Tuberculosis challenges: mycobacterial culture confirmation and disease recurrence

Thesis submitted to the University of Leicester for the degree of Doctor of Medicine

by

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Abstract

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Background

Tuberculosis (TB) is global pandemic. The ongoing challenges of TB control include inadequate diagnostics and treatment. Culture is the reference standard for laboratory confirmation of disease. However, extrapulmonary TB (EPTB) culture confirmation is low. Successful treatment of TB is compromised by TB recurrence. The epidemiology is poorly characterised in the UK.

Methods

Two studies were conducted. The first, a nested case-control study examined the epidemiology of TB recurrence in Leicester. Cases were identified from the Leicester TB service database (TBIT) and matched to controls on a ratio of one-to-two on date of notification. Multivariate conditional logistic regression examined risk factors for recurrence. The second, a pilot diagnostic study, compared the number of mycobacteria isolated from EPTB samples in the presence or absence of resuscitation promoting factor-containing culture supernatant (CS). Diagnostic performance was assessed by a composite reference standard.

Results

From a cohort of 4628 patients, 82 TB recurrences occurred (1.8%); 84% were relapses and 16% reinfections. On multivariate analysis, smoking (OR 3.8; p=0·04), grade 3-4 adverse drug reactions (OR 5.6; p=0·02), ethnicity 'Indian subcontinent' (OR 8·5; p<0·01), ethnicity 'other' (OR 31.2; p=0.01) and receipt of immunosuppressants (OR 6.8; p<0·01) were independent predictors of TB recurrence.

In 8 of 19 samples, CS-dependent differentially culturable tubercle bacilli (DCTB) were present. The peripheral lymphocyte count (PLC) positively correlated (p=0.04) with CS most probable number assay counts. CS increased culture sensitivity from 36 to 43%; specificity remained at 100%.

Conclusion

Within Leicester, TB recurrence was infrequent, predominantly due to relapse. The identification of an elevated recurrence risk amongst the ethnic group contributing most cases to the national TB burden presents an opportunity to improve individual and population health.

CS may improve culture diagnostic performance although larger studies are required. The PLC represents a candidate biomarker of host CS-DCTB populations.

Declaration

I declare that this thesis has been written by myself and the contents of this thesis are solely my own work except where acknowledgment has been given.

I designed the study entitled "A study of tuberculosis recurrence in Leicester". I co-designed the study entitled "Enhancing the culture of extrapulmonary tuberculosis with resuscitation promoting factor". I obtained ethical approval for both studies. All patients recruited into the latter study were personally seen by me and written informed consent obtained as dictated by study ethics. All clinical samples from patients suspected of having TB were processed by me and I undertook all the laboratory experimentation. All study data was collected personally by me and entered into appropriate databases. I undertook all statistical modelling and analysis with some guidance from the Stata forum (https://www.statalist.org/forums/) and a local statistician (Matthew Richardson). I prepared all manuscripts for published papers arising from this MD thesis and these were edited by my co-authors. During the review process, further edits were suggested by reviewers.

Chapter 1 contains subsections published in two papers arising from this thesis:

1. Rosser A et al. Resuscitation-promoting factors are important determinants of the pathophysiology in Mycobacterium tuberculosis infection. Critical Reviews in Microbiology. 2017; 43(5): 621-630
2. Rosser A et al. Recurrent tuberculosis in the pre-elimination era. The International Journal of Tuberculosis and Lung Disease. 2018; 22(2): 139-50

Chapter 2 is an expanded version of the work published as: Rosser A et al. A nested case-control study of predictors for tuberculosis recurrence in a large UK Centre. BMC Infectious Diseases 2018 18(1):94.

Chapter 3 is an expanded version of the work published as: Rosser A et al. Differentially Culturable Tubercule Bacilli are Generated During Non-Pulmonary Tuberculosis Infection. American Journal of Respiratory and Critical Care Medicine. 2018; 197(6): 818-82

No part of this thesis has been submitted in any previous application as part of a higher degree.

Andrew Rosser

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Research output arising directly from this body of work

Oral presentation:

- Title ‘enhancing the culture of extrapulmonary tuberculosis with resuscitation promoting factors’ presented at the Acid Fast Club summer meeting, Porton Down, Salisbury, 7-8th of July 2016.

Publications:

- Rosser A, Stover C, Pareek M, Mukamolova GV. Resuscitation-promoting factors are important determinants of the pathophysiology in Mycobacterium tuberculosis infection. Critical Reviews in Microbiology. 2017; 43(5): 621-630. doi: 10.1080/1040841X.2017.1283485. PMID: 28338360
- Rosser A, Pareek M, Turapov O, Wiselka MJ, Mukamolova GV. Differentially Culturable Tubercule Bacilli are Generated During Non-Pulmonary Tuberculosis Infection. American Journal of Respiratory and Critical Care Medicine. 2018; 197(6): 818-82. doi: 10.1164/rccm.201705-1048LE. PMID: 28880570
- Rosser A, Richardson M, Wiselka MJ, Free RC, Woltmann G, Mukamolova GV, Pareek M. A nested case-control study of predictors for tuberculosis recurrence in a large UK Centre. BMC Infectious Diseases. 2018; 18(1): 94. doi: 10.1186/s12879-017-2933-4. PMID: 29486715
- Rosser A, Marx FM, Pareek M. Recurrent tuberculosis in the pre-elimination era. The International Journal of Tuberculosis and Lung Disease. 2018; 22(2): 139-50. doi: 10.5588/ijtld.17.0590. PMID - 29506610

List of abbreviations

ADC	Albumin Dextrose Catalase
ATT	Anti-tubercular treatment
ART	Anti-retroviral therapy
BCG	Bacillus Calmette–Guérin
CAS	Central African strain
CFU	Colony forming unit
CI	Confidence Interval
CRS	Composite reference standard
CS	Culture supernatant
CSF	Cerebrospinal fluid
CXR	Chest x-ray
DM	Diabetes mellitus
DMARD	Disease modifying anti-rheumatic drug
DNA	Deoxyribonucleic acid
DS-TB	Drug-susceptible TB
EAI	East African / Indian
EBUS	Endobronchial ultrasound
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme linked immunosorbent assay
gDNA	Genomic DNA
HIV	Human immunodeficiency virus
IQR	Interquartile range
LAMP	Loop mediated isothermal amplification

LN	Lymph node
LPA	Line probe assay
LTBI	Latent tuberculosis infection
MDR	Multidrug resistant
MGIT	Mycobacteria growth indicator tube
MIRU-VNTR	Mycobacterial interspersed repetitive unit-variable number of tandem repeat
MODS	Microscopic observation drug susceptibility
MPN	Most probable number
Mtb	*Mycobacterium tuberculosis*
NAAT	Nucleic acid amplification test
NTM	Non-tuberculous mycobacteria
OADC	Oleic Acid Albumin Dextrose Catalase
OR	Odds ratio
PCR	Polymerase chain reaction
PHE	Public Health England
PLC	Peripheral lymphocyte count
PLWH	People living with HIV
PMM	Predictive mean matching
POC	Point-of-care
Rpf	Resuscitation promoting factor
RR-TB	Rifampicin resistant TB
PY	Person years
RFLP	Restriction fragment length polymorphism
SD	Standard deviation

TB	Tuberculosis
TBM	TB meningitis
TLA	Thin layer agar
TTP	Time-to-positivity
UHL	University Hospitals of Leicester NHS Trust
UHL-MD	University Hospitals of Leicester NHS Trust Microbiology Department
UK	United Kingdom
UoL	University of Leicester
VIF	Variance inflation factor
WCC	White cell count
WGS	Whole genome sequencing
WHO	World Health Organisation
XDR	Extensively drug-resistant
ZN	Ziehl-Neelsen

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Chapter 1. Introduction

1.1 Epidemiology

1.1.1 History

Tuberculosis (TB) is a disease long a part of human history (1). From the 17th to the early 19th century in both Europe and North America, TB was responsible for 1 in 5 adult deaths (2). Between 1830-1950 the TB epidemic waned, notably this was prior to both the introduction of TB treatment in the 1940s and the routine use of the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine in the 1950s (3-5). In industrialised countries, rates continued to fall up to 1985 (6). In some developing countries there was evidence of disease stabilisation but in others such as South Africa, TB remained a significant cause of morbidity and mortality (6, 7).

From the mid-1980s, there was an upsurge in TB incidence in many areas worldwide which lead the World Health Organization (WHO) to declare in 1993 that TB was a global public health emergency (8). One of the principle drivers was emergence of Human Immunodeficiency Virus (HIV) infection (9-11). Other factors implicated in the recrudescence of TB included an increase in migrants from high prevalence to low prevalence areas; population aging; transmission in institutions such as prisons, hospitals and homeless shelters; socio-economic breakdown in areas such as the former Soviet Union and an increase in drug resistance (12).

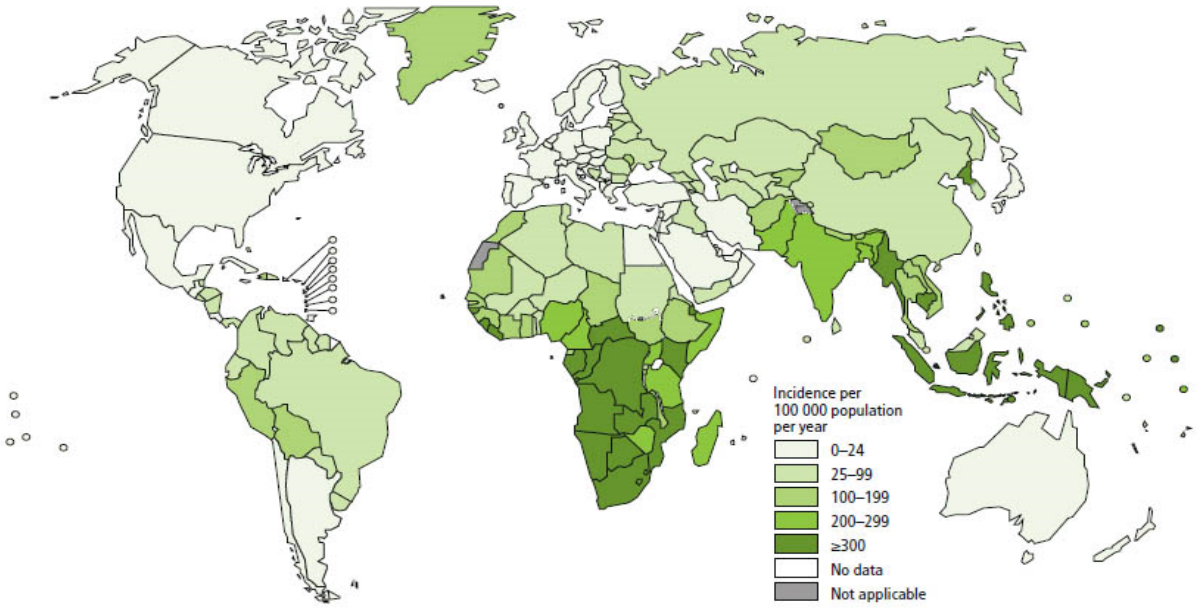
Following the initiation of the TB control programs in 1995 (see section 1.11) global TB incidence eventually began to fall in 2004 from a peak of circa 170 cases per 100,000 population (1). The reduction in TB incidence from 2000-2016 has averaged 1.4% per year (1).

1.1.2 Worldwide epidemiology

In 2016, approximately 10.4 million people worldwide developed active TB of whom 1.7 million died making TB the top cause of death globally from a single infectious organism (1). Worldwide, a total of 476,774 recurrent ('relapse') cases were notified, representing 7.2% of incident cases (13). 1.2 million of new TB cases were HIV positive, of whom 0.4 million cases died (1). Pulmonary TB (PTB) accounted for 85% of notifications, ranging between 76% in the Eastern Mediterranean Region to 92% in the WHO Western Pacific Region (1). In 2015, 83% of 5.9 million new and retreatment patients were successfully treated (1).

There were approximately 490,000 cases of multidrug resistant (MDR) TB and a further 110,000 patients with rifampicin-resistant TB (RR-TB) who were eligible for MDR-TB treatment in 2016 (1). 47% occurred in China, India and the Russian Federation(1). Of the MDR-TB cases examined for second line drug resistance, 20% were resistant to fluoroquinolones and 6.2% of cases were extensively drug resistant (XDR) TB (1). MDR-TB case detection and treatment coverage was insufficient with 153119 (26%) of MDR-TB cases diagnosed and 129689 (22%) of people enrolled in MDR-TB treatment programs. 8511 people started XDR-TB treatment (1).

Figure 1.1 Estimated Global TB incidence rates for 2016 (1)



1.1.3 European epidemiology

323,000 incident TB cases were reported in the WHO European region in 2015 and an estimated 32,000 died (14). 27.2% of notified cases had been previously treated for TB (14). Since 2006, incidence has fallen on average by 5.4% per year up to 2015 (14). Similarly, mortality has declined 7.4% per year between 2006-2015 (14). There were an estimated 74,000 MDR-TB cases, accounting for 16% of new and 48% of previously treated cases (14). Of the MDR-TB cases undergoing second line phenotypic drug susceptibility testing, 23% were XDR (14). Overall, of those completing TB treatment commenced in 2014, 76.0% were classified as cured or treatment completed (14).

Seventy nine percent of notified cases were classified as PTB with bacteriological confirmation (nucleic acid amplification tests (NAAT) / acid fast bacillus (AFB) smear / culture) achieved in 61.4% of cases (14). 12.4% of cases were notified as EPTB with seven

countries reporting more than 30% of incident cases as EPTB (14). No data on bacteriological confirmation of EPTB was provided (14).

The age and sex distribution of TB varies significantly between European countries (14). Rates in males and females are typically similar until the age of 25 with a male predominance in older age groups (14). Of countries reporting HIV -TB co-infection data, 88.3% TB patients were tested of whom 9.0% were HIV positive (14). Foreign born nationals represent 6.3% of cases notified although in certain countries they represent the majority of notified cases, for example Sweden (89.5%) (14). This disproportionate burden in foreign-born persons in high income countries is likely due to an interaction between immigration from high TB burden countries and the reactivation of remotely acquired latent TB infection (LTBI) in the first five years after arrival in the host country (15).

1.1.4 England epidemiology

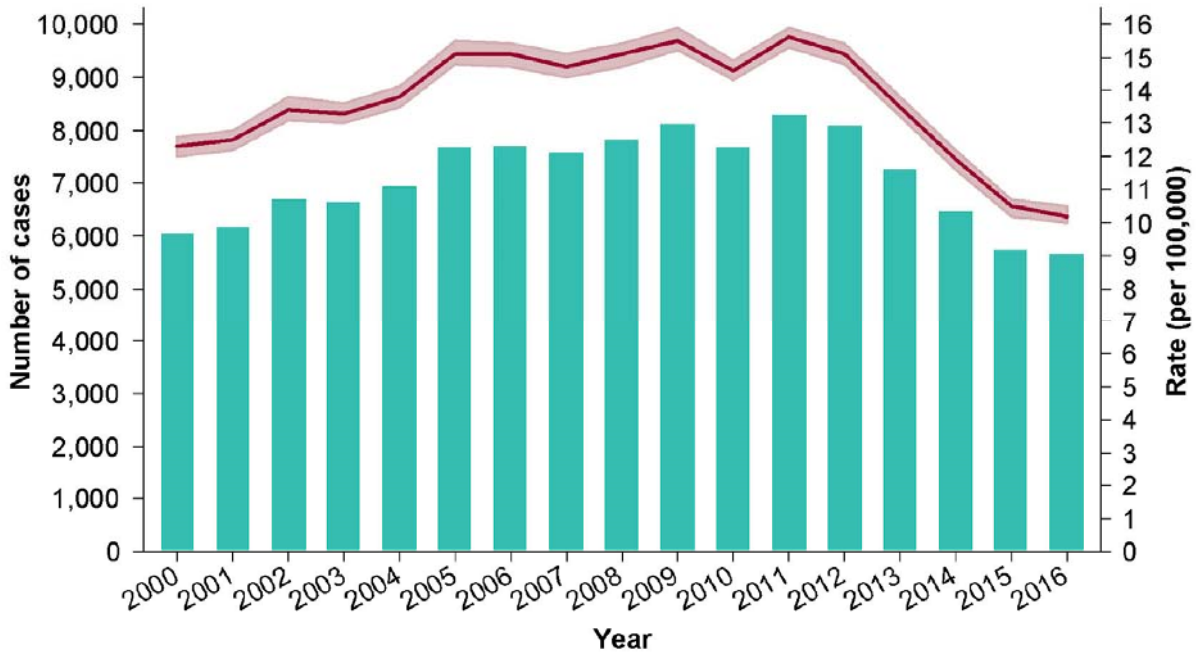
5,664 cases of TB were notified in England in 2016 giving an incidence rate of 10.2 per 100,000 population (16). This represents a continuing fall from a peak incidence of 15.6 per 100,000 population in 2011 (see Figure 1.2). 6.5% of notified cases had a previous diagnosis of TB at least 12 months before their current notification (16). 83.4% of people with an expected treatment duration of less than 12 months in 2015 completed treatment and 5.3% died by the last recorded outcome (16).

1,469 TB cases occurred in the UK-born population with a TB incidence rate of 3.2 per 100,000 population, down 10.5% from 2014 (16). 61.7% of cases were from the White ethnic group, 20.3% were South-Asians and 12.8% from Black-ethnic groups (16). Amongst non-white ethnic groups, the TB incidence was three-to-fourteen times higher than the White population (16).

In the non-UK born population, 4,096 cases were notified representing 73.6% of the total TB burden in England (16). The TB incidence in this population was 49.4 per 100,000 population falling from a peak of 100.7 cases per 100,000 population in 2005 (16). The most common countries of birth for foreign born cases included India (24.8% of total non-UK born cases), Pakistan (15.7%), Somalia (5.2%) and Bangladesh (4.4%) (16).

TB predominantly affected males (59.1% of cases) and those between 25-29 years of age (16). 3.8% of cases were co-infected with HIV (2015 data). PTB accounted for 3,041 (53.9%) of cases. Amongst non-UK born cases, 2103 (51.4%) had extrapulmonary TB (EPTB). 76% of PTB and 48% of EPTB were culture confirmed. 59 cases (1.7%) had MDR-TB/ RR-TB and 10 (0.18%) had XDR-TB.

Figure 1.2 TB case notifications and rates for England 2000-2016 (16)



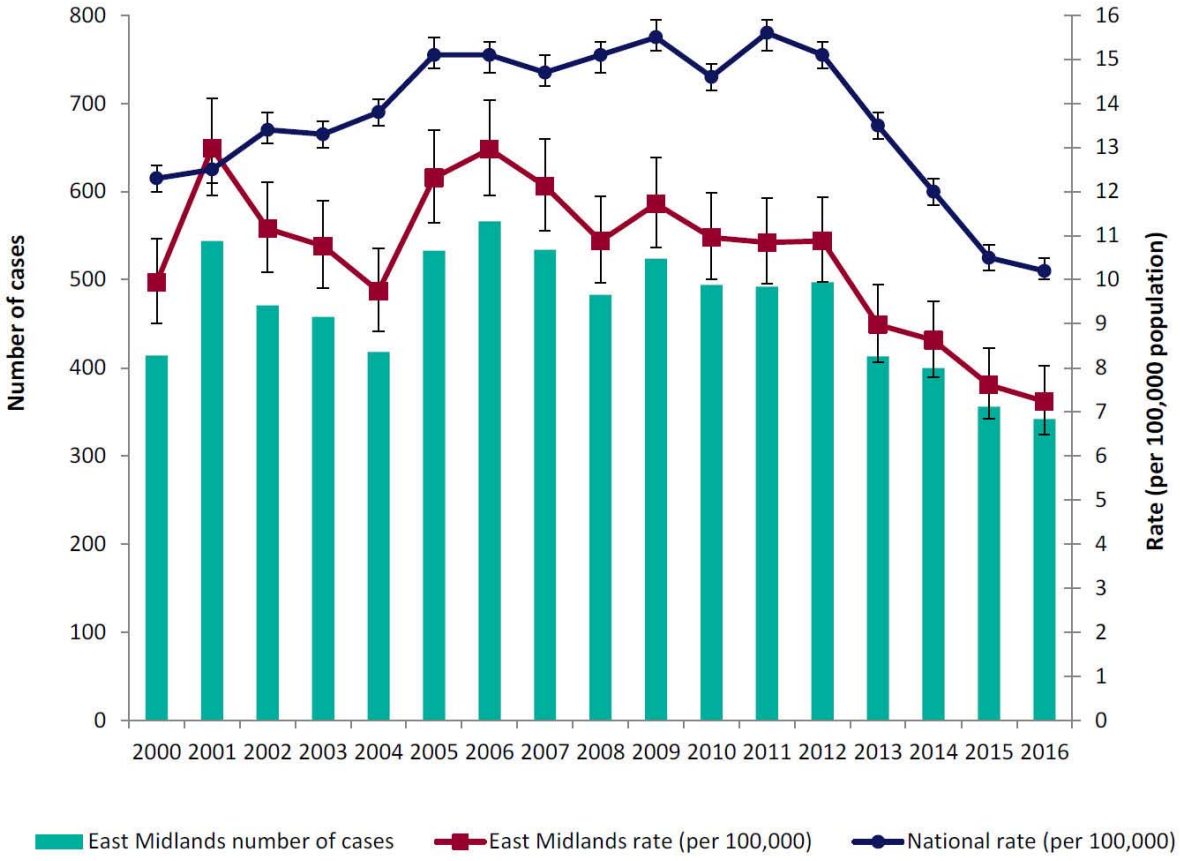
1.1.5 East Midlands epidemiology

In 2016, 342 cases were reported in the East Midlands with an estimated incidence rate of 7.2 per 100000 population (see Figure 1.3) (17). Within the city of Leicester TB rates were 37.3 cases per 100,000 population, a slight increase from 2015 (36.2 cases per 100,000 population) (17). The number of cases in the East Midlands has fallen compared to 2015 (3.9%) however this was lower than the yearly decline from 2012-2015 (10%) (17).

Of cases with a recorded country of birth, 72.0% were non-UK born. Of these, 38.6% were Indian, 9.1% Pakistani and 4.6% from Romania (17). Amongst UK-born, 68.8% of cases were from the White ethnic group and 17.2% were Indian (17). The annual TB incidence in non-UK born was 48.2 cases per 100,000 population, 21 fold higher than that in the UK born population (2.3 per 100,000 population) (17).

TB incidence rates were highest in males (58% of total cases) and those aged 30-39 years old (17). 54.1% of cases had PTB. 78.% of PTB but only 50.3% of EPTB cases were culture confirmed (17). 3.9% of cases had MDR-TB of which 1.5% were XDR (17). 3.8% of cases over 15 years of age were co-infected with HIV (17). 16 (5.2%) of cases were previously diagnosed with TB more than 12 months before their current notification (17). Of the patients diagnosed in 2014 with an expected treatment duration of less than 12 months, 76.5% completed treatment and 5.6% had died by 12 months after treatment initiation (17).

Figure 1.3 TB incidence from 2000-2016 in the East Midlands (17)



1.2 The organism

Mycobacteria are members of the order *Actinomycetales* along with *Rhodococcus*, *Corynebacterium*, and *Nocardia* (18). *Mycobacterium tuberculosis* (*Mtb*) is the member of the *Mtb* complex (MTBC). MTBC is composed of genetically related mycobacterial species which cause TB in discrete hosts (19). *Mtb*, *M. africanum* and *M. cannetti* are human specific pathogens; *M. microtti* infects principally rodents; *M. pinnipedi* seals and sea lions; *M. caprae* goats and sheep; *M. orygis* cows, oryxes, gazelles, rhesus monkeys of South Asian origin and humans; *M. bovis* has an expanded host range including bovids and humans (20, 21).

Mycobacteria are non-motile, non-sporing, Gram positive bacilli with a replication time of 12-24 hours (19). The cell wall is rich in mycolic acids which convey acid fastness, resistance to acidic and alkaline conditions, desiccation and various antibiotics (22). *Mtb* typically respires aerobically utilising carbohydrates however during host infection it switches predominantly to fat metabolism (23). *Mtb* is facultatively intracellular and predominantly infects macrophages during the early stages of infection (24), however *Mtb*

deoxyribonucleic acid (DNA) has been detected in other tissues including adipose tissue, splenic and liver cells (25, 26).

1.3 Pathogenesis

Mtb is transmitted primarily by the inhalation of aerosols measuring less than 5 μm in diameter containing viable bacilli (27). Upon reaching the alveoli, *Mtb* is phagocytosed by resident macrophages and dendritic cells (28). Innate lymphoid cells produce interleukin-17 & interferon γ (IFNγ) and may provide protection against *Mtb* infection (29). Neutrophils are abundant during infection and play a role in restricting early *Mtb* growth (30, 31). Despite internalisation, *Mtb* can continue to replicate within the cell (32)

Favourable outcomes to *Mtb* infection require a fine balance of innate and adaptive immune responses (33). Following initial infection, myeloid cells sense *Mtb* through cell surface receptors producing chemokines leading the further cellular recruitment and IL-10 production which limits macrophage responses to *Mtb* (28). Myeloid cells help initiate an adaptive immune response by facilitating CD4 T helper$_1$ (TH_1) and T helper$_{17}$ (TH_{17}) differentiation (28). TH_1 cells promote inflammatory cell recruitment and enhancing macrophage killing of *Mtb* (28, 34, 35). TH_{17} cells have a central role in protective immunity to *Mtb*, particularly in the early stages of infection (36-38). Macrophages and T-cells are key to granuloma formation which ultimately controls the primary infection in most patients but in 90% of cases fails to kill *Mtb* bacilli and provides a sanctuary site for non-replicating bacilli to latently infect the host, known as latent TB infection (LTBI). (28, 39, 40). At present, the physiological status of these bacilli is unknown, however it is hypothesised that conditions within the granulomas may vary leading to heterogeneous populations consisting of replicating and non-replicating cells (41, 42). Differential killing of *Mtb* at the onset of adaptive immunity may lead to the formation of heterogeneous lesions, some of which may be sterilised whilst others may progress to maximum bacillary load (43). In approximately 5% of newly infected cases, there is rapid progression to active TB within 2 years (44).

Although patients with LTBI are a clinically homogenous group lacking any signs or symptoms of active TB, it is thought that LTBI is in fact a range of infection states ranging from a sterilizing host immune response to subclinical active infection at high risk of reactivation (45). It is hypothesized that the immune system hinders bacterial replication and if this is upset by factors such as immunosuppression due to diabetes mellitus, HIV or malnutrition, then bacillary replication resumes leading to reactivation and clinical disease.

(42, 46). The mechanism(s) by which bacillary replication resumes are as yet unresolved but may involve cell wall alterations by enzymes called resuscitation promoting factors (Rpf) (47).

Table 1.1 Risk groups for TB reactivation

Risk factor	TB relative risk (vs. gen population)	Reference
Advanced HIV infection	9.9	(48)
TNFα blocker therapy	1.6-25.1	(49)
Silicosis	2.8	(50)
Immigrants from high TB incidence countries	2.9-5.3	(51)
Poorly controlled diabetes mellitus	1.7	(48)
Chronic renal failure	2.4	(48)
Healthcare workers	2.5	(52)
Tobacco smoking	2-3.4	(53-55)

Abbreviation: TNFα - tumour necrosis factor α

Rpfs are a family of bacterial proteins, able to resuscitate non-replicating bacteria that were discovered in *Micrococcus luteus* which possesses a single *rpf* gene (56). Studies of sputum using most probable number (MPN) and colony forming unit (CFU) assays have revealed the presence of *Mtb* populations with different cultivation requirements including those growing on solid agar; those growing in 7H9 liquid medium; those only revealed in 7H9 liquid medium supplemented with Rpf-containing culture supernatant (CS) and Rpf-deficient CS respectively (57, 58). These populations are collectively termed differentially culturable tubercle bacilli (DCTB) (58, 59). The physiology of Rpf and the clinical, diagnostic and treatment implications of DCTB are discussed in section 3.1.

1.4 Risk factors for TB acquisition and reactivation

The risk factors for developing TB can be divided into those for acquiring infection and those for progression to active disease (44).

Various factors combine to determine risk of acquiring infection following exposure including the proximity and duration of contact (60-62), infectiousness of the individual case such as smear positivity (63) and behavioural factors such as smoking, alcohol use and indoor pollution (44).

The lifetime risk of latent TB reactivation is approximately 10% (64-66), although may be significantly higher in specific risk groups (see Table 1.1).

1.5 TB and HIV

Approximately 36.7 million people were infected with HIV at the end of 2016, of whom 19.5 million were accessing antiretroviral therapy (ART) (67). 1.2 million people living with HIV (PLWH) developed TB in 2015 of whom 0.4 million died (1).

HIV impacts the natural history of TB in numerous ways: it increases the risk of progression from primary infection to disease (68); in those with LTBI, the risk of reactivation is increased from an approximate 8-10% lifetime risk to about a 10% per year risk (69); HIV elevates the risk of recurrence due to reinfection (70) (see section 1.10 for further information).

HIV predominantly targets activated Thelper$_1$ (TH_1) cells using CD4 as the main receptor and chemokine receptors CCR5 or CXCR4 as co-receptors (71). HIV infection reduces TH_1 cells counts through both cellular destruction and lower proliferation (72). Since, TH_1 cells are central to the control of *Mtb* infection, HIV has a deleterious effect on the host immune control of *Mtb* (73). This occurs shortly after HIV seroconversion (74), correlating with the rapid loss of *Mtb* specific TH_1 cells (75). As TH_1 cells counts decline, the risk of TB increases in a stepwise fashion (76). Those with CD4 counts <100 cells mm^{-3} are at ten times the risk of TB compared to those with ≥500 cells mm^{-3} (77).

At CD4 counts >350 cells mm^{-3}, TB presentations are similar to those patients without HIV (78). HIV positive patients are more likely to be AFB smear negative compared to HIV negative (79). At lower CD4 counts, PTB still predominates (78), however there is an increasing proportion of extrapulmonary disease (80), the risk of which is inversely proportional to the CD4 count (81). At CD4 counts < 200 cells mm^{-3}, TB patients may have atypical chest x-ray (CXR) signs such as middle / lower lobe infiltrates, pleural effusion, fewer cavitations or even a normal CXR (69, 82). Below CD4 counts of 75 cells mm^{-3} , pulmonary findings may be absent and miliary TB characterised by multiple organ involvement and mycobacteraemia is more common (65). Asymptomatic subclinical tuberculosis with negative AFB-smear sputum microscopy and CXR but with positive TB culture is common for HIV-associated TB and may constitute 10% of the total TB burden in endemic areas (83, 84).

In patients without active TB, commencing antiretroviral therapy (ART) reduces the risk of progression to active TB (85). In those with active TB, prompt ART initiation reduces mortality, particularly at lower CD4 counts(86, 87).

1.6 Clinical features

TB is classified by site of disease. PTB is defined by involvement of the lung parenchyma and/or tracheobronchial tree whilst EPTB affects any tissues or organs outside the lungs (88). Classical PTB presents with a collection of symptoms including chronic cough, shortness of breath, expectoration, haemoptysis, night sweats, fever and weight loss (65). The symptoms of EPTB depend upon the site affected, most commonly lymph nodes or lung pleura (82). Patients with LTBI lack any clinical and /or radiological signs of disease (89).

1.7 Diagnosis of tuberculosis

1.7.1 Symptomatic screening

Patients presenting with a cough persistent for more than two to three weeks with/without compatible signs and/or symptoms should be suspected of having TB (90). Symptomatic screening for TB is a simple low-cost method to identify patients requiring further investigation with a CXR, sputum smear microscopy and/or culture where available (91).

1.7.2 Microscopy

Clinical samples may be stained for the presence of AFBs by Ziehl-Neelsen (ZN) or fluorescence auramine phenol methods (92). These methods are cheap, quick, easy-to-use and suitable for low resource settings (93), also identifying those patients who are the most infectious (94). As ZN AFB-smear microscopy has a detection limit of 10,000 cells ml^{-1}, the diagnostic performance for expectorated sputum is low with a sensitivity between 34-80% (95). The sensitivity for EPTB specimens ranges between 0-40% (96).

1.7.3 Culture

Culture is the laboratory standard method for the confirmation of *Mtb* infection (97). It is commonly performed using both solid and liquid modalities (92). Solid culture conventionally uses egg based media such Löwenstein-Jensen (LJ) medium (92, 98). Automated liquid culture methods such as the BacTec™ MGIT™ 960 system detect mycobacterial growth by measuring oxygen consumption or CO_2 production (92). Culture is inherently slow with on average a primary culture taking 2-4 weeks and antibiotic susceptibility another 2-4 weeks, totalling 4-8 weeks for organism identification and phenotypic susceptibility testing (99, 100).

Cultures of *Mtb* require 10-100 bacilli ml^{-1} to become positive (98, 101), exhibiting a sensitivity of 80-93% and specificity of 98% (101, 102). Sample type can influence sensitivity, for example, one study found gastric aspirates were superior to induced sputum in children (103). The diagnostic performance of EPTB culture is lower with sensitivities of 30-80% (104). Reasons for lower EPTB culture sensitivity include the irregular distribution of mycobacteria through the sample, its paucibacillary nature and the lack of an efficient processing technique applicable to all EPTB samples (105).

Using novel approaches, culture can be accelerated whilst maintaining sensitivity. Thin layer agar (TLA) or microscopic observation drug susceptibility (MODS) are quicker than regular culture, inexpensive and accurate (106), although they require specialised laboratory settings (107). One research group has shown that the optimisation of media, atmospheric conditions and bacterial identification through autofluoresence can reduce detection time to 72 hours although this was very labour intensive and was conducted in a well-equipped research laboratory (108). Other authors found that *Mtb* can occasionally be recovered from otherwise culture negative samples by supplementing standard liquid 7H9 culture medium with Rpf-containing CS or recombinant Rpf and can improve time-to-positivity (TTP) of sputum samples cultured in mycobacterium growth indicator tubes (MGIT) (57, 58, 109). However, at present, Rpf are unstable and difficult to produce (57). Currently, no study has evaluated the effect of Rpf supplementation on culture diagnostic performance.

1.7.4 Histology

Histological examination is frequently used for EPTB diagnosis (96). Characteristic appearances include granulomatous inflammation with or without caseation and Langerhans cells (110). Sensitivity ranges between 90-100% although specificity is frequently lower (111-114), as conditions including non-tuberculosis mycobacteria (NTM) infection and sarcoidosis have overlapping histological appearances (96, 110, 115).

1.7.5 Radiological diagnosis

CXR is the initial imaging modality for patients with presumptive TB (101). Although highly sensitive, CXR interpretation is beset by poor specificity and observer inconsistency (116). CXRs may be normal in patients with PTB (117). Computed tomography (CT) of the thorax is an effective diagnostic approach when CXRs are unremarkable providing information on disease activity such as ‘tree-in-bud’ appearance, consolidation and

cavitation (118). However these signs are not restricted to PTB and microbiological evaluation is still required (118).

Radiological imaging is used to investigate EPTB providing radiographic evidence of disease. CT is favoured for lymphadenitis and abdominal TB whilst magnetic resonance imaging (MRI) is preferred for central nervous system (CNS) and musculoskeletal TB (119).

1.7.6 Molecular diagnostics:

Line probe assays (LPAs) are endorsed by the WHO for the detection of drug resistance (1). DNA is extracted from patient specimens or culture isolates; specific regions of DNA are amplified by PCR and amplification products hybridised with probes which detect specific resistance associated mutations (120). First line LPAs detect rifampicin and isoniazid resistance and second line LPAs fluoroquinolone and injectable drug resistance (1). LPAs are rapid but are unable to detect all resistance associated mutations (120).

Xpert® MTB/RIF (Cepheid, Sunnyvale, California, USA) is an automated, simple-to-use, cartridge based NAAT platform that detects *Mtb* directly from clinical samples providing a rifampicin resistance profile within 2 hours (121). It is suitable for point-of-care (POC) testing in low resource settings (122). A Cochrane review of Xpert® MTB/RIF use in adults with PTB showed pooled sensitivity for AFB smear positive patients was 89% and specificity 99% and 67% and 99% in AFB smear negative cases. For rifampicin resistance detection, pooled sensitivity was 95% and specificity 98%, although culture and phenotypic susceptibility testing is still recommended (123).NAATs exhibit several deficiencies including the inability to detect antibiotic resistant subpopulations of *Mtb* present in a larger sensitive population (heteroresistance) (124) and the inability to distinguish live from dead bacilli precluding their use to monitor treatment responses (118).

The performance of Xpert® MTB/RIF in EPTB varies by sample type. A recent meta-analysis concluded that sensitivity when compared to culture in lymph node samples was 81% and 83% for TB meningitis (TBM) although only 46% for pleural TB. This fell to 81%, 63% and 21% respectively when compared to a composite reference standard (CRS) (125). The WHO recommend Xpert® MTB/RIF as the initial diagnostic test for TBM (121).

Xpert® MTB/RIF Ultra is a redesign of Xpert® MTB/RIF with the aim of improving sensitivity in patients with paucibacillary disease or HIV (126). The new assay exhibits a 1

log_{10} lower limit of detection (15.6 vs. 112.6 CFU/ml) (127). Using culture as the gold standard, sensitivity compared to Xpert® MTB/RIF was 78.9% vs. 66.1% for smear-negative disease and 97.8% vs 98.9% for smear positive disease (127). Performance in the detection of rifampicin resistance was equal (127).

A loop mediated isothermal amplification (LAMP) assay was approved by the WHO as replacement for AFB smear microscopy in patients at low risk of MDR-TB (1). The LAMP assay can be read visually within one hour and requires little infrastructure useful for operation in low resource settings (1, 128)

In most settings, the susceptibility of *Mtb* isolates is determined phenotypically, where the growth or metabolic activity of the isolate is evaluated in the presence of anti-tuberculous drugs (129). Whole genome sequencing (WGS) of isolates provides substantial information including the genotypic prediction of drug resistance from known resistance associated loci and the characterisation of *Mtb* DNA strain-type patterns (130). Previously a research tool, implementation in the clinical diagnostic microbiology laboratory is considered feasible (131)

1.7.7 Interferon gamma release assays

Current commercial IFNγ release assays (IGRAs) include QuantiFERON® TB Gold produced by Qiagen and T-SPOT® produced by Oxford Immunotec. IGRAs quantify the *ex-vivo* cellular immune response to culture filtrate protein 10kDa (CFP-10) and ESAT-6 (132). They are primarily used for the diagnosis of latent TB infection (LTBI) and are unsuitable to rule in or rule out active TB using whole blood or extra-sanguineous fluids (133). Unlike the tuberculin skin test, IGRAs are unaffected by BCG vaccination status, however they are at most 80% sensitive and cannot discriminate patients at high risk of progression to active TB (132, 134).

1.7.8 Other diagnostic approaches

Adenosine deaminase is a biomarker used for TB in pericardial, pleural, ascitic and cerebrospinal fluid (104). It exhibits varied sensitivity and specificity depending on the cut off chosen (104).

1.8 TB Treatment

Patients with LTBI who are at increased risk of developing active disease require treatment (65) including PLWH, house hold contacts of PTB cases, those receiving anti-TNF therapies, organ transplants, silicosis and end stage renal disease (135). LTBI treatment has

a low incidence of adverse side effects and reduces disease reactivation by 69% (136). Current UK treatment options include 6 months of isoniazid (preferred in PWLH) or 3 months of isoniazid and rifampicin (137).

The standard regimen in the UK for drug susceptible tuberculosis (DS-TB) is two months of rifampicin, isoniazid, pyrazinamide and ethambutol followed by a continuation phase of 4 months of rifampicin and isoniazid (137). This is extended to 10 months in cases of CNS involvement (137). The standard regimen attains a 95% cure rate under clinical trial conditions and 90% under programmatic conditions (65). Unfortunately, a proportion of apparently successfully treated patients will develop recurrent TB either due to endogenous reactivation (relapse) or exogenous reinfection, which is largely influenced by the effectiveness of local TB treatment and control programs (138).

Treatment guidelines for RR-TB/MDR-TB were updated by the WHO in 2016 (139). Patients with PTB who have not been previously treated with second line drugs, in whom resistance to fluoroquinolones and second line injectables (amikacin, kanamycin or capreomycin) is considered unlikely may be treated with a 9-12 month shorter regimen (139). Patients infected with *Mtb* resistant to components of the shorter regimen, who are pregnant or with EPTB should be treated with the standard longer regimen (139). The longer regimen consists of five active TB drugs including pyrazinamide, a fluoroquinolone, a second line injectable agent and two other drugs such as ethionamide, cycloserine, linezolid or clofazimine with a total treatment duration of 20 months (139). Regimens are optimised following phenotypic drug susceptibility testing (65). XDR TB is more difficult to treat although cure rates of over 80% have been reported (140). New drugs including pretomanid and sutezolid, which are currently being assessed in clinical trials, may improve XDR-TB cure rates (1).

1.9 TB recurrence

1.9.1 Defining TB recurrence

The WHO defines TB 'relapse' (recurrence) as 'relapse patients that have been previously treated for TB, were declared cured or treatment completed at the end of their most recent course of treatment and are diagnosed with a recurrent episode of TB (either a true relapse or a new episode of TB caused by reinfection' (88). However, in practice, the definition of recurrent TB employed varies significantly between studies. For example, the recurrent episode may be defined by: bacteriological confirmation of recurrent disease (141); histological/radiological/clinical evidence of recurrent disease with a compatible response to TB treatment (142, 143); reinitiation of TB treatment by a physician(144); renotification of disease to public health authorities (145). Similarly, studies differ in the length of time that must have passed after the 1st episode before a 2nd episode can be defined as recurrence rather than treatment failure. In some studies, it is at least 3 months(146) or 12 months(147) after treatment completion whereas others count recurrences occurring the first day after treatment finished (148).

At present, despite the publishing of the WHO definition of TB recurrence (88), there is no international consensus definition, thus authors continue to define recurrence differently and heterogeneity persists between studies. This introduces bias the estimation of recurrence rates and hinders comparison between settings (138, 149)

1.9.2 Reinfection and relapse as mechanisms of TB recurrence

Recurrent TB is composed of two distinct although clinically indistinguishable pathological processes, reinfection and relapse (150, 151). Relapse occurs when patients fail eliminate residual *Mtb* bacilli during treatment which subsequently reactivate possibly after many years to cause a second episode of TB and those with reinfection exogenously acquire infection with a new strain of TB (151).

Debate about whether reinfection or relapse is the primary mechanism of recurrence has taken place over the last few decades. Stead proposed in the 1960s the 'unitary concept of pathogenesis' which stated that TB commences with a primary infection and subsequent bouts of active TB are due to the reactivation of dormant bacilli (152). However, others considered exogenous reinfection to play a significant pathogenic role (153, 154). Through the use of molecular genotyping techniques, it has become clear that both processes contribute to the recurrence of TB within a population (70, 141, 144, 147, 148, 155-173).

1.9.3 Molecular genotyping to delineate mechanisms of TB recurrence

A number of molecular genotyping techniques have been developed which generate strain-type DNA patterns for individual isolates. Methods include IS6110 restriction fragment length polymorphism (RFLP) typing (155), spoligotyping (174), mycobacterial interspersed repetitive unit variable number of tandem repeat (MIRU-VNTR) typing (161), and WGS (161).

Comparison of the strain-type DNA patterns generated by these methods is used to differentiate reinfection from relapse (138). Where patterns are indistinguishable, this is considered to be a case of relapse and where they differ, a case of reinfection. Studies (excluding WGS) differ in their definition of reinfection by 1(168), 2(160) or even 3(167) band differences, the cut off set to allow for errors in genotyping methods and strain evolution within the host (175). WGS determines the genetic distance between strains by calculating the number of single nucleotide polymorphisms, with values of ≤6(161) and ≤10 (176) defining relapse and ≥1306 (161) and >100 (176) reinfection in two studies. Of the current methods available, WGS is best able to resolve relapse and reinfection with other methods frequently generating inconsistent results (161).

There are limitations to using differences in strain type patterns between episodes to delineate mechanisms of recurrence. For example, reinfection may be misclassified as relapse where population strain diversity is low or individuals are repeatedly infected from the same source case and relapse misclassified as reinfection due to strain evolution, mixed infection or laboratory cross-contamination (175).

1.9.4 TB recurrence rates

Patients with DS-TB who adhere to the standard four drug course in the clinical trial setting experience recurrence rates of 1-2% by 24 months after treatment completion (177). Outside of clinical trials, proportions vary significantly. For example, Dobbler et al report 0.5% of patients developed TB recurrence in an Australian study that ran over 13 years (178) whereas Li et al reported that 16.7% of patients had recurrent TB in a Chinese study run over a 5 years (179). The level of TB recurrence is also described by the incidence rate represented in person years of follow up (PYs). Rates quoted in studies range between 0·34 to 10·3 per 100 PYs (147, 158). This variability stems from differences in the local epidemiology of recurrence as well as differences in recurrence definitions used (149).

Within the UK setting, data describing the level of TB recurrence is limited (180). Public Health England (PHE) Annual TB reports from 2010-2016 state that 6-7% of

patients notified with TB have had a previous episode of TB more than 12 months before their current notification. Published studies examining TB recurrence in various UK settings show lower proportions of recurrence. For example, Crofts et al examined TB notifications in England and Wales for the period 1998-2005 and found 1.4% of patients developed recurrent TB (145). Two other studies examined TB notifications in Blackburn between 1978-1987 and in Northwick Park Hospital between 2002-2013 and found TB recurrence proportions of 3.1% (143) and 0.4% (180) respectively. Thus, the current understanding of TB recurrence rates in the UK is principally derived from TB notification data. The discrepancies between Annual TB reports and published data as well as the inability to check the veracity of notification data call into question the accuracy of this data and indicates the need for further well conducted studies.

1.9.5 Contribution of relapse and reinfection to the TB recurrence burden

The relative proportion of TB recurrence due to relapse and reinfection varies significantly between studies. Where the TB incidence is less than 20 per 100,000 population, the proportion of reinfection is low including: 16% in the Netherlands (163); 23% in Italy (181); 26% in Denmark (167); 4-31% in the USA(144, 160, 168, 173). Where TB incidences are between 20-50 cases per 100,000 population, the reinfection level was: 23% in Spain (182); 25% in Iran (183); 44% in Gran Canaria (164). Where TB incidences are higher than 50 cases per 100,000 population, the reinfection proportion was: 18% in Uganda (169); 31% in India (155); 31% Brazil (184), 33% in Malawi (166); 35-77% in South Africa (157, 158, 162, 165, 172); 53% in Taiwan (141). Several factors account for these differences including the level of TB incidence, which when higher, raises the likelihood of TB transmission and thus reinfection (141). Additionally, HIV is a known risk factor for reinfection (70, 144, 158, 159, 166, 185), thus population differences in HIV prevalence may affect reinfection rates (141). Finally, where the length of follow up or observation time is longer, the proportion of reinfection rises as relapse rates are higher closer to the end of treatment whereas the reinfection risk is constant over time (138).

At present, there is no published data detailing the relative contribution of relapse and exogenous reinfection to TB recurrence in the UK. This information is important to more precisely direct TB control and prevention efforts at a local level (169).

1.9.6 Risk factors for TB recurrence

The risk factors for recurrent TB are categorised into host and bacillary risk factors after non-MDR-TB treatment. The risk factors among individuals who completed an episode of MDR-TB treatment are also presented.

1.9.6.1 Socio-demographic

There is some but inconsistent evidence about an association between age and recurrent TB. Crofts et al found that children between 0-14 years of age were at lower risk of recurrence compared to 15-44 year-olds (145); Goswami et al demonstrated that patients aged 20-45 years of age were at greater risk than those under 20 years (186); Golub et al reported that those aged 40-49 years were at lower risk compared to those under 30 (187); Kim et al showed that patients aged 65 years or older were at greater risk than those under 45 years (188).

Male gender is a strong independent predictor of TB recurrence. Hung et al found that males were twice as likely to experience recurrence than females and Millett et al found that males experienced up to four times higher risk (189, 190). The former suggested the observed association between male gender and recurrence may be due to residual confounding of other risk factors more prevalent in men such as smoking (190).

Low socioeconomic status is reported as a significant risk factor for recurrence. In a nationwide study conducted in Taiwan, individuals earning less than New Taiwan (NT)$19200 (US$605) a month were found to be at elevated risk of recurrent TB compared to those earning NT$30,300 (US$954) or more a month (190). Other socio-economic determinants including unemployment (173, 191), homelessness (192), use of public transport (173) and inner city residence (147) were independently associated with recurrent TB.

Foreign country of birth and immigration are associated with TB recurrence across a range of studies (160, 189, 193). Migrants to Spain were at elevated risk compared to the native population (189). Among Mexican immigrants in the USA who experienced two consecutive episodes of TB within 12 years of immigration reinfection was a more common cause of recurrence than reactivation compared to native residents (160). Conversely, in a study conducted in California, individuals born in the USA were found to be at greater risk of recurrence than those elsewhere (193).

Particular ethnic groups are at elevated risk of recurrence. These included indigenous groups vs. non-indigenous in Taiwan (194); South Asians (Indians, Bangladeshis and

Pakistanis) in the UK compared to the white population (145), and non-Hispanic white ethnicity vs. non-white ethnicities in the USA (144, 195).

1.9.6.2 Co-morbidities

HIV type-1 infection is consistently associated with recurrence across high and low burden settings (145, 147, 162, 171, 173, 188, 193, 196-198). Several studies demonstrate that HIV infection is associated with recurrence due to reinfection but not relapse (70, 144, 158, 159, 166, 185) although not all studies could confirm this result (168, 169, 181).The degree of immunosuppression as measured by the peripheral CD4 lymphocyte count is inversely associated with recurrence risk. Golub et al found that a CD4 count of between 200-349 cells mm^{-3} measured during the initial TB diagnosis was associated with a lower risk of recurrence compared to patients with counts of less than 200 cells mm^{-3} (187). Similarly Pulido et al found a CD4 count of less than 100 cells mm^{-3} were at greater risk compared to those with higher counts (199). Two studies found no association between CD4 count and recurrence risk (158, 197).

The impact of antiretroviral therapy (ART) on TB recurrence was examined by Golub et al who showed a lower likelihood of recurrence (187). This was supported by a meta-analysis of 7 papers by Khan et al who showed patients not on ART were at greater risk of subsequent relapse (200). The relationship between duration of the initial TB treatment episode and the risk of recurrence in HIV positive patients was examined by in several cohort studies. Receiving more than 37 weeks of TB treatment was associated with a lower recurrence risk (196, 199) compared to shorter durations. Similarly, 12 months of TB treatment vs. 6 months was associated with lower rate of recurrence (1.9% vs. 9.0% p=<0.01) (201). These three studies predated the widespread use of ART. Likewise, Nahid et al showed the extension of the standard 6-month TB treatment course was associated with a reduced risk of recurrence in a cohort where 47% of patients had received no ART (170).

Diabetes mellitus is independently associated with TB recurrence (190, 191, 194). Only Lee et al. explored the degree to which blood sugar control (as measured through glycated haemoglobin) affected recurrence risk. They found that patients with no measurement of glycated haemoglobin were at higher risk of recurrence compared to those with at least one measurement and suggested better glycaemic control through better adherence to diabetes care and improved glucose monitoring may explain their findings (194).

Low peripheral blood haemoglobin levels were an independent predictor of TB recurrence in a study conducted in Uganda with patients with concentrations less than 12g/dl at greater risk compared to those with higher values (169).

Chronic lung disease (CLD) was independently associated with TB recurrence(144, 190). Hung et al found this risk was independent of smoking status (190) whereas Pettit et al found significant interaction between CLD and smoking (144).

Low Vitamin D levels were identified as a risk factor for recurrent TB in one study. Those patients with serum levels <75nmol/L were at greater risk than those with higher levels although no association was in patients with levels <50nmol/L when compared to higher values(202).

Several life-style factors are associated with recurrent TB. Patients who habitually smoke are at greater risk of TB recurrence (203). This was quantified in one study who demonstrated that those who smoke more than 20 cigarettes a day are more likely to experience TB recurrence than those smoking less than 20 a day (191). A combined history of alcohol/injecting drug/non-injecting drug use (188), alcoholism (204) and intravenous drug use (189) are independently associated with recurrent TB.

1.9.6.3 Clinical factors and extent of disease

Bodyweight measured at TB treatment initiation and failure to gain weight during TB treatment are reported to be associated with TB recurrence. One study found that patients of 50-69kg were at lower risk than those under 50kg (194), whilst another demonstrated that weight loss at the time of diagnosis of more than 10% (compared to less than 10%) was associated with recurrent TB (191). Furthermore, the failure to gain more than 5% of bodyweight within the first two months of TB treatment was independently associated in both studies (191, 205).

The presence of pre-treatment pulmonary cavitation as assessed by CXR is associated with TB recurrence (191, 206, 207). In two studies employing genotyping, relapse was described as the underlying mechanism in this association (158, 167). Residual cavitation at the end of therapy is also a risk factor for relapse (158). Post treatment scarring increased the risk of recurrence in one study when three or more predefined vertical zones on a CXR were involved compared to no zones (198).

Site of disease is an important determinant of TB recurrence risk. In one study, compared to patients with PTB, those with EPTB sited in lymph nodes, lung pleura and 2

more or more sites were at lower risk of recurrence (145). In those patients with PTB and concomitant EPTB, the risk of recurrence was elevated (146, 206).

1.9.6.4 Treatment supervision and adherence

Low adherence to the standard 6-month course of TB-treatment and irregular treatment increases the risk of TB recurrence (208). Anaam et al. showed an association between failure to take >80% of prescribed doses in the continuation phase of TB treatment (191) and the risk of recurrence. Thrice weekly dosing compared to daily dosing is also associated with an increased recurrence risk (206).The relationship between supervised (vs. self-administered) therapy and the risk of recurrence was assessed by two studies. El-sahly et al found supervised therapy was associated with a lower recurrence risk (173) whereas Kim et al. found no such association (188).

1.9.6.5 Bacillary risk factors

Infection with specific *Mtb* strains is associated with TB recurrence. A study conducted in Vietnam identified infection with the Beijing strain was associated with an increased risk (209). Similarly, Burman et al found that those infected with the Beijing strain were at greater risk of relapse in a study (195). This finding was limited to individuals of the Asian-Pacific Islander race who were resident in the USA or Canada. A study conducted in Malawi demonstrated increased relapse risk due to the East African Indian (EAI) / Central Asian strain (CAS) (159). Interestingly, they undertook a genome wide association analysis and found no genomic variants significantly associated with relapse.

Pre-treatment drug resistance appears to increase the likelihood of TB recurrence. Isoniazid mono-resistance was inconsistently associated with recurrence risk. Guerra-Assunção et al found an elevated risk of relapse (159) whereas Pascopella et al found a reduced risk of recurrence (193). The authors for the latter study suggested this finding may be due to (intended) intensified or extended TB treatment in patients with known mono-resistance (193). Pyrazinamide mono-resistance was also associated with TB recurrence (193).

Pre-treatment sputum smear-positive compared to smear-negative TB increases the likelihood of TB recurrence (188, 192, 193). Furthermore, a lack of culture conversion after two months of TB treatment is associated with recurrent TB (195). When assessed by meta-analysis, sputum AFB-smear positivity at 2-months of treatment had a sensitivity of 24% (95% confidence interval (CI) 12-42%) and specificity of 83% (95%CI 72-90%) to

predict recurrence. Similarly, 2-month culture positivity displayed a sensitivity of 40% (95%CI 25-56%) and specificity of 85% (95%CI 77-91%) (210).

1.9.6.6 Risk factors in multidrug- and extensively drug-resistant TB cohorts

Few studies examined risk factors for TB recurrence amongst patients with MDR-TB and extensively drug-resistant (XDR) TB in low-burden settings. Among successfully treated MDR-TB patients, pre-XDR and XDR resistance profiles (211), pre-treatment pulmonary cavitation as seen on CXR(211), diabetes mellitus (212) and previous TB treatment (213) were reported to be associated with the risk of TB recurrence.

1.9.6.7 Risk factors present in the UK population

At present, the understanding of risk factors for TB recurrence in the UK is solely derived from a study by Crofts et al (145). They found that HIV co-infection and South Asian ethnicity independently increased the risk of TB recurrence whilst individuals aged 0-14 years or over 65 years were at lower risk (145). This study was derived from notifications of TB cases to the UK enhanced TB surveillance system which at that time collected a limited number of data points (145). They were therefore unable to examine many relevant factors which may predispose to TB recurrence within the UK population, to control for confounding or to check the accuracy of notification data to minimize information bias. Therefore, the risk factors for TB recurrence remain poorly characterised in the UK. This information is needed to identify susceptible groups so enabling medical and public health inventions to be optimally focused (189, 214).

1.9.7 Sequelae of tuberculosis recurrence

Recurrent TB has significant patient and public health implications. On a patient level, individuals diagnosed with recurrent TB may have more extensive pulmonary disease (215) and develop chronic airflow limitation (216). Patients also experience poorer outcomes, for example recurrent TB is associated with lower cure rates (217), an increased likelihood of default (218) and higher mortality (219) than the treatment of new TB.

On a public health level, patients with recurrent TB experience higher levels of drug resistant disease (220). For example, in a study in New York 27% of patients with recurrent TB accrued resistance to additional drugs compared to their first episode of TB (196). This has financial implications for the healthcare system as the treatment of drug resistant TB is expensive compared to drug sensitive disease (221). Furthermore, recurrent TB contributes towards ongoing transmission of *Mtb* (222). For example, in a study

conducted in Cape Town, South Africa den Boon et al reported that previously treated patients made up 56% of smear positive TB cases (223). However, at present, the precise contribution which recurrent TB makes to overall TB transmission is unknown.

Presently, the public health and patient impacts of recurrent TB in the UK are unknown, information crucial to determining whether recurrent TB is a public health priority and should be included as part of a comprehensive TB control strategy.

1.9.8 Recurrence and TB control

One indicator of the effectiveness of a TB control program is the TB recurrence rate (145, 147, 171, 180). Increases above expected levels highlight the need to strengthen core elements of the TB control strategy, namely the effective detection and treatment of TB cases (138, 193). Active detection ensures that infectious cases are promptly identified and treated so minimising transmission (172, 224), although whether this is superior to passive case finding is uncertain (225). Furthermore, ensuring patients are effectively treated reduces recurrence due to relapse, so minimising transmission which consequently decreases recurrence due to reinfection (138).

Understanding the local epidemiology of TB recurrence is crucial to intervening effectively as relapse and reinfection are optimally tackled through specific interventions. In settings where reinfection is the predominant cause of recurrence, measures must be targeted to interrupt transmission (162). These may include infection control interventions in clinical and community settings, particularly necessary where nosocomial MDR and XDR-TB transmission is demonstrated (226). Other measures may include aggressive case detection (141) and in very high incidence settings community wide screening and TB preventative treatment for those without active disease (162). Where relapse is the predominant mechanism, specific measures include the extension of TB treatment in high risk groups (227) and targeted post treatment surveillance (189, 193, 214). Given that the relative proportion of relapse and reinfection vary between settings, the approach taken will depend upon the local epidemiology. Currently, this is poorly understood in the UK and should be studied to inform TB recurrence control interventions.

1.10 Background to the project

The incidence of TB in Leicester is consistently several-fold higher than the national average reflecting local factors including immigration from high incidence countries (228, 229). Routine epidemiological information for TB cases is collected in the East Midlands and programmatic performance analysed yearly (17). However, TB recurrence, a marker of the efficacy of TB control, has not been assessed in Leicester. Analysing the associated risk factors for TB recurrence, the local TB recurrence rate and the contribution of reinfection and relapse will inform local practice. Locally over 40% of TB cases are extrapulmonary however culture confirmation is only successful for half. Previous papers published from the University of Leicester demonstrate Rpf-containing CS can enhance bacillary recovery in clinical specimens (57, 230). This technique may improve TB culture sensitivity with benefits for patient care and TB control.

1.11 Thesis aims and objectives

The research work consisted of two studies examining the areas of TB recurrence and EPTB culture diagnostic performance.

The aims were to:

1. Study the epidemiology of recurrent TB in patients treated at the University Hospitals of Leicester (UHL).
2. Evaluate whether the supplementation of standard EPTB culture with Rpf-containing CS enhances bacillary recovery from samples and informs mycobacterial subpopulations in EPTB.

Chapter 2 contains the study of TB recurrence.

Specific objectives

- To define the risk factors for TB recurrence present during the prediagnostic and TB-treatment periods of patients treated for TB at UHL.
- To characterise the epidemiology of recurrent TB within Leicester.
- To estimate the proportion of TB recurrence due to relapse and reinfection within Leicester.

Chapter 3 describes the study of Rpf-containing CS supplementation of EPTB culture.

Specific objectives:

- To calculate the change in culture sensitivity and specificity with the supplementation of EPTB culture with Rpf-containing CS.
- To measure the change in TTP of Mycobacterium Growth Indicator Tube (MGIT) EPTB cultures with the addition of Rpf-containing CS.
- To quantify CS-dependent bacillary counts present in EPTB samples using MPN and CFU assays.
- To correlate host and bacillary factors with the CS-dependent bacillary counts.

Chapter 2: A study of tuberculosis recurrence in Leicester

2.1 Introduction

TB treatment is often complicated by recurrence (138), the presence of which indicates TB control and treatment activities require strengthening (193). This is best achieved by studying the local mechanisms of recurrence and identifying those groups at risk, so preventative interventions are effectively targeted (169).

Currently, TB recurrence in the UK is poorly understood, being principally derived from the analysis of surveillance data by Crofts et al (145). This study was limited by the narrow range of risk factors examined, the inability to control for confounding and potential inaccuracies in notification data leading to information bias.

In this chapter, I describe the conduct and results of a nested case control study examining TB recurrence in a cohort of TB patients treated at UHL. This study design is considered a robust method for examining uncommon outcomes such as TB recurrence (206). I used verifiable data from medical records and hospital databases collected during the course of routine clinical care. I examined a wide range of clinical and demographic factors encompassing many not present in TB notification data with the aim of controlling for unmeasured confounders and identifying hitherto unrecognised risk factors within our local population. Furthermore, I used DNA strain-typing data to estimate the proportion of recurrence due to relapse and reinfection. The information garnered from this study will aid the formulation and targeting of control interventions towards those populations deemed at risk of TB recurrence.

2.2 Methods

2.2.1 Study setting, design and data source

The University Hospitals of Leicester NHS Trust is a 1700 bedded tertiary referral centre which serves the metropolitan area of Leicester (population 334,000 (231); TB incidence 36.2 per 100,000 population per year (232)) and Leicestershire and Rutland (population, 705,000 (231); TB incidence 3.4 per 100,000 population per year (232)). All TB cases notified at UHL were prospectively recorded on a centralised database (TBIT) which is used by the Leicester TB service for the integrated surveillance and management of TB cases.

Cases of TB recurrence were identified from patients notified twice or more to the TBIT database, whereas controls were defined as any patient notified only once. The incipient episode and first recurrent episode were used in the study and subsequent episodes of recurrence were not included. Cases were matched to controls on the date of notification at a ratio of 1:2 using the IBM SPSS 22.0 statistics package (IBM, Redmond, USA) with cases and controls separated by a maximum of 8 weeks.

2.2.2 Study population and variables

All cases of TB seen at UHL and notified to TBIT between the 1st of January 1994 and the 14th of December 2014 were included in the study. Database entries were excluded where patients cultured NTM or were denotified due to the assignment of an alternative diagnosis other than TB.

The variables selected for analysis were significantly associated with TB recurrence in previous studies or considered biologically plausible. The data collected for variables such as alcohol or smoking status pertained to the prediagnostic and TB-treatment periods. Data were not available for the post-treatment period between the first and recurrent episode. Study data were extracted from the TBIT database, pseudonymized and recorded on an Access 2010 database (Microsoft, Redmond, Washington, USA) situated on the UHL IT system. Reviews of paper and online records were performed to corroborate, supplement and fill in missing data from the TBIT database.

2.2.3 Inclusion and exclusion criteria

Patients were suitable for inclusion in the study if they were a confirmed case of TB at the first episode, completed their allocated treatment course and were declared cured or treatment complete by their treating physician. TB nurse review confirmed completion of the allocated treatment course. Patients were excluded if during the first episode of TB they failed to complete their prescribed treatment course, transferred out of the area before treatment completion, were lost to follow up, died or were classified as a treatment failure. Cases and matching controls with unavailable records were also excluded.

2.2.4 Definitions

Tuberculosis definitions:

- *A case of TB*: Where a biological specimen is positive for *Mtb* by smear microscopy, culture or an approved rapid diagnostic test or a case which has been

diagnosed as active TB by a clinician and a full course of treatment is planned to be administered (88).

- *Pulmonary tuberculosis (PTB):* Disease involving the lung parenchyma or tracheobronchial tree (88).
- *Extrapulmonary tuberculosis (EPTB):* Any case not involving the lungs such as the brain, intrathoracic lymph nodes or pleura (88).
- *Recurrence:* a patient who had been previously treated for TB, declared cured or treatment completed at the end of their most recent course of treatment and was diagnosed with a recurrent episode of TB (either a relapse or a new episode of TB caused by reinfection) (88).
- *Reinfection:* A second episode of TB resulting from exogenous infection with an *Mtb* strain differing from that responsible for the first episode by ≥ 2 MIRU-VNTR loci (70, 138).
- *Relapse:* A second episode of TB resulting from endogenous reactivation of the *Mtb* strain responsible for the first episode as shown by ≤ 1 MIRU-VNTR locus difference (70, 138).
- *Standard treatment regimen:* the use of six months of isoniazid and rifampicin with 8 weeks of pyrazinamide (with or without other drugs) (206).
- *Phenotypic susceptibility:* the determination of the susceptibility of an organism to a drug by the observation of growth or metabolic inhibition in a medium containing the anti-tuberculous drug (129).

Demographics:

- *Ethnicity*: classified by the Office for National Statistics classification (White; Indian Subcontinent; Afro-Caribbean; Oriental / Other Asia; Other). (233). As numbers for ‘Oriental’ / ‘Other Asia’ were small, this was subsumed into ‘Other’.
- *Housed:* Resident at a fixed address.
- *Smoker:* the regular smoking of tobacco at the time of diagnosis (203).
- *Alcohol user:* habitual alcohol consumption at the time of diagnosis (203).

Co-morbidities:

- *Diabetes mellitus:* the documented use of diabetic medication (194), a fasting blood sugar ≥7.0mmol/L, a 2 hour blood glucose of ≥11.1mmol/L after an oral glucose

tolerance test, an HB_{A1C} of ≥6.5% or symptoms of hyperglycaemia with a random blood sugar ≥11.1mmol/L (234) .

- *End stage renal failure:* an estimated glomerular filtration rate (eGFR) of <15ml/min/1.73m^2 or receiving haemodialysis (235).
- *25-OH vitamin D_3 deficiency:* as a number of methods (mass spectroscopy and immunoassay) were used over the twenty-year period covered by the study to determine 25-OH vitamin D3 deficiency; deficiency was defined by the assay used for each individual patient. The serum sample had been drawn within 1 month of treatment initiation.
- *HIV positive:* a positive result from an enzyme linked immunosorbent assay (ELISA) or NAAT for HIV type 1 infection (no cases of HIV type 2 infection were present in the final study cohort).
- *HIV negative:* either a negative ELISA / NAAT or an unknown HIV status.
- *Pulmonary cavitation:* visible on available imaging or if unavailable, recorded in the medical notes.
- *Immunosuppressant use:* receiving immunosuppressive medication (glucocorticoids such as prednisolone, oral immunosuppressants such as ciclosporin or immunosuppressive monoclonal antibody therapy such as infliximab) any time after symptom onset up until the completion of anti TB therapy.

Treatment:

- *Adverse drug reaction:* any unintended adverse response to a drug occurring at a therapeutic dose and resulting in either death, drug withdrawal, change in the administration of the frequency or dose of the drug, or, that no action is required (236). These were graded by the Common Terminology Criteria for Adverse Events v4.0 (237)
- *Treatment interruption:* any period in which anti-TB therapy was discontinued temporarily at the physician's direction or due to patient action.

2.2.5 Molecular strain typing

The identification and typing of isolates was performed by the Health Protection Agency (now PHE) Midlands Regional Centre for Mycobacteriology. Restriction fragment length polymorphism (RFLP) typing of *Mtb* strains was performed upon request until 2004,

whereafter all isolates were analysed by MIRU-VNTR typing. Where available, MIRU-VNTR strain typing data were used to classify cases of recurrence (238). If isolates from both episodes differed by two or more MIRU-VNTR loci, the case of recurrence was ascribed to reinfection and by one or less locus difference, relapse (70)

2.2.6 Epidemiological analysis

The time at risk of TB recurrence was calculated for all cases from the time after completion of TB treatment until renotification or cases were censored at the end of the study (14th of December 2014). Patients were assumed to have completed treatment 24 weeks after the notification date unless otherwise recorded. The recurrence incidence rate was calculated for all patients with recurrence as the numerator and person years of follow up as the denominator (165). The incidence of recurrence with differing periods of follow up after the first disease episode were calculated. The estimated reinfection rate was calculated assuming those patients without typing data had recurrent disease due to reinfection in the same proportions as those for whom typing data was available. The same approach was taken for the estimated relapse rate.

2.2.7 Statistical analysis

Statistical analyses were performed using SPSS version 22 (IBM, Redmond, USA) and Stata 13.1 statistics package (Statacorp, Texas, USA). Graphs were produced using Stata 13.1. Continuous and categorical variables were examined with the Wilcoxon rank-sum and Fisher's exact test respectively

The dataset was first assessed for incompleteness. The percentage of missing data was calculated for variables being considered as predictors of TB recurrence. For those variables missing data, the level varied between 1.6-54.5% (see Appendix section 1.1.1). Complete case analysis may introduce biased parameter estimates (239), thus multiple imputation to account for missing data was performed for the following variables:

- Demographic: weight, habitual alcohol use, habitual tobacco smoking, UK-birth status.
- Laboratory: blood haemoglobin concentration, peripheral white cell counts (WCC) and serum creatinine levels.
- TB specific: AFB smear status, TB culture status and phenotypic antimicrobial susceptibility (sensitive / resistant).

The variables Vitamin D deficiency and BCG vaccination status were missing 55% and 48% of values which is at the upper bound of 50% of missing data recommended when using multiple imputation so these were not included in the model (240). Multiple imputation was performed using Stata 13.1.

Multiple imputation was performed for missing values under the assumption that data was missing at random. The missing data pattern was arbitrary not monotone (239) (see Appendix 1.1.2). All variables used in the analysis model including the dependent variable were included in the imputation model (241). The imputation model contained both continuous and categorical variables therefore multiple imputation by chained equations was chosen (239). Predictive mean matching (PMM) was used for non-normally distributed continuous variables (creatinine, weight and WCC), regression for normally distributed continuous variables (haemoglobin), logistic regression for binary variables (AFB smear status) and augmented logistic regression for binary variables exhibiting perfect prediction (TB culture status, phenotypic antimicrobial susceptibility, habitual tobacco smoking, habitual alcohol use and UK-birth status) (241). A burn in period of 100 was used between imputations (239). As the fraction of missing information (FMI) was 0.539, 60 imputation sets were created for conditional logistic regression analysis (241). No interactions were specified in the imputation model. The median percentage of missing data for imputed variables was 9·6% (interquartile range (IQR) 4·9-12·2%; range 1·6-39·4%).

Post imputation analysis was performed. For categorical variables, the mean and standard deviation (SD) of original and imputed datasets were assessed and frequency charts were compared (see Appendix 1.4.1). For continuous variables, the mean and standard deviations of original and imputed datasets were compared and trace plots and kernel density plots were analysed (see Appendix 1.4.2-1.4.3). Good evidence of convergence was achieved.

Two multivariate conditional logistic regression models were developed, both fitted with variables as potential predictors with recurrence of TB as the main outcome variable. One included all clinically relevant variables irrespective of p-values upon univariate analysis to control for confounding as far as possible within the dataset (240). The second model contained variables identified upon univariate conditional logistic regression with a p-value <0.2 (240) to assess for overfitting in the first model (240).

Before undertaking conditional logistic regression analysis, the presence of multiple collinearity was considered using several approaches. Variance inflation factors (VIF)

were used to detect collinearity (242, 243) (see Appendix 1.5.1). VIFs less than 10 were considered non-significant (244). No variables demonstrated VIFs ≥4. Correlation amongst the independent variables was tested to assess for multiple collinearity (243). The decision on which predictor variable(s) to retain from a group of suspected collinear variables was based upon significance in univariate analysis or degree of suspected collinearity. In two-way contingency table analysis, the variables 'standard treatment' and 'treatment interruption' both exhibited collinearity with 'adverse drug reaction (ADR)' (φ=0.55 & -0.38 respectively). The variable 'other comorbidities' also exhibited collinearity with 'creatinine' (point biserial=0.49). The direction of association for variables 'treatment interruption', 'standard regimen' and 'other comorbidities' varied between univariate and multivariate analysis suggesting the presence of multicollinearity with one or more variables (242, 243) (see Appendix 1.5.2). Thus, these three variables were dropped from multivariate analyses without substantially affecting the overall models. Habitual alcohol consumption correlated with habitual tobacco smoking (φ=0.53) but was retained due to clinical significance. Both models were checked with the Stata 13.1 function 'linktest' and no specification error was detected. Results from the conditional logistic regression analysis were presented as odds ratios (ORs) and 95% confidence intervals with a p-value of <0.05 considered significant. The Strengthening the Reporting of Observational Studies in Epidemiology guidelines were used to guide the reporting of this retrospective observational study (245).

2.2.8 Ethical approval

The study was confirmed by the National Research Ethics Service Committee East Midlands –Leicester as not requiring formal ethical review as the study involved the use of a pre-existing database (see Appendix 12). No patient-specific data or personal identifiers were used in the preparation of this report which was an analysis of routine data collected as part of service evaluation and therefore ethical approval was not required.

2.3 Results

2.3.1 Description of study cohort.

A total of 5122 notifications for *Mtb* disease were identified by a retrospective review of the TBIT database covering the period from 1st of January 1994 to the 14th of December 2014. After the removal of 358 patients, who were denotified or diagnosed with NTM disease, 4764 notifications remained. 120 patients were notified twice and 8 thrice leaving 4628 patients in the study cohort. Following a detailed review of each identified potential recurrence case, 43 were excluded leaving 85 cases of recurrence. In 3 cases, the records of matched control subjects were missing leaving 82 matched case-control units for analysis (see figure 3.1).

Of the study cohort, 51.6% (95%CI 50.1-53.0%) were male and the mean patient age was 40.9 years (standard deviation (SD) 18.8 years; range 0.1-98 years). 69.0% (95%CI 59.5-62.3%) of patients were of Indian subcontinent ethnicity, 11.3% (95%CI 10.4-12.2%) Afro-Caribbean ethnicity, 11.4% (95%CI 10.5-12.4%) White ethnicity and 8.3% (95%CI 7.5-9.1) of any other background. Study cohort HIV prevalence was 7.5% (95%CI 6.8-8.3%).

The case and control group baseline characteristics are presented in Table 2.1. Habitual alcohol and tobacco misuse, AFB smear positivity and the use of immunosuppressing drugs were higher amongst cases of TB recurrence compared to controls. Of note, 64.8% of cases and 69.3% of controls were Vitamin D deficient indicating a high level of vitamin D deficiency amongst the entire cohort. 79.5% of cases and 81.1% of controls were vaccinated with BCG. Using a cut off of 13.0g/dl for men and 11.5g/dl for women (UHL pathology laboratory lower limits of normal), 63.5% of cases and 51.0% of controls were designated anaemic (p=0.087). Immunosuppressive treatment taken included glucocorticoids 26 (79%) patients, glucocorticoids with disease modifying anti-rheumatic drugs (DMARDs) 4 (12%) & DMARDs alone 3 (9%) (see Table A3.1). In 12(36%) of cases, immunosuppressing drugs were given for ongoing management of chronic conditions, 5(15%) for complications of TB disease (e.g. paradoxical reaction), 5 (15%) where evidence suggests a benefit (e.g. tuberculous meningitis), 9 (27%) where the wrong diagnosis was initially suspected (e.g. asthma) and 2 (6%) for unknown indications. 4 (5%) of recurrence cases died of TB during their 2nd disease episode.

Figure 2.1 Flow diagram of study cases and controls included in the data analysis

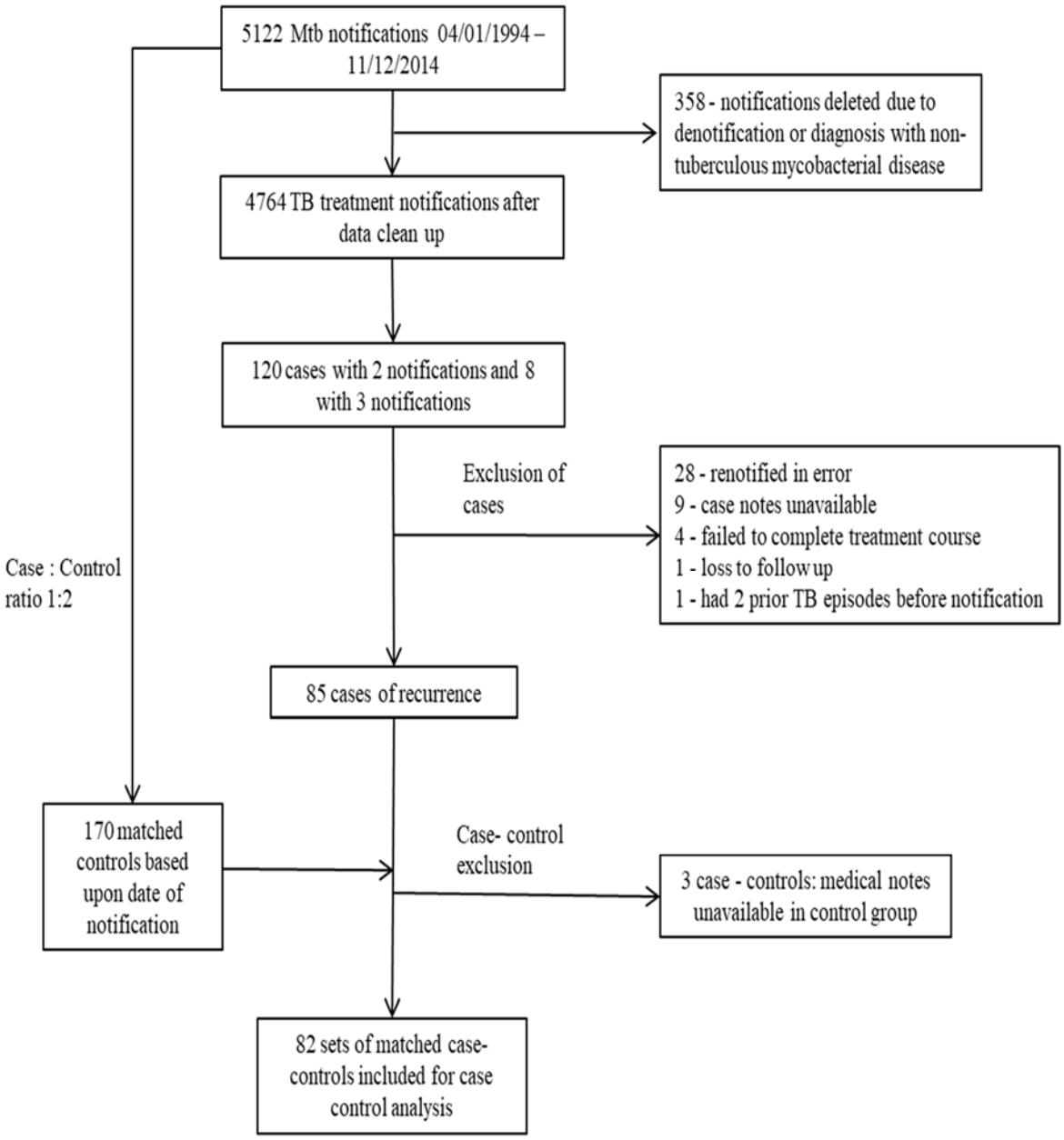


Table 2.1 Cohort demographics

	Case (n=82) N (%)	Control (n=164) N (%)	P value
Age (year, +/-SD)	40.2 +/-17.6	37.4 +/-17.6	0.23
Weight (kg)[a]	56 (45.5-68)**	59 (48-68)**	0.26
Gender			
Male	42 (51.2)	84 (51.2)	-
Female	40 (48.8)	80 (48.8)	1.00
Ethnicity			
White	10 (12.2)	20 (12.2)	-
Afro-Caribbean	7 (8.5)	17 (10.4)	0.78
Indian subcontinent	61 (74.4)	125 (76.2)	1.0
Other	4 (4.9)	2 (1.2)	0.18
Permanent residence	81 (98.8)	163 (99.4)	1.00
UK Born[b]	25 (30.5)	37 (22.5)	0.16
Habitual alcohol consumption[c]	17 (23.6)	20 (12.4)	0.03
Habitual tobacco smoking[d]	20 (26.3)	19 (11.7)	0.01
BCG vaccinated[e]	31 (79.5)	73 (81.1)	0.81
Comorbidities			
Diabetes mellitus	9 (11.0)	15 (9.2)	0.65
Solid organ transplant±	0	1 (0.6)	1.00
Cancer	0	3 (1.8)	0.55
End stage renal failure	1 (1.2)	3 (1.8)	1.00
Rheumatological conditions¶	3 (3.7)	1 (0.6)	0.11
HIV-1 seropositivity	5 (6.1)	6 (3.7)	0.51
Vitamin D deficiency[f]	24 (64.8)	52 (69.3)	0.67
Laboratory			
Haemoglobin[g] (g/dl +/-SD)	11.7+/-1.8	12.2+/-1.9	0.11
White cell count[h] (x10^9 cells/L)	7.5 (6.0-9.6)**	7.7 (5.9-9.5)**	0.91
Creatinine[i] (µmol/l)	70.0 (61.5-79.0)**	71.0 (61.0-83.0)**	0.46
Primary disease site			
PTB	45 (54.8)	68 (41.4)	-
PTB + EPTB	10 (12.2)	31 (19.0)	0.09
EPTB	27 (33.0)	65 (39.6)	0.14
Pulmonary cavitation	19 (23.2)	23(14.0)	0.11
Microbiology			
AFB smear positive[j]	30 (41.7)	42 (27.7)	0.01
***Mtb* culture positive[k]**	56 (76.7)	93 (66.9)	0.16
Drug resistance[l]	7 (12.5)	6 (6.5)	0.24
Treatment			
Standard regimen	68 (82.9)	145 (88.4)	0.24
ADR			
No ADR	64 (78.0)	143 (87.2)	-
Grade 1 - 2	4 (4.9)	7 (4.3)	0.74
Grade 3 - 4	14 (17.1)	14 (8.5)	0.06
Treatment interruption (days)	0 (0-0)**	0 (0-0)**	0.96
Immunosuppressing drugs	17 (20.7)	16 (9.8)	0.03
Duration (days)	168 (168-273)**	168 (168-274)**	0.50

The missing data for case and controls is a: 14&16; b:2&2; c:10&2; d:6&2; e:4374; f:45&89; g: 8&15; h:8&16; i:6&17; j: 10&19; k:9&25; l: 26&71. ±Renal transplant; ¶Psoriasis and rheumatoid arthritis; **median value and interquartile range. Abbreviations: SD - standard deviation; HIV – Human Immunodeficiency Virus; PTB - pulmonary tuberculosis; EPTB- extrapulmonary tuberculosis; ADR – adverse drug reaction; AFB – acid fast bacillus; *Mtb – Mycobacterium tuberculosis;* BCG - Bacillus Calmette–Guérin.

2.3.2 Site of Tuberculosis disease

The pulmonary tract was the major site of disease for those with (67%) and without (60%) TB recurrence (see table 2.2). Lymph node disease was the predominant EPTB form in both cases and controls with and without concomitant pulmonary disease. Spinal disease was more common in controls with EPTB (23%) than cases (4%) (p=0.18).

Table 2.2 Site of disease in the primary episode in patients with and without recurrent disease

Site of disease	Cases (n=82) N(%)	Controls (n=164) N(%)	P†
Pulmonary	45 (55)	68 (41)	-
Pulmonary with concomitant extrapulmonary	10 (12)	31 (19)	0.09
Cutaneous	0	1	
Disseminated	2	9	
Eye	0	1	
Gastrointestinal	1	1	
Lymph node	4	11	
Oropharyngeal	1	0	
Pleural	2	6	
Spinal	0	2	
Extrapulmonary	27 (33)	65 (40)	0.14
Bone & joint	2	2	
Cutaneous	0	2	
CNS	1	0	
Eye	1	2	
Gastrointestinal	0	3	
Lymph node	17	37	
Peritoneal	2	3	
Pleural	2	6	
Psoas	0	1	
Renal	1	0	
Spinal	1	9	

† - P value for the comparison of site of disease using PTB as the reference point.

I compared the site of disease in the recurrence episode with that of the primary episode of TB. Of those with PTB, the majority (64%) recurred as PTB (see table 2.3). Of those with PTB & EPTB, 40% recurred as PTB, 10% as PTB +EPTB (site of EPTB disease changed) and 50% as EPTB (4 cases site of EPTB disease changed, 1 unchanged). Of the EPTB cases, 11% recurred as PTB, 15% as PTB + EPTB (3 site of EPTB disease changed, 1 unchanged) and 74% as EPTB (3 site of EPTB disease changed, 17 unchanged).

Table 2.3 Change in site of disease between primary and recurrent TB episodes

Primary site of disease	Recurrence Site of disease		
	PTB (%)	PTB+EPTB (%)	EPTB (%)
PTB	29 (64)	5 (11)	11 (25)
PTB + EPTB	4 (40)	1 (10)	5 (50)
EPTB	3 (11)	4 (15)	20 (74)

Abbreviations: PTB - pulmonary tuberculosis; EPTB - extrapulmonary tuberculosis

2.3.3 Incidence and duration of recurrence

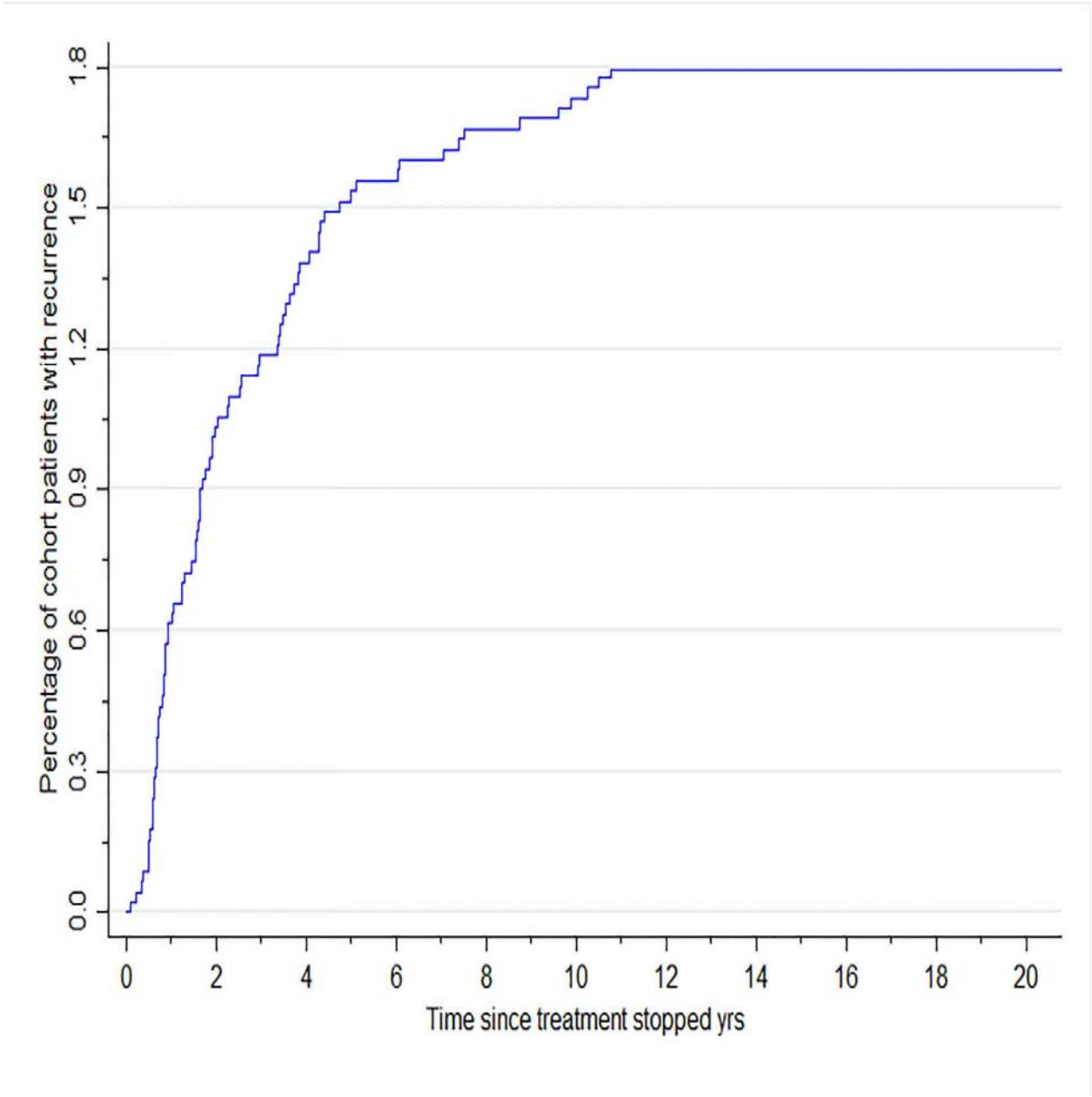
85 patients out of a total study cohort of 4628 (1.8%) experienced recurrence (see figure 2.2). Patients were observed for a median period of 7.8 years (IQR 3.6-11.6 years; range 0.7 -20.8 years). After patients completed treatment, the median time to recurrence was 1.7 years (IQR 0.8 – 3.8 years; range: 0.1 -10.8 years). The majority (57.3%; (95%CI 46.5-67.5%)) of recurrence cases were diagnosed within two years and 76.8% (95%CI 66.6-84.6%) within four years after treatment completion.

The overall recurrence incidence rate was 1.9 (95%CI 1.5-2.3) per 1000 patient years of follow up (PYs) with the incidence highest within 6-12 months post TB treatment (9.9 (95%CI 6.4-14.8) per 1000PYs) and subsequently decreasing during the 1-2 years (4.5 (95%CI 2.8-6.9) per 1000PYs) and 2-4-year periods (2.1 (95%CI 1.2-3.3) per 1000PYs) post TB treatment and eventually plateauing by the 4-8-year period (0.8 (95% CI 0.4-1.5) per 1000PYs) post TB treatment. No recurrence cases occurred more than 12 years after TB treatment completion (see table 2.4)

Table 2.4 Incidence rate of TB recurrence by period of follow up after treatment completion

Period of follow up after treatment completion	Patient years (PY) of follow up	1000PYs	Number of events	Total recurrence (%)	Incidence rate per 1000PYs
0-6 months	2269	2.269	6	7.32	2.64
6-12 months	2221	2.221	22	26.83	9.91
1-2 years	4192	4.192	19	23.17	4.53
2-4 years	7715	7.715	16	19.51	2.07
4-8 years	12645	12.645	10	12.20	0.79
8-12 years	8542	8.542	9	10.98	1.05
>12 years	6259	6.259	0	0.00	0.00

Figure 2.2 Kaplan-Meier curve showing cumulative percentage recurrence in 4628 notified TB patients



A sensitivity analysis was performed to assess the effect of moving the cut off for differentiating TB recurrence from treatment failure on the proportion of total recurrences, relapses and reinfections (see table 2.5). Changing the definition from the designated cut off of 0 months post treatment completion to 3 months reduced the number of recurrences to 80 and to 6 months to 76, a 7% reduction.

Table 2.5 Sensitivity analysis of the recurrence definition cut off

Cut off (months)	Total recurrences	Relapses	Reinfections	'Treatment failures'
0	82	16	3	0
3	80	15	2	2
6	76	14	2	6
12	54	9	1	28

2.3.4 Reinfection versus relapse

Of the 82 TB recurrence cases in the final case control study, paired MIRU-VNTR DNA strain-type profiles were available for 19 isolates. Twelve isolates were typed using 15 locus MIRU-VNTR, 3 with 24 locus MIRU-VNTR and in four cases, the initial isolate was typed with 15 locus MIRU-VNTR and the recurrence isolate with 24 locus MIRU-VNTR (see Appendix 2 for further information). No RFLP data were available from PHE Birmingham. There were three (16%; 95%CI 3.4-39.6%) cases of reinfection and 16 (84%; 95%CI 60.4-96.6%) of relapse (see Appendix 2 for MIRU-VNTR DNA strain typing data).

The clinical and demographic characteristics of relapse and reinfection cases were compared to identify predictors specific to each mechanism of TB recurrence although no significant differences were found (see table 2.6).

The median time to recurrence was examined for relapse and reinfection cases. This calculated at 0.8 years (IQR 0.5-0.9; range 0.2-1.1 years) for confirmed reinfection cases and 1.4 years (IQR 0.7-2.4; range 0.1-5.1 years) for confirmed relapses (p=0.27). The estimated reinfection incidence rate was 0·3 (95% CI 0.2-0.5) per 1000PYs and the estimated relapse rate was 1·6 (95% CI 1.2-2.0) per 1000PYs.

Table 2.6 Demographics of cases of relapse and reinfection

	Relapse (n=16) N (%)	Reinfection (n=3) N(%)	P value
Age (year, +/-SD)	39.8 (+/-18.4)	31.3 (+/-3.2)	0.58
Weight (kg)	48.0 (40.5-67.5)**	50.0 (44.0-57.0)**	0.91
Gender			
Male	8 (50.0)	1 (33.3)	-
Female	8 (50.0)	2 (66.6)	1.00
Ethnicity			
White	2 (12.5)	0	-
Afro-Caribbean	1 (6.3)	0	-
Indian subcontinent	11 (68.7)	3 (100)	1.00
Other	2 (12.5)	0	-
Housed	16 (100)	3 (100)	1.00
UK Born[a]	7 (43.8)	0	0.50
Habitual alcohol consumption[b]	3 (20)	0	1.00
Habitual tobacco smoking[c]	5 (33.0)	0	0.52
BCG vaccinated[d]	6 (85.7)	1 (100)	1.00
Comorbidities			
Diabetes mellitus	3 (1.8)	0	1.00
Rheumatological conditions	1 (6.3)	0	-
Vitamin D deficiency[e]	4 (30.1)	0	1.00
Laboratory			
Haemoglobin (g/dl +/-SD)	11.2 (+/-1.8)	11.16 (+/-3.3)	0.96
White cell count (x10^9 cells/L)	9.2 (6.6-10.4)**	7.8 (7.2-12.)**	0.74
Creatinine (µmol/l)	71.0 (62.0-81.0) **	61.0 (54.0-67.0)**	0.16
Primary disease site			
PTB	12 (75.0)	1 (33.3)	
PTB + EPTB	1 (6.3)	0	1.00
EPTB	3 (18.7)	2 (66.6)	0.17
Pulmonary cavitation	8 (50)	0	0.23
Microbiology			
AFB Smear positive	9 (56.3)	0	0.21
Drug resistance	4 (25.0)	1 (33.3)	1.00
Treatment			
Standard regimen	9 (56.3)	2 (66.6)	1.00
Adverse drug reaction	6 (37.5)	0	0.52
No reaction	10(62.5)	3(100)	
Grade 1 - 2	1(6.3)	0	0.79
Grade 3 - 4	5	0	0.52
Treatment interruption (days)	5.79(+/-15.8)	0	0.43
Immunosuppressing drugs	3 (18.7)	0	1.00
Duration (days)	259 (168-323)**	168 (168-366)**	0.56

Missing data relapse and reinfection: a: 0&1; b:1&0; c: 1&0; d:9&3; e:3&2. **median and IQR.
Abbreviations: AFB - acid fast bacillus; BCG - Bacillus Calmette-Guérin; CXR - chest x-ray; SD - standard deviation.

2.3.5 Drug resistance

I analysed the change in sensitivity profiles for 29 cases where paired *Mtb* cultures were available with associated MIRU-VNTR DNA strain type data and phenotypic drug susceptibility was determined (see Table 2.7). Cases of reinfection demonstrated no change in sensitivity profile between the primary and recurrence episode. Of the 16 cases of

relapse, 13 (81%) displayed no change in sensitivity profile and 3 (19%) decreased susceptibility to one additional drug with one isolate becoming a multidrug resistant strain.

Table 2.7 The change in sensitivity profile in recurrence patients with paired isolates

Recurrence classification	Number of cases (29)	Primary episode sensitivity profile	Recurrence episode sensitivity profile
Recurrence	10	Sensitive	Sensitive
Reinfection	2	Sensitive	Sensitive
	1	H resistant	H resistant
Relapse	11	Sensitive	Sensitive
	1	Sensitive	R resistant
	1	Q & H resistant	H resistant (Q not tested)
	1	H resistant	RH resistant
	1	RHZQ resistant	RHZEQ resistant
	1	RHZE resistant	RHZE resistant

R- Rifampicin; H - Isoniazid; Z - Pyrazinamide; E - Ethambutol; Q - Quinolone
Recurrence: two episodes of tuberculosis characterized by paired cultures positive for *Mtb* without MIRU-VNTR strain typing performed; Relapse: ≤ 1 MIRU-VNTR locus difference between isolates; Reinfection: ≥2 MIRU-VNTR loci differences.

2.3.6 Factors associated with TB recurrence on conditional logistic regression analysis

Univariate conditional logistic regression analysis showed that habitual alcohol consumption, habitual tobacco smoking, AFB smear positivity, receiving immunosuppressants and suffering a grade 3 / 4 ADR significantly increased the risk of TB recurrence.

In the multivariate analysis including all clinically significant variables (see Table 2.8), ethnicity 'Indian subcontinent' (OR 8·5, 95% CI 1·8-39.3, p=<0·01), ethnicity 'Other' (OR 31.2, 95% CI 2.1-471 p=0·01), habitual tobacco smoking (OR 3.8, 95%CI 1·1-13.1, p=0·04), a grade 3 / 4 ADR (OR 5.6, 95%CI 1.3-24.8, p=0·02) and receiving immunosuppressants (OR 6.8, 95% CI 1.9-24.4; p=<0·01) were found to significantly increase the risk of TB recurrence after controlling for confounding.

The second multivariate analysis included variables with p≤0.2 on univariate analysis (see table 2.9). The odd ratios (OR) for the second model were smaller than the first model, particularly for Indian subcontinent (4.3 (95% CI1.3-14.5) vs. 8.5(95%CI 1.8-39.3) and 'Other' ethnicity 31.2(95%CI 2.1-471.0) vs 10.0 (95%CI 1.1-88.3) indicating overfitting was present in the first model (240). Subgroup analysis by recurrence mechanism or by disease site was not possible due to small samples numbers which resulted in model instability.

Table 2.8 Factors associated with tuberculosis recurrence on conditional logistic regression

	Unadjusted Odds ratio (95% CI)	P value	Adjusted odds ratio (95% CI)	P value
Age	1.0 (1.0-1.0)	0.26	1.0(1.0-1.1)	0.09
Weight	1.0 (1.0-1.0)	0.29	1.0 (1.0-1.0)	0.46
Gender				
Male	Reference		Reference	
Female	1.0 (0.6-1.7)	1.00	1.1(0.5-2.7)	0.85
Ethnicity				
White	Reference		Reference	
Afro-Caribbean	0.8 (0.3-2.7)	0.75	5.4 (0.7-43.4)	0.13
Indian subcontinent	1.0 (0.4-2.2)	0.96	8.5(1.8-39.3)	<0.01
Other	3.9 (0.6-24.8)	0.15	31.2(2.1-471.0)	0.01
UK Born	1.5 (0.8-2.8)	0.17	2.8(0.9-8.8)	0.07
Permanent residence	0.5 (0.3-8.0)	0.62	0.2(0.0-4.0)	0.26
Habitual alcohol consumption	2.2 (1.1-4.8)	0.03	1.8(0.6-5.7)	0.30
Habitual tobacco smoking	2.8 (1.4-5.6)	<0.01	3.8(1.1-13.1)	0.04
Co-morbidities				
Diabetes mellitus	1.2 (0.5-2.7)	0.67	0.9 (0.2-2.8)	0.86
HIV-1 seropositivity	1.7 (0.5-5.5)	0.40	4.1(0.7-23.1)	0.12
Other comorbidities±	1.0 (0.3-3.5)	1.00	¶	
Laboratory				
Haemoglobin (g/dl)	0.9 (0.8-1.0)	0.09	0.9 (0.7-1.2)	0.54
White cell count (x10^9 cell/l)	1.0(1.0-1.1)	0.44	1.0 (0.8-1.1)	0.36
Creatinine (µmol / l)	1.0 (1.0-1.0)	0.86	1.0(1.0-1.0)	0.41
Primary disease site				
PTB	Reference		Reference	
PTB + EPTB	0.5 (0.2-1.1)	0.09	0.7 (0.2-2.1)	0.49
EPTB	0.6 (0.4-1.1)	0.12	1.4 (0.6-3.7)	0.49
Pulmonary cavitation	1.8 (0.9-3.6)	0.08	1.3 (0.5-3.9)	0.61
Microbiology				
AFB positive	2.3 (1.2-4.1)	<0.01	1.7 (0.6-4.3)	0.30
Culture positive	1.6 (0.8-2.9)	0.16	1.3 (0.5-3.2)	0.54
Drug resistant	1.5 (0.5-4.1)	0.49	2.6 (0.5-13.8)	0.28
Treatment				
Standard regimen	0.7(0.3-1.4)	0.26	¶	
ADR				
No ADR	Reference		Reference	
Grade 1 - 2	1.2 (0.4-4.1)	0.78	1.3 (0.3-6.1)	0.78
Grade 3 - 4	2.3 (1.0-5.3)	<0.05	5.6 (1.3-24.8)	0.02
Treatment interruption	1.1 (0.4-2.6)	0.88	¶	
Immunosuppressing drugs	3.0 (1.2-7.1)	0.01	6.8 (1.9-24.4)	<0.01

±±Active cancer, end stage renal failure, solid organ transplant & rheumatology conditions. ¶ not included in the analysis due to multiple collinearity

Abbreviations: AFB – acid fast bacillus; ADR – adverse drug reaction; EPTB- extrapulmonary tuberculosis; HIV – Human Immunodeficiency Virus; PTB - pulmonary tuberculosis;

Table 2.9 Factors associated with tuberculosis recurrence on conditional logistic regression (p<0.2 on univariate analysis)

	Unadjusted Odds ratio (95% CI)	P value	Adjusted odds ratio (95% CI)	P value
Ethnicity				
White	Reference		Reference	
Afro-Caribbean	0.8 (0.3-2.7)	0.75	4.3 (0.8-22.7)	0.08
Indian subcontinent	1.0 (0.4-2.2)	0.96	4.3 (1.3-14.5)	0.02
Other	3.9 (0.6-24.8)	0.15	10.0 (1.1-88.3)	0.04
UK Born	1.5 (0.8-2.8)	0.17	1.6 (0.7-3.8)	0.32
Habitual alcohol consumption	2.2 (1.1-4.8)	0.03	1.5 (0.5-4.2)	0.44
Habitual tobacco smoking	2.8 (1.4-5.6)	<0.01	3.0 (1.0-8.5)	0.04
Laboratory				
Haemoglobin (g/dl)	0.9 (0.8-1.0)	0.09	0.9 (0.7-1.1)	0.30
Primary disease site				
PTB	Reference		Reference	
PTB + EPTB	0.5 (0.2-1.1)	0.09	0.6 (0.2-1.6)	0.28
EPTB	0.6 (0.4-1.1)	0.12	1.1 (0.5-2.5)	0.82
Pulmonary cavitation	1.8 (0.9-3.6)	0.08	1.1 (0.4-2.8)	0.86
Microbiology				
AFB positive	2.3 (1.2-4.1)	<0.01	1.5 (0.6-3.6)	0.34
Culture positive	1.6 (0.8-2.9)	0.16	1.1 (0.5-2.3)	0.89
Treatment				
ADR				
No ADR	Reference		Reference	
Grade 1 - 2	1.2 (0.4-4.1)	0.78	1.4 (0.3-5.9)	0.65
Grade 3 - 4	2.3 (1.0-5.3)	<0.05	4.3 (1.4-12.7)	0.01
Immunosuppressing drugs	3.0 (1.2-7.1)	0.01	4.4 (1.6-12.1)	0.01

Abbreviations: AFB – acid fast bacillus; ADR – adverse drug reaction; EPTB- extrapulmonary tuberculosis; PTB - pulmonary tuberculosis.

2.4 Discussion

I conducted this nested case control study within an ethnically diverse population of patients attending UHL for TB treatment in order to delineate risk factors for TB recurrence. I found that for patients who completed standard treatment for their first TB episode, ethnicity (Indian subcontinent and other ethnic groups), habitual tobacco smoking, the receipt of immunosuppressant medication and suffering a grade 3 or 4 ADR independently increased the risk of subsequent TB recurrence. TB recurrence affected 1.8% of the study cohort with incidence peaking at 6-12 months after treatment completion and falling thereafter. Relapse (endogenous reactivation) was the predominant mechanism of TB recurrence affecting 84% of patients for whom paired DNA strain-type profiles were available.

2.4.1 Recurrence incidence

The overall TB recurrence incidence in Leicester of 1·9 per 1000PYs was low. This is comparable to the rate of 4.1 per 1000PYs for England and Wales found by Crofts et al when examining national TB notification data between the periods 1998-2005 (145) and to rates from other low incidence countries such as Australia (0.8 per 1000PYs (178)) and Spain (5.3 per 1000PYs (147)).

I observed that 57% of recurrences occurred within 2 years and 77% of recurrences within 4 years after treatment completion. This contrasts with the findings of Nunn et al who reviewed 15 TB treatment trials and found 78% of recurrences occurred within 6 months of treatment and 91% within 12 months (246). Possible explanations include the longer duration of follow up, that patients were treated outside a clinical trial setting so delaying diagnosis and the substantial proportion of recurrence cases in my study presenting as EPTB (44%), a factor which has been shown to delay diagnosis(247).

2.4.2 Contribution of relapse and reinfection to recurrence

To date, no study has detailed the proportion of TB recurrence due to relapse and reinfection in the UK. I found relapse to be the predominant mechanism accounting for 84% of recurrences. This finding corresponds with the findings from a recent systematic review article arising from this thesis, which demonstrated that in low incidence settings, the median percentage of TB recurrences due to relapse was 81% (248). Mathematical models support this finding suggesting that the extent to which reinfection contributes to

TB recurrence in different settings / populations is a function of the background incidence in that population (141, 249).

In most settings, relapse tends to occur earlier after treatment whereas the risk of reinfection remains constant and proportionately increases over time (159, 162, 167). The converse was found in this study, the median time to relapse being 499 days (IQR248-857) and to reinfection 292 days (IQR 186-338) This is probably a chance finding due to the low number of reinfection cases in this study. Nevertheless, cases of reinfection can occur soon after treatment completion and should not be automatically attributed to relapse.

In this study, MIRU-VNTR was used to DNA strain-type *Mtb* isolates. A cut off of ≥ 2 MIRU loci differences between episodes was chosen to define cases of reinfection. This difference in loci has been used by others (70, 250) however it is an arbitrary cut off which may potentially misclassify recurrence cases. MIRU-VNTR is now considered a low-resolution typing method and has been shown by whole genome sequencing to mistakenly classify relapse as reinfection and vice versa (161). Misclassification may also occur through other mechanisms. For example, where population strain diversity is low or individuals are repeatedly infected from the same source case reinfection may be misclassified as relapse (175). Similarly, relapse may be misclassified as reinfection due to strain evolution, mixed infection or laboratory cross-contamination (175). The routine WGS of mycobacterial isolates has been introduced by PHE (251) and will improve typing resolution so overcoming the limitation of MIRU-VNTR and enable a more precise understanding of the mechanisms of TB recurrence in the UK.

2.4.3 The clinical impact of recurrence

I examined the change in phenotypic drug susceptibility profiles between the primary and recurrent episode. In the current study, 3 (10%) of culture confirmed recurrence (all relapses) acquired drug resistance during the first treatment episode, one cases becoming an MDR-TB case. This study adds to the body of evidence demonstrating that recurrence is associated with increasingly drug resistant strains. However, what drives resistance in patients who have been apparently successfully treated has not been established.

In this study, 5% of recurrence cases died during treatment for their recurrence episode. Unfortunately, due to the retrospective nature of this study, it was not possible to capture data detailing patient morbidity or any financial impact. In the published literature there is limited data on the clinical outcomes of patients with recurrence with one study showing a small (3.1%) increase in mortality in recurrence cases compared to those with no previous

episodes of TB (188). This data demonstrates the impact TB recurrence has for patients and the importance of minimising its occurrence.

Comparison of the site of disease in primary and recurrence episodes showed that 75% of patients with initial PTB and 50% with initial PTB and EPTB recurred with pulmonary involvement and were thus potentially infectious (94, 252). Furthermore, 26% of EPTB patients recurred with new pulmonary involvement, converting from a generally non-infectious to an infectious form of disease. The contribution of recurrent TB to the overall burden of TB transmission is unknown although it is thought that patients with recurrent TB may act as a focus of transmission (226). Modelling of the contribution of recurrent TB to overall transmission may indicate whether it is worthwhile including strategies to address TB recurrence as part of an enhanced TB control strategy to support TB elimination and should be evaluated in the future.

2.4.4 Risk factors for TB recurrence

I established that Indian subcontinent and other ethnicity were significantly positively associated with TB recurrence and Afro-Caribbean ethnicity demonstrated a positive although non-significant trend after controlling for confounding with multivariate analysis. Low representation within the study cohort may have precluded the attainment of statistical significance. Previous studies have demonstrated significant associations between non-white ethnicity and clinical aspects of TB. For example, Pareek et al showed extrathoracic disease was independently associated with Indian subcontinent and Afro-Caribbean ethnicity(253) and in another study, miliary TB was independently associated with Indian subcontinent ethnicity (254).

In the present study, I was unable to determine whether ethnicity (host genotype) itself mediates an individual's risk of recurrence or functions as a proxy for other risk factors(s) which were unaccounted for in the present study. Certain ethnic groups may have a heightened exposure to TB through travel to high incidence areas to visit family and friends(255) or due to social deprivation within migrant communities(51) However in the absence of robust data this remains theoretical. Vitamin D status is associated with both ethnic background (256, 257) and the risk of developing TB (258, 259). However, it remains to be determined whether vitamin D explains the association between ethnicity and TB recurrence. Certain *Mtb* strains including EAI /CAS (159) and Beijing (195, 209, 260) are independently associated with TB recurrence and may be more prevalent in certain ethnic groups. For example, Pareek et al found EAI strains are common in Indian

subcontinent patients studied in Birmingham (253). The proportion of TB cases with paired genotyped cultures was low in the present study precluding adjustment for strain lineage in the multivariate analysis. Studies examining adherence to medication in chronic conditions such as rheumatoid arthritis (261) and diabetes mellitus (262) have demonstrated lower adherence in South Asian patients. Unfortunately, adherence to medication was not reliably or quantifiably recorded and could not be assessed in the present study. Finally, it is known that there are disparities in access to health care between ethnic groups (263) and these conjecturally could impact TB treatment outcomes.

The strong association between TB recurrence and Indian subcontinent ethnicity, a group that are the most important in terms of TB cases nationally (264) and contribute more than 25% of cases worldwide(1), may have significant implications for individual patient management and public health. My results, in concert with those from a previous study by Crofts et al, who similarly showed an association between Indian subcontinent ethnicity and TB recurrence, suggest that interventions targeting this large at-risk group could significantly prevent future TB episodes.

I found habitual tobacco smoking to independently predict TB recurrence. This finding is consistent with epidemiological studies in different settings including Taiwan, China and India (179, 192, 203, 265) and identifies a problem area within the study cohort. Smoking increases baseline disease severity and reduces treatment response through inhibiting macrophage immune function(265, 266) possibly increasing recurrence risk through immunoparesis. One study did demonstrate poor adherence to TB treatment amongst tobacco smokers(265), suggesting this may act as a confounder. Currently the effect of smoking cessation on TB treatment outcomes is unclear (267) however it manifestly reduces cardiovascular and cancer related mortality (268) and should be offered to all TB patients regardless of whether it reduces recurrence or not.

In this study, experiencing a grade 3 or 4 ADR elevated a patient's future risk of TB recurrence. ADRs are considered a major cause of poor adherence to TB treatment (269), which in turn is associated with an increased risk of TB recurrence (270). Patients experiencing grade 1 or 2 ADRs were not at an elevated risk of recurrence, presumably due to better treatment adherence. Unfortunately, data detailing patient adherence was not routinely recorded and was thus not controlled for in multivariate analysis. Although not directly examining TB recurrence, a study conducted in an MDR-TB cohort showed that ADRs did not impact treatment outcomes in those patients adherent to treatment (271). Adherence may therefore mediate the association between ADRs and TB recurrence

although further research is required. Measures to address poor adherence arising from ADRs include pre-treatment counselling, supervised treatment and drugs to mitigate ADR symptoms (269) and should be considered when ADRs arise.

Immunosuppression of any kind predisposes to TB (272). Immunosuppressing therapies including glucocorticoids (273), non-biologic DMARDs (274) and anti-TNFα monoclonal antibodies (275) are associated with an increased risk of TB reactivation. Cases are reported where immunosuppressive therapy was instituted during the primary TB episode and was then followed by recurrence (276, 277). Similarly, recurrent TB has also been documented in renal transplant patients on long term immunosuppression (278). The present study provides for the first-time robust data showing that receiving immunosuppressants anytime from symptom onset until completion of TB treatment in the first TB episode predispose to recurrence. Most study patients received immunosuppressants for appropriate indications including the treatment of co-morbid conditions and the complications of TB, however a minority were for incorrect diagnoses or complications of TB where no significant evidence of benefit existed (see Appendix 3). The data captured within this study cannot quantify the degree to which each individual patient was immunosuppressed or assign a risk of recurrence to different immunosuppressants. Thus, it is unclear which patients may require specific interventions. However, it seems prudent to judiciously use immunosuppressants in patients with suspected or confirmed TB with careful consideration of the risk: benefit ratio.

2.4.5 Study limitations

The completeness and veracity of study data was limited due to several reasons. A number of variables exhibited varying degrees of missing data which was handled through the use of multiple imputation. Whilst this avoids the bias introduced by complete case analysis such as censoring of patients with missing values (279), multiple imputation itself can introduce bias into the results for example if the imputation is misspecified due to incorrect assumptions about the mechanism of missing data, variables in the analysis model and /or the dependent variable is not included in the imputation model or if components are imputed separately (241, 280). Convergence and the appropriateness of the imputed data was checked however imperfections in the imputation procedure or departures from missing at random may have affected variables with large fractions of missing data such as drug susceptibility (241)

The retrospective nature of the dataset impacted the control of confounding in the imputation model and multivariate analysis by several means. The information on several confounding factors (e.g. smoking) was based upon self-reported data and may not represent actual exposure status, although this is likely to have affected cases and controls equally. Likewise, adherence was self-reported and poorly recorded in the medical notes so could not be accounted for. Additionally, several potential confounders such as vitamin D status with high fractions of missing information, lead to imputation model instability and were excluded. It is possible that the omission of potential confounding variables may have misspecified the imputation model and biased subsequent analysis

Misclassification bias may have been introduced into the study and occurs when disease subjects are classified as non-diseased and vice versa (281). Within my study, several factors could misclassify recurrences as non-recurrences and contrariwise. The follow up of patients was passive so patients moving out of the hospital catchment area may not have been captured. Of these patients, those who subsequently experienced recurrence would be misclassified as controls underestimating the total number of recurrences. Similarly, patients who recur but are not renotified could be misclassified as controls. Laboratory cross contamination could misclassify controls as cases but each patient's data was reviewed thoroughly and required a diagnosis of TB from a TB physician, so mitigating this risk. In this study the definition of TB recurrence was independent of bacteriological confirmation and as TB may be mimicked by a variety of conditions (282), the inclusion of culture negative cases could lead to overestimates. For variables which require culture positive results such as drug resistance, the inclusion of culture negative cases could underestimate the association with recurrence leading to a type 2 error. Finally, cases of reinfection and relapse may have been misclassified by the use of low resolution MIRU-VNTR genotyping(161) and future studies of recurrence in the UK should utilise higher resolution whole genome sequencing.

The sample size of the study cohort precluded subgroup analysis including examination of risk factors specific to PTB and EPTB. Similarly, the low proportion of patients with paired DNA strain-typing data prevented the examination of risk factors for relapse and reinfection and lowered the precision of the estimates of both mechanisms of TB recurrence. Low patient numbers may have introduced type 2 errors for several possible risk factors such as age, and UK birth status.

The study was conducted in an urban setting characterised by an over-representation of groups at risk of TB such as immigrants with TB rates higher than the national average,

similar to areas of the country including Birmingham and parts of London (283). The external validity of the study would be improved further by including data from multiple centres, including international settings. Finally, the comparison of this study with other studies is somewhat hampered by several factors including the lack of a standard definition of recurrence across studies and differences in length of follow up after treatment completion.

2.5 Conclusion

In this chapter, I describe a nested case control study examining TB recurrence within a cohort of patients diagnosed with and treated for TB in Leicester. I found the rate of TB recurrence to be low with 1.8% of patients affected over a median 7.8 years of follow up. This is similar to other UK settings (180) suggesting TB treatment and control activities are likely comparable in efficacy to those elsewhere within the UK. However, the presence of TB recurrence within the study cohort suggests that there are still modifiable gaps in TB treatment and control activities (193). Akin to other low incidence settings, relapse was the predominant mechanism of recurrence. Whether targeting of control measures towards relapse is warranted is uncertain.

TB recurrence was deleterious for patient outcomes with a proportion of patients experiencing infection with more resistant strains in the recurrent episode with the attendant issues of longer treatment with often more toxic medication. Some patients died during their recurrent episode. It seems likely that TB recurrence increases morbidity, may economically disadvantage patients and may contribute to overall TB transmission within the community, however since no specific data was collected, this remains conjectural.

I identified several risk factors for TB recurrence within our patient cohort. Factors such as tobacco smoking and ADRs may be addressed by established clinical strategies although whether risk factor modification reduces TB recurrence is unknown although in light of the obvious health benefits, these should nonetheless be pursued. The attendant risk amongst ‘Indian subcontinent’ and ‘other’ ethnic groups, who together comprise the largest burden of TB nationally, has significant implications for individual patient management as well as for public health. Future work should incorporate host genetic, socioeconomic and environmental factors to unpick the association between ethnicity and TB recurrence to inform future preventative strategies.

2.6 Priorities for Further Research

Various studies have been conducted in low-burden countries to improve our understanding of recurrent TB, its frequency, underlying mechanisms and risk factors. Future studies of the epidemiology of recurrent TB should seek to improve comparability, for example by developing and applying consensus definitions of recurrence and standardizing the reporting of the frequency and timing of recurrent TB with total length of follow up provided in person-years. This would permit both comparison between sites as well as facilitating the meta-analysis of recurrent TB data.

While studies to date have focused on establishing risk factors for recurrence and distinguishing relapse from reinfection, future research is needed to better understand the biological, microbiological and pathophysiological mechanisms of disease recurrence. Basic science directed towards understanding these mechanisms may highlight novel therapeutic approaches and potential biomarkers predicting the probability of definite cure vs. disease re-activation and reinfection TB. In Leicester, there exists expertise in basic immunology and mycobacteriology as well as physician scientists with expertise in clinical TB. Immunological profiling of recurrent TB cases and comparison with TB controls with the aim of identifying host markers of susceptibility should be pursed. Similarly, predisposing mycobacterial factors should be examined.

Although individual risk factors of recurrence have been identified, to date, little is known about suitable strategies to effectively prevent recurrence among high-risk individuals. Research is needed to understand the benefits of individualized and intensified TB treatment towards achieving long-term cure. This may entail research on the effect of adjuvant therapies to reduce lung destruction and inflammation (284), the modification of other individual risk factors (285), and the possibility of extending the duration of TB treatment for certain groups of patients at high risk of recurrence (193). To properly assess interventions to minimize recurrence will require prospective studies enrolling large numbers of patients, particularly in low incidence settings given the infrequency of the condition. Although Leicester contributes significantly to the national TB burden, this is not a feasible endeavour for a single centre and would require a multicentre involvement

More research is also needed to better understand the long-term consequences of recurrent TB to individuals and health-care systems. There is currently a lack of knowledge about the clinical, social and socio-economic consequences that TB and recurrent TB imposes on patients, their families, and the health-care system. At the health-care level,

more information is also needed to understand the extent to which recurrent TB contributes to the overall TB burden in different populations. Particularly in populations where drug-resistant and MDR-TB is highly prevalent, the role of TB recurrence towards the acquisition and transmission of drug-resistance, and in reverse, the role of drug-resistance towards increasing recurrent TB, should be investigated. Finally, former TB patients should be re-considered as an important high-risk group for TB control. As low-burden countries will scale up control efforts among high-risk groups in order to reach TB elimination in the future, implementation research and mathematical modelling could help determine the circumstances under which interventions targeted at former TB patients, such as increased efforts to prevent or early detect TB recurrence, may be a cost-effective element of comprehensive TB elimination strategies in low-burden countries and beyond.

Chapter 3: Enhancing the culture of extrapulmonary tuberculosis with resuscitation-promoting factor

3.1 Introduction

Worldwide, EPTB accounted for only 15% of notified cases in 2016 (1). However, the burden of disease in fact varies significantly between settings as shown in a 2013 review of EPTB in Europe which reported that the proportion of EPTB in European countries varied between 4-48% with prevalence highest in the UK (105). Of the TB cases notified in the UK during 2016, 46.1% had EPTB and amongst foreign-born this was 51.4% (16). More locally in the East Midlands, EPTB affected 45.9% of TB cases in 2016 (17). Studies show EPTB is particularly prevalent in certain groups including females (286-288), patients under 25 (288), those with HIV infection (289), and Vitamin D deficiency (290).

EPTB does not feature significantly in TB control strategies as it contributes little to the transmission of disease (291, 292). Nonetheless, it a significant cause of morbidity and mortality (293, 294), for example mortality rates of 17.1-41.2% are reported in cases series of tuberculous meningitis (295, 296). However, the diagnosis of EPTB is often delayed for reasons including the wide spectrum of clinical manifestations of EPTB, the broad range of other conditions with similar clinical features, presentation to non-specialist clinicians who may have a low index of suspicion of TB and the difficulty obtaining an adequate clinical sample for microbiological diagnosis (105, 297).

Once a diagnosis of EPTB is contemplated, confirmation is often difficult due to the limited range of effective diagnostic tests available (298). The sensitivity of these assays is commonly low due to the paucibacillary nature of many extrapulmonary samples and interference/inhibition of molecular amplification assays from the sample matrix (297, 299) (see Section 1.7 for information on the performance of diagnostic tests in EPTB).

Despite advances in the molecular detection of *Mtb*, culture is still considered the gold standard for diagnosis, including that of EPTB (300, 301). Successful cultivation is important for clinical management (302), enabling drug susceptibility testing, typing for outbreak investigations and advance 'omic' studies (118, 300, 303). However in EPTB, culture confirmation rates are consistently lower than those for PTB both nationally and in the East Midlands (16, 17). Furthermore, culture is too slow taking typically 2-4 weeks for bacteriological confirmation (108) which is frequently too slow to influence patient management (304). By optimising culture and bacterial detection, organism isolation and

phenotypic susceptibility can be achieved within five days although these approaches are technically challenging, resource intensive and currently limited to the research setting (108). Currently, there is the lack of an easy approach to increase culture positivity in the era of increasing EPTB prevalence (305).

Rpfs are a family of bacterial proteins, able to stimulate the regrowth of non-replicating bacteria and were first discovered in *Micrococcus luteus* (56, 306). *Mtb* possesses 5 *rpf*-like genes whose products exhibit similar biological activity to that of Rpf from *M. luteus* (306). Rpf are able to resuscitate 'quiescent' *Mtb* although the mechanism is poorly understood with studies suggesting their enzymatic activity is essential for their biological effects (307, 308). Clinical studies where Rpf was added to standard culture either as recombinant proteins or CS demonstrated the presence of dominating populations of *Mtb* bacilli in sputum which could not be detected by standard culture in liquid 7H9 or solid 7H10 agar (57, 58). A recent study additionally showed an *Mtb* population only detectable with the use of Rpf-deficient CS (58). Collectively these populations were designated differentially culturable tubercle bacilli (DCTB) (58, 59). There is increasing experimental evidence that the presence of DCTB has ramifications for clinical and treatment outcomes. CS-dependent and Rpf-dependent *Mtb* were apparently enriched in treated patients and showed high level tolerance to killing by antimicrobials including rifampicin (57), streptomycin and isoniazid (309). Furthermore, the eradication of CS-dependent *Mtb* prevented relapse in a Cornell Mouse model (310). Finally, the application of recombinant Rpf or Rpf-containing CS has often increased bacterial yield in a substantial number of sputum samples and in some cases enabled the recovery of *Mtb* from specimens that would be otherwise culture negative by standard culture methods (57, 58) and also reduced the TTP of sputum culture positive after more than 20 days by standard culture methods (109). These findings highlight the potential for Rpf to improve culture diagnostic performance.

To date, three independent studies have highlighted the presence of DCTB in sputum samples (57, 58, 109), however it is unknown whether DCTB are commonly present in EPTB and if Rpf-containing CS can stimulate CS-dependent DCTB populations so potentially improving EPTB culture sensitivity and TTP. Given the consistently lower culture confirmation rate of EPTB compared to sputum, I focused specifically upon EPTB samples. The work presented in this chapter details a novel clinical diagnostic study evaluating the impact of Rpf-containing CS supplementation on the standard culture of EPTB samples compared to a composite reference standard. Given the likely impact of

DCTB on clinical outcomes, I also evaluated the presence of DCTB populations in EPTB samples and host factors which may modulate CS-dependent populations.

3.2 Methods

3.2.1 Study setting and design

This pilot diagnostic study was conducted at UHL between 1st of January 2015 and 29th of February 2016. Patients suitable for screening were identified from those referred to the rapid access TB clinic and from inpatients and outpatients of the respiratory medicine and infectious diseases services.

Patients were eligible for inclusion in the study if they were ≥ 18 years of age, suspected to have EPTB or EPTB with concomitant pulmonary tuberculosis (PTB) and were willing and able to give written informed consent for inclusion in the study. Where patients were identified as potential EPTB cases and recruited prior undergoing diagnostic sampling, consent was sought to collect an additional sample for research purposes. In some cases, patients were only identified as potential EPTB cases after they had already undergone diagnostic sampling. Where there was a surplus of clinical material in the clinical diagnostic laboratory, consent was sought from the patient to use this for research purposes. In some cases, samples were identified in the microbiology laboratory as coming from patients with suspected EPTB however consent for inclusion in the study could not be sought from the patient. Ethical approval was obtained to use material (acellular fluid or tissue samples) from such patients as long as the sample was provided during the course of an investigation planned as part of the patient's routine medical care, it had undergone routine processing by the UHL microbiology department (UHL-MD) and was surplus to requirements. It was specified by the ethics committee that samples obtained by this last route be pseudonymised. If the patient was unable to provide a clinical sample and/or did not meet the inclusion criteria, they were excluded.

Patients recruited to the study received a unique study number which was used throughout the study. Numbers were not allocated consecutively due to a concurrent study utilising the same study file. Spent samples included in the study were allocated a letter as an identifier. Data was collected from consented patients by a review of the clinical notes. Data for spent samples was obtained from the TBIT database. Haematology and biochemistry values utilised in the study were those closest (<1 month) to the date of EPTB sample acquisition.

3.2.2 Study definitions

The definitions used in this study are described in section 2.2.4. The definition of Vitamin D deficiency used in this study differs as one assay was in use for the duration of the study.

Co-morbidities:

- 25-OH Vitamin D_3 deficiency: serum concentration of <30nmol / L within 1 month of EPTB diagnosis.

Growth assays:

- Differentially culturable tubercule bacilli (DCTB): the population of bacteria that is elucidated when the bacterial count, as calculated by the MPN assay, exceeds the number of plateable bacilli (CFU count) (58)

3.2.3 Sample storage and processing

Samples were received and stored in the UHL-MD category 3 laboratory prior to transfer to the category 3 laboratory at the University of Leicester (UoL) using an approved UN 3373 transport box. Samples collected prospectively by me from patients during the course of their planned diagnostic procedure were stored at 4ºC prior to transport. Samples processed by UHL-MD prior to their retrieval for research purposes were either stored at either 4ºC or -20ºC. For those samples stored at -20ºC, some were directly frozen and some were decontaminated according to UHL-MD protocol (see figure 3.1) and then frozen to 20ºC. The handling of samples other than those prospectively collected by me was at the behest of UHL-MD biomedical scientist staff. Packed clinical samples were transported to UoL on ice

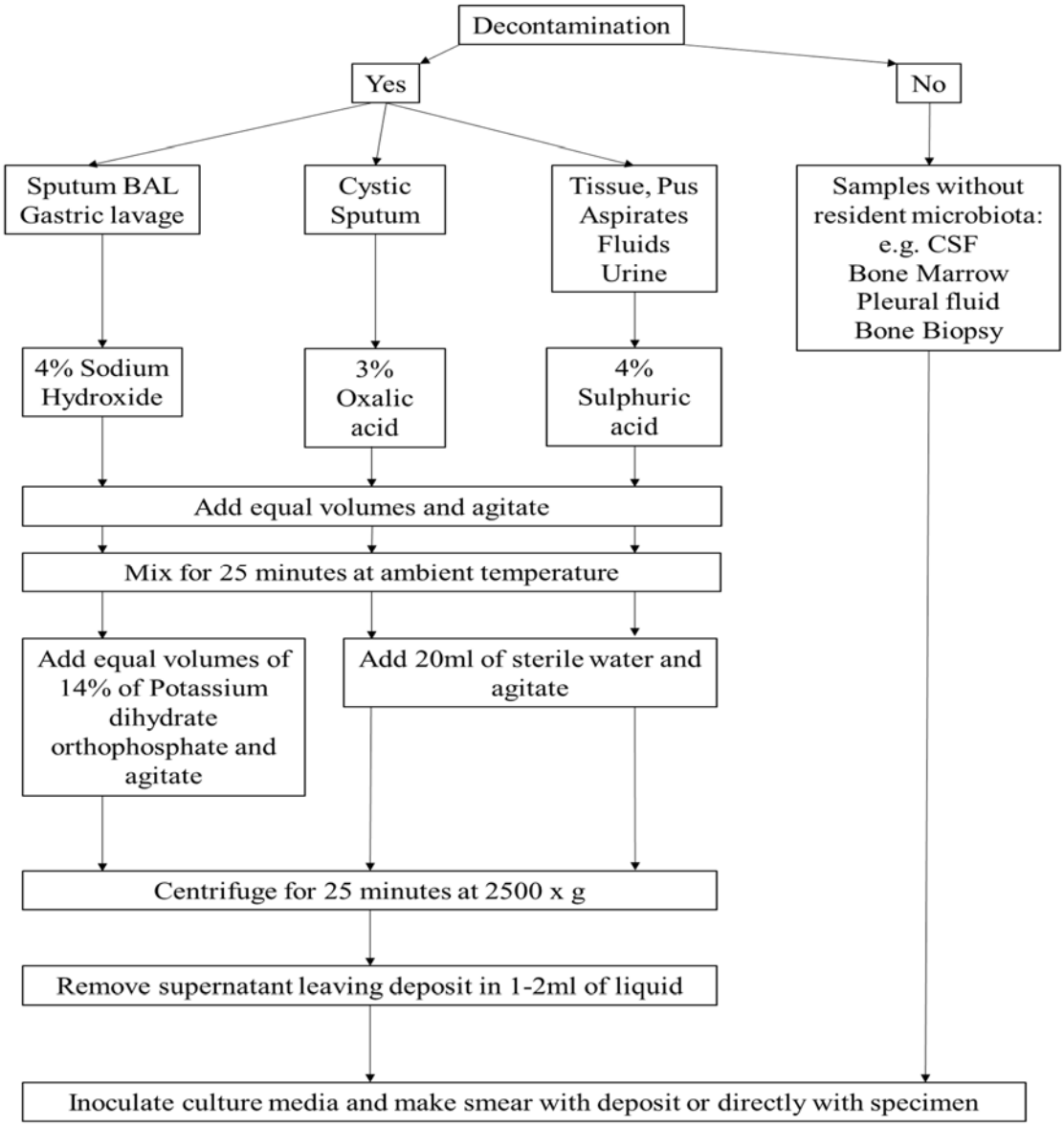
Samples received at the UoL category 3 laboratory were stored avoiding additional freeze-thaw cycles. The processing of samples used UHL-MD protocols based upon the UK Standards for Microbiology Investigations published by PHE (92). To minimize the risk of contamination, all media preparation was performed in a class II biological safety cabinet. All clinical samples were from patients suspected to have EPTB therefore all sample manipulation, decontamination and culture were performed under biosafety level 3 conditions.

Samples were prepared for decontamination using the following methods: tissues were homogenised by repeated vortexing for 10 minutes in a sterile 20ml Universal container with sterile ceramic beads and 5ml of 0.9% NaCl; bone samples were similarly homogenised using sterile glass 20ml Universal containers with glass beads and 5ml of 0.9% NaCl; fluid specimens were centrifuged in a Heraeus™ Megafuge™ 40R (Thermoscientific) at 2500g for 25 minutes and the deposit resuspended in 1ml of fluid from the original clinical sample by vortexing. To make the sample safe for ZN staining

under class 2 conditions,100µl of deposit or 10% of tissue sample was fixed in 4% (w/v) paraformaldehyde overnight and then stained using a cold ZN staining method as previously described (311). *Mycobacterium. smegmatis* and *Escherichia coli* were used as positive and negative staining controls respectively. ZN stain results for samples decontaminated by UHL-MD were copied from pathology records.

Decontamination of samples prepared as previously described followed the standard UHL-MD protocol (see figure 3.1). Those samples already decontaminated by UHL-MD were not further decontaminated. Non-cerebrospinal fluid (CSF) sterile site samples contaminated with blood were decontaminated using 4% H_2SO_4 (Fisher Scientific, Loughborough, UK) to reduce false positive signalling with the BACTEC 960 system (BD Biosciences, San Jose, California, USA) (92). Lymph node samples obtained by endobronchial ultrasound (EBUS) were decontaminated using freshly prepared 4% (w/v) NaOH (Fisher Scientific). Samples were resuspended by vortexing (see figure 3.1 for details). The resuspended deposit / non-decontaminated sample was then used for growth assays.

Figure 3.1 Sample decontamination flow chart



Adapted from UHL-MD laboratory protocol for the processing of samples for mycobacterial culture.

3.2.4 Mycobacterial growth assays

For each clinical sample, 2 MPN assays were performed 1) MPN assay performed using Rpf-containing CS supplementation (MPN_CS) 2) MPN with no CS supplementation (MPN_7H9). These two assays permitted the detection of 'CS-dependent' DCTB and 'CS-independent' DCTB respectively. The number of CS-dependent DCTB were calculated by $\log_{10}$ (MPN_CS / CFU) and CS-independent DCTB by $\log_{10}$ (MPN_7H9 / CFU). Rpf-depleted CS from a quintuple *rpf* deletion *Mtb* mutant was unavailable for this project thus 'Rpf-independent' DCTB populations could not be examined. EPTB sample volumes were insufficient to permit multiple technical replicates of CFU assays thus mean values and 95% confidence intervals could not be constructed for CFU counts. Differences of greater than the order of one $\log_{10}$ were considered significant to account for the lack of confidence values and the potential for mycobacterial clumping.

For the MPN growth assays, Middlebrook 7H9 medium (BD Biosciences) was prepared with supplementary 10% (v/v) OADC supplement (oleic acid, albumin, dextrose and catalase; BD Biosciences), 0.05% (w/ v) Tween 80 (Fisher Scientific), 0.2% (v/v) glycerol and 1.5% (v/v) reconstituted BBL PANTA solution (polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin; BD Biosciences). For the preparation of the MPN_CS, freeze dried CS was obtained from laboratory stocks which had been stored at -80ºC for ≤6 months (the preparation of freeze dried culture supernatant is described in Appendix 4). Freeze dried CS was reconstituted in sterile deionised water on ice and dissolved over 30 minutes. Reconstituted CS was added 50% (v/v) to the supplemented 7H9 medium. The sterility of CS was checked with each experiment. Middlebrook 7H10 agar (BD Biosciences) with 10% (vol/vol) Middlebrook OADC supplement was used for the CFU assay. To reduce contamination, 100µl of reconstituted PANTA solution was spread evenly on 7H10 agar plates and allowed to dry before the plates were used.

The precision and repeatability of the MPN and CFU assays were assessed. Six technical replicates were performed for both MPN and CFU assays of one laboratory *M. smegmatis* mc2155 and 2 laboratory *Mycobacterium bovis* BCG cultures. The isolates were grown at 37ºC in a modified 7H9 medium. This differed from the supplemented 7H9 medium described previously in that OADC supplement was replaced with 10% (v/v) ADC supplement (Albumin Dextrose Catalase; BD Biosciences) and no PANTA was used. At an optical density of ≥0.8 at 580nm as determined by CO8000 cell density meter (Biochrom Ltd, Cambridge, UK), the culture was diluted 10,000-fold in modified 7H9 medium. MPN assays were done in quadruplicate in a 48-well microtitre plate (Greiner

Bio-One Ltd, Stonehouse, UK) by adding 50µl of diluted culture to 450µl of modified 7H9 medium and serially diluting tenfold. For the CFU assay, the diluted culture was further serially diluted tenfold in modified 7H9 medium and 10µl of each dilution placed on the 7H10 agar plate (no PANTA applied). Dilutions were plated in triplicate. *M. smegmatis* plates were incubated for ≤5 days and *M. bovis* BCG for ≤5 weeks. Contamination was assessed by plating the original culture on Lysogeny agar (Fisher Scientific). MPN counts were calculated using the U.S. Food and Drug Administration method (312). CFU counts were derived by counting colonies from appropriate dilutions. Mycobacterial aetiology was confirmed using ZN staining.

DCTB populations in patient samples were assessed using MPN_CS, MPN_7H9 and CFU assays. MPN_CS and MPN_7H9 assays were performed in quadruplicate in 48-well microtitre plates by adding 50µl of neat decontaminated sample to 450µl of media and serially diluting tenfold. For the CFU assay, the neat decontaminated sample was serially diluted tenfold in supplemented 7H9 medium and 10µl of the neat sample and of each dilution placed on a 7H10 agar plate in triplicate. 100µl of neat processed sample was also placed upon a 7H10 agar plate to increase culture sensitivity. MPN and CFU assay plates were incubated for approximately 12 weeks at 37ºC under standard atmospheric conditions. Those samples negative for growth at 12 weeks were discarded or earlier if fungal contamination was visualised. Cultures were reviewed on a weekly basis for growth. MPN and CFU counts were calculated as previously described. The limit of detection for MPN assay was 5 x 10^0 CFU ml^{-1} and the CFU assay was 1 x 10^1 CFU ml^{-1} (for patients recruited before 25/03/2015, only 3 x 10µl of neat sample was plated making the limit of detection 3.33 x 10^1 CFU ml^{-1}).

3.2.5 *Mycobacterium tuberculosis* identification and typing

Where growth was observed in the CFU assay, colonies were transferred from the solid agar plate and suspended in 0.5ml of standard 7H9 medium in a 1.5ml Eppendorf tube (Fisher Scientific). For positive MPN assays, 0.5ml of liquid culture medium was transferred from the relevant MPN microtitre plate well to a 1.5ml Eppendorf tube. Wherever possible, culture was taken from the MPN_7H9 assay to avoid genomic DNA (gDNA) contamination from CS. Samples were spun at 13000g in an Espresso microcentrifuge (Thermoscientific) for 10 minutes. The supernatant was removed and the pellet washed with 1ml of unsupplemented 7H9 medium. This process was repeated twice. The final pellet was resuspended in 500µl of unsupplemented 7H9 medium. 50µl was used

for ZN staining as previously described. The sample was then sterilised by heating to 100ºC for 1 hour in a dry heat block (Grant Boekel) for *Mtb* gDNA extraction.

Table 3.1 Primer sequences for *Mycobacterium tuberculosis* identification

Gene	Primer name	Primer sequence
rpoB	*rpoB* forward primer	5'GGAGGCGATCACACCGCAGACGTT 3'
	rpoB reverse primer	5'ACCTCCAGCCCGGCACGCTCACGT 3'
16S *rRNA*	16S *rRNA* forward primer	5' GAGATACTCGAGTGGCGAAC 3'
	16S *rRNA* reverse primer	5' GGCCGGCTACCCGTCGTC 3'

To identify whether the isolate was mycobacterial in origin a polymerase chain reaction (PCR) targeting 16S *rRNA* gene was performed (313). A PCR targeting the *rpoB* gene was used to confirm membership of the *Mtb* complex. Primer sequences were those published by El- Hajj et al (313) (see Table 3.1). Primers were produced by Sigma-Aldrich (Dorset, UK). The reaction mixture contained 4.6µl PCR grade water (Sigma Aldrich), 2.0µl 5x Green GoTaq® reaction buffer (Promega, Madison, USA), 1.0µl of dNTPs (2nM) (Promega), 0.5 µl of forward primer (10µM), 0.5 µl of reverse primer (10µM), 0.4 µl of GoTaq G2 DNA polymerase (Promega) and 1.0 µl of sample DNA. 16S *rRNA* and *rpoB* PCR reactions were performed on a T100 PCR thermocycler (Biorad, Hemel Hempstead, UK). Cycling conditions were 95ºC for 5 minutes, 30 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72.5 ºC for 60 seconds. A final cycle of 72.5 ºC for 10 minutes was performed. PCR amplicons were separated using gel electrophoresis with a 2% (w/v) agarose gel (Fisher Scientific) and visualised. Amplicon size was determined using a DNA ladder (Hyperladder 100bp plus (Bioline, London, UK) or Generuler 100bp-10kbp (Fisher Scientific)). The 16S *rRNA* amplicon was predicted to be 208bp and *rpoB* amplicon 189bp (313). For each PCR, *Mtb* H37Rv gDNA was used as a positive control and PCR grade water as a negative control.

Any isolates confirmed by PCR as a member of the *Mtb* complex were verified by 24 locus MIRU-VNTR typing as described by Supply et al (238). This was to identify cross contamination with the *Mtb* laboratory strain H37Rv and to identify individual strains. For each isolate, individual PCRs using primer sets for each locus were run (for primer sequences for each MIRU-VNTR locus see Appendix 5). PCR reaction mixture, thermocycling parameters and amplicon visualisation on agarose gel electrophoresis followed the method previously described for 16S and *rpoB* PCR. The number of repeats for each locus was calculated by the amplicon size (see Appendix 6). The strain lineage was confirmed using the MIRU-VNTRplus web resource (314). MIRU-VNTR sequences

generated at UoL were compared with the reference laboratory typing data when available (see Appendix 7).

3.2.6 Effect of culture supernatant on clinical sample time-to-positivity determined using a BacTec MGIT 960 system

The effect of Rpf-containing CS supplementation on the TTP of MGIT culture was measured using a standard 7ml BBL MGIT (MGIT_7H9) and one prepared with 50% (v/v) culture supernatant (MGIT_CS). The MGITs were prepared in a class II biological safety cabinet to minimise contamination. For the preparation of MGIT_7H9, 115µl of reconstituted PANTA (1.5% v/vl) and 0.8ml of BACTEC MGIT growth supplement (BD Biosciences) were added aseptically to a standard MGIT from which 115µl of 7H9 medium had been removed. For the preparation of the MGIT_CS, sterile deionised water was added to freeze dried CS and left on ice for 30 minutes to dissolve. 3.515ml of 7H9 medium was removed from the MGIT tube. 0.4ml of BACTEC MGIT growth supplement, 115µl of reconstituted PANTA (1.5% v/v) and 3.8ml of reconstituted CS were then added to the MGIT. The MGITs were transferred immediately to the biosafety level 3 laboratory. 0.5ml of processed patient sample was inoculated into each MGIT as soon as possible after MGIT preparation. The inoculated MGITs were then transported to UHL-MD in a secured transport box and loaded onto the BACTEC 960 system. MGITs were incubated at 37ºC for ≤6 weeks and the growth index measured automatically by the BACTEC 960 system (BD Biosciences). Positive cultures were confirmed as mycobacteria by ZN staining. When no growth occurred after 6 weeks, MGITs were classified as negative as recommended by the manufacturer. Where growth only occurred in MGITs and not in MPN / CFU assays, identification of *Mtb* complex by PCR and 24 locus MIRU-VNTR typing was performed. The difference in TTP was calculated between the paired MGITs. As per the agreed standard operating procedure (SOP), only one pair of MGITs (MGIT_CS & MGIT_7H9) was transported per week from UoL to UHL. This mandated some patient samples to be frozen / refrozen at -80ºC pending MGIT inoculation.

3.2.7 Reference standard and units of analysis

Culture is known to be a poor reference standard for EPTB (315), thus a composite reference standard (CRS) was used as the reference standard for this diagnostic study to define subjects as disease positive and disease negative. Patients were categorised into proven TB (culture positive, smear negative & culture positive or smear positive & culture positive), probable TB (culture negative but symptoms and signs of tuberculosis and

radiological and / or histopathological / cytological evidence of TB), possible TB (symptoms and signs compatible with active TB with a clinical response to TB treatment at 3 months) or not TB (symptoms and signs compatible with TB without diagnostic evidence of TB and improvement without TB treatment) (315). Possible, probable and proven TB patients were classified as CRS positive and not TB as CRS negative.

Solid and liquid culture media are typically both inoculated when TB is suspected (33) thus liquid and solid culture were analysed together as one unit of analysis in this study. The MPN_7H9 and CFU assay from each sample were analysed together and classified as the standard culture method. MPN_CS and CFU assay were analysed together and represented the effect of Rpf-containing CS on culture performance.

3.2.8 Statistical analysis

This study was a pilot therefore did not require formal statistical powering (316). Continuous variables were examined using the Wilcoxon rank-sum and Kruskal-Wallis test. Fisher's exact test was used to examine categorical variables. Spearman rank correlation with Bonferroni correction was used for continuous variables. The sensitivity, specificity, positive predictive value, negative predictive value and their respective 95% confidence intervals were calculated for each unit of analysis against the CRS standard using the 'diagt' command of the Stata 13.1 statistics package (Statacorp, Texas, USA). Graphs were produced using Microsoft Excel 2013 (Microsoft, Redmond, USA), Prism® version 6 (Graphpad Software, California, USA) and Stata 13.1. The study was conducted using the principles outlined in the STARD initiative (317).

3.2.9 Ethical approval

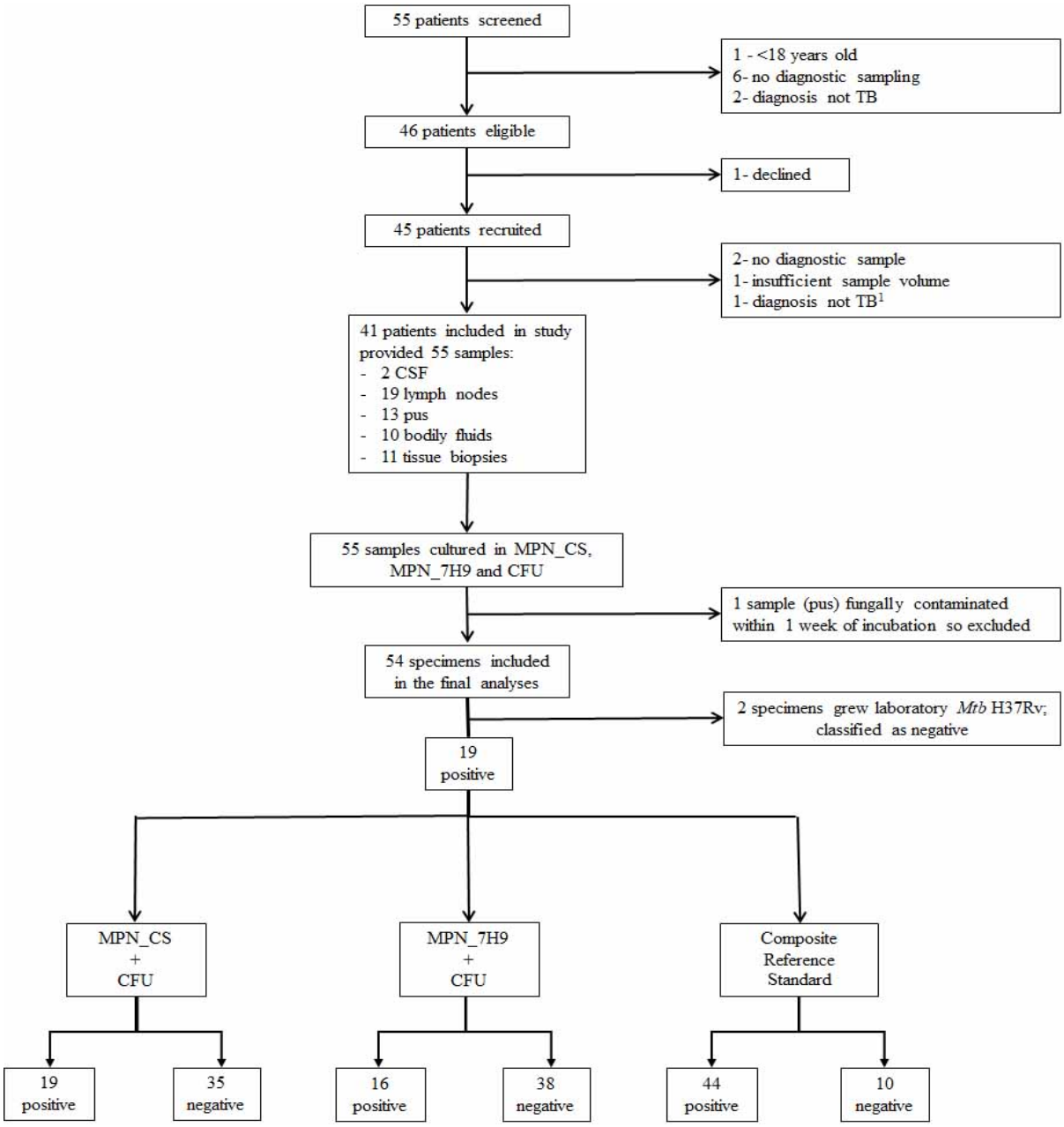
The study was approved by National Research Ethics Service Committee East Midlands Leicester (07/Q2501/58).

3.3 Results

3.3.1 Patient recruitment and study population

Between the 1st of January 2015 and 29th of February 2016, 55 patients were screened and 46 patients were eligible (see Figure 3.2). After exclusions 41 patients remained in the study, of whom 31 patients provided 1 sample, 7 patients 2 samples, 2 patients 3 samples and 1 patient 4 samples. One sample was excluded for early fungal contamination making 54 samples in the final analysis. (For a breakdown of samples by tissue type and anatomical location, see Appendices 8&9).

Figure 3.2 Flow diagram describing patient flow in the study



1 - Diagnosis re-evaluated after recruitment to study.

The baseline characteristics of the study participants are shown in table 3.2. The age of patients without TB was significantly higher than those with TB (p=0.034). Otherwise no significant differences were found between the two groups. There was a high prevalence of Vitamin D deficiency in both groups and no samples were positive upon AFB smear microscopy indicating all samples were paucibacillary. 25 patients were classified by the CRS as proven TB, 8 as probable TB, 1 as possible TB and 7 as not TB (for individual patient classifications see appendices 8&9).

Table 3.2 Patient baseline characteristics

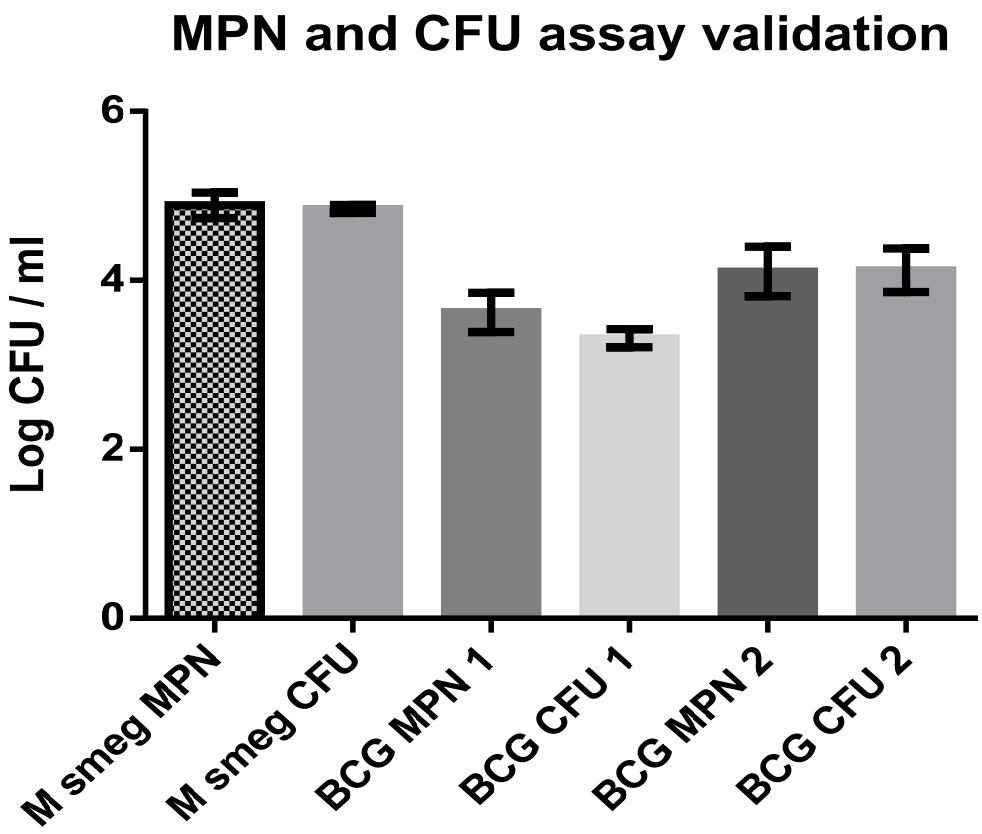
	Tuberculosis n=34 N (%)	**Not Tuberculosis n=7 N (%)**
Age (year, +/-SD)	38.9 (+/-14.2)	55.0 (+/-19.4)
Gender		
Male	18 (52.0)	4 (57.2)
Female	16 (47.1)	3 (42.8)
Weight (kg)[a]	65 (59-91)**	49
Ethnic group[†]		
White	1 (3.0)	2 (28.5)
South Asian	28 (82.3)	5 (71.5)
Afro-Caribbean	5 (14.7)	0
Tobacco smoking	4 (11.7)	0
Alcohol consumption[b]	7 (21.8)	1 (20.0)
Co-morbidities		
HIV type 1 infection	5 (14.7)	1 (14.3)
Anti-retroviral therapy	4 (11.8)	1 (14.3)
Vitamin D deficiency[c]	26 (86.6)	2 (66.6)
Laboratory		
Haemoglobin (g/dl)[d]	11.5 (10.0-12.4)**	11.6 (9.6-12.6)**
Neutrophils (x10^9 cells/L)[d]	4.3 (3.4-7.5)**	5.7 (4.4-10.5)**
Lymphocytes (x10^9 cells/L)[d]	1.7 (1.4-2.0)**	1.7 (0.6-1.9)**
Monocytes (x10^9 cells/L)[d]	0.5 (0.4-0.6)**	0.4 (0.3-0.7)**
Lymphocyte: Monocyte ratio[d]	3.1 (2.7-3.9)**	2.7 (2.3-4.7)**
Creatinine (µmol/L) [d]	57 (51-62)**	75 (55-321)**
C-reactive protein (mg/L)[e]	39 (13-92)**	75 (16-77)**
Primary disease site		
PTB +EPTB	10 (29.4)	n/a
EPTB	24 (70.6)	n/a
Clinical symptoms		
Night sweats[f]	17 (53.1)	1 (20)
Fever[g]	21 (63.6)	2 (33.3)
Weight loss[h]	12 (40)	0
AFB smear positive	0	0
***Mtb* culture positive[†]**	18 (48.7)	0

Missing data: a=8&6; b=2&2; c= 4&4; d=1&0; e=4&0; f=2&2; g=1&1; h=4&2. †8 (42%) of isolates belonged to the Delhi/CAS lineage, 4 (21%) East African-Indian, 2 (11%) Haarlem, 1(5%) Beijing, 1 Turkish, 1 S-type and 1 H37Rv-like as determined by MIRU-VNTR typing. ** median value and interquartile range. South Asian ethnicity - Indian, Pakistani and Bangladeshi; Afro-Caribbean ethnicity - Black African and Black Caribbean. List of abbreviations: AFB – acid fast bacilli; n/a – not applicable; SD – standard deviation.

3.3.2 Assay validation

The MPN and CFU assay technical replicates are shown in Figure 3.3. The results show concordance between CFU and MPN assays as demonstrated by the overlapping 95% CIs for both methods (see Figure 3.3).

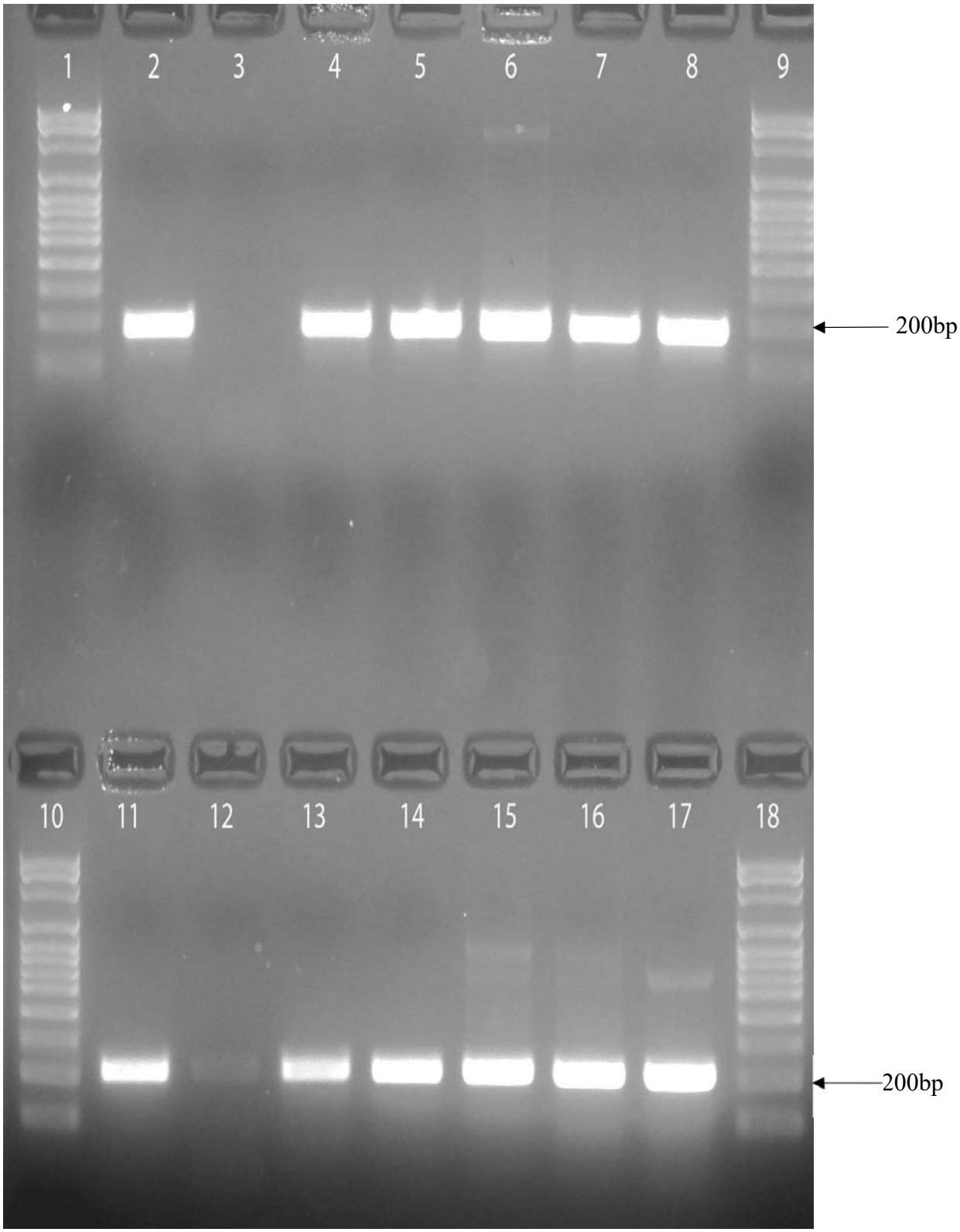
Figure 3.3 Concordance demonstrated between the culture cell counts as determined by MPN and CFU assays



The mean values with 95% confidence intervals are shown for the 6 technical replicates of each assay.
List of abbreviations: BCG - *M. bovis* BCG; M smeg - *M. smegmatis* MC2155

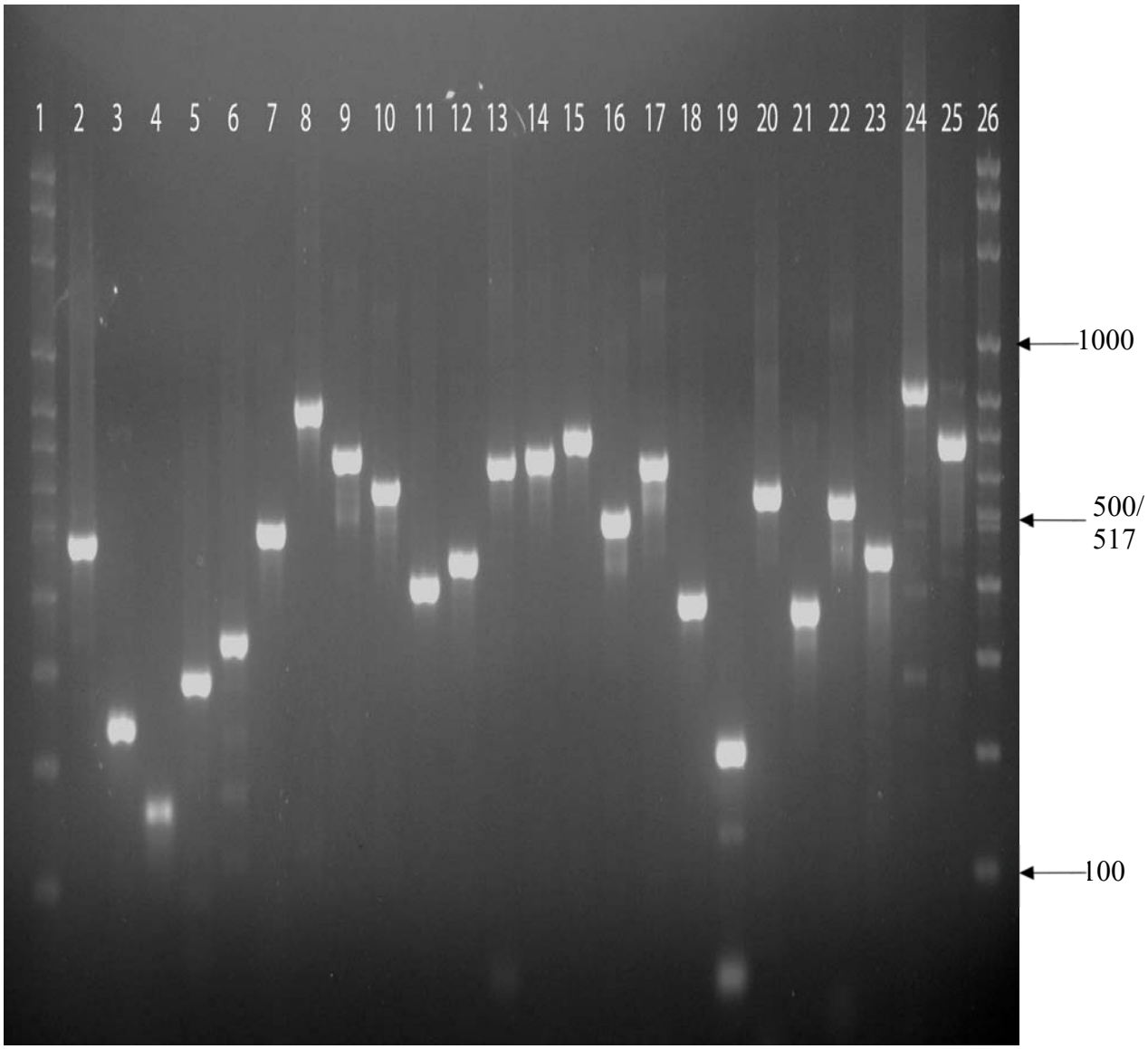
The *rpoB* and 16S *rRNA* PCR amplified gDNA from the laboratory *Mtb* strain H37Rv confirming correct specification of the PCR and thermocycler. The 'no-template' control excluded extraneous nucleic acid contamination (see figure 3.4). The specificity of the PCR primer set had been previously verified and was not repeated (313). The strain identification generated by the 24-locus MIRU-VNTR performed at UoL (see figure 3.5) showed concordance with that from the PHE reference laboratory in Birmingham (see Appendix 7).

Figure 3.4 A gel showing amplification products from PCRs targeting 16S *rRNA* & *rpoB*



Hyperladder 100bp plus DNA ladder: Lane 1,9,18 & 18; *rpoB* PCR: Lane 2 – *Mtb* H37Rv gDNA positive control; Lane 3 –no template negative control; Lane 4-8 clinical samples; 16S *rRNA* PCR: Lane 11 Mtb H37Rv gDNA positive control; Lane 12: no template negative control; Lane 13-17 clinical samples.

Figure 3.5 Example 24 locus MIRU-VNTR gel from patient 36

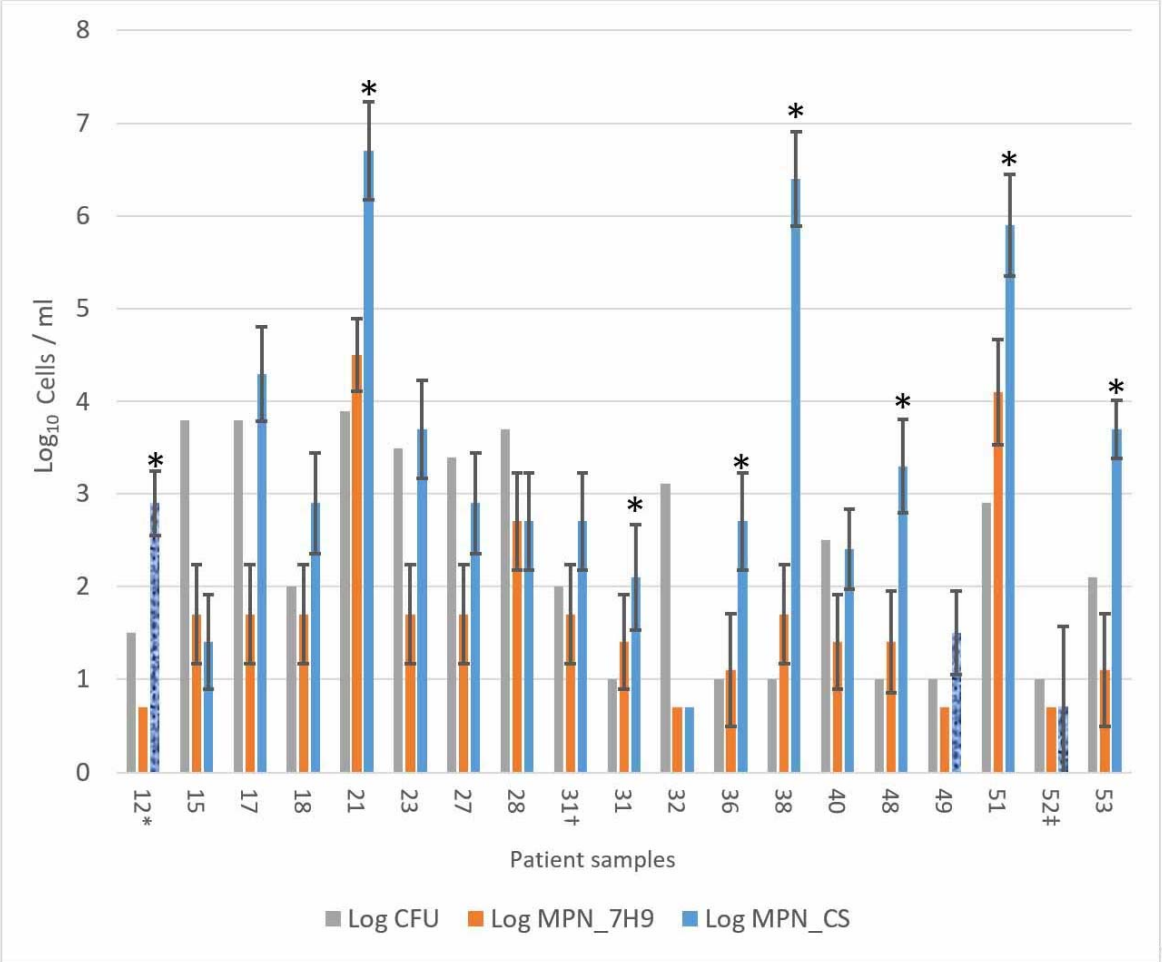


Lane 1&26: DNA ladder - Hyperladder 100bp plus. Lane 2 -6: ETR-A – ETR-E; 7-16: MIRU02-MIRU40; 17-25: Mtub04 - QUB-4156

3.3.3 MPN and CFU growth assays

The bacillary counts for MPN_CS, MPN_7H9 and CFU assays of the 19 culture positive samples are graphically displayed in figure 3.6. Numerical values are presented in appendices 8&9. Bacillary counts ranged over $\log_{10}$ 0.69-6.7 for MPN_CS, $\log_{10}$ 0.69-4.5 for MPN_7H9 and $\log_{10}$ 1-3.9 for the CFU assay. In 42% (8/19) samples the MPN_7H9 counts were lower than MPN_CS and CFU counts. In 42% (8/19) samples CS-dependent *Mtb* were dominating populations, including 3 samples which produced *Mtb* cultures only with CS-supplementation. Interestingly in 9 out of 19 samples (42%) there was no significant difference between CFU and MPN_CS counts (taking in consideration limit of confidence) and in 2 samples plateable *Mtb* exceeded those grown in liquid media.

Figure 3.6 Mycobacterial subpopulations present in extrapulmonary tuberculosis samples



MPN_CS bars topped with * indicate samples with dominating CS-dependent populations. The error bars indicate the 95% confidence intervals for the MPN values. Patterned bars- samples recovered only with CS supplementation. The CFU assay limit of detection for sample 12 is 33 cfu/ml; for all other samples is 10 cfu/ml. MPN assay limit of detection was 5 cfu/ml. †This sample was collected 10 days before the 2nd sample from patient 31. ‡ The 95% confidence interval for this sample crosses the x-axis and is not shown below it. Due to small sample volumes, only a single CFU assay technical replicate was performed per sample meaning 95% confidence intervals were not calculable

3.3.4 The diagnostic impact of culture supernatant supplementation

The combined sensitivity of the standard method (MPN_7H9 and CFU) was 36% and specificity 100% when compared to the CRS (see Table 3.3). The supplementation of liquid culture with Rpf-containing CS increased the combined sensitivity to 43% and specificity remained 100%. The overlapping of 95% confidence intervals indicated this increase was statistically not significant. Analysis by sample type indicated Rpf-containing CS improved sensitivity for lymph node by 7% and tissue biopsy culture by 25% although this was not statistically significant. Patients recruited into the study also had samples processed by the UHL-MD as part of routine clinical management. The combined sensitivity of MGIT and LJ culture was 64% and specificity 100% compared to the CRS.

Table 3.3 The impact of Rpf-containing CS on EPTB culture diagnostic performance

Method vs CRS		Lymph Nodes % (95% CI)	Tissue biopsies % (95% CI)	Fluids (%) 95% CI	Pus (%) 95% CI	Pooled Analysis (%) 95% CI
MPN_CS + CFU	Sens	40 (18-67)	63 (26-90)	25 (5-64)	55 (25-82)	43 (29-59)
	Spec	100 (31-100)	100 (31-100)	100 (20-100)	100 (20-100)	100 (66-100)
	PPV	100 (52-100)	100 (46-100)	100 (20-100)	100 (52-100)	100 (80-100)
	NPV	25 (7-57)	50 (14-86)	25 (5-64)	29 (5-70)	29 (15-47)
MPN_7H9 + CFU	Sens	33 (13-61)	38 (10-74)	25 (5-64)	55 (25-82)	36 (23-52)
	Spec	100 (31-100)	100 (31-100)	100 (20-100)	100 (20-100)	100 (66-100)
	PPV	100 (46-100)	100 (31-100)	100 (20-100)	100 (52-100)	100 (75-100)
	NPV	23 (6-54)	38 (10-74)	25 (5-64)	29 (5-70)	26 (14-43)
LJ + MGIT†	Sens	80 (51-95)	50 (18-83)	50 (17-83)	72 (39-93	64 (48-77)
	Spec	100 (31-100)	100 (31-100)	100 (20-100)	100 (20-100)	100 (66-100)
	PPV	100 (70-100)	100 (40-100)	100 (40-100)	100 (60-100)	100 (85-100)
	NPV	50 (14-86)	43 (12-80)	33 (6-76)	40 (7-83)	38 (21-59)

CSF is included in the pooled analysis but is not analysed separately as only 2 samples present. † LJ agar and MGIT culture performed at UHL-MD. Abbreviations: CFU – colony forming unit; CRS – composite reference standard LJ – Lowenstein-Johnson agar; MGIT – mycobacterial growth indicator tube; MPN_CS – most probable number assay (MPN) with 7H9 medium supplemented with Rpf-containing culture supernatant; MPN_7H9 – MPN assay with 7H9 medium; NPV – negative predictive value; PPV – positive predictive value; sens – sensitivity; spec – specificity

Table 3.4 The effect of storage conditions on culture diagnostic performance

		Refrigerated (n/t)	95% CI	Frozen (n/t)	95% CI	Decontaminated and Frozen (n/t)†	95% CI
MPN_CS+CFU	Sensitivity	71% (15/21)	48-88	40%(4/10)	14-73	0% (0/13)	0-28
	Specificity	100% (9/9)	63-100	n/a	n/a	100% (1/1)	5-100
MPN_7H9+CFU	Sensitivity	62% (13/21)	39-81	30%(3/10)	8-65	0% (0/13)	0-28
	Specificity	100% (9/9)	63-100	n/a	n/a	100% (1/1)	5-100

Storage conditions: refrigerated – storage at 4ºC; frozen – storage at -20ºC; decontaminated and frozen: decontamination as per standard protocol and storage at -20ºC. Abbreviations: n/t – number of total; n/a – not applicable

Analysis of pre-analytical sample handling (see Table 3.4) revealed that culture of those samples refrigerated at 4ºC prior to processing in MPN_CS & CFU exhibited a combined sensitivity of 71% for the diagnosis of TB using CRS as the reference standard (for detailed information of sample storage conditions see Appendices 8 and 9). Culture of those samples frozen to -20ºC prior to study inclusion exhibited a sensitivity of 40% and those decontaminated and frozen prior to study inclusion 0%. A similar effect was seen for culture in MPN_7H9 combined with CFU.

3.3.5 The impact of Rpf-containing CS on MGIT time-to-positivity.

14 samples from 13 patients were inoculated into MGIT_CS and MGIT_7H9. One sample was contaminated with fungi and discarded. Six paired MGITs were culture positive for *Mtb* (see Table 3.5). In two samples (patient 31 & H), *Mtb* was only grown in the MGIT_CS. The sample from patient H was negative by MPN_CS, MPN_7H9 and CFU assays. The isolate was typed and identified as a clinical strain (see Appendix 8). Two samples exhibited growth stimulation with CS and two - inhibition. Patient 51's sample was heavily blood stained and frequently flagged positive despite no growth seen on ZN staining potentially introducing error. There were no significant differences in TTP between MGIT_CS and MGIT_7H9 (p=0.42). There was no significant correlation between the CS-DCTB population calculated by growth assays and the differential TTP (p=0.22).

Table 3.5 The effect of culture supernatant supplementation on MGIT time to positivity.

Patient	MGIT_CS TTP	MGIT_7H9 TTP	Differential TTP
31†	15 days 19 hours	neg	pos CS MGIT
32	neg	neg	neg
36	neg	neg	neg
38	24 days 14 hours	38 days 3 hours	pos 13 days 13 hours
40	10 days 15 hours	10 days 8 hours	neg 7 hours
46	neg	neg	neg
50	neg	neg	neg
51*	21 days 23 hours	15 days 0 hours	neg 6 days 23 hours
52	23 days 18 hours	24 days 15 hours	pos 21 hours
53	neg	neg	neg
54	neg	neg	neg
B	neg	neg	neg
H‡	16 days 7 hours	neg	pos CS MGIT

* heavily blood stained; † later 2nd pleural fluid sample from patient 31; ‡ This sample was culture negative at UHL-MD

3.3.6 Measures of bacterial load and their correlation with host factors

MPN_CS counts were correlated with host and bacillary factors (see Table 4.8). Only the host peripheral blood lymphocyte count (PLC) correlated significantly the MPN_CS count ($p=0.04$). No other significant correlations were found. Similarly, no correlation between host and bacillary factors and either the MPN_7H9 or CFU bacillary count were found. Multivariate analysis was not performed.

Table 3.6 The correlation of host and bacillary factors to MPN_CS log_{10} bacterial counts.

Factors	Proportion n=18 n (%)	P value
Age (year, +/-SD)	38.6 (+/-14.3)	0.52
Gender		
Male	Reference	
Female	6 (33.3)	0.85
Weight (kg)[a]	64.5 (60-83)**	0.91
Ethnicity		
Indian subcontinent	Reference	
Afro-Caribbean	3 (16.7)	0.81
Habitual tobacco smoking	2 (11.1)	0.53
Habitual alcohol consumption	4 (22.2)	0.83
HIV type 1 infection	3 (16.7)	0.37
Anti-retroviral therapy	2 (11.1)	1.00
Vitamin D deficiency[b]	14 (82.3)	0.23
Haemoglobin (g/dl +/-SD)	12.1 (+/-15.2)	0.18
Neutrophils ($x10^9$ cells/L)	4.4 (3.4-5.9)**	0.20
Lymphocytes ($x10^9$ cells/L)	1.5 (1.0-2.0)**	**0.04**
Monocytes ($x10^9$ cells/L)	0.5 (0.4-0.6)**	0.31
Monocyte: Lymphocyte ratio	0.35 (0.28-0.45)**	0.11
Creatinine (µmol/l)	58 (51-62)**	0.88
C-reactive protein (mg/L)	87 (29-118)**	0.85
Primary disease site		
PTB +EPTB	Reference	
EPTB	12 (66.6)	0.85
Strain lineage		0.45
Sample storage temperature		
4°C	Reference	
-20 °C	4 (22.2)	0.92
Storage duration (days)	7 (5-9)**	0.97
Decontamination		
None	Reference	
H_2SO_4	9 (50.0)	0.45
NaOH	4 (22.3)	0.16
Granulomas visible upon histology[c]	9 (56.2)	0.29

Missing data: a=4; b=1; c=2.**Median value and interquartile range. List of abbreviations: EPTB – extrapulmonary tuberculosis; PTB – pulmonary tuberculosis; SD – standard deviation.

3.4 Discussion

In this pilot study, CS-DCTB were isolated from a range of anatomically and physiologically diverse sites ranging from colonic tissue to vertebral bone (see Appendix 9). This indicates CS-DCTB are not exclusive to sputum, rather their formation is a ready occurrence during the course of *Mtb* infection of the human host as exemplified by the presence of dominating CS-DCTB populations in 42% of EPTB culture positive samples.

By examining associations between host factors and MPN_CS bacillary counts I was able to demonstrate a significant positive correlation with the host peripheral blood lymphocyte count. This adds to the growing evidence that the host immune response plays a significant role in CS-dependent DCTB formation.

The addition of Rpf-containing CS to standard 7H9 liquid culture medium combined with solid 7H10 agar enabled the recovery of *Mtb* from three samples which would otherwise have yielded negative growth. Although this increased the sensitivity of standard culture, due to the small sample size no statistically significant effect was demonstrated.

3.4.1 EPTB disease results in CS-DCTB formation

Previous studies have demonstrated the presence of CS-dependent and Rpf-dependent *Mtb* bacilli (57, 58) in the sputum of humans suffering from PTB. Through the use of MPN and CFU growth assays I found dominating CS-dependent DCTB populations were present in EPTB disease. The range of anatomical sites sampled was broad (see Appendix 9) including intrathoracic and extrathoracic lymph nodes, vertebral bone, intestinal sites and pleural fluid. This evidence indicates that CS-dependent and Rpf-dependent bacilli are not exclusive to sputum, rather their formation is an important manifestation of *Mtb* infection and host interactions. This concurs with the findings of Turapov et al (318) who infected mice intranasally with *M. bovis* BCG and found that Rpf-dependent bacilli were formed by 2 weeks of infection whereas *ex vivo* incubation with lung homogenates and serum failed to induce their formation. Similarly, Manina et al found non-replicating but metabolically active *Mtb* bacilli as well as actively growing bacilli were generated during the course of wild type mouse infection whereas only actively replicating *Mtb* bacilli were found in IFNγ-deficient mice, so indicating the importance of the host immune response in generating bacterial phenotypic heterogeneity(319).

The proportion of EPTB samples containing CS-dependent DCTB was lower than that reported for in other studies examining DCTB populations in sputum (57, 58). Whether a

true difference in the formation of DCTB population exists between EPTB and PTB is uncertain as I did not recruit patients with PTB as a comparator group.

In this study, I did not elucidate CS-independent DCTB (where the number of organisms in MPN_7H9 is greater than in CS-supplemented MPN assays and similarly exceeds the plateable cell count). These were present in 1.8% of sputum samples in a study by Chengalroyen et al (58). This finding may simply reflect the small number of samples recruited into this pilot study. I noted the MPN_7H9 bacillary counts were less than either the MPN_CS and CFU counts in 42% (8/19) of samples. Mukamolova et al (57) noted the presence of inhibitory activity in sputum, reflected by MPN_7H9 counts lower than CFU counts which was abrogated by washing steps. Inhibitory activity present in samples may account for the lower MPN_7H9 counts however small sample volumes prevented further investigation.

There is accumulating evidence that the host immune response is a critical factor in formation of DCTB populations and the heterogeneity witnessed between hosts. I examined host and bacillary factors finding a significant positive correlation between the host peripheral blood lymphocyte count and the MPN_CS bacillary count. This finding supports the findings of others that the cellular immune response is a key determinant of the population proportions of CS-dependent bacilli (58, 318). Unfortunately, lymphocyte immunophenotyping was not routinely performed in the clinical setting thus I was unable to determine if specific lymphocyte populations were responsible for the observation observed. Additionally, due to the small sample size, I was unable to conduct multivariate to adjust for confounding as typically 10-15 observations per predictor are required (320). Factors such as diabetes mellitus (321) and smoking (322) adversely affect TB treatment responses. It was recently hypothesised that diabetes mellitus may affect TB treatment outcomes in part through inducing the formation of *Mtb* persister cells (323). To assess the effect of host factors on CS-DCTB populations, larger, sufficiently powered studies are required. Studies should also assess whether any improvement in TB treatment outcomes through the modification of host risk factors such as diabetes mellitus is mediated by modulation of DCTB populations.

3.4.2 Effect of culture supernatant on culture performance

Previous studies on sputum have demonstrated increased culture yield when 7H9 liquid media is supplemented with either recombinant Rpf or Rpf-containing CS and in some samples, this enabled the recovery of *Mtb* bacilli from samples that would otherwise be

negative by standard culture (57, 58). To date, the effect of culture supplementation with recombinant Rpf or Rpf-containing CS on culture sensitivity and specificity has not been evaluated within a diagnostic trial. I compared standard 7H9 liquid media and 7H10 solid agar with 7H9 supplemented with Rpf-containing CS and standard 7H10 using a composite reference standard (315). Overall CS increased the sensitivity of culture by 7% and when analysed by sample type, lymph node exhibited a 7% increase in sensitivity and tissue biopsies 28% whilst specificity remained 100% (see table 4.5). However, the 95% confidence intervals of supplemented and unsupplemented cultures overlapped indicating this was not a statistically significant finding.

This pilot study was not designed to determine definitively whether CS improves the sensitivity of culture. Whilst the findings are not statistically significant, for 3 samples, *Mtb* bacilli were recovered from otherwise culture negative samples using CS. This is impacts patient management as phenotypic drug susceptibility testing can be performed so TB-treatment can be tailored to patient management and typing of isolates may reveal chains of transmission. Larger appropriately powered studies are required to delineate the precise impact of CS on culture diagnostic performance.

Currently there are significant barriers to the routine use of CS and recombinant Rpf in the clinical diagnostic microbiology laboratory. The production, freeze drying and storage of CS and its reconstitution for use (see Appendix 3) are laborious. Furthermore, CS contains *Mtb* DNA complicating WGS direct from positive cultures. Recombinant Rpf is unstable and difficult to produce (57). Until these issues can be resolved, the routine use of Rpf or CS within in the clinical microbiology laboratory setting is not feasible.

3.4.3 Mycobacterium growth indicator tubes

In the present study, the effect of Rpf-containing CS on the TTP of MGIT culture was compared by the simultaneous inoculation of two MGITS, one supplemented with CS. The inoculation of MGITs was performed at UoL and transported to UHL-MD. These were designated UN3373 category A specimens and approval for this section of the project was delayed. Consequentially, only 14 EPTB samples were available for this section of the study of which 6 were culture positive for *Mtb*. I found the effect of CS on the MGIT TTP to be inconsistent. For one sample from patient 38, TTP was reduced by 2 weeks, one from patient 51 raised by 1 week and for two others the effect was negligible. For the remaining two samples, only MGIT_CS was culture positive consistent with findings from MPN and CFU assays that for some samples, CS enables the recovery of *Mtb* from otherwise culture negative samples. Given the very small sample size, it is impossible to draw any

conclusion on the impact of CS on MGIT performance. Differences in MGIT performance may simply reflect the irregular distribution of mycobacteria in clinical samples or mycobacterial clumping and consequentially differences in the number of bacilli inoculated into MGIT_CS and MGIT_7H9. Future studies should be powered to detect a clinical meaningful reduction in MGIT TTP and consequentially whether it is worthwhile pursuing the development of an easy to use stable recombinant form of Rpf. Determination of what constitutes a clinically meaningful reduction in TTP will require a review with TB clinicians and potentially modelling to determine the level at which a reduction in TTP improves patient outcomes. In light of the effect of sample decontamination and freezing (see Table 4.6) on *Mtb* recovery, all samples should be frozen in 10% glycerol after decontamination which Turapov et al have demonstrated to cryopreserve DCTB populations (309).

3.4.4 Study limitations

There were several limitations to this pilot study. Firstly, the external validity of the study was restricted. The study was conducted in a first world setting with Indian subcontinent and Black African ethnic patients recruited from a single tertiary centre. Secondly, the standard culture method employed in clinical diagnostic laboratories includes liquid culture in MGIT often with concurrent culture on solid agar. In this study, the impact of CS on culture diagnostic performance was assessed using MPN assays to represent liquid culture and 7H10 agar representing solid culture which may not be equivalent and thus may bias the assessment of CS. Thirdly, as this study was conducted by a sole investigator in a single centre over 18 months, the number of patients available for recruitment was low. This limited the ability of the study to detect significant differences between standard culture and culture supplemented with CS and whether CS improved performance in specific sample types. Furthermore, when correlations between MPN_CS counts and host and bacillary variables were examined, multivariate analysis was not possible to adjust for confounding. Fourthly, the sample volume obtained from EPTB patients was often small. This is because invasive sampling is often required to obtain clinical specimens in patients with EPTB and repeated biopsying may lead to increased patient morbidity. Small sample volumes precluded multiple technical replicates of CFU assays and consequentially deriving mean values and 95% confidence intervals. Fifthly, some patients were recruited into the study after their diagnostic procedure had already been performed. Some samples therefore had to be retrieved from the UHL-MD laboratory. However, the handling of these samples was detrimental to the optimal recovery of *Mtb*. For example, some were

either frozen to -20ºC or decontaminated as per national guidance and then frozen to -20ºC. As shown in Appendix 10, 9 samples decontaminated and subsequently inoculated into MGITs and on LJ agar slopes were culture positive for *Mtb* in UHL-MD whereas aliquots of decontaminated samples that were then frozen to -20ºC were all culture negative in this study. Sixthly, I did not have access to CS from *Mtb* from which all 5 *rpf* had been deleted. Subsequent experiments by Chengalroyen et al (58) demonstrated the presence of Rpf-independent DCTB in sputum samples and the inclusion of this study may have revealed additional complexity to DCTB populations in EPTB specimens. In retrospect, I should have developed and validated an assay to quantify CS activity to ensure the absence of resuscitation was due to the lack of a CS-dependent DCTB population rather than an inactive batch of culture supernatant. Finally, there was the potential for observer bias. As I had frequent contact with patients during my clinical work, I was aware of their likelihood of having TB potentially influencing my handling and processing of the growth assays and subsequent interpretation of sensitivity & specificity.

3.5 Conclusion

In this pilot study, I found that CS-dependent DCTB are common in patients with EPTB affecting diverse anatomical sites such as the colon and cervical spine. This evidence indicates that CS-DCTB are formed as a consequence of infection of the human host by *Mtb* rather than being a phenomenon restricted to human sputum. The host peripheral lymphocyte count correlated with MPN_CS bacillary counts highlighting the suspected role of the host immune response in modulating host CS-DCTB proportions. It is hypothesised that host DCTB populations may affect response to TB treatment and the subsequent risk of relapse although currently there is little firm clinical data to support this contention. Monitoring of DCTB populations currently requires the use of highly laborious growth assays which require many weeks until a final result is achieved. The development of host biomarkers of DCTB populations may enable a quick determination of host DCTB burden, facilitate the monitoring of DCTB population dynamics during treatment and potentially eliminate the need for repeat sampling, difficult in EPTB. The host PLC represents a candidate biomarker of host CS-DCTB populations and warrants assessment in a larger trial. It has been proposed in the past that immunomodulators may reduce persister formation and may abrogate host DCTB formation (18). Future studies should examine whether modulation of host immune responses may minimise DCTB formation although noting that in we found in a case control study (see Chapter 2) that taking

immunosuppressant medication was associated with an increased risk of subsequent TB recurrence in multivariate analysis. Further advances in the understanding of the interactions between the host immune system and *Mtb* may permit such an intervention.

The impact of adding Rpf-containing CS to 7H9 culture medium was assessed within a clinical diagnostic trial for the first time. Rpf-containing CS increased the sensitivity of EPTB culture whilst maintaining specificity although as this was a pilot study, it was underpowered to detect any statistically significant differences. This study does highlight that adding Rpf-containing CS to standard culture enables to the recovery of *Mtb* from samples that would otherwise be culture negative by the 'standard method' which permits the detection of drug resistance and isolate strain typing. The effect of CS supplementation of MGIT liquid culture was inconclusive although in one sample TTP was reduced by approximately 2 weeks. These findings suggest that the methods used in this pilot study are feasible and Rpf-containing CS demonstrates sufficient effect that is worth following up in an adequately powered larger study. However, there are barriers to the introduction of CS or recombinant Rpf into the routine clinical microbiology laboratory. CS requires storage at -80ºC, takes time to prepare and contains *Mtb* H37Rv DNA; recombinant Rpf are unstable and difficult to produce (57). A durable easy-to-use form of recombinant Rpf should be pursued alongside further a larger clinical diagnostic trial so that use in the routine clinical diagnostic microbiology laboratory is feasible.

3.6 Future Directions

Based upon the findings of this pilot study, a larger clinical trial is warranted to test the possible effect of Rpf-containing CS on culture diagnostic performance. To improve the external validity, this should be multicentre including patients from a wide range of settings. The 'real world' impact of Rpf-containing CS should be assessed using standard BACTEC MGITs and solid agar used in the routine clinical diagnostic laboratory rather than extrapolating diagnostic performance from growth assays. When examining DCTB populations in samples, a validated assay to adjust for differences in the 'resuscitation activity' of CS batches should be considered, to in effect quality control each CS batch. Furthermore, only samples that have been stored at 4ºC should be used and if storage is required, samples must be frozen in 10% glycerol after decontamination. If Rpf-containing CS or Rpf demonstrate statistically and clinically significant effects in a larger trial, it may be worth combining with rapid culture methods such as MODS or TLA.

The host PLC was identified in this pilot trial as a candidate biomarker of host CS-DCTB population proportion however peripheral blood lymphocyte immunophenotyping should be undertaken to see if one specific population underpins the observed association. This may also provide insights into the generation of DCTB populations in the host. Furthermore, multivariate regression analysis of associations between other host factors and DCTB populations should be performed to see if variables such as diabetes or bacterial strain type impact DCTB formation.

Chapter 4: Final discussion and conclusions

In this thesis, I describe two studies examining specific issues of TB diagnosis and treatment, two areas highlighted by the WHO as requiring further innovation to improve TB control (1): 1) a nested case control-study of TB recurrence in Leicester; 2) a clinical diagnostic accuracy study examining the change in EPTB culture diagnostic performance with Rpf-containing CS supplementation.

The understanding of TB recurrence in the UK is incomplete (180). The nested case control study was undertaken with the objectives of: 1) describing the epidemiology of TB recurrence in Leicester; 2) identifying specific at-risk groups; 3) measuring the proportion of TB recurrence due to relapse and reinfection. I found that the level of TB recurrence in Leicester was low, in accordance with other published data from the UK (143, 145, 180). This study identified that non-white ethnic groups, those who smoke tobacco, patients on immunosuppressive treatment and those with ADRs are specific at-risk groups, information lacking prior to this study. Furthermore, the study showed that relapse is the predominant mechanism of recurrence in Leicester, typical for low incidence settings as shown by a systematic review arising from this thesis (248). The findings from this study should be taken forward by a number of parties: basic and clinical science researchers should look into the underlying host and mycobacterial factors which predispose these at-risk groups; public health authorities should examine these findings with a view to incorporating TB recurrence as part of an overall TB control strategy; physicians managing TB patients identified as at-risk of recurrence should factor this information into decisions of treatment duration, planned follow up and addressing concomitant comorbidities.

The diagnosis of EPTB has been identified as a major challenge, particularly due to the poor sensitivity of diagnostic tests (298). Previously published papers describe the recovery of *Mtb* from otherwise culture negative samples with the use of recombinant Rpf or Rpf-containing CS (57, 58). I examined the impact of EPTB culture supplementation with Rpf-containing CS with the objectives of: 1) quantifying the effect on culture sensitivity and specificity; 2) evaluating the effect on MGIT TTP; 3) measuring CS-dependent *Mtb* bacillary counts by MPN and CFU assays; 4) correlating host factors with CS-dependent bacillary counts. The study demonstrated the presence of CS-dependent DCTB populations from diverse anatomical sites including the colon and vertebrae. This finding shows that DCTB populations are formed as a consequence of infection of the host rather than a phenomenon limited to the respiratory tract. Previous studies have implicated

the host immune response in the modulation of host DCTB populations (58, 318), which is supported by the finding of a significant association between MPN_CS bacillary counts and the PLC. The present pilot study was underpowered to look for associations with host factors in multivariate analysis. As CS-dependent and Rpf-dependent DCTB are implicated in the requirement for extended treatment for TB (309) and relapse (310), a greater understanding of the host factors which modulate DCTB populations may ultimately prove therapeutically useful. Future studies should examine the host factors modulating DCTB populations and whether their modification abrogates DCTB formation. The study showed that the sensitivity of EPTB culture was increased by supplementation with Rpf-containing CS, although this was not statistically significant. No effect on MGIT TTP was elicited although sample numbers were small. Assuming a stable form of Rpf can be developed, this work should be furthered within larger clinical studies and potentially combined with other techniques seeking to improve culture diagnostic performance.

In conclusion, the work undertaken for this thesis has extended our understanding of mycobacterial subpopulations in EPTB, highlighted the impact of the host immune response and shown that stimulation of CS-dependent DCTB subpopulations has the potential to improve culture sensitivity. Those groups at-risk of TB recurrence were identified in Leicester which should provoke research into understanding why these groups are susceptible and how to best intervene therapeutically to prevent future TB episodes. The results provided in this thesis can be used by both basic scientists and clinicians seeking to improve TB diagnosis and treatment

Appendix 1: Multiple imputation diagnostics

1.1 Missing data

1.1.1 Missing data summary

Figure A1.1 Missing data summary prior to imputation of missing values

				Obs<.		
Variable	Obs=.	Obs>.	Obs<.	Unique values	Min	Max
Weight	30		216	58	9	96
ukborn	4		242	2	0	1
Tobacco use	8		238	2	0	1
Alcohol use	12		234	2	0	1
AFB Smear	29		217	2	0	1
Mtb culture	34		212	2	0	1
Susceptibility	97		149	2	0	1
hb	23		223	73	7.5	16.2
wcc	24		222	92	2.2	30.1
Creatinine	23		223	70	31	906

1.1.2 Missing data pattern

Figure A1.2 Pattern of missing data prior to imputation of missing values

Missing-value patterns
(1 means complete)

Percent	Pattern 1	2	3	4	5	6	7	8	9	10
45%	1	1	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1	0
5	1	1	1	1	1	1	0	1	0	0
4	1	1	1	1	1	1	1	0	1	0
4	1	1	1	1	1	1	1	0	1	1
2	1	1	1	0	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	0	0
2	1	0	0	1	1	1	1	1	1	1
2	1	1	1	0	0	0	0	1	0	0
2	0	1	1	1	1	1	1	1	1	1
1	1	1	1	1	0	0	1	1	1	1
<1	1	1	0	1	1	1	1	1	1	1
<1	1	1	1	0	0	0	0	0	0	0
<1	1	1	1	0	0	0	1	0	1	1
<1	1	1	1	0	0	0	1	1	0	0
<1	1	1	1	0	0	0	1	1	1	0
<1	1	1	1	0	0	0	1	1	1	1
<1	1	1	1	1	0	0	0	1	0	0
<1	1	1	1	1	1	1	0	0	0	0
<1	1	1	1	1	1	1	0	1	1	1
<1	1	0	0	0	1	1	1	0	1	1
<1	1	0	0	1	1	1	1	0	1	1
<1	1	0	0	1	1	1	1	1	1	0
<1	1	1	0	1	1	1	0	1	0	0
<1	1	1	0	1	1	1	1	1	1	0
<1	1	1	1	0	1	0	0	0	0	0
<1	1	1	1	1	0	0	0	1	1	1
<1	1	1	1	1	0	0	1	1	0	0
<1	1	1	1	1	0	0	1	1	1	0
<1	1	1	1	1	1	1	0	0	1	0
100%										

Variables are:
(1) ukborn (2) Tobacco use (3) Alcohol use (4) Creatinine (5) hb
(6) wcc (7) AFB Smear(8) Weight (9) Mtb culture (10) Susceptibilty

1.2 Distribution of imputed continuous variables

Figure A1.3 Distribution of data for the variable 'Haemoglobin'

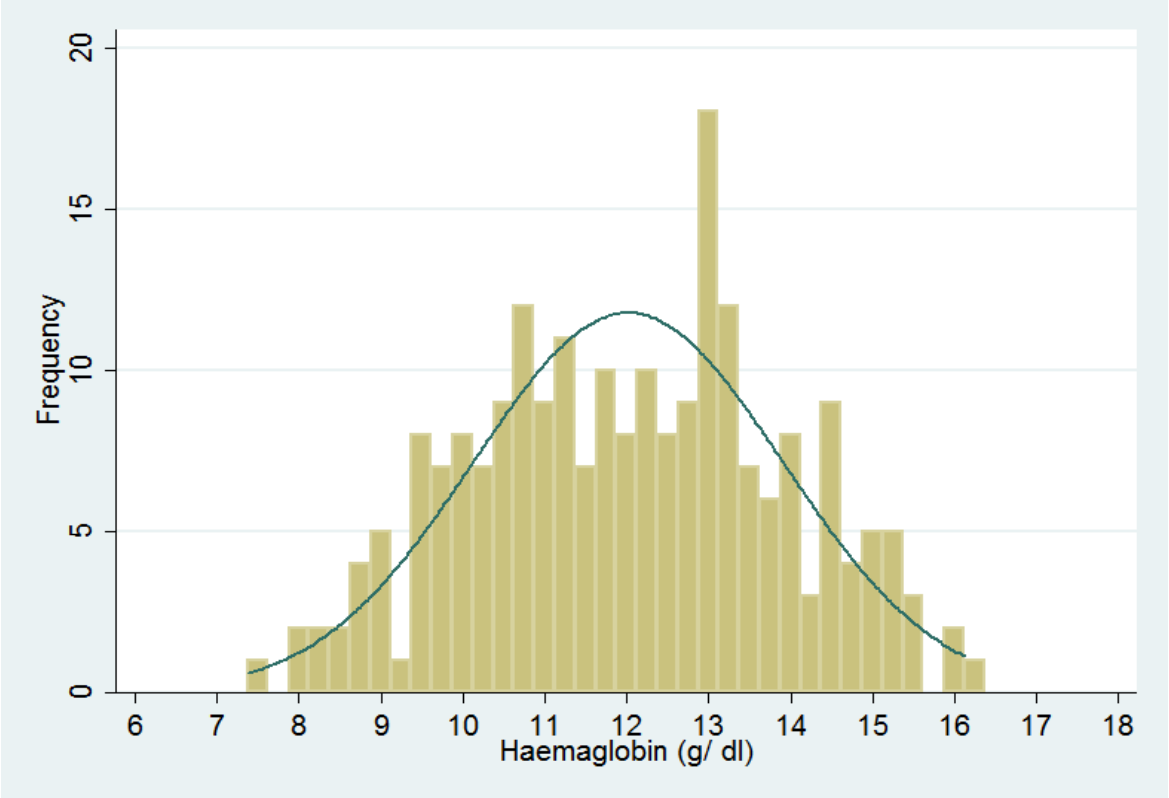


Figure A1.4 Distribution of data for the variable 'Weight'

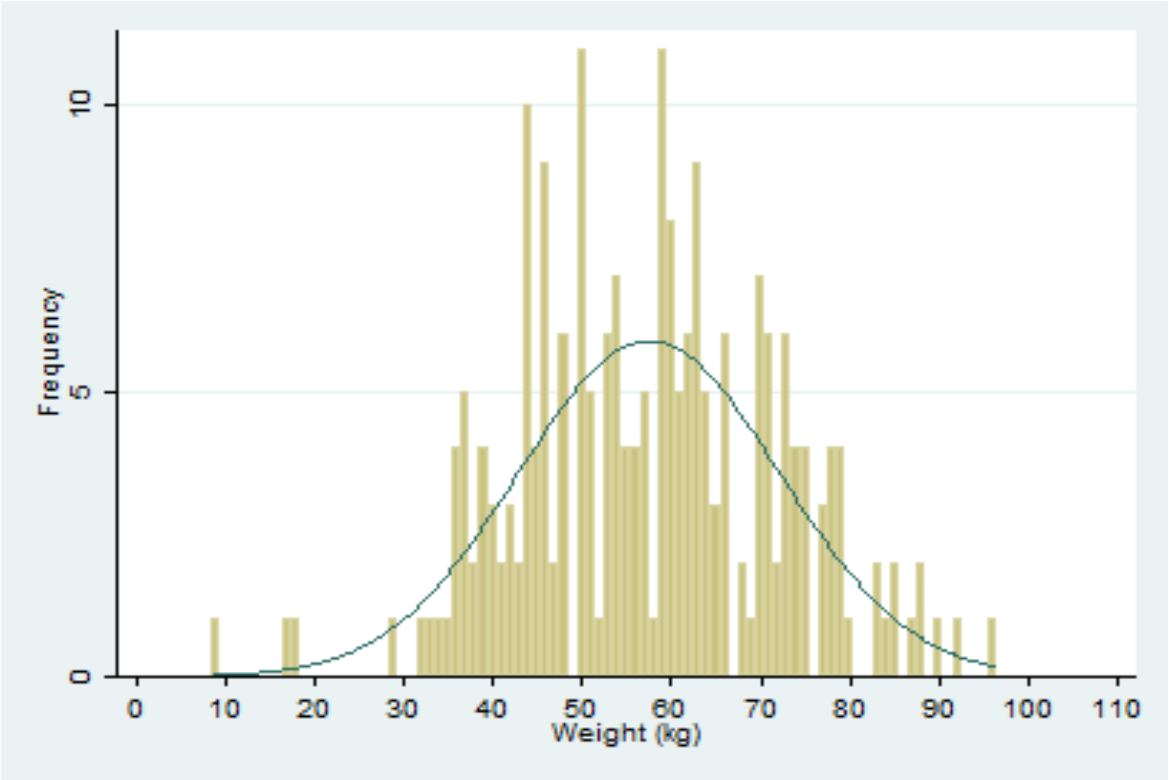


Figure A1.5 Distribution of data for the variable ‘White Cell Count’

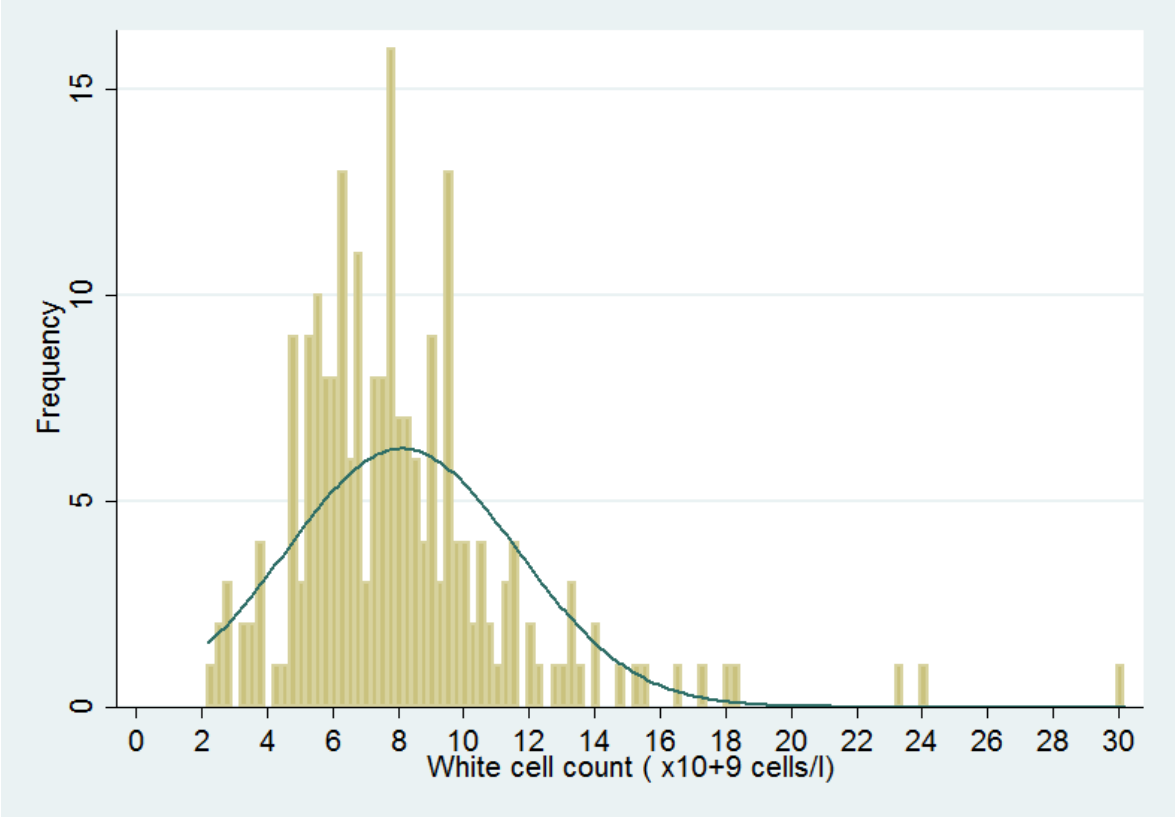
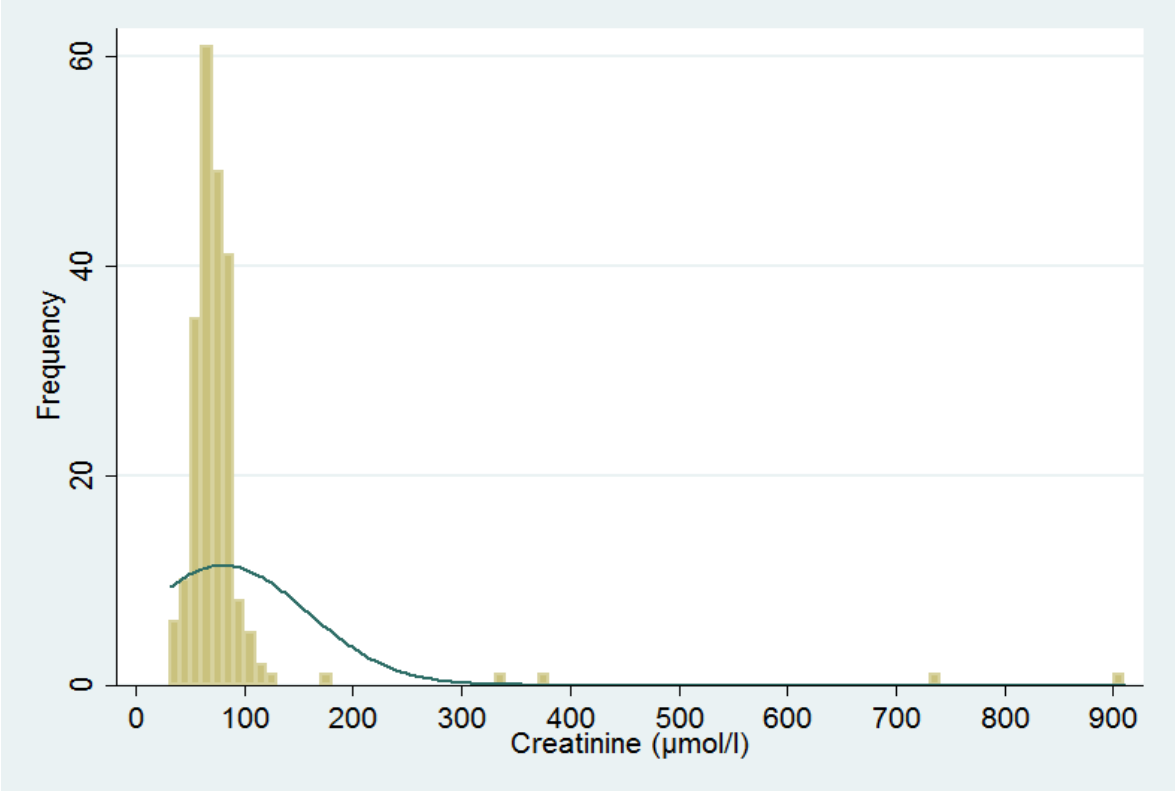


Figure A1.6 Distribution of data for the variable ‘Creatinine’



1.3 Multiple Imputation Code

1.3.1 Stata command

mi impute chained (pmm, knn (10)) wcc creatinine (pmm, knn (3)) weight (regress) hb (logit, augment) afbsmear mtbculture alcoholuse smoker ukborn sensorres= recurrence age sex housed i.ethnicgrpcat immcompdis diabetes hivpos i.tbsitecat cxrcavitate immunosuprx standardrx treatinterupt drugreact, add (60) dots rseed (2323) burnin (100) savetrace(r1)

1.3.2. Multiple imputation output

Figure A1.7 Stata output generated from imputation of variables missing data

```
Multivariate imputation                      Imputations =       60
Chained equations                                 added =       60
Imputed: m=1 through m=60                       updated =        0

Initialization: monotone                     Iterations =     6000
                                                burn-in =      100
```

```
                 wcc : predictive mean matching
          Creatinine : predictive mean matching
              Weight : predictive mean matching
                  hb : linear regression
          AFB Smear  : augmented logistic regression
          Mtb culture : logistic regression
          Tobacco use : augmented logistic regression
          Alcohol use : augmented logistic regression
              ukborn : augmented logistic regression
          Susceptibility : augmented logistic regression
```

	Observations per m			
Variable	Complete	Incomplete	Imputed	Total
wcc	222	24	24	246
Creatinine	223	23	23	246
Weight	216	30	30	246
hb	223	23	23	246
AFB smear	217	29	29	246
Mtb culture	212	34	34	246
Alcohol use	234	12	12	246
Tobacco use	238	8	8	246
ukborn	242	4	4	246
Susceptibility	149	97	97	246

(complete + incomplete = total; imputed is the minimum across m of the number of filled-in observations.)

1.4. Post imputation analysis

1.4.1 Pre and post imputation mean and SD

Table A1.1 The pre and imputation mean and standard deviation values

	Pre-imputation mean	Pre-imputation SD	Pre-imputation min	Pre-imputation max	Post-imputation mean	Post-imputation SD	Post-imputation min	Post-imputation max
Weight	57.5046	14.6385	9	96	57.6728	14.6132	9	96
UK-born	0.25619	0.43743	0	1	0.25907	0.43900	0	1
Tobacco use	0.16386	0.37093	0	1	0.17188	0.37800	0	1
Alcohol use	0.15811	0.36563	0	1	0.16443	0.37135	0	1
AFB smear	0.29032	0.45496	0	1	0.28428	0.45191	0	1
***Mtb* culture**	0.70283	0.45809	0	1	0.69241	0.46227	0	1
Susceptibility	0.08724	0.28315	0	1	0.12418	0.32993	0	1
Hb	12.0143	1.89091	7.5	16.2	11.9839	1.91027	7.07261	16.43
WCC	8.11441	3.53061	2.2	30.1	8.12124	3.51845	2.2	30.10
Creatinine	80.6636	77.9091	31	906	79.7456	74.4140	31	906

1.4.2 Trace plots

Trace plots from the imputation of continuous variables are displayed. The first twenty (of sixty) imputation traces are presented for clarity.

Figure A1.8 Imputation trace plot for variable 'Creatinine'

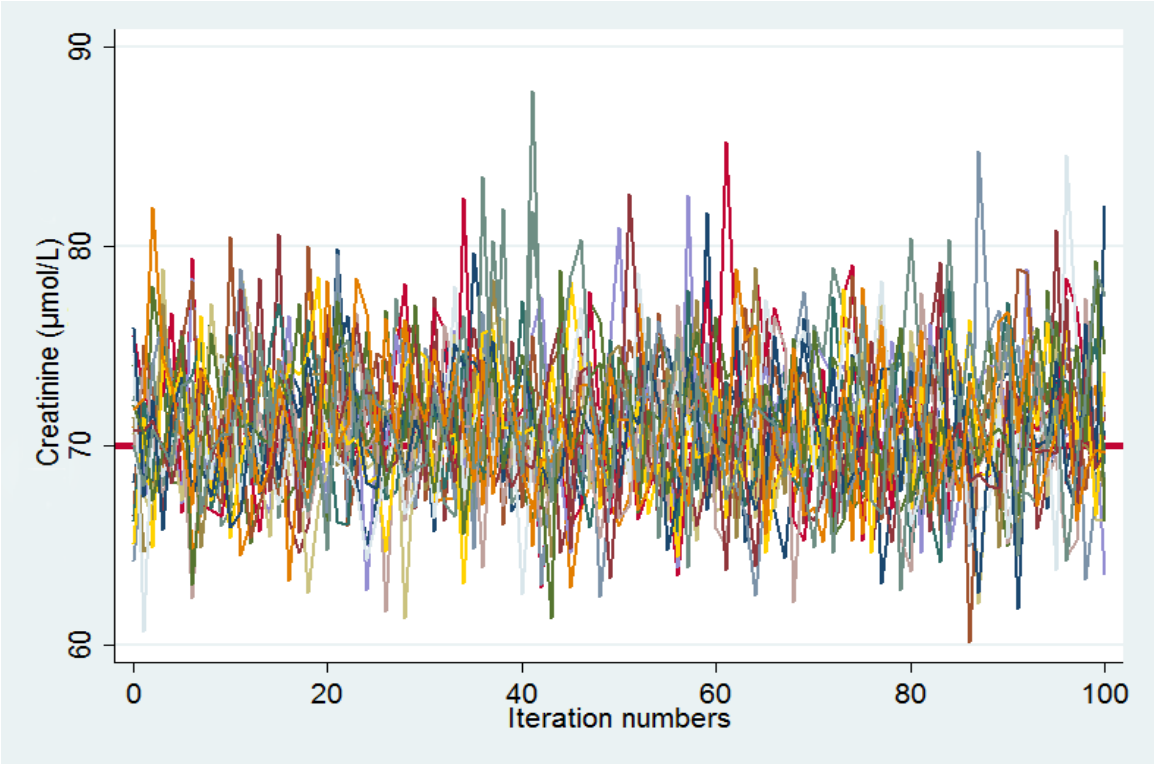


Figure A1.9 Imputation trace plot for variable 'Haemoglobin'

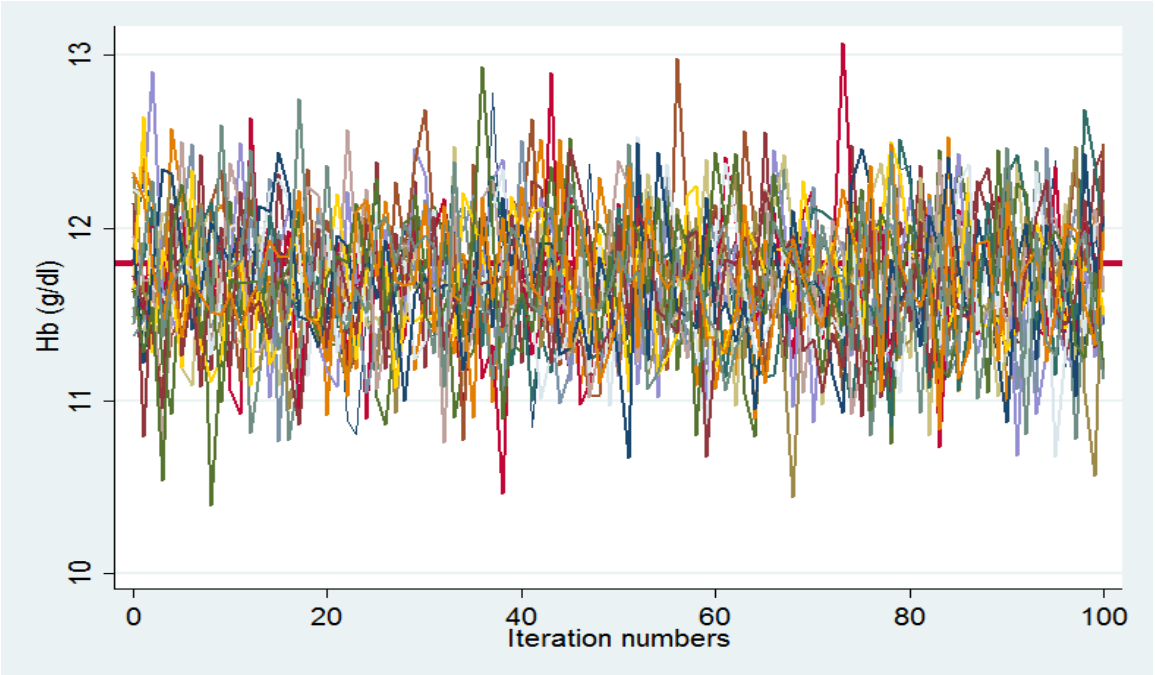


Figure A1.10 Imputation trace plot for variable 'White Cell Count'

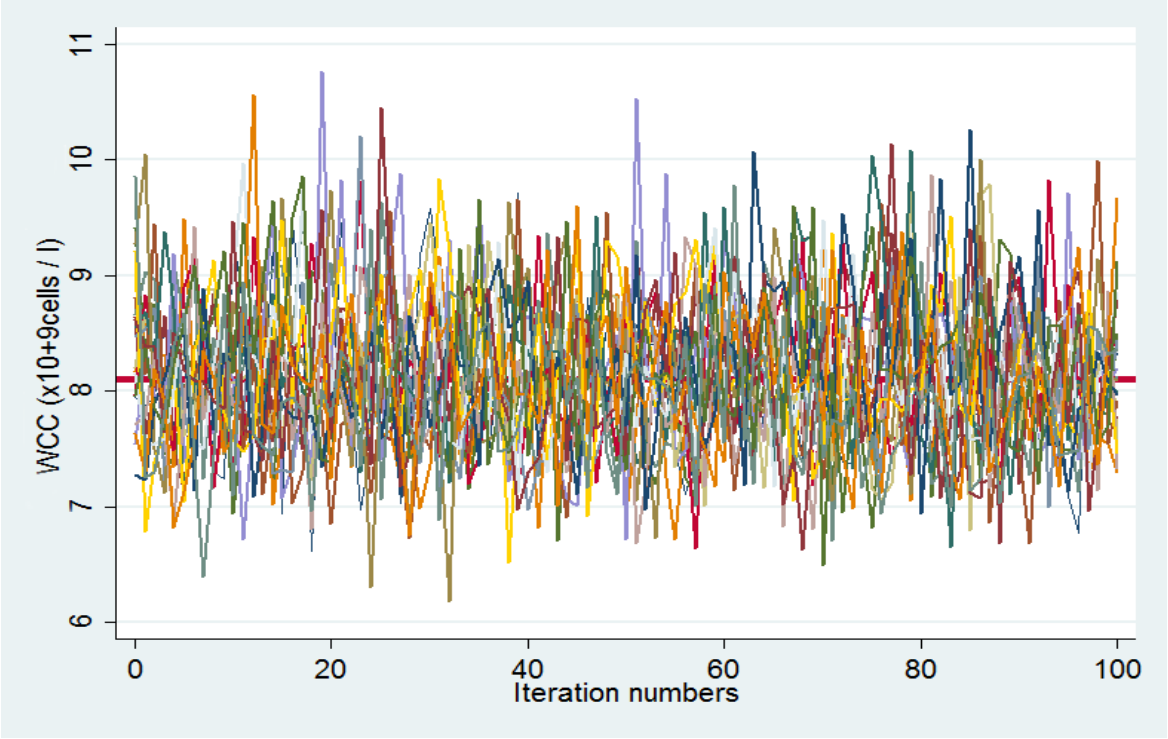
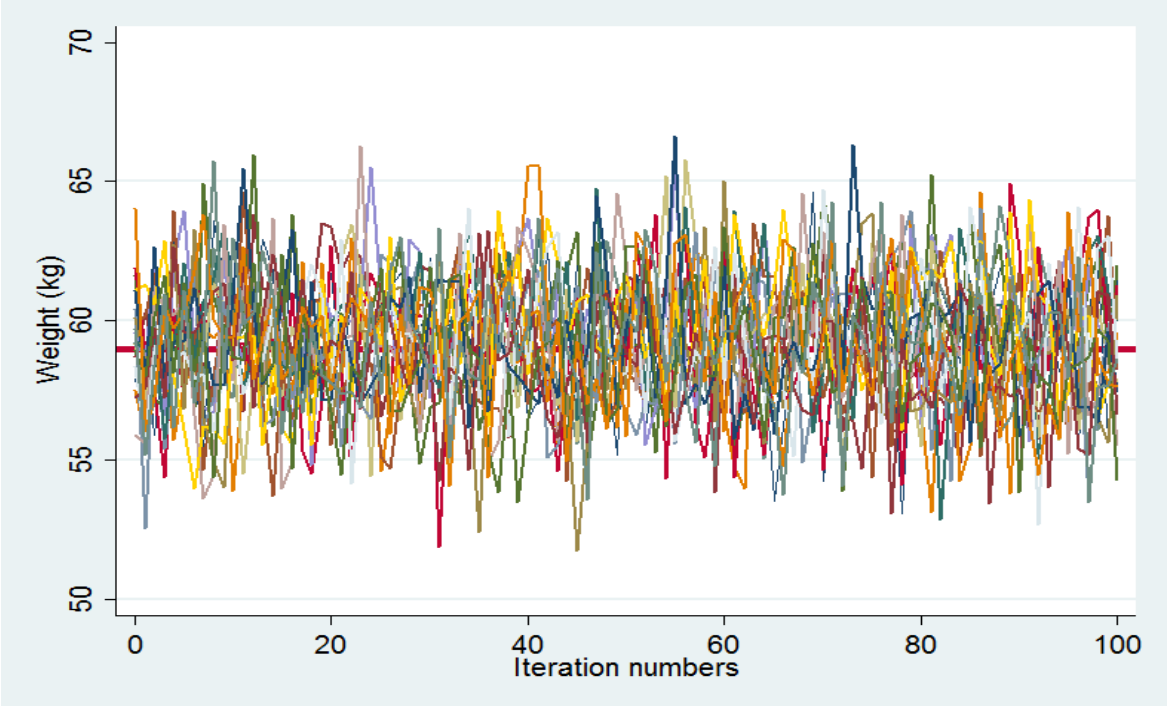


Figure A1.11 Imputation trace plot for variable 'Weight'



There are no apparent trends for any of the chains in the trace plots of the imputed continuous variables Creatinine, Haemoglobin, White Cell Count, and Weight. The chains

appear to oscillate around the average value suggesting convergence of the algorithm (324).

1.4.3 Kernel Density Plots

The Kernel Density plots for four imputed continuous variables are presented, showing good evidence of convergence, twenty of sixty imputations are displayed for clarity.

Figure A1.12 Kernel Density plot of imputations 1-20 for variable 'Creatinine'

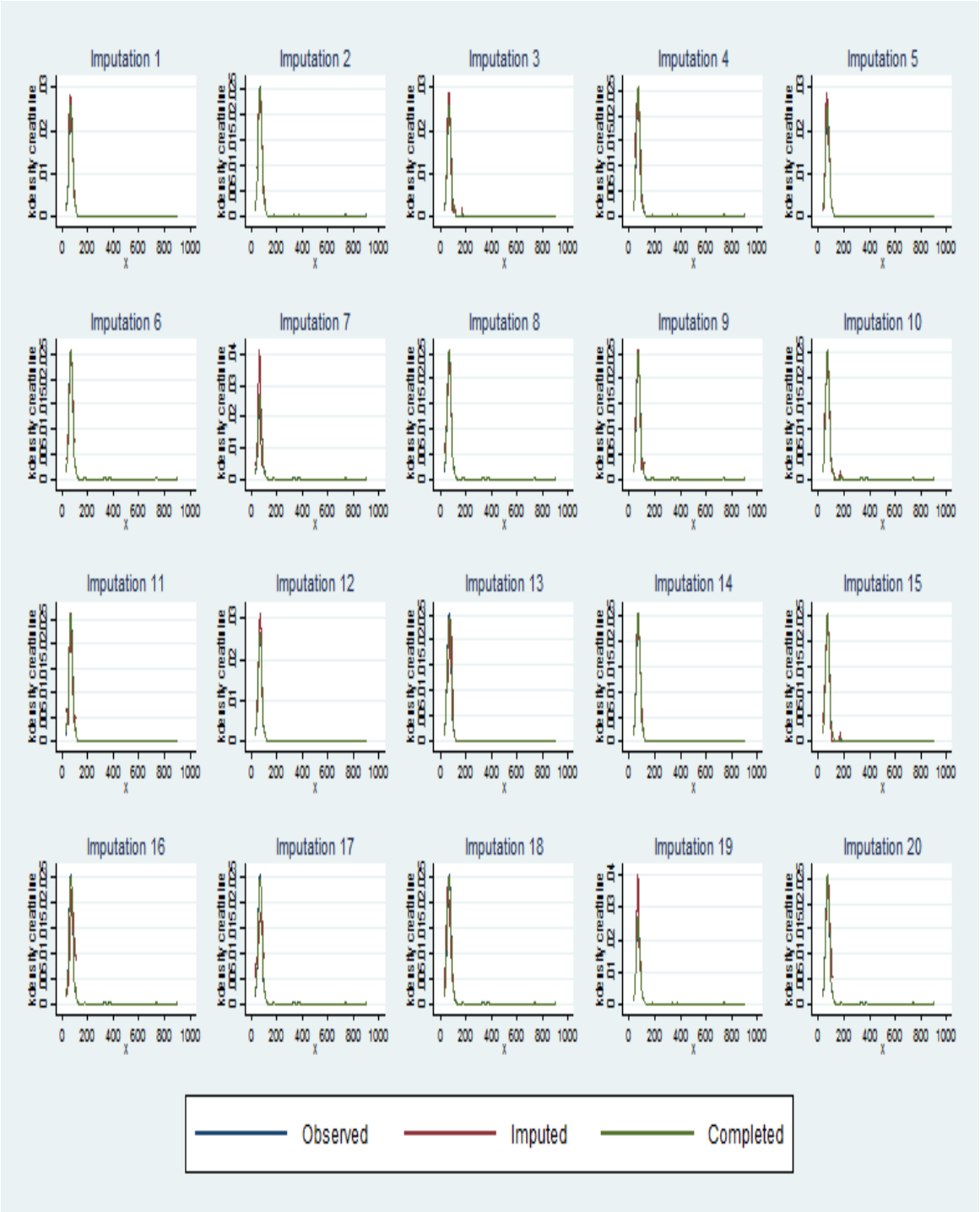


Figure A1.13 Kernel Density plot of imputations 1-20 for variable 'Hb'

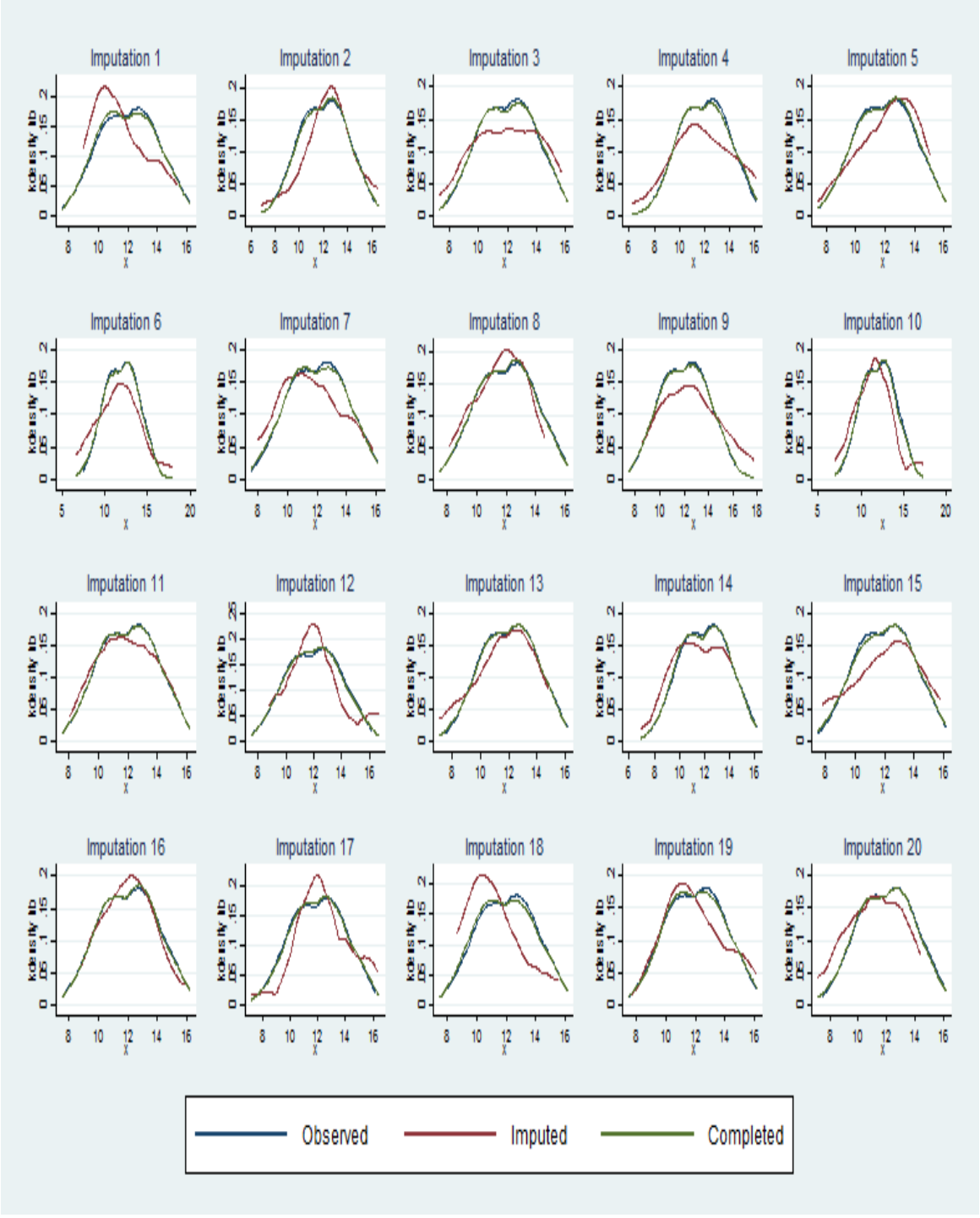


Figure A1.14 Kernel Density plot of imputations 1-20 for variable ‘White Cell Count’

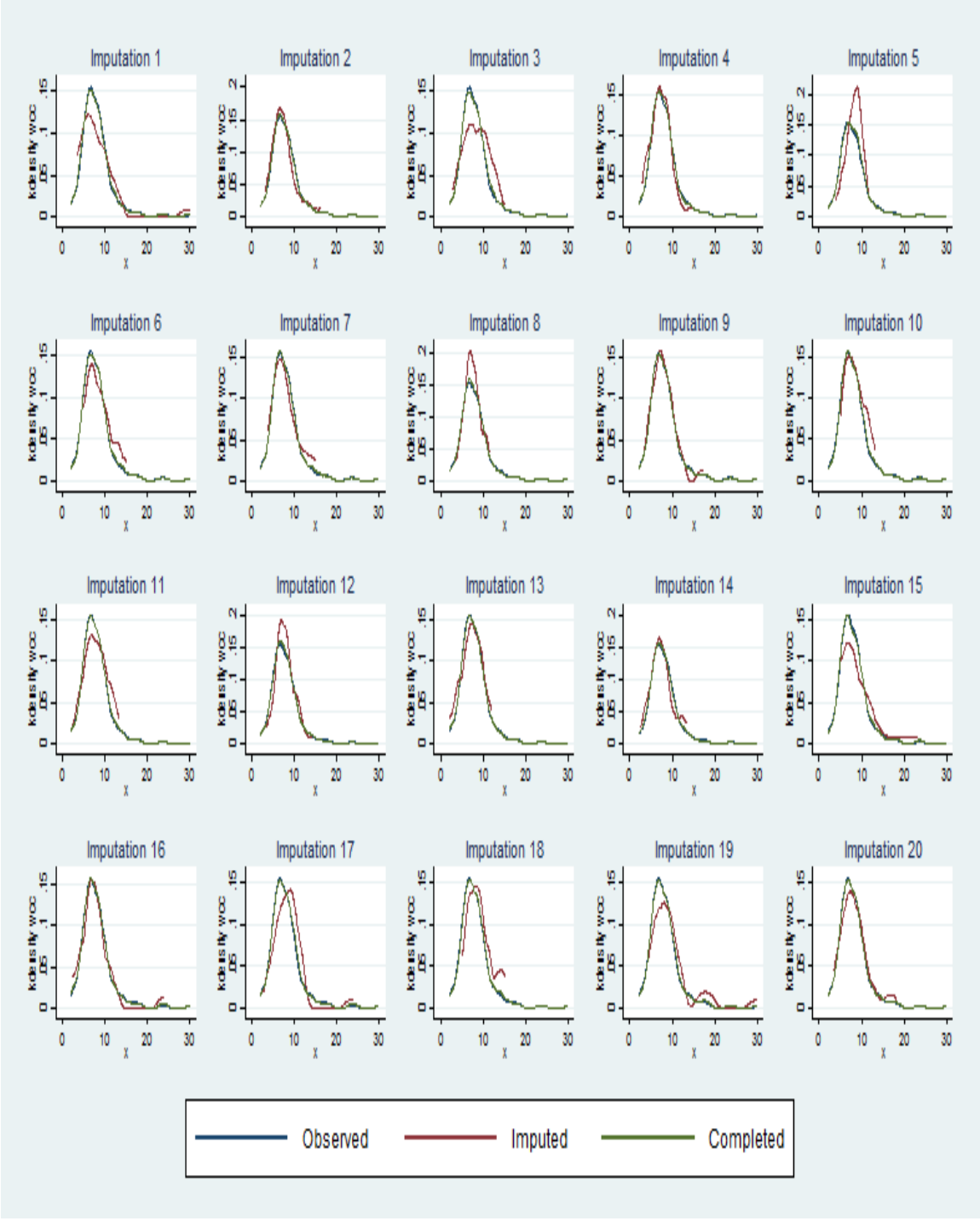
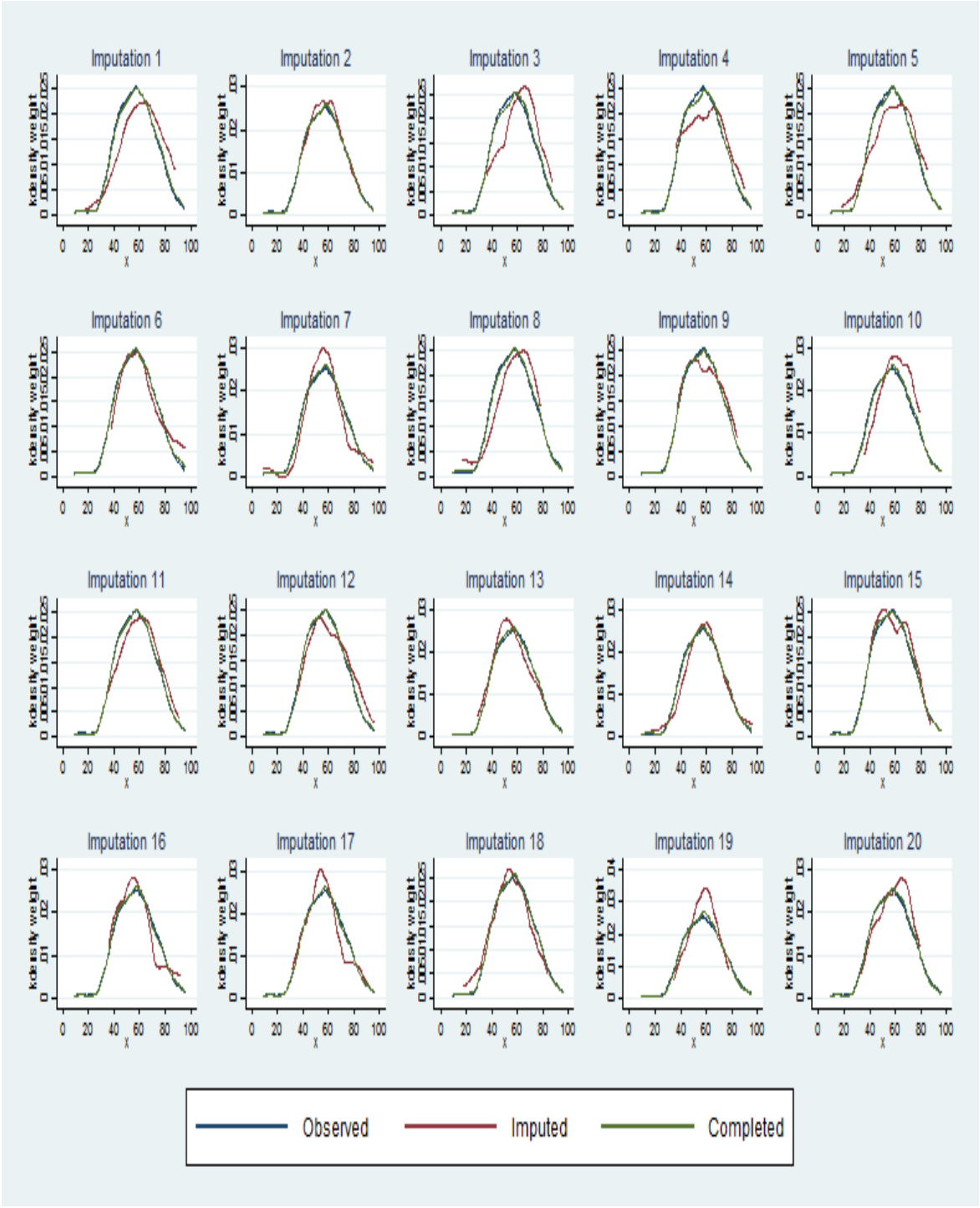


Figure A1.15 Kernel Density plot of imputations 1-20 for variable 'Weight'



1.5 Conditional logistic regression diagnostics

1.5.1 Variance inflation factors

Table A1.2 The variance inflation factors for variables considered for multivariate conditional logistic regression analysis

Variable	VIF	1/VIF
Ethnicity (South Asian)	3.29	0.3
Ethnicity (Afro-Caribbean)	2.88	0.35
UK-Born	2.02	0.49
Tobacco Use	1.96	0.51
Drug reaction	1.85	0.54
Site of disease (EPTB)	1.75	0.57
Other comorbidities	1.65	0.61
AFB smear	1.64	0.61
Sex	1.63	0.61
Creatinine	1.61	0.62
Site of Disease (PTB + EPTB)	1.61	0.62
Treatment pause	1.58	0.63
Weight	1.56	0.64
Age	1.54	0.65
Hb	1.53	0.65
Alcohol use	1.52	0.66
Standard Treatment	1.47	0.68
HIV seropositive	1.41	0.71
CXR Cavitation	1.41	0.71
Drug resistance	1.38	0.72
WCC	1.37	0.73
Ethnicity (Other)	1.33	0.75
***Mtb* culture**	1.26	0.79
Immunosuppressive drugs	1.26	0.79
Diabetes mellitus	1.19	0.84
Housed	1.09	0.92
Mean VIF	1.65	

1.5.2 Table of dropped variables

Table A1.3 Variables dropped from the multivariate conditional logistic regression analysis due to suspected multiple collinearity

Variable	Univariate analysis	P value	Multivariate analysis	P value
Treatment pause	1.07(0.44-2.64)	0.877	0.32 (0.07-1.43)	0.134
Standard treatment	0.66(0.32-1.35)	0.256	1.23(0.34-4.30)	0.741
Conditions predisposing to active tuberculosis	1.0 (0.29-3.50)	1.00	0.38 (0.05-2.69)	0.333

Appendix 2: MIRU-VNTR data for case control study

Table A2.1 MIRU-VNTR genotyping data of paired isolates

Type of recurrence	Lineage	Episode	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	Miru 02	Miru 10	Miru 16	Miru 20	Miru 23	Miru 24	Miru 26	Miru 27	Miru 39	Miru 40	Mtub04	Mtub21	QUB-11b	Mtub29	Mtub30	Mtub34	Mtub39	QUB-26	QUB-4156
Relapse	Delhi / CAS	1st	4	2	2	3	5	2	6	4	2	5	1	7	3	3	3									
		2nd	4	2	2	3	5	2	6	4	2	5	1	7	3	3	3									
Relapse	Delhi / CAS	1st	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
		2nd	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
Relapse	Delhi / CAS	1st	-	2	2	3	5	2	5	4	2	5	1	7	3	3	4									
		2nd	-	2	2	3	5	2	5	4	2	5	1	7	3	3	4									
Relapse	Delhi / CAS	1st	4	2	2	3	4	2	6	4	2	5	1	7	3	2	3									
		2nd	4	2	2	3	4	2	6	4	2	5	1	7	3	2	3									
Relapse	Delhi / CAS	1st	4	2	2	3	5	2	5	4	2	5	1	7	3	2	3									
		2nd	4	2	2	3	5	2	4	4	2	5	1	7	3	2	3									
Reinfection	EAI & EAI	1st	5	6	2	2	6	2	6	3	2	6	2	2	3	1	3									
		2nd	4	7	2	5	6	2	6	3	2	6	2	2	3	1	3									
Relapse	Delhi / CAS	1st	2	2	2	3	5	2	8	6	2	3	1	7	3	3	2	4	4	2	4	2	3	3	8	4
		2nd	2	2	2	3	5	2	8	6	2	3	1	7	3	3	2	4	4	2	4	2	3	3	8	4
Relapse	Beijing	1st	4	2	4	3	5	2	3	3	2	5	1	7	3	3	3									
		2nd	4	2	4	3	5	2	3	3	2	5	1	7	-	3	3	4	4	-	4	4	3	3	8	2
Reinfection	Delhi / CAS	1st	4	2	2	3	5	2	4	5	2	5	1	7	3	3	4	4	4	2	4	2	3	3	1	4
		2nd	4	2	2	3	5	2	4	5	2	5	1	7	3	3	-	4	4	2	4	2	3	3	7	4
Relapse	Delhi / CAS	1st	4	2	2	3	5	2	8	4	2	5	1	7	3	3	2	3	4	2	4	2	3	3	7	4
		2nd	4	2	2	3	5	2	-	4	2	5	1	7	3	3	2	3	4	2	4	2	3	3	7	4

Table A2.2 Remaining MIRU-VNTR genotyping data of paired isolates

Type of recurrence	Lineage	Episode	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	Miru 02	Miru 10	Miru 16	Miru 20	Miru 23	Miru 24	Miru 26	Miru 27	Miru 39	Miru 40	Mtub04	Mtub21	QUB-11b	Mtub29	Mtub30	Mtub34	Mtub39	QUB-26	QUB-4156
Relapse	Delhi / CAS	1st	4	2	2	3	5	2	6	4	2	5	1	3	3	3	3									
		2nd	4	2	2	3	5	2	6	4	2	5	1	3	3	3	3									
Relapse	EAI	1st	-	4	2	6	5	2	4	3	2	6	2	2	3	1	3									
		2nd	-	4	2	-	5	2	4	3	2	6	2	2	3	1	3	2	-	3	3	2	3	7	4	1
Relapse	Delhi / CAS	1st	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
		2nd	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4	4	3	2	4	2	2	2	5	4
Relapse	Delhi / CAS	1st	3	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
		2nd	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
Relapse	Delhi / CAS	1st	4	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
		2nd	-	2	2	3	4	2	7	4	2	5	1	1	3	3	4									
Relapse	EAI	1st	4	2	4	5	4	2	5	3	2	6	2	2	3	3	5									
		2nd	4	2	4	5	4	2	5	3	2	6	2	2	3	3	5									
Reinfection	EAI & EAI	1st	6	1	4	6	5	2	4	2	2	6	2	2	3	3	4									
		2nd	7	1	4	4	4	2	4	2	2	5	2	2	3	3	4									
Relapse	Delhi / CAS	1st	-	2	2	3	4	2	7	5	2	5	1	7	4	3	3									
		2nd	-	2	2	3	4	2	7	5	2	5	1	7	4	3	3	4	4	-	4	4	3	3	8	2
Relapse	EAI	1st	4	4	2	6	5	2	4	3	2	6	2	2	3	1	3									
		2nd	4	4	2	6	5	2	4	3	2	6	2	2	3	1	3									

Appendix 3 Indications for immunosuppression

Table A3.1 Indications for treatment with immunosuppressants

Tuberculosis recurrence	Corticosteroid used	Immunosuppressant used	Stated indication for drug treatment
Yes	Yes		Wheeze (GP initiated prior to TB diagnosis)
Yes	Yes		ATT-induced urticaria
Yes	No	Methotrexate	Comorbid psoriatic arthropathy
Yes	Yes		Wheeze (GP initiated prior to TB diagnosis)
Yes	Yes		Colitis suspected before TB diagnosed; paradoxical reaction
Yes	Yes		Comorbid ulcerative colitis
Yes	Yes	Infliximab	Comorbid rheumatoid arthritis
Yes	Yes		Wheeze (GP initiated prior to TB diagnosis)
Yes	No	Methotrexate and cyclosporine	Psoriasis
Yes	Yes		Tuberculosis meningitis
Yes	Yes		Spondyloarthropathy suspected before TB diagnosed
Yes	Yes		Brittle asthma
Yes	Yes		Prevention of ureteric stricture in a renal TB case
Yes	Yes		Suspected Addison's disease
Yes	Yes		TB IRIS
Yes	No	Methotrexate and cyclosporine	Rheumatoid arthritis suspected before TB diagnosed
Yes	Yes		Not stated
No	Yes		Pleural TB
No	Yes	Cyclophosphamide	Granulomatosis with polyangiitis suspected before TB diagnosed
No	Yes		Unknown
No	Yes		Wheeze (GP initiated prior to TB diagnosis)
No	Yes		Chronic obstructive pulmonary disease
No	Yes		Disseminated TB
No	Yes		Wheeze (GP initiated prior to TB diagnosis)
No	Yes		Ocular TB
No	Yes		Suspected Addison's disease
No	Yes		Addison's disease
No	Yes	Leflunomide	Comorbid rheumatoid arthritis
No	Yes	Tacrolimus	Solid organ transplant
No	Yes		Addison's disease
No	Yes		Tuberculoma
No	Yes		Tuberculoma
No	Yes		Slow to resolve pleural TB

Abbreviations: ATT - Anti-tuberculous therapy; GP - General practitioner; IRIS - Immune reconstitution inflammatory syndrome

Appendix 4: Preparation of freeze dried culture supernatant

4.1 Culture supernatant production

CS preparation is performed within a class 1 biological safety cabinet in a biosafety level 3 laboratory. *Mtb* H37Rv stock culture is inoculated into Middlebrook 7H9 medium supplemented with 10% (vol/vol) OADC supplement (BD Biosciences) and 0.05% (wt / vol) Tween 80 (manufacturer) in roller bottles (manufacturer). Cultures are incubated at 37ºC in the roller incubator (Wheaton, Millville, USA) until an optical density at 600nm of 0.6-0.9 indicating mid exponential phase is obtained. At this point, *Mtb* culture from each roller bottle is aliquoted into 50ml sterile falcon tubes (manufacturer) using a 25ml disposable plastic pipette (manufacturer). A maximum of 30ml is allocated to each tube. The tubes are centrifuged for 15min at 2000 x g.

4.2 Culture supernatant filtration

A cartridge A 0.22 μm filter is placed between the vacuum pump and the disposable filtration unit to prevent contamination of the pump. The pump is switched on and using a 25ml plastic pipette, supernatant is transferred from each Falcon tube to the reservoir of the filtration unit. The first volume is allowed to pass through the reservoir to detect any set up issues. The pellet is not to be disturbed in the Falcon tube. The filtration procedure is repeated using a second filter unit resulting in twice filtered CS. The CS is checked for sterility by incubation of 5ml for 8 weeks at 37ºC with shaking.

4.3 Freeze drying

Figure A4.1 Freeze drying machine

The CS is then transferred aseptically to sterile falcon tubes with 0.22µm filter. These are placed in an AdVantage 2.0 BenchTop Freeze Dryer (SP Scientific, Warminster, USA) at -40ºC. This process takes between 2-4 weeks depending upon ambient temperature. At the end of drying, the material is a light brown colour (dark brown indicates that the CS is spoiled). The end product is transferred and stored in the -80ºC freezer

Appendix 5: 24 locus MIRU-VNTR primer sequences by locus

Table A5.1 MIRU-VNTR forward and reverse primer sequences by locus

Locus	Sequence (5'-3')
ETR-A	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
ETR-B	GCGAACACCAGGACAGCATCATG GGCATGCCGGTGATCGAGTGG
ETR-C	GTGAGTCGCTGCAGAACCTGCAG GGCGTCTTGACCTCCACGAGT
ETR-D	CAGGTCACAACGAGAGGAAGAGC GCGGATCGGCCAGCGACTCCTC
ETR-E	CTTCGGCGTCGAAGAGAGCCTC CGGAACGCTGGTCACCACCTAAG
MIRU 2	TGGACTTGCAGCAATGGACCAACT TACTCGGACGCCGGCTCAAAAT
MIRU 10	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
MIRU 16	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
MIRU 20	TCGGAGAGATGCCCTTCGAGTTAG GGAGACCGCGACCAGGTACTTGTA
MIRU 23	CTGTCGATGGCCGCAACAAAACG AGCTCAACGGGTTCGCCCTTTTGTC
MIRU 24	CGACCAAGATGTGCAGGAATACAT GGGCGAGTTGAGCTCACAGAA
MIRU 26	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
MIRU 27	TCGAAAGCCTCTGCGTGCCAGTAA GCGATGTGAGCGTGCCACTCAA
MIRU 39	CGCATCGACAAACTGGAGCCAAAC CGGAAACGTCTACGCCCCACACAT
MIRU 40	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
Mtub04	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC
Mtub21	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
QUB-11b	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
Mtub29	GCCAGCCGCCGTGCATAAACCT AGCCACCCGGTGTGCCTTGTATGAC
Mtub30	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA
Mtub34	GGTGCGCACCTGCTCCAGATAA GGCTCTCATTGCTGGAGGGTTGTAC
Mtub39	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
QUB-26	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCGAT
QUB-4156	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT

Appendix 6: 24 locus MIRU-VNTR number of repeats by locus

Table A6.1 MIRU-VNTR numbers of repeats by locus

Locus	0	1	2	3	4	5	6	7	8	9	10
ETR A	195	270	345	420	495	570	645	720	795	870	945
ETR B	121	178	235	292	349	406	463	520	577	634	691
ETR C	44	102	160	218	276	334	392	450	508	566	624
ETR D	56	133	210	287	364	441	518	595	672	749	826
ETR E	65	118	171	224	277	330	383	436	489	542	595
Miru 02	402	455	508	561	614	667	720	773	826	879	932
Miru 10	482	535	588	641	694	747	800	853	906	959	1012
Miru 16	565	618	671	724	777	830	883	936	989	1042	1095
Miru 20	437	514	591	668	745	822	899	976	1053	1130	1207
Miru 23	148	201	254	307	360	413	466	519	572	625	678
Miru 24	395	449	503	557	611	665	719	773	827	881	935
Miru 26	285	336	387	438	489	540	591	642	693	744	795
Miru 27	498	551	604	657	710	763	816	869	922	975	1028
Miru 39	540	593	646	699	752	805	858	911	964	1017	1070
Miru 40	354	408	462	516	570	624	678	732	786	840	894
Mtub04	537	588	639	690	741	792	843	894	945	996	1047
Mtub21	92	149	206	263	320	377	434	491	548	605	662
QUB-11b	67	136	205	274	343	412	481	550	619	688	757
Mtub29	335	392	449	506	563	620	677	734	791	848	905
Mtub30	247	305	363	421	479	537	595	653	711	769	827
Mtub34	326	380	434	488	542	596	650	704	758	812	866
Mtub39	272	330	388	446	504	562	620	678	736	794	852
QUB-26	163	274	385	496	607	718	829	940	1051	1162	1273
QUB-4156	563	622	681	740	799	858	917	976	1035	1094	1153

The values indicate the size of the amplicon in base pairs and correspond to the number of repeats as designated in the first row of the table

Appendix 7: MIRU-VNTR typing of reference laboratory compared to UoL

Table A7.1 MIRU- VNTR typing for Birmingham reference laboratory compared to UoL

Patient	Typing	Lineage	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	Miru 02	Miru 10	Miru 16	Miru 20	Miru 23	Miru 24	Miru 26	Miru 27	Miru 39	Miru 40	Mtub04	Mtub21	QUB-11b	Mtub29	Mtub30	Mtub34	Mtub39	QUB-26	QUB-4156
12	UoL	EAI	6	4	4	6	5	2	5	3	2	6	2	2	3	2	2	2	6	3	2	1	3	3	3	1
	Ref Lab	EAI	-	4	4	6	5	2	5	3	2	6	2	2	3	2	2	2	6	3	3	1	3	4	3	1
15	UoL	Beijing	4	-	4	3	5	2	3	2	2	5	1	6	4	3	3	4	4	-	4	4	3	3	7	2
	Ref Lab	Beijing	-	2	3	4	5	2	3	2	2	5	1	6	3	3	3	4	4	6	4	4	3	3	7	2
18	UoL	Haarlem / S	2	2	4	3	4	3	3	4	2	5	1	5	3	2	2	2	2	2	4	2	3	3	5	2
	Ref Lab	Haarlem	2	2	4	3	3	2	3	4	2	5	1	5	3	2	2	2	2	2	4	2	3	3	5	2
23	UoL	EAI	9	5	4	6	3	2	5	4	1	6	2	3	4	4	4	3	6	-	3	2	3	6	8	1
	Ref Lab	EAI	1	5	4	-	3	2	4	3	1	6	2	2	3	3	3	2	-	-	3	2	3	6	-	1
36	UoL	Delhi / CAS	4	2	2	3	5	2	5	2	2	5	1	7	3	3	3	2	5	2	4	2	4	3	6	2
	Ref Lab	Delhi / CAS	4	2	2	3	5	2	6	2	2	5	1	7	3	3	3	2	5	2	4	2	4	3	6	2
40	UoL	Delhi / CAS	4	2	2	3	6	2	6	4	2	4	1	7	3	2	2	3	5	2	4	4	3	3	8	2
	Ref lab	Delhi / CAS	4	2	2	3	6	2	6	4	2	5	1	7	3	2	3	4	5	2	4	4	3	3	8	2
49	UoL	Delhi / CAS	4	2	2	3	3	2	6	4	2	5	1	4	3	3	4	3	4	1	4	2	3	3	8	2
	Ref Lab	Delhi / CAS	4	2	2	3	3	2	6	4	2	5	1	5	3	3	4	3	4	1	4	2	3	3	8	3
51	UoL	Delhi / CAS	-	2	2	3	5	2	4	3	2	5	1	8	4	4	3	-	4	-	4	2	3	3	7	5
	Ref Lab	Delhi / CAS	-	2	2	3	5	2	4	3	2	5	1	7	3	3	3	4	4	-	4	2	3	3	7	4
53	UoL	S	4	2	4	4	3	2	3	4	2	5	1	5	3	2	5	3	1	4	4	2	3	3	8	2
	Ref lab	S	4	2	4	4	2	2	3	3	2	5	1	5	3	2	5	3	1	4	4	2	3	3	-	2

Cells highlighted in yellow indicate differences between Birmingham reference laboratory and UoL typing results. List of abbreviations: CAS – Central African Strain; EAI – East Africa Indian; UoL – University of Leicester; ref lab – reference laboratory.

Appendix 8: Study isolates MIRU-VNTR typing results

Table A8.1 Results of UoL MIRU-VNTR typing of study isolates

Patient	Lineage	Distance	ERT-A	ETR-B	ETR-C	ETR-D	ETR-E	Miru 02	Miru 10	Miru 16	Miru 20	Miru 23	Miru 24	Miru 26	Miru 27	Miru 39	Miru 40	Mtub04	Mtub21	QUB-11b	Mtub29	Mtub30	Mtub34	Mtub39	QUB-26	QUB-4156
12	EAI	0.33	6	4	4	6	5	2	5	3	2	6	2	2	3	2	2	2	6	3	2	1	3	3	3	1
15	Beijing	0.24	4	\	4	3	5	2	3	2	2	5	1	6	4	3	3	4	4	\	4	4	3	3	7	2
16	H37RV	0.08	3	3	4	3	3	3	3	2	2	6	1	3	3	2	1	2	2	5	4	2	3	5	5	2
17	Turkey	0.33	3	2	4	3	4	3	5	3	2	5	1	1	3	2	2	1	3	2	4	4	3	3	8	3
18	Haarlem / S	0.38	2	2	4	3	4	3	3	4	2	5	1	5	3	2	2	2	2	2	4	2	3	3	5	2
21	Delhi / CAS	0.29	3	2	2	3	5	2	6	3	2	5	1	5	3	3	3	4	4	2	4	2	3	3	6	3
23	EAI	0.44	9	5	4	6	3	2	5	4	1	6	2	3	4	4	4	3	6	\	3	2	3	6	8	1
27	Delhi / CAS	0.38	4	2	2	3	5	2	6	5	2	5	1	8	4	4	3	2	2	2	4	2	3	3	8	1
28	Delhi / CAS	0.42	4	2	2	3	5	2	5	5	2	3	1	6	2	2	1	2	4	2	5	2	3	3	9	2
29	H37RV	0.08	3	3	4	3	3	3	3	2	2	6	1	3	3	2	1	2	2	5	4	2	3	5	5	2
31§	Haarlem	0.46	3	3	4	3	2	2	4	3	1	5	1	6	3	2	1	3	2	4	4	4	2	2	5	3
31*	Haarlem	0.46	3	3	4	3	2	2	4	3	1	5	1	6	3	2	1	3	2	4	4	4	2	2	5	3
32	EAI	0.5	10	5	2	6	5	2	6	3	2	6	2	2	3	0	3	1	8	6	2	2	3	4	3	1
36	Delhi / CAS	0.25	4	2	2	3	5	2	5	2	2	5	1	7	3	3	3	2	5	2	4	2	4	3	6	2
48	Delhi / CAS	0.32	\	2	2	1	3	1	6	4	2	5	1	6	3	3	2	3	4	\	4	2	3	3	8	4
40	Delhi / CAS	0.38	4	2	2	3	6	2	6	4	2	4	1	7	3	2	2	3	5	2	4	4	3	3	8	2
48	EAI	0.29	6	4	3	6	5	1	4	3	2	6	2	2	3	2	2	1	5	3	3	1	3	4	6	1
49	Delhi / CAS	0.29	4	2	2	3	3	2	6	4	2	5	1	4	3	3	4	3	4	1	4	2	3	3	8	2
51	Delhi / CAS	0.38	\	2	2	3	5	2	4	3	2	5	1	8	4	4	3	\	4	\	4	2	3	3	7	5
52	H37Rv	0.25	3	2	4	3	3	2	3	4	2	6	1	5	3	2	2	2	2	4	4	2	3	5	5	2
53	S	0.17	4	2	4	4	3	2	3	4	2	5	1	5	3	2	5	3	1	4	4	2	3	3	8	2
H	Delhi / CAS	0.42	3	2	2	3	5	2	5	5	2	5	1	4	3	3	2	8	4	2	4	2	2	3	9	3
Lab isolate	H37Rv	0.08	3	3	4	3	3	3	3	2	2	6	1	3	3	2	1	2	2	5	4	2	3	5	5	2

§ - First isolate from patient 31; * - Second isolate from patient 31; Abbreviation list: CAS – Central African Strain; EAI – East African Indian.

Appendix 9: Culture positive samples laboratory & processing data

Table A9.1: Culture positive sample data

Patient number	Sample type	Storage duration (days)[#]	Storage (°C)[#]	Sample amount	Decon[‡] method	CS Batch	Sample to ATT (days)	Duration of incubation (days)	AFB smear	UHL culture result	CRS
12	T9 bone Bx	5	4	Few bone fragments	None	1	1	104	Neg	Pos	Proven
15	L axillary LN	7	4	Sliver of tissue	H_2S0_4	1	10	84	Neg	Pos	Proven
16	Pleural tissue	2	4	Sliver of tissue	None	1	n/a	103	Neg	Neg	Not TB
17	Spinal abscess	6	4	1ml	H_2S0_4	1	1	49	Neg	Pos	Proven
18	L axillary LN	4	4	2 FNA cores	None	1	1	61	Neg	Pos	Proven
21	R cervical abscess	2	4	1ml	H_2S0_4	1	21	84	Neg	Pos	Proven
23	R cervical abscess	8	4	5ml	H_2S0_4	1	2	91	Neg	Pos	Proven
27	Pleural tissue	6	4	Sliver of tissue	H_2S0_4	1	7	85	Neg	Pos	Proven
28	Psoas abscess	6	4	5ml	H_2S0_4	1	1	85	Neg	Pos	Proven
29	ILN station 7R	1	4	1 FNA core	NaOH	2	n/a	85	Neg	Neg	Not TB
31	Pleural fluid	2	4	10ml	None	2	12	84	Neg	Neg	Proven
31	Pleural fluid	0	4	5ml	None	2	12	84	Neg	Pos	Proven
32	C3 spinal abscess	5	4	5ml	H_2S0_4	2	2	84	Neg	Pos	Proven
36	ILN station 4R	9	4	2 FNA cores	NaOH	3	0	109	Neg	Pos	Proven
38	Colon tissue	7	4	4 slivers of tissue	NaOH	3	-1	109	Neg	Pos	Proven
40	Pleural tissue	13	-20	1ml mashed up tissue	None	3	6	84	Neg	Pos	Proven
48	ILN station 4R	7	4	1ml saline & floating tissue	NaOH	3	1	84	Neg	Pos	Proven
49	ILN station 7	15	-20	5ml saline & floating tissue	NaOH	3	15	84	Neg	Pos	Proven
51	Ileocaecal abscess	42	-20	5ml	H_2S0_4	3	0	104	Neg	Pos	Proven
52	Vertebral Bx	9	4	Few bone fragments	None	3	5	97	Neg	Neg	Prob TB
53	Mediastinal LN	71	-20	5ml saline & floating tissue	?NaOH	2	22	84	Neg	Pos	Proven

- storage conditions and duration before sample enrolled into the study; ‡ - standard LN decontamination by H_2SO_4 method; EBUS LN by NaOH; abbreviations: Bx – biopsy; ILN – intrathoracic lymph node; LN – lymph node;

Table A9.1 continued…

Patient number	MPN_CS*	MPN_CS SD*	MPN_7H9*	MPN_7H9 SD*	CFU*	Resuscitation index	Inhibition Index	CS-dependent DCTB$^{\Delta}$	Lineage	Distance†
12	2.9	0.18	<0.7	n/a	<1.5	>1.4	n/a	1.5 $^{\Pi}$	EAI	0.33
15	1.4	0.26	1.7	0.27	3.8	-2.4	-2.1	0§	Beijing	0.24
16	n/a	n/a	n/a	n/a	2.8	n/a	n/a	n/a	H37Rv	0.08
17	4.3	0.26	1.7	0.27	3.8	0.5	-2.1	0.5	Turkey	0.33
18	2.9	0.28	1.7	0.27	2.0	0.9	-0.3	0.9	Haarlem / S	0.38
21	6.7	0.27	4.5	0.2	3.9	2.8	0.6	2.8 $^{\Pi}$	Delhi / CAS	0.29
23	3.7	0.27	1.7	0.27	3.5	0.2	-1.8	0.2	EAI	0.44
27	2.9	0.28	1.7	0.27	3.4	-0.5	-1.7	0§	Delhi / CAS	0.38
28	2.7	0.27	2.7	0.27	3.7	-1.0	-1	0§	Delhi / CAS	0.42
29	n/a	n/a	n/a	n/a	1.5	n/a	n/a	n/a	H37Rv	0.08
31	2.7	0.27	1.7	0.27	2.0	0.7	-0.3	0.7	Haarlem	0.46
31	2.1	0.27	1.4	0.26	<1	>1.1	>0.4	1.1 $^{\Pi}$	Haarlem	0.46
32	<0.7	n/a	<0.7	n/a	3.1	>-2.4	>-2.4	0§	EAI	0.5
36	2.7	0.27	1.1	0.31	<1	>1.7	>0.1	1.7 $^{\Pi}$	Delhi / CAS	0.25
38	6.4	0.26	1.7	0.27	<1	>5.4	>0.7	5.4 $^{\Pi}$	Delhi / CAS	0.32
40	2.4	0.22	1.4	0.26	2.5	-0.1	-1.1	0§	Delhi / CAS	0.38
48	3.3	0.26	1.4	0.28	<1	>2,3	>-0.4	2.3 $^{\Pi}$	EAI	0.29
49	1.5	0.23	<0.7	n/a	<1	>0.5	n/a	0.5	Delhi / CAS	0.29
51	5.9	0.28	4.1	0.29	2.9	3.0	1.2	3 $^{\Pi}$	Delhi / CAS	0.38
52	0.7	0.44	<0.7	n/a	<1	>-0.3	n/a	0§	H37Rv-like	0.25
53	3.7	0.16	1.1	0.31	2.1	1.6	-1	1.6$^{\Pi}$	S	0.17

Those samples with MPN or CFU values with a '<' sign implies that no growth occurred and the value inserted indicates the limit of detection. * - $\log_{10}$. Δ CS-dependent DCTB as calculated by Log_{10}(MPN_CS/CFU) and where the CFU value is zero, the limit of detection is inserted the denominator. §- if calculation of DCTB yielded a negative result, this was adjusted to 0 as we consider only the growth stimulatory effect of CS. Π – CS-DCTB values of ≥1.0 are considered significant due to the limits of confidence of MPN and CFU assays. † - value generated by www.MIRU-VNTRplus.org describing the 'distance' genetically from the strain type, the smaller the value the closer the isolate is to the reference strain. ‡ - standard LN decontamination by H_2SO_4 method; EBUS LN by NaOH. List of abbreviations: ATT – anti-tuberculous therapy; CFU – Colony forming unit assay; EAI – East Africa Indian; CAS – Central African Strain; CRS – composite reference standard; EBUS – endobronchial ultrasound; FNA – fine needle aspirate; LN -lymph node; MPN_CS - Most probable number assay with CS; MPN_7H9 – Most probable number assay with standard 7H9 media SD – standard deviation; Rpf – resuscitation promoting factor; UHL – University Hospitals of Leicester

Appendix 10: Culture negative samples laboratory & processing data

Table A10.1 Culture negative sample data

Patient number	Sample type	Storage duration before processing (days)	Storage before processing (°C)	Sample amount	Decon method‡	Storage duration after processing (days)	Storage after processing (°C)	CS batch	Duration of incubation	AFB smear	UHL culture result	CRS
11	CSF	15	-20	1.5ml	None			1	92	Neg	Neg	Poss TB
13	ILN station 7	7	4	Sliver of tissue	H_2S0_4			1	93	Neg	Neg	Not TB
14	Supraclavicular LN	6	4	Sliver of tissue	H_2S0_4			1	93	Neg	Neg	Prob TB
16	Pleural fluid	1	4	5ml	None			1	83	Neg	Neg	Not TB
16	Pleural fluid	2	4	40ml	None			1	83	Neg	Neg	Not TB
24	R foot abscess	4	4	2ml	H_2S0_4			1	92	Neg	Neg	Not TB
24	R foot bone Bx	4	4	Small fragments	H_2S0_4			1	85	Neg	Neg	Not TB
26	T8-9 vertebral Bx	2	4	1ml decon sample	H_2S0_4	20	-20	1	85	Neg	Neg	Not TB
27	Pleural fluid	10	4	5ml	H_2S0_4			1	85	Neg	Neg	Proven
30	ILN station 7	21	4	1 biopsy core	NaOH			2	84	Neg	Neg	Not TB
33	CSF	2	4	2ml	None			2	84	Neg	Neg	Proven
33	Mediastinal LN	3	4	1ml decon sample	NaOH	24	-20	2	102	Neg	Pos	Proven
33	Mediastinal LN	3	4	1ml decon sample	NaOH	35	-20	3	91	Neg	Pos	Proven
34	ILN station 7	2	4	1ml decon sample	H_2S0_4	50	-20	2	84	Neg	Pos	Proven
35	Pleural fluid	2	4	1ml decon sample	H_2S0_4	32	-20	2	84	Neg	Neg	Proven
35	Pericardial fluid	4	4	1ml decon sample	H_2S0_4	14	-20	2	84	Neg	Pos	Proven
35	Pericardial fluid	2	4	1ml decon sample	H_2S0_4	14	-20	2	84	Neg	Pos	Proven
35	Pericardial fluid	2	4	1ml decon sample	H_2S0_4	57	-20	3	85	Neg	Pos	Proven
37	Supraclavicular LN	3	4	1ml decon sample	H_2S0_4	28	-20	3	84	Neg	Pos	Proven
38	ILN station 7	10	4	1ml saline & tissue sliver	NaOH			3	102	Neg	Pos	Proven
39	Pelvic abscess	9	4	4ml	H_2S0_4			3	102	Neg	Neg	Not TB
41	Axillary LN	14	4	0.5ml saline & tissue sliver	H_2S0_4			3	91	Neg	Neg	Prob TB

Table A10.1 Culture negative sample data continued

Patient number	Sample type	Storage duration before processing (days)	Storage before processing (ºC)	Sample amount	Decon‡ method	Storage duration after processing (days)	Storage after processing (ºC)	CS Batch	Duration of AFB incubation	smear	UHL culture result	CRS
41	Bone marrow aspirate	1	4	1.5ml	None			3	99	Neg	Neg	Prob TB
42	Inguinal abscess	2	4	1ml decon sample	H_2S0_4	54	-20	3	85	Neg	Neg	Prob TB
43	Pleural fluid	2	4	1ml decon sample	H_2S0_4	39	-20	3	85	Neg	Neg	Prob TB
43	Pleural tissue	21	-20	0.5ml homogenised	None			3	85	Neg	Neg	Prob TB
44	L cervical abscess	2	4	1ml decon sample	H_2S0_4	19	-20	3	85	Neg	Pos	Proven
45	L cervical abscess	4	4	1ml decon sample	H_2S0_4	36	-20	3	100	Neg	Neg	Prob TB
46	R cervical abscess	4	4	1ml decon sample	H_2S0_4	137	-20	3	97	Neg	Pos	Proven
50	Cervical LN	63	-20	1ml homogenised	H_2S0_4			3	104	Neg	Pos	Proven
54	Cervical LN	79	-20	sliver of tissue	H_2S0_4			4	92	Neg	Neg	Prob TB
B	Cervical abscess	50	-20	0.25ml saline & pus	H_2S0_4			4	92	Neg	Neg	Prob TB
H	Pleural tissue	55	-20	4 slivers of tissue	None			4	92	Neg	Neg	Proven

‡ - standard LN decontamination by H_2SO_4 method; EBUS LN by NaOH. List of abbreviations: ATT – anti-tuberculous therapy; CRS – composite reference standard; CSF – cerebrospinal fluid; decon – decontaminated; EBUS – endobronchial ultrasound; FNA – fine needle aspirate; ILN – intrathoracic lymph node; LN -lymph node; SD – standard deviation; UHL – University Hospitals of Leicester.

Appendix 11: Mycobacterial Growth Indicatory Tube Data

Table A11.1 Mycobacterial Growth Indicator Tube information.

Patient number	Sample type	Storage before processing (UHL) (ºC)‡	Storage (UHL) (days)	2nd stage storage before processing (UoL) (ºC)#	Storage (UoL) (days)	Decon method	Post processing storage conditions (UHL)(ºC) §	Post processing duration (days)	CS Batch	Strain	Distance*
31†	Pleural fluid	4	2	-80	94	None			3		
32	C3 spinal abscess	4	5	-80	63	H_2S0_4			3		
36	ILN station 4R	4	9	-80	120	NaOH			3		
38	Colon tissue	4	7	-80	49	NaOH			3		
40	Pleural tissue	-20	13	-80	112	None			3		
46	R cervical abscess	4	4			H_2S0_4	-20	137	3		
50	Cervical LN	-20	63			H_2S0_4			3		
51	Ileocaecal abscess	-20	42			H_2S0_4			3		
52	Vertebral Bx	4	9			None			3		
53	Mediastinal LN	-20	71	-80	160	?NaOH			4		
54	Cervical LN	-20	79			H_2S0_4			4		
B	Cervical abscess	-20	50			H_2S0_4			4		
H	Pleural tissue	-20	55			None			4	Delhi / CAS	0.42

† - First pleural fluid sample from patient 31. ‡ - Storage conditions at the UHL microbiology laboratory before sample transferred to the UoL for growth assays. # - Some samples were frozen / refrozen at -80ºC after use in growth assays for future use in MGIT experiment. § - Sample decontaminated and then frozen to-20ºC at UHL prior to enrolment in the study. * - value generated by www.MIRU-VNTRplus.org describing the 'distance' genetically from the strain type, the smaller the value the closer the isolate is to the reference strain. List of abbreviations: CAS – Central African strain; CS – culture supernatant; EBUS – endobronchial ultrasound; LN – lymph node; UHL – University Hospitals of Leicester; UoL – University of Leicester

Appendix 12: Verification of ethical status of TB recurrence project

From: Leicester NRESCommittee.EastMidlands- (HEALTH RESEARCH AUTHORITY) <nrescommittee.eastmidlands-leicester@nhs.net>
Sent: 12 November 2014 10:38
To: Rosser Andrew - ST3 - IDU & Micro
Subject: Project advice

Dear Dr Rosser

Thank you for your letter dated 4 November 2014 addressed to the Leicester REC Committee.

I can advise that as the proposed project is using a pre-existing database then ethical review by the committee will not be required.

Any permissions necessary will be those between the research team and the database management team.

I hope this answers your query but please let me know if you required any further information.

Kind regards

Ellen

Ellen Swainston | REC Manager and Joanne Unsworth | REC Assistant
Health Research Authority
National Research Ethics Service (NRES)
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