistent INF-ϒ release and granuloma formation.

1.1.6 Mtb and HIV co-infection

HIV infection has a profound effect on the capacity to resist Mtb infection, and renders Mtb-infected individuals susceptible to develop active TB [10, 11]. The hallmark of the HIV-infection is the impairment of T cell functions and the ultimate loss of $CD4^+$ T cells resulting in impaired ability of the host to mount an effective Mtb specific T cell response [11, 48-51]. Deficient maturation and function of CD8+ cytolytic T cells as well as deficient granuloma formation may reduce the control of latent TB [48, 52]. With regard to the innate immune response, several studies have addressed the ability of macrophages to control the growth and/or kill intracellular Mtb. However, data in this respect has been quite controversial [11].

HIV infection is associated with impaired recruitment and function of macrophages to sites of TB infection resulting in deficient granuloma formation depending on the degree of immunosuppression [10]. HIV and TB infections fuel each other.

1.1.7 Human leukocyte antigen

One of the most intriguing aspects of this disease is that the outcome of an infection with Mtb is highly variable between individuals. There is evidence of multifactorial genetic predisposition in humans that influences the susceptibility to TB. The majority of infected persons (about 90%) will never develop the disease [53], the immune system apparently being capable of permanent containment of the infection. In the 10% of individuals who do develop active TB disease, this is usually after a latent interval that can vary from weeks to many decades. There is a whole spectrum of clinical presentations, ranging from subclinical to rapidly fatal and almost any organ can be involved. Evidence shows that there is multifactorial host genetic predisposition in humans that influences the susceptibility to TB [54].

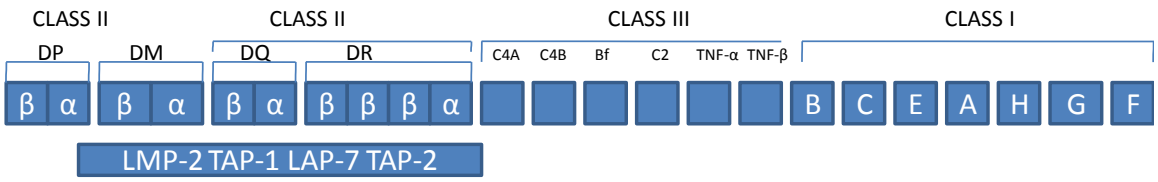
The adaptive immune system is regulated by the Major Histocompatibility complex (MHC), a locus on chromosome 6, of highly pleomorphic genes that encode for Human Leukocyte Antigens (HLA). The MHC or HLA in humans comprises three sub-regions (Figure 3): the HLA class I region, encoding for HLA-A, -B, and -C antigens; the HLA class II region, encoding for HLA-DR, -DQ, and -DP antigens; and the HLA class III region, encoding for the second and fourth component of complement C2 and C4, factor B, tumor necrosis factors (TNF)-α and -β, heat shock protein 70, and 21-hydroxylase [55]. Class I and class II genes code for cell-surface glycoproteins that present antigenic peptides to CD8+ and CD4+ T cells respectively, and execute a pivotal function in the homeostasis of the immune response [56].

Histocompatibility genes are inherited as a group (Haploid) from each parent, and are co-dominantly expressed in each individual. A heterogeneous human being inherits one paternal and one maternal haplotype each with three Class I (A, B, C) and three Class II (DQ, DR DP) loci.

In the class II locus, a person inherits a pair of HLA-DP genes (DPA1 and DPB1 genes which encode α and β chains respectively), a pair of HLA-DQ genes (DQA1 and DQB1 which encode α and β chain respectively), one gene of HLA- DRα (DRA1) and one or more genes of HLA DRβ (DRB1 and DRB3, DRB4, DRB5.

HLA II present longer exogenous peptides > 11 amino acid to CD4+ T Cells as compared to MHC I which present endogenous shorter peptides 8-10 amino acids [57] to CD8+ T Cells. The MHC class II is synthesized in the endoplasmic reticulum. Lysosomal proteases break down TB antigens into smaller peptides that bind with MHC class II. These peptide-MHC class II complexes are transported to the plasma membrane, where they are presented to CD4+ T cells. The T cell receptor recognition of the presented peptide initiates the generation of adaptive cellular immunity against TB [58]. Various HLA have been suggested to confer susceptibility or resistance to TB [59-61], and the genetic influence associated with the HLA system appears to influence the development of TB [61]. Much interest has focused on the HLA genes (notably the HLA-DR2 and HLA-DQB1 loci) that determine which mycobacterial antigens are presented to the helper T cells [62, 63].

The HLA system is a highly polymorphic biological system. The HLA system is a T cell restriction component associated with regulation of immune-responsive and immune-suppressive genes [64]. Antigen-specific T cell responses require the presentation of processed peptides in context of MHC class I and class II cell-surface glycoprotein. Proteins encoded by class II MHC genes mediate the recognition of the antigen by regulatory T cells and are associated with immune recognition and response [65].



Major histocompatibility complex

Figure 3. Genomic structure of the Major Histocompatibility Complex region

Although an association between TB and HLA has been reported in many populations, results are inconsistent, the HLA associations with the different Mtb genotypes have not been explored and no studies have been done in the African population. Studies using serology methods reported an increase of HLA-DR2 in TB [66-68].

1.1.8 Clinico-pathological features of TB

The clinical outcome of TB infections depends on a number of factors: i) host factors including immune status, age, sex, co-existing diseases and nutrition and ii) pathogen factors like virulence of infecting bacilli and host- bacilli interaction.

More than 85% of all tuberculous infections are pulmonary only, while the remaining 15% are extra-pulmonary or both pulmonary and extra pulmonary [69]. One important factor for the clinic-pathological outcome of the disease is HIV co-infection. One previous study of TB on patients with advanced HIV disease [70] revealed variable disease manifestations comprising 38% pulmonary involvement alone, 30% extra pulmonary sites alone while 32%

showed both extra pulmonary and pulmonary manifestations. Extrapulmonary dissemination of TB disease increases with declining immune status [71].

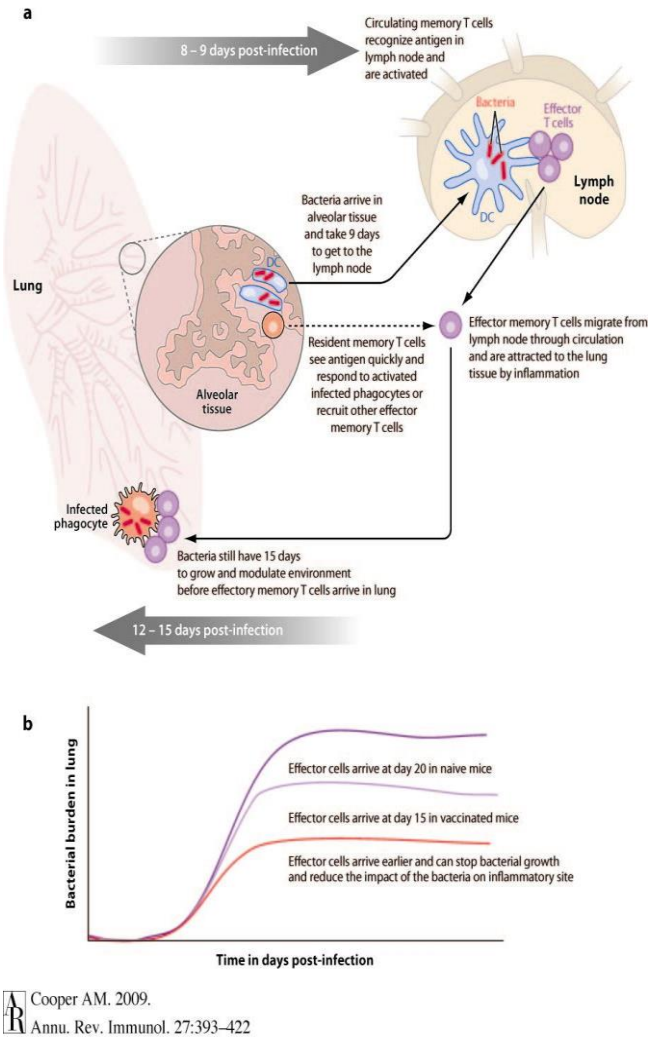
Table 1. Clinical feature of TB with and without HIV [72].

	HIV negative	**HIV positive**
Constitutional symptoms	Common in both groups	
Extrapulmonary disease	0-30%	60%
Pulmonary features		
Sub-apical disease	Common	Uncommon
Cavitation	Common	Uncommon
Lower lobe involvement	Rare	Common
Diffuse or miliary	Rare	Common
Diffuse or miliary	Rare	Common

In a majority of people infected with tubercle bacilli, the disease is controlled resulting in a subclinical primary lesion (Figure 4) undetectable by chest X-ray but adequate to produce a tuberculin reaction. Progression of this primary lesion is halted by development of a delayed type hypersensitivity (DTH) reaction and cell mediated immunity (CMI). Secondary TB occurs as a result of reactivation of a primary tuberculous lesion or from exogenous source in individuals previously exposed to Mtb antigens [73].

Post primary TB develops from the exudative reaction and is typically restricted to the upper lobes of the lung. The exudative reaction is followed by a lipid pneumonia phase in which there is localized accumulation of foamy histiocytes in the alveoli at the periphery of the

upper lobe of the lung [74, 75] The alveoli increasingly become foamy and eventually degenerate leaving lipid debri [76].



Cooper AM. 2009.
Annu. Rev. Immunol. 27:393–422

Annual Reviews

Figure 4. Pathogenesis of pulmonary TB as per Cooper AM et al [30]. Permission granted by Cooper AM et al and Elsevier publishers.

Endobronchial TB with consequential bronchial obstruction is a constant clinical and pathological feature of post primary TB [77, 78]. Lipid pneumonia may progress to caseous pneumonia following necrosis [77, 79-81]. This necrosis is acellular, homogenized and associated with a marked increase in acid fast bacilli [80].The alveolar septa also undergo necrosis.

1.1.8.1 Pulmonary TB

The systemic signs and symptoms include fever, night sweats, anorexia and weight loss [82]. The local symptoms include chest pain and cough which is the commonest local manifestation of TB. Cough may be initially unproductive but as inflammation and tissue necrosis ensues, it becomes productive. Occasionally patients may present with haemoptysis. Haemoptyisis may arise from a ruptured dilated vessel (Rasmussen's aneurysm) in a tuberculous cavity wall, tuberculous bronchiectasis or erosion into an airway (broncholithiasis).

1.1.8.1.1 Radiological features

Radiological abnormalities are common in TB; however in HIV infection a normal X-ray is common. In primary TB, the abnormal radiological lesions are seen as middle or lower lung zone infiltrate associated with ipsilateral hilar lymphadenopathy. Secondary TB typically presents with radiological abnormalities in the upper lobe occasionally with cavitation. Hilar and mediastinal adenopathy is rare in immunocompetent adults but common in children. As the disease progresses, the infected material may spread through airways to produce patchy bronchopneumonia or may be aspirated downstream to produce bronchopneumonia. The parenchymal caseous lesion may erode into blood vessels and disseminate systemically as miliary TB. In HIV/AIDS patients, the radiological abnormalities depend on the degree of immunosuppression [83], and may present with both typical and atypical chest radiograph pattern, though patients with CD4+ T lymphocytes counts less than 200 cells/µl are likely to present with atypical radiographic appearance of pulmonary TB [83]. In more advanced HIV disease, atypical findings become more common, and lower lung zone infiltrates and intra-thoracic adenopathy are common. Previous studies based on genotyping Mtb isolates showed similarities in radiological features between patients with primary TB and those with reactivated TB [84, 85]. Therefore, the prediction of the radiological appearance of TB does not depend of the duration between the acquisition of the infection and development of disease. The independent predictor of radiological appearance may be integrity of the host immune response, severely immune compromised patients tend to manifest with primary TB, whereas immune competent patients tend to have reactivated forms[84, 85].

1.1.8.1.2 Primary pulmonary TB

Radiologically primary PTB manifests as four main entities: lymphadenopathy, parenchymal disease, miliary disease and pleural effusion.

Lymphadenopathy: Radiological feature of lymphadenopathy is detected in 96% of children and 43% of adults [86]. Lymphadenopathy is typically right sided and unilateral in the hilum and right para-tracheal region (Figure 5). In a third of cases, lymphadenopathy may be bilateral. Any lymph node greater than 2 cm is suggestive of active disease [87].

Parenchymal disease: At radiology, parenchymal disease in primary TB manifests as dense, homogeneous opacity predominantly in the lower and middle lobes. It can be distinguished from bacterial pneumonia on the basis of radiological evidence of lymphadenopathy.

Miliary disease: Miliary TB clinically manifests in 1% to 7% of patients with all forms of TB [88]. It is more commonly seen in children, malnourished, individuals on immune suppressants, elderly and immune compromised person [88]. The classic radiological manifestation is evenly distributed small 2-3mm nodules randomly distributed throughout both lungs with intra and interlobular septal thickening [86], with slight lower lobe predominance.

Pleural effusion: Pleural effusion occurs in one fourth of patients with primary TB and 18% of post primary TB [86]. Pleural effusions are usually observed in association with parenchymal or nodal disease, though occasionally they may be the only radiological finding indicative of PTB in approximately 5% of adults [89].

1.1.8.1.3 Post primary TB

Secondary or post primary TB refers to reactivation with or without re-infection of TB. The radiological features of primary and post primary TB may overlap. At radiology, the common manifestations of secondary TB include parenchymal disease, airway involvement and pleural extension.

The earliest manifestation of parenchymal disease is patchy, poorly defined consolidation particularly in the apical or posterior segments of the upper lobe or superior segments of the lower lobe [90, 91].

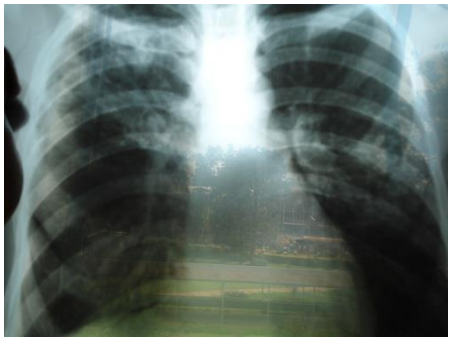


Figure 5. Para-tracheal lymph nodes. Photo Wamala D.

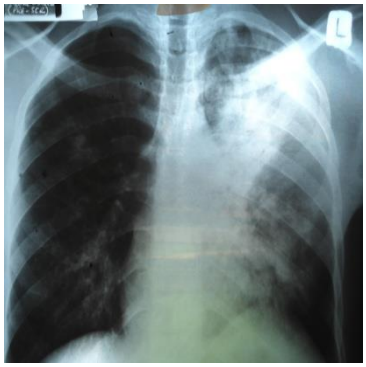


Figure 6. Cavitation. Photo Wamala D.

Cavitation affects about 50% of patients with post primary TB (Figure 6). The cavities have thick irregular walls. These cavities are usually multiple and may resolve to result in emphysematous changes or scarring. Lymphadenopathy and pneumothoraces manifests in 5% of patients with post primary TB [91]. Tuberculous pleurisy is more common in primary TB than post primary TB [91].

1.1.8.2 Extrapulmonary TB

Extrapulmonary TB arises as a result of contiguous spread of Mtb bacilli to adjacent structures like pleura or pericardium, or by lympho-haematogenous spread during primary or chronic disease.

1.1.8.2.1 Potential pathways for TB dissemination

It is well established that Mtb can migrate from its primary infection site to the lymphatic and blood circulatory system. However the mechanism to achieve this is not clear. Haematogenous dissemination is a vital step in the pathogenesis of extrapulmonary TB. The bacilli traverse across the alveolar epithelial membrane by direct invasion and lysis of the epithelial cells. Mtb is capable of replicating intraepithelially and spread from cell to cell through the epithelial monolayer [92, 93]. There is evidence that Mtb is cytotoxic and causes cell necrosis which facilitates bacterial cell to cell spread [94, 95]. Bacterial cytotoxic factors have been identified in cellular modules. Early secretory antigenic target 6kDa (ESAT-6) and culture filtrate protein 10kDa (CFP-10) encoded by RD1 genes have been linked to macrophage and pneumocyte cytolysis [96-99]. Mycobacteria bind to sulphated glycoconjugates on epithelial cells using haemoglobulin binding haemagglutinin adhesion (HBHA) [100]. Mtb is also capable to invade and proliferate within endothelial cells [101]. Dissemination of Mtb is facilitated by mononuclear phagocytic cells, which traffic Mtb across alveolar walls [102].

A number of factors including the innate and adaptive immune response influence mycobacterial dissemination and development of extrapulmonary TB [103]. Dissemination of TB frequently occurs in people with single nucleotide pleomorphism in genes that encode for TLR-2 and Toll-interleukin 1 domain containing adaptor protein (TIRAP), a protein that mediates signals from Toll-like receptors activated by macrophages [104, 105]. Lipomannan and the 19kDa lipoprotein are agonists of TLR-2 pro-inflammatory cytokine signaling [106].

Extrapulmonary TB follows organ seeding with Mtb from mucosal or lympho-haematogenous spread. Mucosal spread occurs especially in pulmonary TB patients with high bacillary loads. In chronic cavitary TB disease, highly infectious respiratory secretions that bathe the upper respiratory mucosa and gastrointestinal tract facilitate TB spread to laryngeal or gastrointestinal tracts respectively. Tuberculous pleurisy is the result of a delayed hypersensitivity response to Mtb antigen that gain access to the pleural space after rupture of a sub pleural caseous focus or tubercle bacilli access into the pleural cavity [107].

1.2 THE PATHOGEN

Host and environmental variables are important variables that determine the TB disease outcome. However, it is becoming evident that pathogen determinants and their interaction with the host and environment are important in the understanding of the pathogenesis of TB [108].

Previous studies have clearly demonstrated the restriction of certain Mtb complex species to particular hosts and localities, Mtb, the primary causative agent of human TB; *M. africanum*, the main causative agent of human TB in West Africa [109]; *M. bovis*, which is responsible for bovine TB, *M. microti*, which is a pathogen of voles and rarely infects humans [110, 111]; *M. canettii*, an Mtb strain isolated from the horn of Africa [12, 112]; *M. pinnipedii*, also known as the seal bacillus [113-115]; and *M. caprae* which is primarily isolated from goats [116].

Previous observations also strongly suggest that clinical strains of Mtb vary in their virulence and immunogenicity [108, 117, 118], and these variations in pathogenesis of the various genotypes of Mtb have been associated with phenotypic consequences [119]. Some Mtb virulence factors may subvert the host innate immune response and augment the bacterial replication and pathogenicity. Macrophages infected with an Mtb strain which caused an outbreak in Leicester, UK produced less protective IL-12p40 and more anti-inflammatory IL-10. Mtb Beijing lineage strain HN878 caused several outbreaks of TB in Houston, Texas, USA between 1995 and 1998. This strain was found to cause rapid death in immunocompetent mice[120], while in rabbits it caused increased Mtb dissemination, more severe TB clinical manifestations and higher bacillary load [121]. The hyper virulence of HN878 was associated with inability to induce Mtb-specific T cell proliferation and IFN-γ production by the spleen and lymph nodes of infected mice. HN878 was also associated with 2- to 4-fold lower levels of TNF-α, IL-6, IL-12 [120].There is also evidence to show that the early interaction between Mtb and the host depends on the Mtb lineage of the infecting strain [122].

1.2.1 Mtb complex and Mtb genomic diversity

Mtb genomic evolution has been attributed to mutation, deletions and transposition of chromosomal regions [126]. Through genetic characterization, various lineages or genotypes

of Mtb have been demonstrated (Figure 7) [127-130]. The Mtb complex comprises seven human-adapted distinct lineages which show strong phylogeographical population structuring [119]. The different lineages are associated with specific geographical areas (Figure 8) [131-136]. For Mtb, more recent studies have further shown that specific genotypes are restricted to particular geographical areas, a phenomenon referred to as geographical sub-structuring [128, 137]. In addition, some genotypes of Mtb have been reported to be associated with TB affecting different organs in the host [138]. Despite high homology of the genome of Mtb complex members, these observations strongly suggest variations in pathogenicity of the various genotypes. In a study to assess differences in virulence between Mtb genotypes, a marked difference was observed in survival, lung bacterial load, lung histopathology, and cellular immune responses after infection of mice with different isolates from a spectrum of Mtb complex genotypes [54, 139].

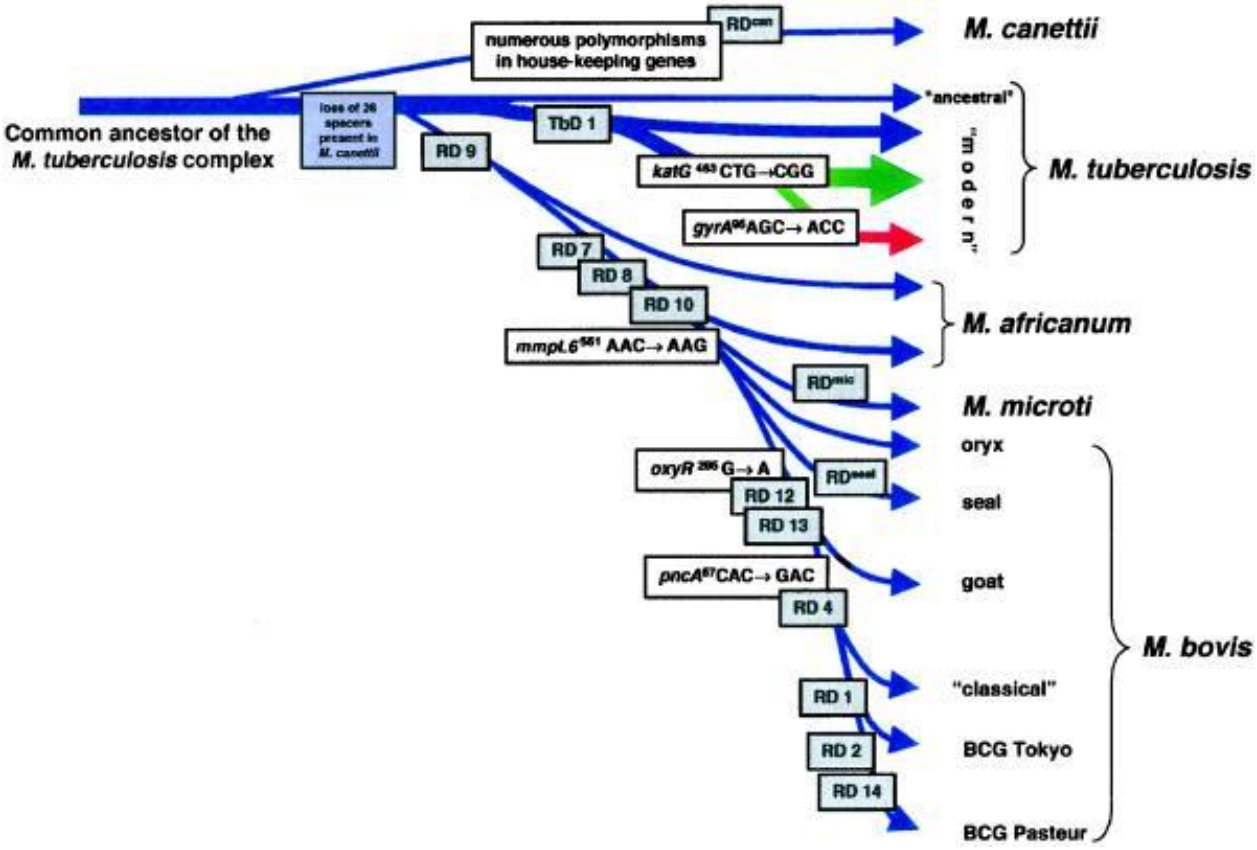


Figure 7. Evolutionary pathway of tubercle bacilli highlighting loss of DNA as shown by Brosch et al [123]. Permission from Diane Sullenberger Executive Editor, PNAS.

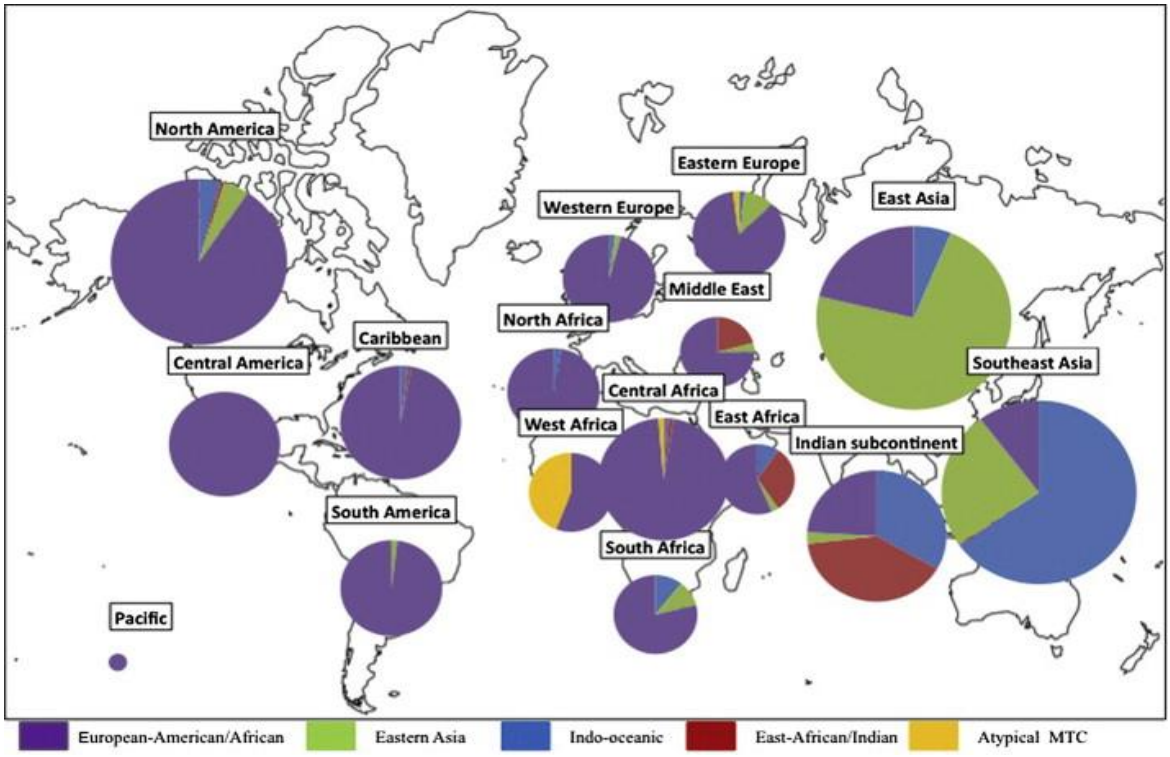


Figure 8. Distribution of the 5 lineages among the different regions of the world by geographic origin of the patients, picture by Perrin et al [124] based on data from Reed et al [125]. With permission from Pascale Perrin and Elsevier limited.

Mtb genotypic diversity has been associated with variable clinical outcomes. The Mtb Beijing genotype can elude the host innate immune system which may explain why this genotype is associated with haematogenously disseminated disease[140] The Euro-American or East African lineages were significantly less associated with extrapulmonary TB [141]. Studies in Vietnam indicated that the East Asian/Beijing and Indo-Oceanic lineages were significantly more likely to cause disseminated TB than the Euro-American lineages and East Asian/Beijing and Indo-Oceanic lineages induced more macrophage TNF-α and IL-1β than the Euro-American strains in experimental models [122].

The Mtb complex, the aetiological agent of TB exhibits genomic diversity comprising seven phylogenetically related lineages [142]. Lineage 2 and lineage 4 (Euro American) are the most geographically widespread lineages [119]. The Euro-American Lineage is most prevalent in Europe and in the Americas. The Euro-American Lineage comprises 10

sublineages, each characterized by large sequence genomic deletions, namely RD 115, RD122, RD174, RD182, RD183, RD193, RD219, RD724, RD726 and RD761. The 11th group has no RD deletion (the so-called H#&Rv-like) [133]. The Euro-American lineage characteristically exhibits spoligotype deletion of spacer 33-36 in the DR locus [143, 144]and comprises the major families T, Haarlem, Latin American-Mediterranean (LAM), S and X, including a total of 34 subfamilies.

By the phylogeny scheme proposed by Gagneux and colleagues [128], RD724 defines a major sub division within the Euro-American lineage of Mtb. Given the geographical long term predominance of RD724-positive Mtb in Kampala over the past two decades [145], the findings suggest that these strains may be specifically adapted to a Central African human host population, Ugandans included. These findings together with other studies [128, 132, 135] indicate that each of the major lineages of Mtb have evolved to most efficiently transmit within an original human population. Some degree of co evolution has occurred as a result of long term association between Mtb complex and its human host [146].

Previous studies have revealed a strong phylogeographical population structure exhibited by the human-adapted Mtb complex, with different lineages associated with particular geographical regions [132, 133, 136, 147-149]. In a study to determine the Mtb lineages of isolates from PTB patients from four continents, the distribution of Mtb lineages and sublineages varied by geographic region, Euro-American sublineage RD724 predominated among African region participants, whereas Euro-American other than RD724 predominated among non-African region participants. Previous literature suggests that distinct Mtb complex phylogenetic lineages cause disease in individuals of a particular genetic background.

Mtb strains have adapted to original human populations, epidemiological studies suggest that different phylogenetic lineages of MTC might have adapted to different human population [146]. Studies have shown low levels of anti-TB drug resistance among the T2 MTC lineages [150]. Success of MTC lineages has been correlated with hypo-inflammatory phenotype in macrophages probably depicting their virulence [117, 119]. Previous work show that Lineages 2-4 exhibited a lower early inflammatory response compared to lineages 1 and 6 [151]. This implies increased virulence of lineages 2-4(Euro-American lineage) and may explain their global dominance over other strains. A delayed inflammatory response implies increased virulence and may be associated with successful predominance of these strains.

With evidence of phylogeographical structuring of Mtb [128], it is no longer feasible to consider TB as a homogenous disease, but rather a spectrum. With increasing evidence that strain-to-strain variation can have important phenotypic consequences, phylogeographical strain variation might affect the development of new diagnostics, drugs, and vaccines [152]. This, therefore, may have both clinical implications as far as patient management and future diagnostic product design are concerned.

Studies using single nucleotide polymorphisms (SNP) as molecular typing tool revealed a higher genetic diversity among Mtb complex than previously assumed, with potential variation in pathobiological phenotype outcome [153, 154].

1.2.2 Phylogeography of Mtb Uganda genotype

In a study of a global collection of strains of Mtb specific deletions [128], TbD1 deleted strains were grouped into three families: the East-Asian (RD105-deleted), the East-African-Indian (RD750-deleted) and the largest lineage, the Euro-American, with several deletions defining various sub-lineages. Among these Euro-American sub-lineages, strains collected from central Africa (3 from Uganda, 2 from Rwanda, 2 from Burundi and 2 from Central African Republic) were deleted for RD724. In Uganda, 139 strains that formed the 5 largest clusters of 344 spoligotypes from Kampala were analyzed at four loci: RD105, RD711, RD724 and RD750 [155]. All the strains evaluated were deleted for RD724 but conserved for the other three regions, confirming the predominance of a spoligotype and RD724-defined Mtb lineage in Kampala [127].

Mtb Uganda genotype has dominated in Kampala periurban region for over two decades [145]. Previous studies have revealed that Mtb Uganda genotype is the predominant cause of TB in Kampala, Uganda [127], but no evaluation has been done of its immunopathological phenotypic characteristics

1.2.3 Molecular typing of mycobacteria

MTBC comprises seven mycobacterial species causing TB, of which Mtb is a major causative agent of TB. Members of the Mtb complex share identical 16SrRNA sequence and nucleotide identity higher than 99.9% [156]. A number of molecular techniques have been developed over the past decade to characterize Mtb complex species and strains. Polymorphism of genomic DNA of Mtb complex has been exploited to differentiate clinical isolates [157].

The most popular molecular method used to differentiate MTBC was DNA finger printing by southern blotting of genomic DNA using a mobile entity IS*6110* as probe [158]. Restriction fragment length polymorphism (RFLP) is based on variation in copy numbers and the differential genomic location of insertion sequence IS*6110* and was previously the gold standard for Mtb complex genotyping [159]. IS*6110*-RFLP has limitations that include low reproducibility between different laboratories and requirement of large amount of good quality DNA. This has motivated the development of several PCR-based typing methods like spoligotyping that requires little DNA.

Spoligotyping (spacer oligotyping) is a simple, cheap and quick PCR based method used to simultaneously detect and type Mtb exploiting previously observed polymorphism at the Mtb direct repeat (DR) locus [160]. This locus is composed of 36-base pairs (bp) DR interspersed with non-repetitive spacer sequences 34 to 41bp long [160]. Strains have variable numbers of DR and presence or absence of particular spacers [161]. In spoligotyping, DR region are targets for in vitro DNA amplification. The amplicons variable spacers are then visualized after hybridization to multiple spacer oligonucleotides covalently bound to a membrane and data digitalized.

Genomic deletion analysis of regions of difference (RD analysis) has been used in supplementation with spoligotyping phylogenetic lineages [132, 133, 162, 163].

2 THE PRESENT INVESTIGATION

2.1 PROBLEM STATEMENT

Previous pulmonary TB studies have shown the predominance of Mtb Uganda genotype in urban Uganda [127, 164]. However, a comparison of the clinical, radiological and pathological features of the Uganda genotype strains with other genotypes of Mtb as well as their association with specific HLA types in the human population has not been studied. The role of the pathogen in the pathogenesis of EPTB and the clinical consequences of Mtb genomic diversity has not been fully explored. This knowledge would be locally relevant in developing suitable vaccines and formulate locally appropriate disease prevention policies. The knowledge will also be valuable in patient management and prognostication.

2.2 THESIS AIMS

The main goal of this thesis was to gain an insight in the pathogenesis and clinico-pathological features of infection caused by Mtb Uganda genotype and other circulating non-Uganda genotypes, with the hypothesis that Mtb Uganda genotype causes clinical pathological presentations different from those of other circulating Mtb strains.

Specifically the aims are:

- To study how the clinical, radiological and pathological features of disease caused by the Uganda genotype compare with other genotypes of Mtb in Uganda (Paper I).
- To study the phylogenetical structure of Mtb isolated in patients with tuberculous lymphadenitis (EPTB) and how it differs from that of pulmonary Mtb isolates previously studied (Paper II).
- To identify HLA II DQ and DR alleles that may confer resistance or susceptibility to Mtb in general and Mtb Uganda genotype in Ugandan patients (Paper III).
- To study the contribution of the different types of mycobacteria to mortality associated with mycobacterial disease (Paper IV).
- To explore the diagnostic potential of CD4 T/CD8+ T Cell ratios in pleural tuberculous effusions (Paper V).

2.3 METHODS

Table 2. Summary of studies and methods used.

	Main aim	Study population	Specimen	Diagnostic methods	Genotyping
Paper 1	To study the clinical, radiological and pathological features of disease caused by the Uganda genotype and other genotypes of Mtb in Uganda	283 patients with lymphadenopathy	FNA of lymph nodes	Clinical, radiological assessment Cytopathological analysis, ZN and culture	Spoligotyping RD analysis
Paper 2	To investigate the phylogenetical structure of Mtb isolate in patients with tuberculous lymphadenitis	121 patients with tuberculous lymphadenopathy	FNA of lymph nodes	ZN and culture	Spoligotyping RD analysis
Paper 3	To identify HLA II DQ and DR alleles in Ugandan patients with TB	43 PTB 42 healthy household controls	Blood and sputum	ZN and culture PCR based sequence specific oligonucleotide primers	SpoligotypingRD analysis Immunogenetic typing
Paper 4	To study the contribution of the different types of mycobacteria to mortality associated with mycobacterial disease	49 mycobacteria culture positive autopsy cases with suspected TB	Autopsy tissue biopies	Pathological analysis. ZN and culture	Spoligotyping RD analysis
Study V	To explore the diagnostic potential of CD4 T/CD8+ T Cell ratios in pleural tuberculous effusions	35 TB patients 43 non-TB controls	Pleural effusion	Culture and histopathological analysis	Immuno-cytochemical double staining.

2.3.1 Study area, patients and samples

The studies were performed at Mulago Hospital Complex. Mulago Hospital is the National Referral Hospital with the largest number of TB patients. It also runs a Fine Needle aspiration clinic to which patients suspected to have extra-pulmonary tuberculosis lymphadenitis are referred from medical or surgical units.

The laboratory studies for study I, II, III and IV were done in the Makerere University College of Health Sciences, Microbiology and Pathology Laboratories. The FNAs and autopsies were done in Mulago Hospital. Study V was conducted in Mulago-Kampala Hospital (patients) and Karolinska University Hospital, Huddige (Controls).

Patients and samples are summarized in Table 2, and recruitment plan of patients in Figure 9.

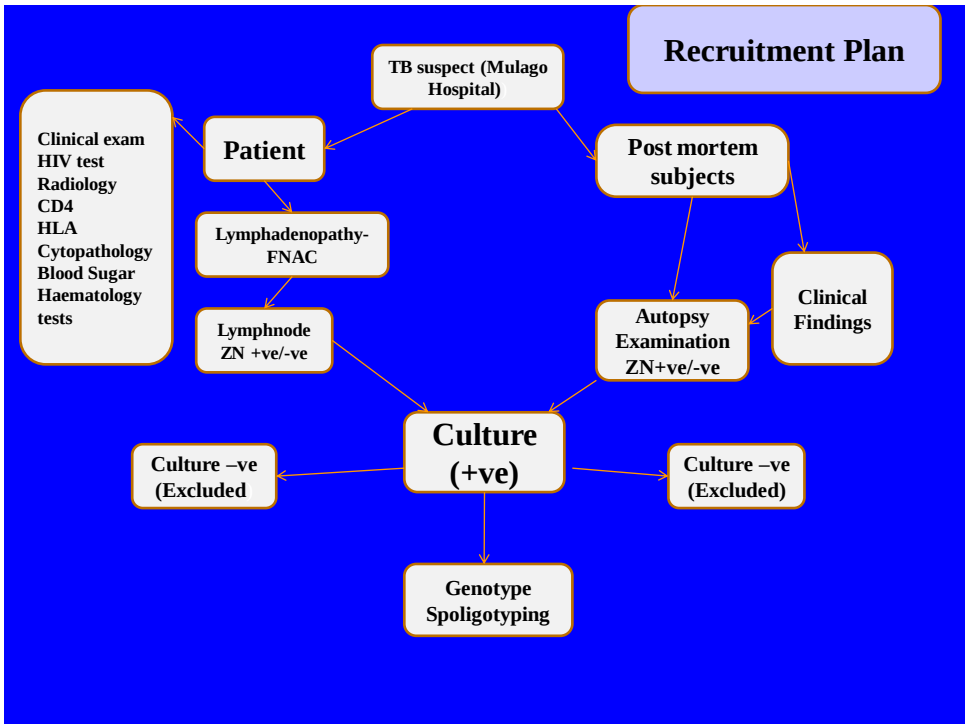


Figure 9. Recruitment plan of patients for studies I, II and IV

The ethical approval for all studies was granted by the Institutional Review Board of Makerere University College of Health Sciences. Written informed consent was obtained

from all enrolled study participants or their legal guardians including permission to use their samples' isolates.

2.3.2 Abdominal ultrasound scans

Scans were performed with a MedisonSonoAce 9900 ultrasound machine (Seoul, Korea) using a 7.5mHz convex probe and a 7.5 mHz linear probe. The presence of abdominal lymphadenopathy was considered significant if there were more than three lymph nodes or at least one lymph node 12 mm or more in size. Lymph nodes were sought in the paraaortic region, the porta hepatis region, the mesentery and the splenic hilum. The role of abdominal ultrasound in diagnosis of extrapulmonary or disseminated TB was previously evaluated, and a significant correlation was found between abdominal lymphadenopathy and active TB as diagnosed by smear or culture [165], thus abdominal ultrasound was found valuable in diagnosing EPTB [166].

2.3.3 Chest x-ray

Posteroanterior chest x-ray was done in all TB cases and was performed with patients in standing posture and holding their breath, using a Schimadzu machine (Shimadzu-Japan), with the patient´s chest positioned 150cm away from the x-ray tube. The chest X-ray was considered abnormal when it showed any of the following: lobar/segmental consolidation, cavitation, fibronodular lesions, pleural effusion, hilar and/or mediastinal lymph nodes, linear interstitial disease or miliary disease.

2.3.4 Immunocytochemistry of pleural fluid

Thoracocentesis was performed for all patients with pleural effusion and symptoms of TB, including evening fevers, unintentional weight loss, cough and night sweats. Five ml of the pleural fluid was taken for cytology and immunocytochemistry, and a pleural biopsy was obtained. The biopsy was fixed in 4% buffered formalin, dehydrated in graded alcohol, embedded in paraffin, cut and stained with haematoxylin/eosin and ZN stain.

The pleural fluid was centrifuged at 1000 rpm, making four slides of each sample with the cytospin technique. Three of these were stained with Diff Quick and according to Papanicolaou and ZN, respectively. The forth slide was fixed overnight in a PEG/ethanol fixative (60 g polyethylene glycol 1500, 500 mL ethanol, 500 mL methanol and 1000 mL distilled water). The slides were then stored at -20°C for later immunocytochemistry.

Immunocytochemical double staining was carried out using the Leica Bond Immunostaining Instrument III. CD4 + cells were immunostained using the Bond polymer refine detection kit and CD8+ cells were immunostained with the Bond polymer refined red detection kit (Leica Biosystem Newcastle Ltd, Newcastle, UK). Briefly, the cytospin slides were fixed in 4% buffered formalin for 30 minutes and rinsed in PBS. Antigen retrieval was performed with citric acid buffer (pH 6) at 98°C for 5 minutes. The primary antibody to CD4 (NCL-L-CD4-368, Novocastra, diluted 1:400) was then added and left to react for 30 minutes, followed by a secondary antibody 15 minutes. Endogenous peroxidase was blocked with $H_2O_2$for 10 minutes with the peroxidase is attached via an antibody-polymer complex. Bound CD4 antibodies were then visualized with diaminobenzidine (DAB)/$H_2O_2$for 10 minutes. Presence of the CD8 epitope is then demonstrated by 15 minutes incubation with the primary antibody, (Anti-human CD8clone C8/144B, M7103, DAKO, Copenhagen, Denmark, diluted 1:100), followed by incubation with a secondary antibody labelled with alkaline phosphatase for 20 minutes, developing the reaction products according to the kit with Fast Red as chromogen. Slides were then counterstained with haematoxylin, dehydrated and mounted. Positive lymphocytes were counted manually.

After immunocytochemistry analysis, CD4+, CD8+ and CD4+/CD8+T lymphocytes were determined in pleural effusions from patients with and without tuberculous pleural effusion (TPE) in order to establish a potential role for CD4+/CD8+ T cell ratio in establishing a diagnosis of TPE.

2.3.5 Fine needle aspiration

FNA was performed on consenting participants fulfilling inclusion criteria. Under aseptic conditions, the lymph node was immobilized with left hand to entirely access it while the right hand was used to aspirate. Using a 23G cutting needle and a 2 ml syringe lymph node aspiration was done with constant suction in a fan like fashion until the material appeared in the hub. The suction was then released, the needle withdrawn an. Lymph node aspirates were cultured for mycobacteria and cytologically analysed for features of TB including granuloma formation, necrosis, presence of AFBs as previously described [167].

2.3.6 HLA Allele Typing

Peripheral venous samples (4ml) were collected in EDTA vacutainers and stored at -80C. Plasma and peripheral blood mononuclear cell (PBMCs) were prepared according to standard techniques. DNA was extracted using a DNA purification kit, Epicenter

Biotechnology and was quantified to ascertain its presence using a GeneQuest as per the manufacturer's instructions. (Model Number CE2302), and confirmed per extraction batch by agarose gel electrophoresis and bioimage visualization.

HLA Class II typing by sequence-specific oligonucleotide probe hybridization was performed on the products. To analyze for the presence of a given allele, polymerase chain reaction sequence (PCR) for HLA- DR and DQ alleles was performed [168, 169] using an MJ-96 well thermocycler. Flanking sequences were amplified using alleleic primers based on a sequence-specific oligonucleotide primers (SSP) principle. This was done using the One lambda Micro SSP DNA Typing kit according to the kit manufacturer's instructions. The PCR-SSP methodology is based on the principle that completely matched oligonucleotide primers are more efficiently used in amplifying a target sequence than a mismatched oligonucleotide primer by recombinant Taq polymerase. Primer pairs are designed to have perfect matches only with a single allele or group of alleles. Under strictly controlled PCR conditions, perfectly matched primer pairs result in the amplification of target sequences (i.e., a positive result) while mismatched primer pairs do not result in amplification (i.e., a negative result).

2.3.7 Post mortem procedure

A complete body postmortem examination was performed on each of the subjects to study the pathological features of the inpatients suspected to have died of TB. The examination was performed within 24hrs after obtaining consent to avoid autolysis and delay in burial. All relevant information was reviewed before performing the postmortem examination including written clinical history, laboratory results and radiology. After external examination, careful attention was paid to ensure aseptic technique. Culture samples were collected in situ immediately after entering the bodies using sterile scalpel and forceps. Different sets of instruments were used for each culture. En masse (Letulle) evisceration was done followed by organ dissection and weighing as described [170]. Organs and regional especially matted caseating lymph nodes were inspected for disease, and representative tissue samples taken. Lungs were examined fresh for extent of disease by cutting sequentially along the arteries, airways and veins following the McCulloch and Rutty method [171]. Culture samples were put in sterile container containing 5ml of distilled sterilized water and transported on ice to the mycobacteriology laboratory at Makerere University School of Biomedical science.

2.3.8 Tissue analysis

Samples for histopathology were taken from the lung, the spleen, the liver, lymph nodes, kidney, adrenals, brain and pancreas. All histopathology tissues were fixed with 10% formal saline, sectioned, dehydrated with graded alcohol, and cleared with xylene. The tissues were then embedded with paraffin wax to produce tissue paraffin blocks from which 4µm tissue sections were cut. The tissue sections were stained with haematoxylin and eosin (HE) for morphological analysis. Tissue sections were screened for AFB after staining with flourochrome (auramine) and ZN stains. Diagnosis of mycobacterial disease in the tissue sections was based on HE histological findings including chronic granulomatous inflammation, caseous necrosis and AFB positivity on ZN and auramine-rhodamine tissue staining. Diagnostic criteria for NTM diagnosis in tissue samples was based on recognition of mycobacterial histopathologic features and positive NTM growth on tissue culture [172].

2.3.9 Mycobacterial identification and genotyping

2.3.9.1 Culture and species identification

In the mycobacteriology laboratory clinical samples were homogenized and disinfected with sodium hydroxide containing N-acetylcysteine prior to inoculation into Mycobacteria Growth Indicator Tube (MGIT 960) liquid culture for six weeks and on solid Loewenstein Jensen media for up to eight weeks culture. Once the culture was declared positive, preliminary smears were made and stained with ZN and Auramine-Rhodamine stain to confirm the isolate as AFB.

DNA was harvested from growth following standard protocols [159] (Reagents from Sigma life Science, USA). DNA was the extracted from the cultured mycobacteria isolate following standard protocol [173, 174]. Isolates were identified by performing polymerase chain reaction (PCR) using 16s reverse and 16s forward primers (Integrated DNA Technologies) targeting the *16s rRNA* region with a conserved sequence typical for the genus Mycobacteria [175-177]. The Capilia TB assay (TAUN, Numazu, Japan) was used to distinguish Mtb complex isolates from NTM [178], as per manufacturer's instructions [179]. Mtb complex isolates were additionally identified by amplification of the insertion sequence IS*6110* using an in-house PCR with aid of reverse and forward IS*6110* primers (Integrated DNA Technologies). Gel bands of approximately 500 bp signified positive results [180].

2.3.9.2 Spoligotyping

Molecular characterization was done of Mtb isolates using DR analysis and spoligotyping following standard protocol [175, 181] and manufacturer's instructions (reagents from Ocimum Biosolution, custom Master Mix from ABgene). Spacers were visualized on film as black squares after incubation with streptavidin-peroxidase and ECL chemiluminescence detection reagents (RPN 2105 Amersham, GE Healthcare Bio-sciences). The spacer hybridization patterns were converted into binary and octal format as previously described [182]. The 43-digit binary code was converted to 15-digit octal code (base 8, having the digits 0-7) [182]. The binary codes of the isolates were entered into the SITVIT2 database of the Pasteur Institute of Guadeloupe and assigned specific shared international spoligotype signatures (SIT) according to the SITVIT2 database [183].

2.3.9.3 Region of difference (RD) analysis

Mtb complex isolates were typed using a PCR based typing method [175] which depends on chromosomal region of difference (RD) deletion loci. The patterns of amplification products are visualized by agarose gel electrophoresis. RD 9 confirmed that the cases were Mtb and ruled out other species, RD4 and RD 14 ruled out M. bovis, the RD724 deletion is characteristic of Uganda genotype.

2.3.9.4 Identification of Uganda genotype

The Uganda genotype, a sublineage of the T2 lineage, was identified by deletion of RD724 on RD analysis [184], and absence of spacers 33-36 and spacer 40 and/or 43 on spoligotyping [185, 186].

2.3.10 Statistical methods

Paper I Crude and multivariable logistic regression analysis was used to explore clinical, radiological and pathological features of Mtb Uganda genotype compared with those of M.tb non-Uganda genotype. Independent sample t-test was used to compare means of continuous variables.

Paper II. Spoligotyping data were digitized and analyzed with the BioNumerics software, version 5.0 (Applied Maths, Kortrijk, Belgium).

Paper III, Fisher's exact test was used to find associations between the patients belonging to certain HLA types and the risk of clustering with "Mtb Uganda genotype". P-values were adjusted for multiple comparisons using the Benjamini and Hochberg method.

Paper IV. Univariate and multivariate data analysis was performed and logistic regression models were used to adjust for confounders like age, sex and HIV status. Chi square and Fisher's exact test were used. The independent-samples t test was used to analyze quantitative data for a two sample case to compare means.

Paper V. The utility of the test to distinguish the TPE from the non-tuberculous effusions was monitored by ROC plot. Comparisons of group means were performed with the Student's t-test. A two-by-two contingency table test with Yate's correction for continuity was used to evaluate a possible correlation between the proportions of CD4+ reactive cells and patient group. The differences between TPE and non tuberculous effusion CD4:CD8 T Cell ratios were adjusted for confounders using linear regression.

3 RESULTS AND DISCUSSION

3.1 PAPER I

The study population comprised patients presenting with clinical features of TB and peripheral lymphadenopathy. Mtb genotyping was done using spoligotyping and RD analysis of Mtb culture isolates.

283 patients with superficial lymphadenopathy were enrolled. Of those 150 (53%) lymph node aspirate cultures turned out negative, 121 were culture positive for Mtb, 9 samples were ZN positive but culture negative, 6 samples were contaminated, and six samples were culture positive for mycobacteria other than Mtb.

Mtb Uganda genotype, a member of the T2 Euro-American lineage, was the predominant genotype isolated from Uganda patients with tuberculous lymphadenitis, accounting for 46% of all cases. This frequency was lower in comparison to the 70% prevalence of the Uganda genotype seen in PTB patients in the same setting [127].

Tuberculous lymphadenitis patients infected with Uganda genotype strains were significantly less prone to present with abdominal lymphadenopathy ($p = 0.014$) than those patients infected with non-Uganda strains (Table 3), even after adjusting for confounding factors including age, sex and HIV co-infection ($p = 0.046$). Abdominal lymph node enlargement was itself significantly associated with abnormal chest X-ray findings ($p = 0.027$) (Table 4).

Table 3. Clinical and immunopathological parameters of patients infected with Uganda genotype and those infected by non-Uganda genotypes of Mtb.

Patient characteristics	**Uganda/Non-Uganda n (%)**	**Crude**		**Adjusted**	
		OR (95% CI)	***P*-value**	**OR (95% CI)**	***P*-value**
Clinical presentations					
Fever less than 6 months	39(70%)/44 (69%)	1.0 (0.5-2.3)	0.916	1.1 (0.5-2.5)	0.870
Night sweats	43(77%)/55(86%)	0.5 (0.2-1.4)	0.200	0.5 (0.2-1.4)	0.213
Cough	27(48%)/30(46%)	1.1 (0.5-2.2)	0.821	1.4 (0.7-3.1)	0.435
Radiological Presentations					
Abnormal Chest X-ray	20(43%)/27(47%)	0.8 (0.4-1.8)	0.624	0.8 (0.4-1.9)	0.644
Abdominal lymphadenitis	21(38%)/39(60%)	0.4 (0.2-0.8)	0.014	0.5 (0.2-1.0)	0.046
Lymph node Pathology					
Single site lymphadenitis	34(62%)/32(50%)	1.6 (0.8-3.4)	0.197	1.4 (0.6-2.9)	0.435
Generalized lymphadenitis	5(9%)/11(17%)	0.5 (0.2-1.5)	0.204	0.7 (0.2-2.1)	0.474
Lymph node ≥ 4 cm	15(28%)/28(45%)	0.5 (0.2-1.0)	0.055	0.6 (0.3-1.3)	0.157
Necrosis	52(93%)/61(94%)	0.9 (0.2-3.6)	0.827	0.8(0.2-3.4)	0.741
Giant cells	28(50%)/25(38%)	1.6 (0.8-3.3)	0.203	1.3 (0.6-2.8)	0.569
Macrophages	53(95%)/58(89%)	2.1 (0.5-8.7)	0.290	1.5 (0.4-6.9)	0.577
Granulomas	47(84%)/46(71%)	2.2 (0.9-5.3)	0.091	1.9 (0.8-4.8)	0.196
ZN positive smear	29(52%)/42(65%)	0.6 (0.3-1.2)	0.152	0.7 (0.3-1.5)	0.348

Adjusted* = Adjusted for Age, Sex and HIV status; OR = Odds ratio, Cl = Confidence Interval. Logistic regression.

Table 4. Association of abdominal lymph node enlargement with features indicative of TB dissemination (i.e. generalized lymphadenopathy, abnormal chest X-Ray, AFB positivity and absence of granuloma).

	Abdominal Lymphadenopathy n = 60 (49.6%)	**Non-Abdominal lymphadenopathy n = 61 (50.4%)**	**P-Value**
Generalized Lymphadenopathy:			
Present	30 (57%)	23 (43%)	0.170
Absent	20 (39%)	19 (30%)	
Granuloma:			
Present	46 (50%)	47 (50%)	0.960
Absent	14 (50%)	14 (50%)	
Abnormal findings on Chest X-ray:			
Present	30 (64%)	17 (36%)	0.027
Absent	24 (41%)	34 (59%)	
AFB smear positivity: (ZN or fluorochrome stain)			
Positive	39 (65%)	29 (47%)	0.053
Negative	21 (35%)	32 (53%)	

Chi-square test.

In line with these findings, lineage 4 (Euro-American lineage), to which Uganda genotype belongs, has been associated with pulmonary TB [105], while the East Asian/Beijing and Indo-Oceanic lineages were significantly more likely to cause disseminated TB [105]. Lineage 4, to which the Uganda sub-lineage belongs shows enhanced transmissibility in human populations [117, 119].

3.2 PAPER II

Lymph node isolates from the 121 patients with tuberculous lymphadenopathy in study I were further analysed by spoligotyping. and the evolutionary relationship and worldwide distribution of the spoligotypes was assessed (Fig 10). The isolates were exclusively Mtb and lineage 4, also known as the Euro American lineage, was the predominant cause of EPTB in Uganda, As shown in paper I the Uganda genotype was the predominant genotype, although at a lower frequency than in studies of PTB in the same setting. The spoligotypes were in most cases similar to those causing PTB with the exception of SIT420 and SIT53 that predominated in this study but are mainly lacking in other studies of PTB.

Exploring the Mtb diversity is essential in appreciating the clinical manifestation and pathogenesis of the disease as well as the rationale for development of new diagnostic strategies. The phylogenetical analysis and the study of the worldwide distribution of clustered spoligotypes indicate an ongoing evolution of the Uganda genotype, with Uganda at the center of this evolution.

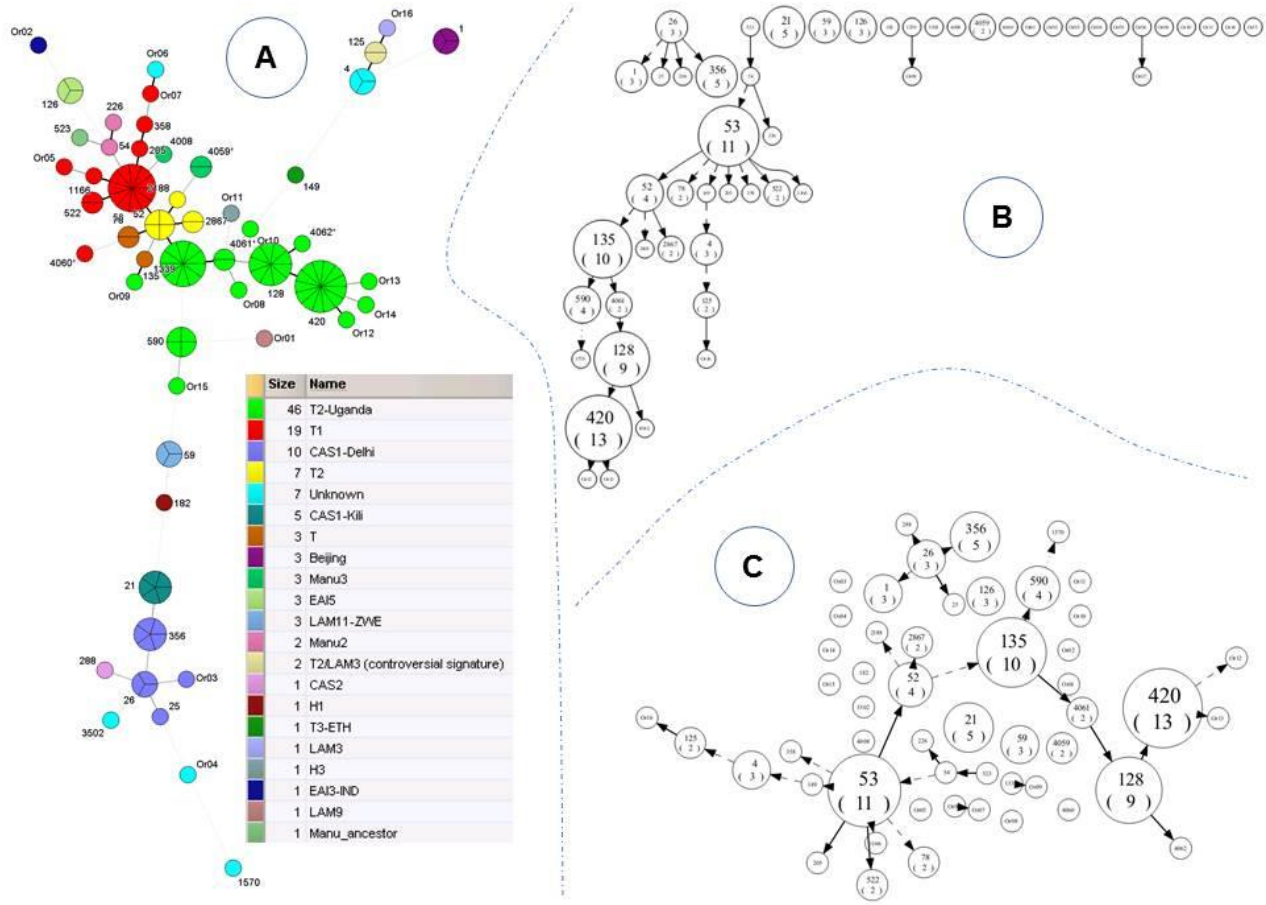


Fig 10. Phylogenetical analysis illustrating evolutionary relationships between Mtb spoligotypes in Kampala, Uganda (n=121 isolates). **(A)**. Minimum Spanning Tree (MST) constructed on all isolates. Separations between the nodes represent the number of strains shared by a given spoligotype pattern. The links between nodes indicate the distance (darker and bolder lines mean a unique change whereas finer gray lines, continued, dotted or dashed, indicate more changes). **(B)**. Spoligoforest tree drawn as a hierarchical layout; and **(C)** spoligoforest tree drawn using the Fruchterman-Reingold algorithm. Loss of spacers is represented by directed edges between nodes, and the arrowheads point to descendant spoligotypes. Solid black lines link patterns that are very similar, i.e. loss of one spacer only (maximum weight being 1.0), while dashed lines represent links of weight comprised between 0.5 and 1, and dotted lines a weight less than 0.5.

3.3 PAPER III

In this matched case control study, HLA class II (HLA-DR and -DQ) allele frequencies in TB patients were compared with those in healthy, ethnically and geographically matched controls.

Two groups, patients and controls were recruited. HIV-negative patients (n=43) were selected from newly presenting ZN smear positive patients. Controls (n=32) were recruited from people previously exposed to TB having neither signs /symptoms of TB nor ever developed clinical signs of TB visiting the same facility for minor complaints.

There was a negative correlation between the HLA- DQB1*03:03 allele and susceptibility to TB (P = 0.003) indicating that this allele may confer protection against TB in the study population Even after correction for multiple testing the absence of the HLA-DQB1*03:03 allele in all TB patients was still found significant.

The more common HLA II alleles isolated here were not associated with TB susceptibility or resistance may imply that the Mtb bacilli and the local host have undergone a genetic co-evolution to ensure that the Mtb pathogen thrives and persist in the local population. The HLA II DQB1:03:03 allele which apparently confers protection against TB are few meaning probably that they are new emerging alleles in the community. Host-pathogen co-evolution favors Mtb pathogens that avoid presentation by the most common HLA molecules in the host population, this results in selection for the host with rare HLA alleles conferring resistance to the Mtb pathogen. The frequency of rare but protective HLA alleles will increase whereas the dominant HLA alleles will become less frequent resulting in dynamic polymorphism [187, 188]. Unfortunately Mtb pathogens mutate faster than the MHC of their host [189].

The finding of a negative correlation between the HLA- DQB1*03:03 allele and susceptibility to TB (P = 0.0025) indicate that this allele may confer protection against TB in the study population. This is supported by previous observations that an effective immune response in terms of IFN-γ production was elicited when peptide T1, a component of CFP-10

was presented to CD4 T cells in the context of DQB1*03 [190]. CFP-10 is a potent T cell antigen that is recognized by many individuals infected with Mtb [190].

MHC polymorphism imposes a limitation of developing a universal vaccine against infectious diseases, because an effective T cell response is dependent on an individual MHC phenotype [191]. Knowledge of the HLA types present in a target population and the particular epitopes that are restricted by these HLA types enables synthesis of optimally immunogenic MHC-based vaccines [192], specifically tailored for the target population [193]. The anti-Mtb immune response is a bias towards CD4+ T cells [194] viewed as crucial for anti-Mtb immune responses.

3.4 PAPER IV

In an autopsy study of patients (n=49) with presumptive TB who were culture positive for mycobacteria at autopsy a majority of cases (75%) were caused by Mtb, of which 16 (55%) were Uganda genotype. In 25% of patients NTM were identified. Although the histopathological findings in most TB cases were severe, 38 (76%) of the patients that were diagnosed with TB on autopsy had not been diagnosed before death. As expected extrapulmonary TB was significantly more prevalent among patients with HIV than among those without, while cavitating TB lung disease was significantly less prevalent among patients with HIV. Histopathology was similar in TB and NTM patients, although NTM patients had significantly reduced tendency to cause pleural effusions.

The genus Mycobacterium comprises more than 120 species including human pathogens [195]. Mtb is considered to be the principal cause of mycobacteriosis in humans [24]. Although speciation of the NTM isolates could not be performed, the results indicate that some NTM species may contribute to mortality. NTM has also been reported as an important cause of mortality in the United States with a significant increase in NTM related death from 1999 through 2010 [196]. In the same study, pleural effusions were not significantly associated with NTM. This is in agreement with a previous study in Taiwan in which tuberculous pleural effusion was significantly more associated with PTB than NTM pulmonary infection [197]. Also in Portugal and Brazil an increase in NTM infections has been observed [198].

NTM in general cause clinical disease in individuals with acquired and inherited immunodeficiency disorders. However, in this study we found mycobacteriosis due to NTM not only in HIV positive patients but also in HIV negative patients. Previous studies have also reported mycobacteriosis due to NTM in HIV negative patients [199].

The pathological findings at autopsy were similar in patients infected with Mtb and those infected with NTM, though pleural effusion was significantly associated with Mtb infection. NTM in general exhibit low virulence and pathogenicity compared to Mtb, with a low tendency to elicit an immunological reaction needed to generate a pleural effusion.

The presence of AFB in ZN stained smear or tissue may signify preliminary diagnosis of Mtb infection but can also represent NTM [200]. A positive AFB test is thus not specific for Mtb complex [201-203]. Mtb is the leading cause of infectious morbidity due to bacterial pathogen globally. Mortality in our case was mainly attributed to late or missed TB diagnosis. The diagnosis of disseminated and extrapulmonary TB is quite elusive because of the lack of classical features of TB. It is important for physicians to have a high degree of TB suspicion in patients present with pyrexia of unknown origin. Early diagnosis is pivotal to strategies designed to reduce transmission and morbidity due to Mtb. NTM should also be identified in all patients with mycobacterial infection because of the clinical implications associated with NTM. In comparison to Mtb NTM require a more prolonged course of therapy with a combination of drugs [204].

3.5 PAPER V

Thirty-five adults suspected of having TB and 43 control subjects without TB but with pleural effusions from other causes were recruited. The diagnosis of TPE was established when the ZN stain or Lowenstein-Jensen culture was positive for TB or when granuloma were shown in the pleural biopsy.

Pleural fluid CD4+ T cells were significantly more abundant in individuals with TB and TPE patients had a significantly higher CD4+/CD8+ ratio compared to non-TB patients and controls at unadjusted analysis. However age and ethnicity were found to be important confounders and the CD4+/CD8+ T cell ratio varied considerably among those with TPE.

Analysis of pleural fluid for the quantity of CD4+ and CD8+ T cells may be useful for establishing a diagnosis of TB in suspicious cases. The gold standard for diagnosis of TPE is detection of Mtb in pleural fluid or pleural biopsy specimens by microscopy or culture. In

pleural tissue biopsy, one needs to demonstrate granulomatous inflammation along with AFB. The diagnostic yields of pleural effusion is 12 -70% for culture [205-207] and 65 -82% for histological examination [208-211], but pleural biopsy is expensive in most of low resource countries.

Previous studies have assessed the use of serum CD4+ and CD8+ immunoprofile in diagnosing TPE. The typical pathogenesis model involves a rupture of sub-pleural caseous foci followed by a delayed hypersensitivity reaction to Mtb antigens [212-214]. Previous work has established that there are distinct differences between the circulating immune profile and the pleural fluid in HIV/TB patients [215]. The TPE is generated from a localized immune response occurring in the lung; hence analysis of the peripheral blood may not be representative of the host immune response, particularly at the acute stage of disease. Evidence shows that immune cells sequester to the lung during TB [216-220]. In conclusion this study indicates that the CD4+/CD8+T Cell immunoprofile may be used to discriminate between TPE and non-TPE though the sensitivity may be reduced by confounders like age and ethnicity.

4 CONCLUDING REMARKS AND REFLECTIONS ON LEARNING OUTCOMES

This thesis set out to explore the influence of strain genomic diversity, and in particular the Mtb Uganda genotype, on the clinical phenotypic outcome of infection with Mtb in Uganda. The studies here further support the dominant role of the Uganda genotype in the ongoing TB epidemic in Uganda.

The finding that Mtb Uganda genotype was less frequent in EPTB cases than previously described in PTB patients in Uganda [127], in combination with the observation that tuberculous lymphadenitis patients infected with isolates of Mtb Uganda genotype were significantly less prone to have abdominal lymphadenopathy may indicate a reduced ability of Mtb Uganda genotype to cause extrapulmonary disease. Since efficient Mtb transmission depends on lung damage, bacterial genotypes that are more prone to cause pulmonary disease may transmit more efficiently and tend to be dominant.

There was a negative correlation between the HLA II DQB1:03:03 allele and susceptibility to PTB, even after correction for multiple testing, indicating that this allele may confer

protection against PTB in the study population. The dominant HLA II alleles were not associated with TB susceptibility or resistance implying that the Mtb bacilli and the local host have undergone a genetic co-evolution to ensure that the Mtb pathogen thrives and persists in the local population.

The study adds more evidence to previous observations that genetic diversity within Mtb has clinical and phenotypic consequences. This has practical consequences for designing diagnostic and drug susceptibility testing molecular methods, and for the use of current antibiotics. Knowledge of the locally prevalent HLA profiles could be used in designing locally customized effective HLA based vaccines. Analysis of pleural fluid for the quantity of CD4+ and CD8+ T cells may be useful for establishing a diagnosis of TB in suspicious cases.

The work has increased awareness of the importance of molecular methods in the management of TB among physicians, and has built both technical and human capacity in molecular diagnostic methods. The molecular methods I learned here will be extended to improve the diagnostic accuracy of cancer and cancer gene mutations at the Pathology Department in order to offer personalized oncology treatment.

As Mulago Hospital prepares for a renal transplant program, this work has demystified immunogenetics and has generated protocols for the new hospital Transplant and Immunogenetics laboratory.

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