A comparison of solid and liquid culture in tuberculosis diagnosis and treatment monitoring

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I, Michael Edward Murphy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Tuberculosis (TB) poses a significant challenge for global health. Developing countries most burdened by disease have insufficient laboratory resources and limited capacity to conduct clinical trials. This thesis aims to investigate microbiological methods currently employed in TB programmes to compare their value in diagnosing TB and monitoring treatment using data from smear-positive patients enrolled in the REMoxTB study.

As TB culture remains out-of-reach in many settings, diagnosis continues to depend on sputum smear microscopy. Early morning samples are considered better than spot samples. However, the data presented show that spot samples have a higher positive yield and greater sensitivity for solid and liquid culture. This evidence does not support guidelines requiring early morning samples which inconvenience patients and complicates trial enrolment.

The data also show a reducing correlation between smear microscopy and culture on solid and liquid media as treatment progresses. These findings question the use of smear microscopy as a proxy for culture during treatment which may prompt inappropriate treatment extensions or retreatments.

Comparing solid and liquid culture, the analyses show that liquid culture is faster and more sensitive. The clinical significance of this increased sensitivity throughout treatment is uncertain, and some samples negative in liquid culture remain positive on solid culture. The relationship between solid and liquid

culture changes during treatment suggesting they differentially support the metabolic requirements of changing mycobacterial populations.

The value of measures of pre-treatment mycobacterial load are investigated and show they are poorly predictive of microbiological responses during treatment in either culture media. Better indicators of treatment response are required which reflect mycobacterial population dynamics.

Increasing availability of TB culture would of great benefit. The data show that the incubation times for liquid cultures, particularly for diagnostic samples, may be significantly reduced without loss of sensitivity, which could increase laboratory capacity and remove barriers to implementation.

Future work will investigate whether these findings are generalisable to smear-negative patients and assess their value in predicting long-term treatment outcomes.

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Abbreviations used in this thesis

AFB	acid fast bacilli
Ag	Antigen
AIDS	acquired immunodeficiency syndrome
ATS	American Thoracic Society
AUROC	area under receiver operating characteristic curve
BC	before Christ
BS	British standard
BSC	biological safety cabinet
Bsl	Baseline
CD4	cluster of differentiation 4; a glycoprotein found on the surface of immune cells such a T-helper cells
CDC	Center for Disease Control
CFP10	10 kDa culture filtrate protein
CFU	colony forming units
CI	confidence interval
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
Ct	cycle threshold; number of PCR cycles until sample positive
CT	computed tomography
CXR	chest x-ray
DNA	deoxyribonucleic acid
DST	drug sensitivity testing

E	Ethambutol
EBA	early bactericidal activity
embB	gene encoding arabinosyl transferases; mutations may confer resistance to ethambutol
EMS	early morning sputum sample
EPTB	extra-pulmonary TB
ESAT6	early secretory antigen target 6
FDA	US Food and Drug Administration
FDG	Fluorodeoxyglucose
FIND	Foundation for Innovative New Diagnostics
FQ	Fluoroquinolone
GU	growth unit
H	Isoniazid
HIV	human immunodeficiency virus
HR	hazard ratio
Hsp	heat shock protein
IFN (γ) (αβ)	interferon (gamma) (alpha-beta)
Ig	Immunoglobulin
IGRA	interferon gamma release assay
INH	Isoniazid
inhA	gene encoding a target for isoniazid and ethionamide; mutations may confer resistance
IQR	interquartile range
IS	insertion sequence
IUATLD	International Union Against Tuberculosis and Lung Disease

katG	gene encoding catalase peroxidase
LAM	Lipoarabinomannan
LED	light-emitting diode
LJ	Lowenstein Jensen; solid culture medium
LMIC	low and middle income countries
log_10	logarithm to base 10
log_n	natural logarithm
LTBI	latent TB infection
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. bovis BCG	*Mycobacterium bovis BCG; vaccine strain*
M. canetii	*Mycobacterium canetii*
M. fortuitum	*Mycobacterium fortuitum*
M. malmoense	*Mycobacterium malmoense*
M. microti	*Mycobacterium microti*
M. tuberculosis	*Mycobacterium tuberculosis*
MBL	Molecular Bacterial Load assay; UCL designed PCR to detect mycobacterial rRNA
MDR	multi-drug resistant
MGIT	mycobacteria growth indicator tube; liquid culture medium
MGIT FP	MGIT false positive
MHC	major histocompatibility complex
MIRU	mycobacterial interspersed repeat units; TB typing methodology
MPY	maximum positive yield; result defined as positive if positive in either or both culture media

(B)MRC	UK Medical Research Council
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MTD	Mycobacterium tuberculosis direct test (Gen-Probe, Inc, San Diego, USA)
NAAT	nucleic acid amplification test
NALC	N-acetyl-cysteine
NaOH	sodium hydroxide
Neg	Negative
NICE	National Institute for Health and Care Excellence
NIH	US National Institute of Health
NPV	negative predictive value
NTM	nontuberculous mycobacteria
NTP	national TB programme
OR	odds ratio
p value	probability of finding the observed or more extreme result when the null hypothesis is true
PANTA	polymixin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin; antibiotic mixture for MGIT
PAS	para-amino salicylic acid; 2nd line anti-TB drug
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	positron emission tomography
pH	numeric scale to specify the acidity or alkalinity of an aqueous solutions

pncA	gene encoding pyrazinamidase enzyme which converts the prodrug pyrazinamide to its activate form
pos	Positive
PP	predictive probability
PPV	positive predictive value
QFT	quantiferon test; interferon gamma-release assay
R	Rifampicin
R	coefficient of correlation
R^2	coefficient of determination
REMoxTB	Rapid Evaluation of Moxifloxacin in Tuberculosis
RFLP	restriction fragment length polymorphisms; TB typing methodology
RNA	ribonucleic acid
ROC	receiver operating characteristic curve
Rpf	resuscitation promotion factor
rpoB	gene encoding the B-subunit of bacterial RNA polymerase; mutations may confer resistance to rifampicin
rRNA	ribosomal ribonucleic acid
Scr	Screening
SMS	spot morning spot
T.SPOT TB	T-spot test; interferon gamma-release assay
TB	Tuberculosis
TBTC	TB Trials Consortium
TDR	totally drug resistant
TDR	Special Programme for Research and Training in Tropical Diseases

TFN	time to first negative culture
TNFα	tumour necrosis factor (alpha)
tRNA	transfer ribonucleic acid
TSN	time to sustained negative culture
TST	tuberculin skin test
TTD	time to detection
TTP	time to positivity
UCL	University College London
UK	United Kingdom
US	United States of America
VNTR	variable number tandem repeat; TB typing methodology
WGS	whole genome sequencing
WHO	World Health Organisation
Wk	Week
XDR	extremely drug resistant
Xpert (MTB/RIF)	rapid test for simultaneous detection of *M. tuberculosis* and rifampicin resistance
Z	Pyrazinamide
ZN	Ziehl-Neelsen stain

Chapter 1 Introduction

The identification of *Mycobacterium tuberculosis* (*M. tuberculosis*) as the cause for tuberculosis by Robert Koch in 1882 was momentous (1). Simultaneously explaining the cause of tuberculosis, which had infected man since antiquity at least, and displacing the predominant miasma theory of disease at the time, Koch might be considered the father of modern microbiology. Having already confirmed *Bacillus anthracis* as the cause of disease, and going on to later discover *Vibrio cholera*, the contribution of this preeminent scientist to the field of infectious diseases is largely unsurpassed (2). It remains, however, his work on tuberculosis which has cemented his place in medical history.

At the advent of the third millennium, tuberculosis continues to exact an enormous toll on global health (3). Largely a disease of poverty, the *M. tuberculosis* bacteria thrives in dark, damp living conditions, and overcrowding and lack of access to healthcare delays treatment and supports ongoing transmission. Those affected have the fewest resources to manage the condition and the socioeconomic consequences on patients and their families can be immense (4-7). Efforts to control TB have historically had limited success, hampered by inadequate resources and unstable healthcare infrastructures. Considerable research investment since the Millennium Declaration (8), however, is reaping rewards; the Millennium Development Goal to halt and reverse the TB epidemic by 2015 has been achieved, and the world is on track to reduce the burden of disease by 50% by 2015 (3). Some areas, however, are failing to achieve these goals in their region, and drug resistant

tuberculosis, HIV co-infection and the growing epidemic of diabetes mellitus present additional hurdles for the future.

Tuberculosis retains a high profile in global health research and attempts to develop new drugs and vaccines, and exploit technological advances continue at a faster pace than ever before. It is increasingly recognised that robust biomarkers predicting TB treatment responses will be crucial to the success of these efforts (9). Few studies have compared different microbiological methods prior to and during TB treatment.

Scientific advances can achieve only so much, however, and political engagement and socially cohesive policies will be required if the scientific community are to stop TB (10).

1.1 Historical Perspectives

Prior to the discovery of the organism *Mycobacterium tuberculosis (M. tuberculosis)* in 1882, accounts of clinical tuberculosis were to be found in the literature of ancient Greece, Rome and Egypt. Hippocrates (460 BC) described phthisis in his work "*Of the Epidemics"(11)* as the most common cause of death of his time and provided an accurate description of the disease:

"…many of those who had been long gradually declining, took to bed with symptoms of phthisis …consumption was the most considerable of the diseases which then prevailed ... most of them were affected by these diseases in the following manner: fevers … constant sweats … sputa small, dense, concocted, but brought up rarely and with difficulty … they were soon wasted and became worse … Many, and, in fact, the most of them, died; and of those confined to bed, I do not know if a single individual survived for any considerable time…."

DNA analyses have provided crucial information about the evolution of the *M. tuberculosis* complex. It has been postulated that an earlier progenitor organism comparable to *Mycobacterium canetii* may have been present in East Africa 3 million years ago (12, 13). Given the association with sites of animal domestication and agriculture, it had been proposed that *M. tuberculosis* affecting humans was an evolution of *Mycobacterium bovis* in animals. Genome studies, however, revealed *M. bovis* represented a later lineage than *M. tuberculosis* (14). The role of animals in the history of human TB through farming and/or domestication may not, therefore, be through cross-species

transmission but through the concentrating of human populations in agriculturally advantageous settings and, later, those industries supporting agriculture. This is supported by a recent study using whole genome sequencing which proposes that *M. tuberculosis* emerged about 70,000 years ago, accompanied migrations of anatomically modern humans out of Africa and expanded as a consequence of increases in human population density during the Neolithic period; *M. tuberculosis* adapted to low and high population densities supporting a long co-evolutionary history (15)

Examining paleo pathological specimens, morphological changes associated with TB have been identified in a five hundred thousand year-old skeleton of Homo Erectus from Turkey (16), but the diagnosis has not been confirmed. By applying molecular methods to similar specimens, researchers have identified the presence of *M. tuberculosis* DNA from fossilised bison in North America dating from around 15000 BC (17). The earliest identification of *M. tuberculosis* in humans dates from 9250-8160 years ago. A woman and infant with typical bony lesions were recovered from the now submerged East Mediterranean village of Altit-Yam near modern day Haifa which is considered to be one of the earliest examples of animal domestication and agriculture (18). Ancient DNA and bacterial lipid analyses and deletion analyses have dated the *M. tuberculosis* discovered as the modern TB lineage containing the TbD1 deletion existing 9000 years ago. More recent examples have been discovered using molecular methods in Egypt in 3500-2650 BC (19), and also in Sweden at a site considered among the earliest sites associated with cattle breeding in 3200-2300 BC (20).

That *M. tuberculosis* was contagious was an idea first proposed even during the time of Aristotle 384 BC, but this idea was largely discounted at the time in favour of a hereditary acquisition, and indeed given the likely shared exposure, whole families were often affected. Later mystical theories predominated, and gave rise to the romanticism afforded 'consumption' in poetry and literature. With the industrial revolution in the latter part of the 18th century came population density, urban growth, and poverty not seen previously which proved excellent conditions for the transmission of *M. tuberculosis*.

By the turn of the 20th century, tuberculosis was the cause of a quarter of deaths in Europe. Sanatoria were considered the best possible treatment and many low rise hospitals with adequate ventilation were built to offer this 'treatment', though there was little evidence for their use even before drug treatments were available (21). A recent systematic review has estimated that in the pre-chemotherapy era, mortality from smear positive TB was around 58% at 5 years and 73% at 10 years, with an average duration of illness of 3 years (22).

During this time there remained strongly held beliefs in the spontaneous theory of diseases - those arising spontaneously from within - and the miasma theory of tuberculosis caused by 'bad air' emanating from rotting organic matter. Neither theory offered any realistic hope of treatment. Despite John Snow's work on the epidemiology of cholera in 1857 showing the water supply as the source of infection (23), neither he, nor Louis Pasteur who also believed a microorganism to be the causative agent of cholera, were able to identify a specific bacteria as causal.

Discovery of Mycobacterium tuberculosis

Robert Koch had come to believe in the germ theory of disease during his research work into outbreaks of anthrax in animals. Through this work, he developed what he considered as the cornerstones of evidence required to prove the germ theory of disease:

- *The microorganism must be found in abundance in all patients or animals suffering from the disease, but should not be found in healthy populations*
- *It must be isolated from a diseased organ and grown in pure culture.*
- *The cultured microorganism should cause disease when introduced into a healthy patient or animal*
- *The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.*

In the late 1800s, having carried out extensive work on the isolation and culture of bacteria, Koch turned his attention to identifying the cause of TB. Working with guinea pigs, rabbits and cats, Koch discovered the causative agent of "consumption" as *Mycobacterium tuberculosis* (24). He was able to identify *M. tuberculosis* from tuberculosis patients' sputum samples using methylene blue staining, isolate the organism in culture and administer the organism to animals who developed the disease and from whom the organism was again isolated, thereby satisfying all four postulates.

Koch presented his findings to the Physiological Society of Berlin on 24 March 1882. Paul Erlich attended this lecture and was inspired to perfect the staining methodology. Within a short period of time, Erlich had discovered the acid fast nature of *M. tuberculosis* and employed this as a method of identification. This method was further refined by Franz Ziehl, Frederich Neelsen and Eduard von Rindfleisch.

Despite their success in having isolated the organism, there was no cure on the horizon and Koch's early attempts to utilise tuberculin as a treatment proved unsuccessful (25). Whilst disastrous for Koch, who had announced tuberculin as a cure, Clemens von Pirquet, having discovered 'allergy' to repeated exposure to the smallpox vaccine, realised the diagnostic potential of tuberculin; patients already infected with tuberculosis displayed delayed hypersensitivity reactions to tuberculin. Such theories surrounding the immunology of TB were proposed by Rudolf Virchow who asserted that tuberculin must not kill TB but rather induce the host to necrose those infected tissues and thereby effectively 'starving' the mycobacterium. Charles Mantoux refined von Piquet's technique, giving rise to the Mantoux test currently used to diagnose latent TB.

Erlich's own work on staining continued in an effort to selectively identify specific groups of cells or organisms, including tuberculosis and malaria. This led him to the 'magic bullet' theory that an agent used to selectively identify a particular organism could also be used to deliver a selective toxin to that organism, so inspiring the notion of antimicrobial chemotherapy. It would not be until many years after Koch's death in 1910 that the first agent of antituberculous chemotherapy, streptomycin, would be discovered.

1.2 Current TB Epidemiology

In 2012, there were 8.6 million incident cases of tuberculosis; a global incidence of 122 per 100,000 population (26). It is estimated that 1.1 million cases occurred in patients co-infected with HIV. The global distribution of TB (figure 1.1) is concentrated in Asia and Africa which contribute 58% and 27% of all cases, with 8%, 4% and 3% occurring in Eastern Mediterranean region, European region and the region of the Americas respectively. Of the total number of global TB cases, 82% occurred in 22 high burden countries which have been a global health priority since 2000.

The five countries with the highest number of incident TB cases were India (2-2.4 million), China (0.9-1.1 million), which together accounted for 38% of all incident TB, South Africa (0.4–0.6 million), Indonesia (0.4–0.5 million) and Pakistan (0.3–0.5 million).

Of the cases of TB in patients co-infected with HIV, 80% were concentrated in the WHO African region (figure 1.2). HIV prevalence in newly diagnosed TB case were greater than 50% in Botswana, Namibia, South Africa, Zambia and Zimbabwe. Estimates for India and China, by contrast, were between 0 and 4%.

Figure 1.1. Map of TB incidence rates per country, 2012 (26)

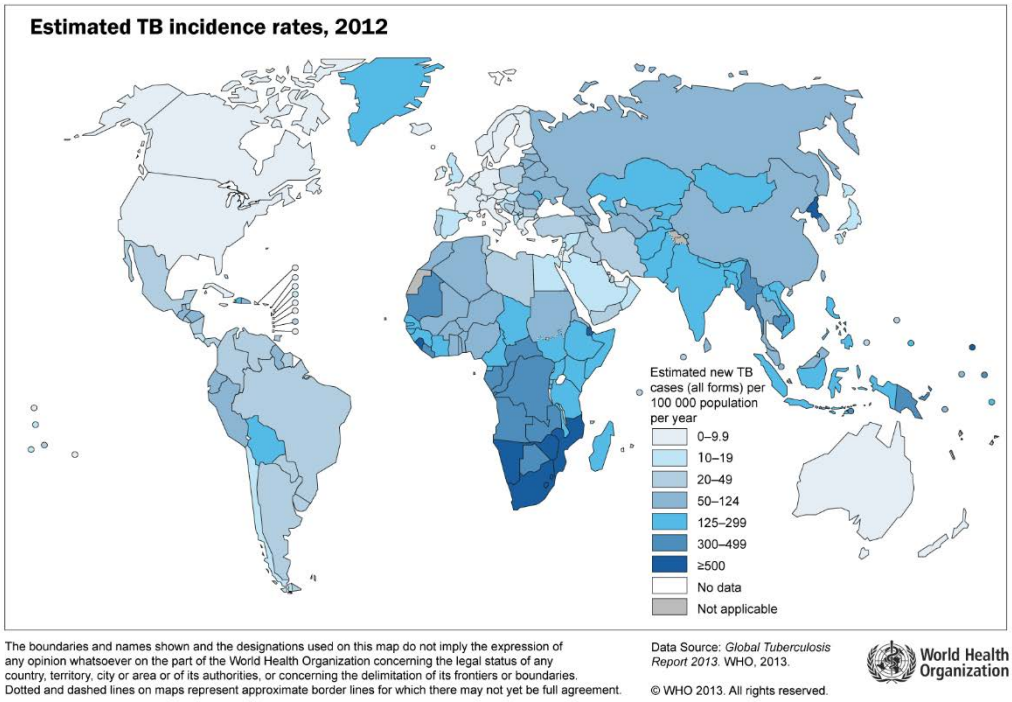
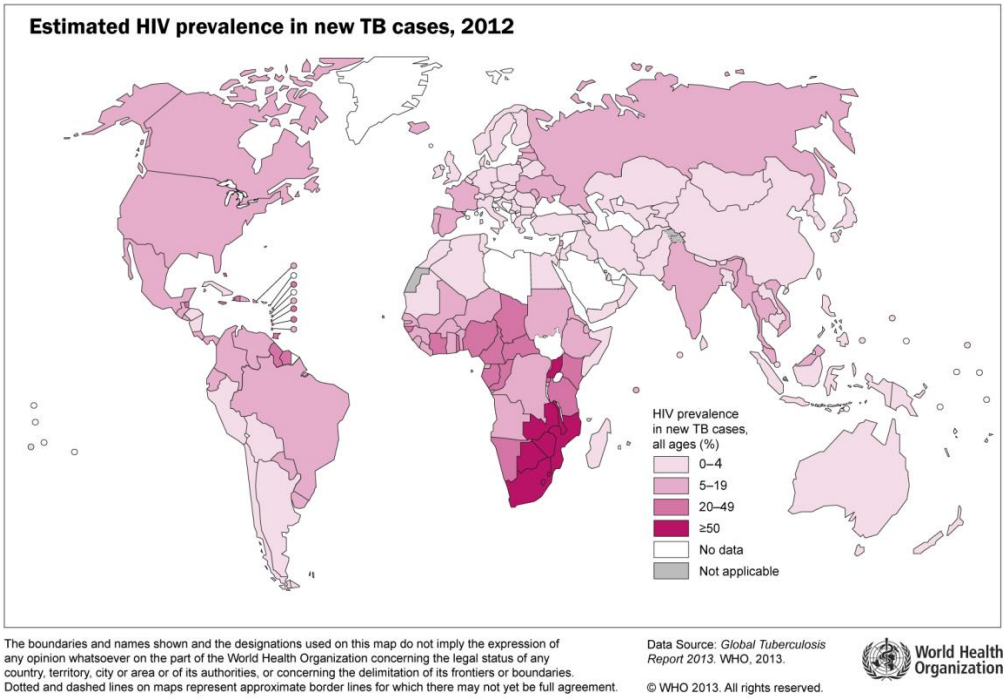


Figure 1.2. Estimated prevalence of HIV amongst new cases, per country (26)



Total deaths attributable to TB in 2012 were 1.3 million, making TB second only to HIV as the cause of death attributable to a single infectious agent, although overall diarrhoeal and respiratory infections were more commonly associated with all-cause mortality (27). Of the TB deaths, 320,000 were reported in patients co-infected with HIV. It is widely accepted that the contribution of TB to deaths attributed to HIV may be under-estimated. A review of autopsy studies have found evidence of TB in 21-54% of such cases and identified TB as the cause of death in 32-45%, yet the diagnosis is commonly missing from death certificates which state only HIV (28, 29).

Recommended practice to test all TB patients for HIV is improving globally, with 46% of cases notified in 2012 being aware of their HIV status, compared to 8% in 2005. This reflects great success in this area, especially in the African region bearing the largest burden of TB/HIV co-infection where rates have reached 74%.

Drug resistant tuberculosis

Drug resistance presents major challenges to TB diagnosis and treatment monitoring and making it possible to identify this at diagnosis has been at the forefront of research efforts which have recently borne fruit with the WHO endorsement of the Gene Xpert test which simultaneously detects *M. tuberculosis* and rifampicin resistance (30). Resistance was identified early in the history of antituberculous chemotherapy. In the first clinical trial of streptomycin monotherapy for the treatment of pulmonary tuberculosis in 1947, the mortality benefit seen after 1 year were largely lost at 5 years with the majority of TB strains having developed streptomycin resistance. Combination

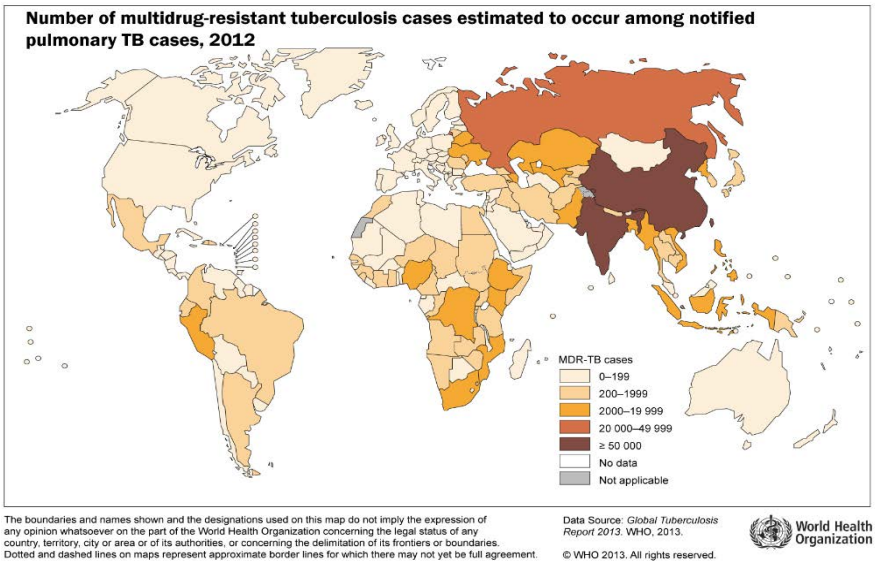
therapy reduced the risk of drug resistance, but poor patient compliance with the required prolonged duration of treatment and interruptions to supply of medication over the years created a selection pressure for the development of drug resistance. Such resistant strains are known to be transmitted both nosocomially and in community settings (31).

Resistance to rifampicin and isoniazid denotes a case of multiply drug resistance TB, so called MDR TB. Additional resistance to the fluoroquinolones and at least one of the injectable agents amikacin, kanamycin or capreomycin defines extensively drug resistant diseases (XDR TB). Cases of totally drug resistant TB (TDR), with resistance to all known classes of antituberculous drugs, have been reported in the published literature (32), but there is no WHO agreed case definition.

Data on the epidemiology of drug resistance are sorely lacking owing to the lack of mycobacterial culture and drug sensitivity testing (DST), either by molecular testing or molecular methods. Additionally much data is based on inferior sub-national surveys, including India and Russia. It is estimated that there were 310,000 cases of MDR TB in 2011 of which just 19% were enrolled on National TB Programmes (NTP) for MDR TB. In India and China where, along with Russia, the greatest numbers of MDR TB are to found, the notification rates were less than ten percent. Of new TB cases, 5% had culture and first line DST. More worryingly, only 9% of retreatment cases are subject to culture and DST despite relapse being the biggest indicator of potential drug resistance and MDR TB (3).

There are 27 countries with a high burden of MDR TB. Estimated proportions of MDR TB among all incident TB cases is 3.7% amongst new cases and 20.2% amongst retreatment cases. India, Russia and China collectively account for 60% of MDR TB cases globally (figure 1.3). In Eastern Europe and Asia the proportion of MDR TB among new cases is as high as 35% and 69% in previously treated cases. Clearly these estimates must be interpreted with caution owing to under-reporting of MDR TB in areas lacking robust drug sensitivity testing data which is rarely performed at all for diagnostic purposes, normally being reserved for those failing treatment after 2 months, and in only a small number of retreatment cases. On the contrary, incentives to diagnose MDR TB may support over-reporting MDR TB to increase funding for healthcare in some countries where all patients with MDR TB are hospitalised; this may be inappropriate in areas of suboptimal control and risks infection of healthcare workers who are disproportionately affected with MDR TB (33, 34).

Figure 1.3 Number of multidrug resistant cases estimated to occur among notified pulmonary TB cases, 2012 (26)



Given low rates of first and even lower rates of second line DST, there are scant data on the epidemiology of XDR TB. XDR TB was first identified during a rapidly fatal outbreak in Tugela Ferry area of South Africa in which 54 HIV co-infected patients were infected with TB (35). Half of the affected patients had never received previous treatment suggesting nosocomial acquisition. Since then, XDR TB has been reported in all countries for whom data is available (26). Of countries who report continuous epidemiological data or representative surveys of second line drug resistance, around 10% of MDR TB cases are considered to be XDR TB; Eastern Europe has the highest burden with rates of 12-14% in Belarus (36, 37), 16% in Latvia and 25% in Lithuania (26).

The lack of data on overall drug sensitivity makes it unclear whether MDR TB and XDR TB are increasing, stabilising or decreasing. Few patients globally are tested for drug resistance; this presents a major challenge for future laboratory strengthening and a focus for capacity building. It is clear, however, that MDR and XDR are being transmitted both nosocomially as described above, and in the community (31, 35, 38). It remains unclear as to whether HIV infection is a risk factor for drug resistant TB but TB/HIV co-infection presents further management challenges given the potential TB/HIV drug-drug interactions.

Case detection and treatment

The recording of reporting of TB has greatly enhanced knowledge of the burden of TB disease and remains one of the five elements of TB control strategies endorsed by the Stop TB partnership (10). In 2012 6.1 million patients diagnosed with TB were engaged in National TB Programmes (NTPs). Of those, 0.4 million had previously received treatment for TB. Treatment success

was 87% in new cases diagnosed with smear positive TB. Much lower rates of 72% were achieved in the European region, likely due to unidentified drug resistant disease in countries of the former Soviet Union (26).

NTPs maintain records of reported TB cases but additional efforts are being made to engage private providers in NTPs to gather more accurate data. This is vitally important in global TB control. A survey of Indian TB patients found around 50% attended private clinics (39). The case notification rate in private practices ranges from 10-40% compared to 67% public NTP providers. A small study of such private providers in India revealed low adherence to international guidelines (40). Public-private mix clinics have been shown to achieve good success rates in India (41) as well as in Africa (42).

Enhanced surveillance systems allow continuous monitoring of TB incidence and HIV prevalence. This helps to focus future control efforts to areas of greatest need and provides data against which the impact of global interventions can be assessed (43). There have been calls to extend TB surveillance to include data on smoking and the emerging epidemic of diabetes, both sharply rising in low and middle income countries which may impact significantly on TB control efforts in future (44-46).

TB Laboratory capacity

Current laboratory capacity is a challenging area of global TB control. Eight of the 22 high burden countries fail to meet the target of one TB microscopy laboratory per 100,000 population and 20 of the 36 combined high burden TB/MDR TB countries did not have the recommended minimum of one

laboratory per 5 million population able to process samples for culture and DST (3). The roll out of automated liquid culture in resource limited settings is continuing at a pace, however, the value of this has yet to be confirmed in terms of cost effectiveness (47). The increasingly complex landscape of TB diagnostics may further burden TB laboratories.

Low cost, low technology tests with favourable health and safety profiles limiting risks of TB transmission to staff are of great benefit. These must be balanced with the needs of TB clinical services and the capacity to conduct epidemiological surveillance. Moreover, laboratory capacity must be advanced to allow sites to conduct of regulatory trials of new TB drugs which has thus far proved a considerable barrier. Such facilities benefit not only TB patients and the global scientific community but also provide high level employment within the respective communities. In providing high level jobs, such trials also help to retain local expertise within the affected communities (48).

Risk factors for tuberculosis

There are a number of factors predisposing patients to active tuberculosis infection. HIV co-infection plays an important part of the TB epidemic globally with incidence ranging from 720 per 100,000 in the US (49) to 9700 per 100,000 in South Africa (49, 50). Patients living with HIV are 20-37 more likely to develop active TB (51). Such risk is reduced by 80% in patients receiving antiretroviral therapy (49, 52), however the additional risk remains even after many years of treatment (53, 54). As patients with HIV may present atypically with few or perhaps even no symptoms, in countries of high TB and HIV co-infection, there is a proven benefit in TB screening in patients with HIV (55, 56).

The WHO has updated its guidelines to encourage intensified TB case finding in patients with HIV and isoniazid preventive therapy where appropriate regardless or the degree of immunosuppression, or whether or not they have previously received TB treatment (57).

Attention has focussed more recently on other causes of relative impairment of cell mediated immunity, including diabetes mellitus and advancing age (45, 58). A disease of poverty, tuberculosis also more commonly affects poorly nourished patients and those who misuse alcohol, cigarettes and illicit drugs (59). These populations may delay seeking healthcare and in the meantime live in damp and dark conditions which support the growth of *M. tuberculosis* and necessitate close contact with others in confined and poorly ventilated spaces, including prisons (60), where prolonged exposure to infected cases increases the risk of transmission. A recent study of molecular epidemiology employing whole genome sequencing identified 'super-spreaders' in just these sort of conditions at the centre of almost all non-school outbreaks (61).

Iatrogenic immunosuppression is increasingly employed in the management of malignancy, autoimmune disease and may include bone-marrow ablative chemotherapy or anti-TNF therapies. TB in these groups may also present atypically and/or with disseminated disease. Screening and preventive therapy may reduce the risk of reactivation of latent disease in these circumstances (62). Engaging TB patients with chronic concomitant health and/or socioeconomic or psychiatric comorbidities presents additional challenges to presentation, diagnosis and management. The relative risks for TB for a number of conditions is shown in table 1.1. The attributable risks are not known

and thus it is unclear to what extent TB disease in a population may be attributable to any specific risk. Without this information, public health interventions to reduce the risk of TB by improving modifiable risk factors, e.g. achieving normoglycaemia in diabetics, may be difficult to prioritise.

Table 1.1 Risk factors for the development of active TB among persons infected with *Mycobacterium tuberculosis* relative to infected persons with normal CXR and no known risk factors

Risk Factor	Relative Risk
High risk	
• AIDS (not on anti-HIV therapy) • HIV (not on anti-HIV therapy)	110-170 50-110
• Transplantation (related to immunosuppressive therapy)	20-74
• Silicosis	30
• Chronic renal failure requiring dialysis	10-25
• Recent TB infection (<2 years)	15
• Abnormal chest x-ray (upper lobe fibronodular disease typical of healed TB infection	
• TNF-α inhibitors	2-9
Medium Risk	
• Treatment with steroids	5
• Diabetes mellitus (all types)	2-4
• Young age when infected (0-4 years)	2-5
Slightly increased risk	
• Underweight (<90% ideal body weight; BMI <20)	2-3
• Cigarette smoker (1 pack/day)	2-3
• Abnormal chest x-ray	2
Low risk	
• Infected person, normal chest x-ray, no known risk factor	1

Adapted: Vernon A, Treatment of Latent TB Infection. Seminars Resp Critical Care Med 2013 (63)

1.3 TB mycobacteriology and immunopathology

TB mycobacteriology

Mycobacterium tuberculosis is one of the Mycobacterium tuberculosis complex species of the genus Mycobacteria, and the major cause of the human disease tuberculosis. It is an obligate aerobe and a facultative intracellular parasite of macrophages in humans, who are the only reservoir for the organism.

M. tuberculosis is rod shaped and may be considered weakly Gram positive owing to its basic cell wall structure; plasma membrane, thick peptidoglycan layer and absent outer membrane. The cell wall of mycobacteria, however, are far more complex lipid laden structures containing mycolic acids. Characteristically, they resist decolourisation with acid-alcohol after staining with auramine phenol or carbol fuchsin. This property gives rise to the common method of identifying *M. tuberculosis* presumptively in patient samples by identifying 'acid-alcohol fast bacilli' (AFB), as shown in figure 1.4.

Figure 1.4 Photomicrograph showing *Mycobacterium tuberculosis* bacteria using acid-fast Ziehl-Neelsen stain; Magnified 1000 X

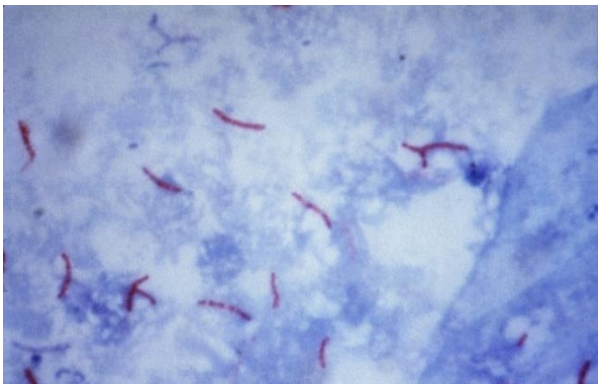


Image: Centers for Disease Control and Prevention's Public Health Image Library, #5789

The *M. tuberculosis* cell wall is composed of an inner cytoplasmic membrane to which is anchored proteins, phosphatidylinositol mannosides and lipoarabinomannan (LAM). Mannose-capped LAM is a major immune stimulator during infection. Above the plasma membrane lies the peptidoglycan layer to which arabinogalactans are attached. The terminal D-arabinose residue is esterified to mycolic acids with glycolipid surface proteins (figure 1.5). Together with additional lipids, glycolipids and peptidoglycolipids, lipids together comprise 60% of the total cell wall weight. Proteins are interspersed throughout the cell wall and form antigens which may be used to measure previous exposure to *M. tuberculosis,* e.g. tuberculin.

Figure 1.5. Diagram of mycobacterial cell wall structure

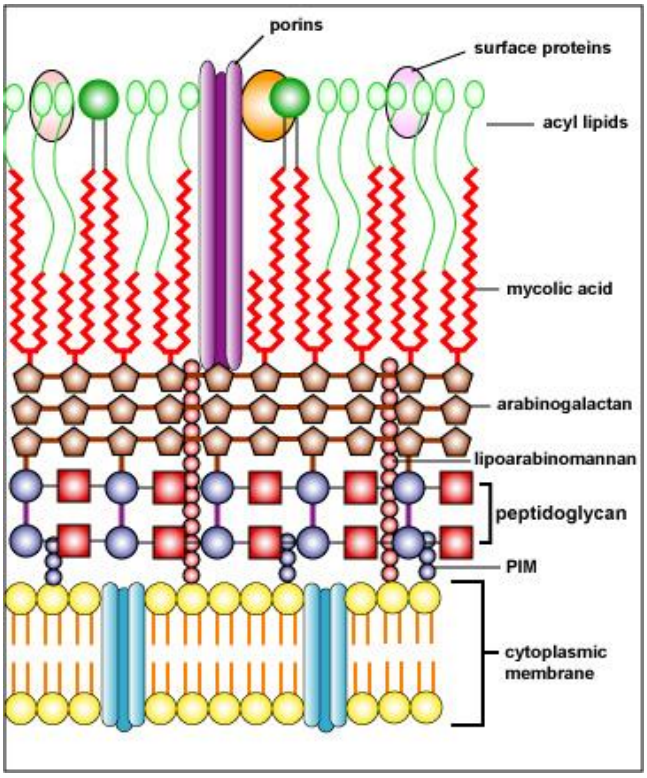


Image © Dr Gary Kaiser

Members of the *M. tuberculosis* complex are slow growing organisms, with a generation time of 15-20 hours (64), with white or buff coloured colonies appearing on solid culture media within 6-8 weeks (figure 1.6).

Figure 1.6. Close-up of a *Mycobacterium tuberculosis* culture showing the colourless rough surface, which are typical morphologic characteristics of colonial growth

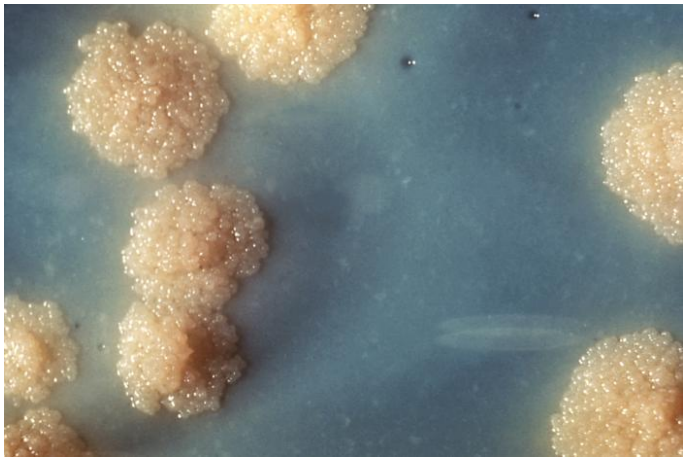


Image: Centers for Disease Control and Prevention's Public Health Image Library, #4428

Colonies in both solid and liquid media may appear as serpentine cords owing to the presence of cord factor (trehalose dimycolate; TDM) associated with virulent strains of *M. tuberculosis* (65). TDM is the most abundant lipid extractable from the surface of *M. tuberculosis* and also the most toxic and granulomagenic (66, 67). It has multiple roles in different stages of TB disease. In early stage infection, TDM prevents phagosome-lysosome fusion and thereby protecting *M. tuberculosis* from killing within the macrophage, and is a specific target of activated macrophages which inactivate TDM using reactive nitrogen intermediaries restoring phagosome maturation (68, 69). In established infection, TDM may be highly antigenic and toxic when interacting with lipid in

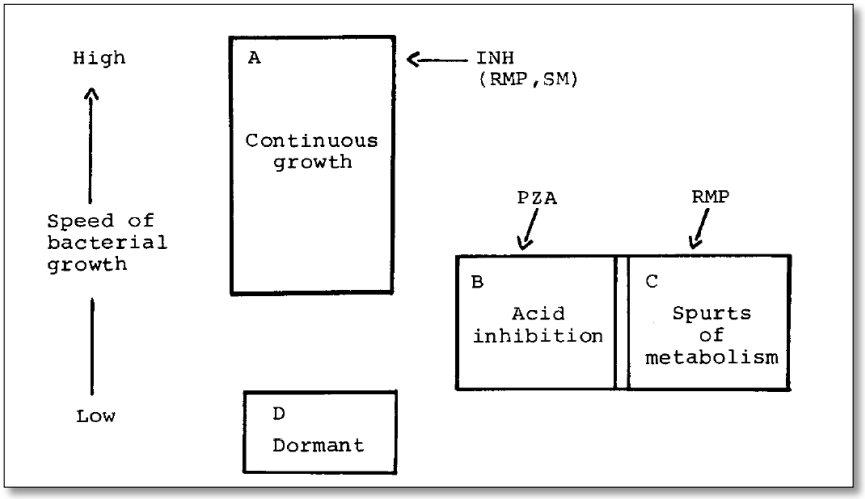
the granuloma initiating necrosis resulting in characteristic caseation (67, 70). It is just one of the many structural and physiological virulence factors present in *M. tuberculosis*.

Populations of Mycobacterium tuberculosis

Tuberculosis sufferers have long been considered to harbour heterogeneous populations of *M. tuberculosis*. Four such populations are proposed (71):

1. Actively growing organisms (killed mainly by isoniazid)
2. Semi-dormant organisms inhibited by an acid environment, (killed mainly by pyrazinamide)
3. Semi-dormant organisms with spurts of active metabolism (killed preferentially by rifampicin)
4. Completely dormant organisms (not killed by standard drugs)

Figure 1.7. Reproduction of figure 1 from 'The action of antituberculosis drugs in short-course chemotherapy'. Mitchison D, *Tubercle* 66 (1985) 219-225



More recent studies have identified the adaptable nature of *M. tuberculosis in vivo*. Large populations of dormant organisms have been discovered in patients' sputum samples prior to treatment which fail to grow routinely in culture. These cell may, however, be resuscitated with the addition of bacterial pheromones denoted resuscitation promoting factors (Rpfs) first discovered in *Micrococcus luteus* (72, 73). These cells may be identified prior to resuscitation as lipid-body positive on sputum smears stained with nile red. This fits with research showing that triacylglycerol (lipid) accumulates in *M. tuberculosis* in a dormant/non-replicating state during hypoxia and other stresses (74). Recent studies show the gene encoding triacylglycerol-synthase is a member of the DosR regulon encoding genes linked to survival in *M. tuberculosis,* supporting the hypothesis that these lipid-body positive cells may have a role in mycobacterial persistence (75-77).

Through the process of resuscitation, it is clear that *M. tuberculosis* within the human host is adaptable and can undergo periods of both dormancy and steady growth. Presumably there are mycobacterial, environmental and host factors at play, but the triggers for this transformation remain poorly understood. It may, however, be possible to exploit such factors in the laboratory to identify the different populations of mycobacteria present in samples which may better correlate with treatment responses.

Persistence may be considered crucial in maintaining the evolutionary advantage of *M. tuberculosis*, but it is important that *M. tuberculosis* remain sufficiently antigenic in some hosts to induce the formation of the granuloma,

the classic histopathological finding in tuberculosis, in which organisms may persist away from the immune system (78).

Immunopathology

The TB bacillus is able to enter macrophages directly via mannose binding receptors which bind LAM and indirectly via complement and Fc receptors. Some macrophages will process *M. tuberculosis* sufficiently and present protein antigens onto the cell surface in the context of Major Histocompatibility Complex class II (MHCII) molecules. As some of these infected macrophages will be transported to local lymph nodes, they will encounter CD4+ helper T cells which bind to antigens presented on MHC II molecules and become primed. On encountering *M. tuberculosis* again, these T cells will release interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα) resulting in macrophage activation and improved mycobacterial killing.

More recent research has also identified a considerable role of B cells and antibody in TB pathogenesis through direct antibody effects, enhanced antigen presentation, cytokine production, and modulating the associated inflammation and immunopathology associated with TB disease (79).

Most commonly, *M. tuberculosis* is thought able to evade the lethal effects of phagocytosis by preventing phagosome-lysozyme fusion, down regulating the production of oxygen free radicals and producing catalase and superoxide which counteracts the damage caused by such free radicals. *M. tuberculosis* can therefore divide unchecked in pulmonary macrophages and induce apoptosis. As the macrophages burst and release the TB bacilli, more

macrophages extravasate toward the site of infection and these too will become infected.

Macrophage activation induced by T cells results in cell death, and the formation of caseating necrotic foci, although several cycles of mycobacterial replication occur before macrophage activation (80). These foci become surrounded by activated macrophages which develop into epithelioid giant cells forming a granuloma. Caseating granulomas are the pathological hallmark of TB infection. Whilst their formation has historically been considered a protective mechanism to contain the infection in the human host, and indeed this is notably impaired with impaired cell mediated immunity, granulomas also provide a niche for the persistence of *M. tuberculosis* within the human host (78).

Patients able to contain TB bacilli in granulomas are considered to be latently infected with TB. Most will remain free from active disease, but 5-10% will progress to clinical tuberculosis, and more if immunosuppressed. In these patients, through poorly understood mechanisms, *M. tuberculosis* contained in the centre of the granulomas will begin to divide and liquefy. This provides an excellent environment for the uncontrolled extracellular proliferation of TB bacilli resulting in lung or other tissue necrosis and potential spread beyond the primary site, both of which will lead to symptomatic disease.

1.4 Clinical tuberculosis

Tuberculosis results from respiratory infection with *M. tuberculosis,* as shown definitively in two year study in 1959 in which an average of 156 guinea pigs were exposed to air in the exhaust duct from a tuberculosis ward with closed circuit ventilation; 71 became infected, largely with lesions in the lungs (81). Respiratory droplets containing the bacteria are produced intermittently when an infected patient talks, sings, coughs and/or sneezes (82). The droplets are small and may remain airborne for some time. In the guinea pig study, despite exposure to large volumes of air from patients with active infection, only a small number of bacteria were capable of causing infection. This group also reported that infectivity can be reduced in the presence of UV light, a concept again being studied in efforts to prevent nosocomial transmission of MDR and XDR TB with some success (83).

Close contact for prolonged periods of time is therefore required to inhale the droplets causing infection; the largest of these will be filtered in the upper respiratory tract with only the smallest reaching the alveoli. Interestingly, of 22 cases in the guinea pig study where the source patient could be identified by drug sensitivity testing, 19 of these cases were generated by 2 source patients despite most patients expectorating large numbers of TB bacilli; it is unclear which of the heterogeneous populations of *M. tuberculosis* are most transmissible. Once in the alveoli, the pathogenic TB bacilli are able to invade macrophages and so begin the process of infection.

The vast majority of patients will either clear *M. tuberculosis* or contain the bacilli with granulomas; these patients are considered latently infected. Most of these patients will remain asymptomatic. Lymphohaematogenous spread may occur to almost any organ where the TB may similarly lie dormant without causing symptoms.

A small proportion of patients will be unable to clear or contain *M. tuberculosis* and will develop primary active tuberculosis. Most active disease, however, is as the result of reactivation in the latently infected.

Pulmonary tuberculosis

The classical symptoms in patients presenting with active pulmonary tuberculosis are cough, sputum expectoration, fever, presenting sub-acutely with accompanying night sweats and weight loss. The predictive value of such classical symptomatology, although supported by some studies (84, 85), may be overestimated. In a study of TB patients in London, fever, sweats and weight loss were each absent in around 40% of culture confirmed new TB cases (86). All three were absent in a quarter of patients.

In resource limited settings, the duration of cough has been investigated. In Lima, a low yield of smear positivity was found in patients with cough duration less than two weeks compared to longer duration; 3.2% compared to 12.4% (87). A study in Malawi studied 90 patients with a short duration of cough, 1-3 weeks, unresponsive to standard antibiotic treatment and diagnosed TB in 35%; 10 cases were sputum smear positive and 24 were smear negative but culture positive (88). Many NTPs mandate smear microscopy of patient's sputum if they

present with cough of longer than 3 weeks of duration not responding to antibiotics and this forms the basis of many public health advertisements to improve case finding.

In high burden settings, a high index of suspicion for TB should be maintained in the presence of any suggestive symptoms. Much stigma is attached to TB disease in these settings, where it may be assumed that the patient is also co-infected with HIV, and thus patients may avoid presenting to healthcare facilities thereby risking more extensive disease and onward transmission in the community.

Extra-pulmonary tuberculosis

There are three main routes for extra-pulmonary dissemination of *M. tuberculosis*: contiguous spread from, usually, the lungs during reactivation to the pleura or pericardium or adjacent lymph nodes; reactivation of bacilli spread haematogenously or via lymphatics during primary infection; lymphohaematogenous spread from pulmonary or extra-pulmonary foci during reactivation due to overwhelming infection and/or immunosuppression.

Extra-pulmonary tuberculosis may affect almost any organ hence the possible symptoms are legion. It is more common in immunosuppressed patients, especially those co-infected with HIV, and may present less typically, with constitutional symptoms and/or symptoms reflecting the any of the target organs affected.

1.5 TB diagnostics

Diagnosing latent TB

Patients latently infected with *M. tuberculosis* are at risk of developing active disease. There is a 5-10% lifetime risk in immunocompetent patients (89), weighted to much higher incidence in the 2-5 years post exposure, with a much greater risk in the immunocompromised; those co-infected with HIV have an annual risk of <10% (90-92). Given there are 3 billion people globally infected with latent TB, there is a very large pool of potentially active disease. Identifying those patients latently infected is therefore beneficial as preventive therapy has been shown to be valuable in reducing the risk of active disease in many patient groups (63).

Delayed type hypersensitivity to tuberculin has been employed in the tuberculin skin test (TST); a small amount of purified protein antigens of TB are injected into the skin and the level of induration measured at 48 hours. This test is confounded by prior BCG vaccination and a positive result may require further investigation. Results in the immunocompromised may be more difficult to interpret as they may not mount an appropriate reaction; this must be considered when reading the induration (table 1.2).

Table 1.2 Interpretative guidelines for Mantoux testing

Patient group	**Mantoux positive at:**
HIV TB contact Fibrotic chest x-ray changes consistent with TB Organ transplant recipient Steroid use >1 month	>5mm
Immigrant from high burden country in UK <5 years Injecting drug user Occupational risk, e.g. prison, hospital, homeless TB laboratory personnel At risk group: diabetes, renal impairment, silicosis, malignancy Children <5 years, or infant exposed to high risk adult	≥10mm
No known risk factors	≥15mm

Adapted from US CDC guidelines (93)

A chest x-ray may provide evidence for latent TB infection in patients with difficult to interpret TSTs. There may be evidence of calcified granulomas in the lower lobes known as Ghon foci. Few guidelines advise chest x-ray as first-line screening in asymptomatic patients owing to poor sensitivity and specificity and a lack of cost effectiveness (94, 95) but this is very much dependent on the background prevalence of TB, MDR TB, the risk factors for transmission and acquisition in settings such as prisons.

In order to overcome the potential issues surrounding chest x-ray and TST, recent research has focussed on interferon-gamma release assays (IGRAs) which measure the production of γ-interferon when patient blood containing T-cells are stimulated by TB antigens in the laboratory. There are three tests

currently on the market; QuantiFERON-TB Gold test (QFT-G), QuantiFERON-TB Gold In-Tube test (QFT-GIT) (Cellestis Limited, Carnegie, Victoria, Australia) and T-SPOT.TB test (T-Spot) (Oxford Immunotec Limited, Abingdon, United Kingdom). These tests use antigens, including ESAT6 and CFP10, which are not present in *M. bovis* BCG and therefore should not be affected by previous BCG vaccination. Quantiferon testing additionally uses the TB 7.7 antigen (Rv2654c), although in a study of differential *ex-vivo* responses in T cell reactivity to stimulation by the individual antigens, none of the LTBI donors responded to TB 7.7 antigen (96).

All these IGRA tests have shown suitable specificity for implementation in NTPs. Conflicting results of the sensitivity of QFT vs T-SPOT.TB are inconclusive and all are based on active TB in the absence of a suitable gold standard for LTBI (97, 98). Studies to date have enrolled heterogeneous populations using varying protocols and thus many questions remain unanswered. In particular, questions remain as to the application of the tests in populations with high rates of HIV co-infection especially with low CD4 counts (57), or other immunocompromise, high burden of TB, both drug sensitive and resistant TB, and their predictive value for identifying those patients who will go on to develop active disease for which no test seems to have a high accuracy (99).

The latter point of is of great importance to any cost benefit analysis. In the UK at present, IGRA is used to confirm a positive TST, although this will miss TST negative IGRA positive patients, or where the TST may be considered unreliable due to immunocompromise. Research is underway to include

additional TB antigens and cytokines to improve the value of this test using next generation IGRAs.

All tests for latent TB infection are, however, indirect measures of adaptive immune responses to mycobacterial antigens; they are unable to differentiate the presence of viable TB bacilli with potential to reactivate, which may require treatment, from evidence of long lasting immunity to TB (100). The current tests for latent TB may therefore identify a large group of patient who may benefit from treatment, but the vast majority of whom would never have developed active disease. There is particular interest in using these to discriminate long-lasting TB immunity from latent TB infection and to identify the small number of patients truly 'at risk' of progressing to active disease.

Diagnosing active TB

The diagnosis of tuberculosis is best achieved by identifying *M. tuberculosis* in clinical specimens using culture. This provides mycobacteria for speciation, typing and phenotypic and genotypic resistance testing. It is recognised, however, due to both the nature of disease and the resource constraints in many settings, this may not always be possible. The WHO provides useful case definitions for classification (table 1.3), although it should be noted that some of these definitions are for pragmatic use in locations where culture is not available or in widespread use, e.g. the 'case of TB' definition allows for inclusion of cases where the clinician initiates TB treatment without culture confirmation of mycobacterial disease. This may have significant impact on the epidemiology of TB, particularly in countries where TB is prevalent and treatment may be commenced more frequently on the basis of risk without laboratory diagnosis;

only half of the reported TB cases in low and middle income countries are culture confirmed (101). Unexpected changes in TB incidence, which has been shown to fall slowly even in successful control programmes, should therefore prompt an initial investigation of diagnostic and surveillance methods. For the studies presented in this thesis, all patients are smear and culture positive and have a definite case of TB according to WHO definitions.

Table 1.3. WHO Definitions of TB cases (3)

Definite case of TB	A patient with *M. tuberculosis* complex identified from a clinical specimen, either by culture or by a newer method In countries not routinely identifying *M. tuberculosis*, a pulmonary case with one or more initial sputum specimens AFB positive is considered "definite"
Case of TB	A definite case of TB OR one in which a health worker has diagnosed TB and decided to treat the patient with a full course of anti-TB treatment.
Case of pulmonary TB	A patient with TB disease involving the lung parenchyma.
Smear-positive pulmonary case of TB	A patient with one or more initial direct sputum smear examinations AFB-positive; or one sputum examination AFB-positive plus radiographic abnormalities consistent with pulmonary TB as determined by a clinician.
Smear-negative pulmonary case of TB	A patient with pulmonary TB who does not meet criteria for smear-positive disease. Diagnostic criteria should include: • two or more AFB-negative sputum smears • x-ray abnormalities consistent with active TB • no response to broad-spectrum antibiotics (except HIV positive) • a decision by a clinician to treat with anti-TB chemotherapy. A patient with positive culture but negative AFB sputum examinations is also a smear-negative case of pulmonary TB.
Extrapulmonary case of TB	A patient with TB of organs other than the lungs diagnosis; based on culture-positivity, or histology or strong clinical evidence consistent with TB and a decision by a clinician to treat with anti-TB chemotherapy. A patient in whom both pulmonary and extra-pulmonary TB has been diagnosed should be classified as a pulmonary case.
New case of TB	A patient who has never had treatment for TB or who has taken anti-TB drugs for less than one month.
Retreatment case of TB	There are three types of retreatment case: (i) a patient previously treated for TB who is started on a retreatment regimen after previous treatment has failed (treatment after failure); (ii) a patient previously treated for TB who returns to treatment having previously defaulted; and (iii) a patient who was previously declared cured or treatment completed and is diagnosed with bacteriologically-confirmed (sputum smear or culture) TB (relapse)

Adapted from WHO Global Tuberculosis Report 2012 (3)

Chest x-ray

Chest x-ray findings attributable to tuberculosis are common and range from small areas of calcification in the lung parenchyma or the pleura, airspace shadowing, cavities, lymphadenopathy and/or pleural or pericardial effusions.

Patients suspected of active TB are routinely offered a chest x-ray. The wide ranging pathological changes make interpretation highly subjective with high levels of intra- and inter-observer disagreement. The role of chest x-ray in the diagnosis of TB is therefore limited and a bacteriological diagnosis is still required, however, they may be valuable in diagnosing disease in possible and probable smear negative TB where pragmatic treatment decisions may be required. In addition, chest x-ray may indicate the mycobacterial burden (102), and the extent of lung disease.

More importantly given the wide differential diagnoses which must be considered in patients presenting with symptoms of TB, CXR may pick up important non tuberculous pathology, e.g. lung cancer, as the cause of patient symptoms.

Blood tests

Blood tests commonly employed to differentiate infectious from non-infectious diseases, e.g. leucocyte count, C-reactive protein, have proven largely ineffectual in the diagnosis of TB, being neither sensitive nor specific as single tests. However, one study used proteomic analyses to identify appropriate targets to measure in the blood of patients with TB. They found that by applying a support vector machine classifier to the combined measurement of serum

amyloid, transthyretin, C-reactive protein and neopterin had a diagnostic accuracy of 78%, much higher than a single sputum smear (103).

Serological antigen testing has been extensively studied in small trials and indeed many are used in field settings. It has, however, been universally agreed that such commercial tests have no value in the investigation of TB (104) (105). In 2011 this led the WHO to issue a statement warning against the use of current commercially available tests (106). In non-commercial studies not included in the review of serological tests however, several antigens have shown potential value.

A study measuring 38kDa IgG, IgM and IgA antibodies and circulating immune complexes showed a greater than 95% sensitivity in smear positive and negative culture positive TB (107). In another study, measuring serum antibodies to LAM had the highest diagnostic value with sensitivity and specificity of 67% and 81% respectively and, significantly, differentiated smear negative and smear positive patients (108). Serum testing remains an attractive option given there are fewer laboratory safety issues than culture and the ease of blood collection; further research is required to ascertain the value of serology in TB diagnosis.

IGRAs for active TB diagnosis

Immunological testing of patients γ-interferon response to TB antigens is unable to differentiate active from latent TB (109). Meta-analyses of studies in the published literature found pooled sensitivities between 60-88% and generally concluded these as too low to employ IGRA testing to confirm or to rule out

active TB (109-111). This was confirmed in a UK based prospective study of patients with a middle or high pre-test probability of having active TB which concluded a NPV of 83% in patients with highest pre-test probability (112). Lower sensitivity of around 60% was reported in a study of 126 patients in Poland and found IGRA no better than TST, with levels of IFN detected the same in both culture negative and positive patients (113). In contrast a study in China supported the use of IGRA in BCG-vaccinated populations and were able to show increasing IGRA conversion from positive to negative during treatment (114).

IGRAs therefore have no role in the diagnosis of TB other than in situations where culture is unavailable. However, despite no NICE guidance supporting a role for IGRA in diagnosing active TB, a recent audit of UK respiratory physicians found that IGRAs are commonly being utilised for the investigation of active TB (115). Ongoing research is likely to answer outstanding questions surrounding the use of IGRAs in the work up of patients with possible active TB (116). All efforts for diagnosis should be concentrated on obtaining material for mycobacterial culture.

1.5 Mycobacterial diagnosis

Smear microscopy

The diagnosis of TB continues to be based largely on the identification of acid-fast bacilli in patient sputum samples. The yield of a single sputum smear in identifying AFB in patients with culture confirmed pulmonary tuberculosis is low (117-121), and even lower in patients co-infected with HIV (122-126). The low standard of equipment, poor conditions and inexperienced staff result in smear detecting culture confirmed *M. tuberculosis* in around 20-35% of samples (127). Multiple specimens are therefore advised with each serially tested sample improving the sensitivity without reducing the specificity.

Efforts have been made to improve detection of TB in field settings by optimising smear microscopy method as this is the only method currently available in many low and middle income settings (121, 128, 129). The WHO advises that smear positive samples should be confirmed as culture positive thereby requiring incubation for up to 8 weeks. This incubation time has been used for many years on the basis of studies conducted in the middle of the 20th century showing very little detection of *M. tuberculosis* after 8 weeks (130, 131).

TB culture

The culturing of *M. tuberculosis* has traditionally employed the egg-based solid Lowenstein-Jensen (LJ) media. Colonies may be identified after 4-8 weeks. Earlier results may be due to the presence of contamination or non-tuberculous mycobacteria. More recently, there has been roll out of automated liquid media systems which are able to more quickly isolate *M. tuberculosis* compared to LJ;

11 days vs 30 days (132, 133). Samples in liquid MGIT media are routinely incubated for 6 weeks, with early studies showing significant reductions in time to positivity compared to LJ media (134-136) and the ability to detect mycobacteria present in samples even when diluted to 10^{-8} (137).

Colonies in both solid and liquid media appearing as serpentine cords is often considered characteristic of *M. tuberculosis* in context, but has proven insufficiently specific for use in TB diagnosis in automated liquid culture systems (138). Organisms isolated should be confirmed as TB using molecular methods and standard DST should be performed to at least first line drugs.

Isolates may be tested for drug sensitivity on LJ media by the agar proportion method, and also the absolute concentration and resistance ratio methods. MGIT DST uses critical concentration method. The critical concentration is that where sufficient antituberculous drug is present to prevent growth detection in the Bactec. There is continuing controversy of the ability of this method for use in clinical practice as the variability in growth of drug resistant strains may increase the proportion of negative results. The methods for DST are best evidenced to detect resistance to first line drugs, with less robust data available for resistance to second and third line drugs.

Molecular diagnostics

Molecular tests have long been used in TB diagnosis as they offer rapid results allowing for more timely clinical management and infection control. The first commercially available nucleic acid amplification test (NAAT) was introduced in 1996, MTD (Gen-Probe, Inc, San Diego, USA). Since then numerous

commercial and in house NAATS kits have been developed using different targets and different methodologies to allow detection and differentiation of organisms of the *M. tuberculosis* complex and their genotypic resistance. Sensitivities and specificities between methods vary widely and the complexity of testing generally requires specialised equipment and highly skilled labour lacking in many resource limited settings where easy use point of care testing may be favourable.

The WHO has recently endorsed the Xpert MTB/RIF (Cepheid, USA) which is used to test patient sputum samples directly. Automated sample processing and real time PCR superfast technology amplifies genes specific to *M. tuberculosis* in addition to the rifampicin resistance determining region of the rpoB gene. A results is available in 2 hours. Biosafety facility requirements are minimal as organisms are subject to >6-log-unit killing within the initial 15 minute processing step even after reagent storage between 4-45C for 3 months. In contrast, the preparation of smears for microscopy generates culturable bio aerosols and thus Xpert is relatively safer than smear microscopy (139).

The sensitivity for smear positive and culture positive *M. tuberculosis* was 98.2% and 99.2% respectively (30). Crucially, the test was also better in smear negative patients identifying culture positive *M. tuberculosis* with sensitivity of 72.5% for a single sample, rising to 85.1% and 90.2% for second and third samples. Since this study, Xpert has been subject to a number of Cochrane systematic reviews, most recently analysing results of 27 studies of 9557 patients of which 59% were conducted in low and middle income countries

(140). This review confirmed high sensitivity and specificity of 89% and 99% with sensitivity for smear and culture positive TB rising to 99%. In consideration as a replacement for smear microscopy, Xpert increased the detection of *M. tuberculosis* by 23%. This presents a potential further benefit to TB diagnostics in areas of high HIV burden where patients are more likely to have smear negative disease (141)

The sensitivity and specificity for detecting rifampicin resistance were similarly impressive at 97.6% and 98.1% (30). The assay has been tested for use in decentralised settings in resource limited settings confirming its value in field settings (142, 143). A cost effective analysis, considering not only rapid diagnostic in field settings, but the increased ability of NTPs in active case finding favoured the rapid scale up of Xpert MTB/RIF (143).

A study in Peru, however, found lower specificity of rifampicin resistance of 91% with a positive predictive value of just 67% (144) which prompted some assay modification. There have been numerous case reports of false positive results (141, 145-147). Such reports highlight the inherent problem of interpreting positive and negative predictive values and may impact the assessment of the Xpert MTB/RIF; the value itself in not inherent in the test, instead it is related to the prevalence of the disease in the population and results, therefore, may not be generalised where prevalence fluctuates, as in TB. Falling prevalence results in increasing false positives which has significant implications; TB patients falsely identified as rifampicin resistant may be treated with drugs with lower efficacy for unnecessarily prolonged durations.

Moreover the test identifies only rifampicin resistance which is largely interpreted as a proxy for MDR TB. Susceptibility to isoniazid is found variably in 0.5%-11.6% of studies of rifampicin resistance. This also again has significant clinical implications; patients identified as having MDR TB on the basis of rifampicin resistance but who have *M. tuberculosis* sensitive to isoniazid may be prescribed an inferior regimen with significantly worse adverse event profiles while withholding the most bactericidal drug available (148).

There are other nucleic acid amplification tests for identifying *M. tuberculosis* which may have advantages in identifying drug resistance other than rifampicin. These tests usually require the isolation of *M. tuberculosis* in culture and can confirm organisms of the *M. tuberculosis* complex. The Genotype MDR TBplus (HAIN Lifescience GmbH, Nehren Germany) additionally identifies high and low level isoniazid resistance associated with mutations in the katG gene and promoter region of the inhA gene. More recently, this test has been developed to include amplifiers for genes involved in resistance to ethambutol, fluoroquinolones and other second line TB drugs (149). As these complex resistance mechanisms are not yet fully elucidated, and may have variable effects on outcome these results must currently also be confirmed using standard susceptibility testing (150).

Molecular tests have been developed for other resistance associated genes for first and second line drugs. Whilst these methods will identify the resistance determining regions selected for amplification, the penetrance of all such genes is currently unknown. Moreover, not all phenotypic resistance will be due to

these mechanisms. In addition, there may be efflux and other mechanisms which may not be identified by current molecular resistance testing.

Recent investigation of the predictive value of whole genome sequencing to detect *M. tuberculosis* and predict resistance to first, second and third line TB drugs have been reported (151). There are likely to be further developments in the near future given the rapid progress in this field (152). Rapid whole genome sequencing allows clinicians to direct initial treatment and prioritise standard resistance testing of those drugs found to be genotypically sensitive. As standard DST to many agents is not validated and may take many weeks or months, it may be best to choose first those agents showing least likelihood of phenotypic resistance on whole genome sequencing. Falling costs of whole genome sequencing to around £50 are not much greater than drug sensitivity testing using MGIT at $56 which only provides resistance data for current first line drugs (61, 153), however, it remains to be seen whether these will fall to within the reach of public health budgets in resource limited settings. TB culture will still be required for treatment monitoring as currently studied molecular methods do not reflect changes in mycobacteria during chemotherapy (154, 155).

Diagnosing extra-pulmonary tuberculosis

The principles of diagnosing EPTB are the same as pulmonary disease; a mycobacterial diagnosis is preferable. All attempts should be made to obtain material for culture. This may include lymph nodes, pleural or pericardial fluid, surgical site samples not fixed in formalin, and bone marrow aspirates. The yield of positive smear and culture, however, are generally lower. Where fluid

samples are collected, spinning down a large volume of fluid and culturing the concentrated pellet may provide additional yield of positive results.

Given the highly variable clinical presentation, diagnosing EPTB can be challenging, and histological examination plays a prominent role. Histologists should be alerted to the possibility of TB and look for the typical, or typically atypical, lesions of EPTB, and stain samples appropriately for AFB. Histological samples may be tested by molecular methods but clearly negative, and indeed positive, results must be interpreted with caution. Given the lower sensitivity and specificity of mycobacterial diagnosis in EPTB, additional tests have been evaluated, e.g. adenosine deaminase. Such evaluations are complicated by the lack of a definitive gold standard.

Central nervous system (CNS) disease may be diagnosed by examining the cerebrospinal fluid (CSF) which may show a predominantly lymphocytic pleocytosis, elevated protein and low glucose. AFB are seen in around 10-20% of cases (156), although there is considerable variation. CSF culture is positive in 60-70% of cases (157, 158) but considered the gold standard as it allows for both detection and evaluation of drug resistance. More rapid PCR techniques have been trialled with variable but largely poor sensitivity (159). Ongoing studies are assessing the application of Xpert in diagnosing tuberculosis meningitis.

Diagnostic delays due to complexity of sampling sites of suspected EPTB are likely to impact on morbidity and mortality and may lead to patients receiving empirical treatment without confirmation of *M. tuberculosis* or drug sensitivity.

1.6 TB Treatment

Treatment for Latent TB

In immunocompetent patients, treatment with 6 months isoniazid, 3 or 4 months rifampicin, 3 months of daily isoniazid and rifampicin or 3 months of weekly isoniazid and rifapentine can be recommended (160) (161). The benefits of reduced incidence of TB, however, must be balanced against the risks of adverse events, particularly hepatotoxicity. As liver damage is higher in advancing age, it has become common practice not to give prophylaxis to otherwise healthy patients over the age of 35 years, unless they are a healthcare worker (160).

The treatment of latent tuberculosis infection (LTBI) is advised for all patients with HIV infection regardless of age. Most guidelines advise 6-9 months treatment with isoniazid, however, a study in Botswana showed a benefit in 36 months of treatment with a 50% reduction on TB incidence compared to those receiving 6 months(162). As TB transmission is high in this setting and any effect may have been related to pre-exposure prophylaxis rather than preventing TB diseases, it was important to know that the beneficial effect of prolonged treatment was most marked in patients initially TST positive >5mm, with a non-significant reduction in patients with TST <5mm. A post trial observational study with uncontrolled access to antiretroviral therapy failed to confirm the results of the initial study, even in the TST >5mm in the post-trial period (163). In multivariable analyses, HIV treatment was found to reduce the

risk of death, but not TB. This treatment regimen is therefore not recommended in more recent WHO guidelines (164).

Short course multidrug regimens for latent TB using 2 months rifampicin and pyrazinamide have been trialled (165) (166). Many regimens containing rifamycins show considerable benefit over isoniazid monotherapy (167). There are concerns however that rifampicin and pyrazinamide containing regimens have higher rates of discontinuation due to adverse events particularly hepatotoxicity in HIV uninfected patients (168), although the concomitant risks of viral hepatitis, alcohol etc. were not assessed. Interestingly, hepatotoxicity was significant higher than in quadruple TB therapy in which both drugs are used during the initial 2 month intensive phase treatment (169). Differences in efficacy between HV infected and uninfected patients remains unexplained (170).

Rifampicin containing regimens may interact with antiretroviral medication and regimens containing rifabutin have been trialled with considerable success, but little is known of the incidence of hepatotoxicity (167). While the treatment of latent TB undoubtedly lowers the incidence of active TB, concerns remain that mortality benefit in HIV patients has yet to be definitively proven for the recommended regimens.

The treatment of latent TB should be balanced against risk of developing tuberculosis in patients with relative immunocompromised (see table 1.1).

Concerns that preventive therapy may result in drug resistant disease if active disease has not been excluded have been largely unfounded (164).

There are no data on prophylaxis in TB contacts, either HIV positive or HIV negative, of patients with MDR TB. Present guidelines advise patients should be closely monitored for symptoms and regularly tested with IGRAs. TB drugs in the research pipeline may provide solutions to this problem in future clinical trials.

Treatment of active TB

The initial trials of streptomycin in the treatment of TB conducted by the British Medical Research Council heralded the beginning of randomised controlled trials as we now understand them (171). The drug was made available on a compassionate basis. Patients were randomly allocated to receive streptomycin or placebo in the first such drug treatment trial in TB in 1947. The result at 6 months were unequivocal; 4/55 deaths in the Streptomycin group compared to 14 deaths in the control group. However, resistance developed in the streptomycin in 35 of 41 relapsing patients and the 5 year outcome was similar in both arms. With the advent of Para-aminosalicyclic acid (PAS), combination treatment trials commenced and, as new drugs became available, showed improved results compared to monotherapy and identified the most efficacious dosing schedules.

Currently employed triple drug combination short course antituberculous chemotherapy emerged as a result of a series of clinical trials performed by the

Medical Research Council (MRC) and its collaborators internationally in East Africa, Hong Kong and India (171). Importantly, the Madras trial demonstrated conclusively that home therapy for tuberculosis was as effective as sanatorium treatment and, since then, there has been a continuing trend towards ambulatory treatment at home. Studies conducted in nomadic populations in Algeria were able to confirm the relatively forgiving nature of the regimen when a few doses were missed. With the emerging threat of MDR TB and XDR TB, however, inpatient treatment of patients with drug resistant disease has again risen to prominence to monitor patients on potentially toxic treatment regimens and for infection control to prevent the onward transmission. Evidence is now emerging of better outcomes in home treatment in patients with drug resistant disease but the impact on transmission has yet to be established (172).

Current standard TB drug treatment

The standard TB regimen consists of two phases: a 2 month intensive phase followed by a 4 month consolidation phase. The initial phase is with rifampicin, isoniazid and pyrazinamide, with the addition of ethambutol if isoniazid resistance is likely. In practice, given the programmatic nature of antituberculous treatment and the lack of availability to DST, ethambutol is normally given for the entire intensive phase. In the consolidation phase, patients continue with isoniazid and rifampicin for 4 months. The previously advised continuation phase comprising isoniazid and ethambutol for 6 months has proven inferior in randomised controlled trial (173) and standard 6 month therapy has become the standard recommended by WHO and the International Union Against Tuberculosis and Lung Disease (IUATLD). The actions of the TB drugs is shown in table 1.4.

Table 1.4. Drugs, symbols, mechanisms of action and molecular resistance genes for WHO/IUATLD recommended for standard antituberculous chemotherapy

Drug	Symbol	Mechanism of action	Molecular resistance
Rifampicin	RMP; R	Inhibit DNA dependent polymerase	rpoB
Isoniazid	INH;H	Inhibit mycolic acid synthesis; inhibit catalase peroxidase	katG inhA
Pyrazinamide	PZA;Z	Unknown; activates at acid pH	pncA
Ethambutol	ETB;E	Inhibits arabinosyl transferase involved in cell wall arabinogalactan and lipoarabinomannan production	embB

Treatment of drug resistant TB

Managing MDR TB is much more complex with less evidence for the most appropriate drugs and duration of treatment. WHO guidelines are based on largely very low quality evidence and, in the absence of randomised control trials, is based on the outcomes of three systematic reviews (174-176). Such reviews are inherently biased towards those drugs tried first and most frequently. Further high quality studies evaluating broader ranges of existing and novel antituberculous agents are required to identify the most effective regimen.

Patients are generally prescribed 5 drugs, in accordance with any available drug sensitivity testing and potential risk for adverse events. No single regimen can be recommended for all, however the following principles are advised:

- include at least four second-line anti-tuberculous drugs likely to be effective as well as pyrazinamide during the intensive treatment phase
- drugs to which the *M. tuberculosis* appear to be sensitive are advised, although the limitations in DST for second and third line drugs should be considered
- a fluoroquinolone should be included if possible; moxifloxacin is best (177)
- The regimen should include pyrazinamide, a fluoroquinolone, a parenteral agent, ethionamide (or prothionamide), and cycloserine, or else PAS if cycloserine cannot be used.
- Ethambutol may be used but is not included among the drugs making up the standard regimen.
- Group 5 drugs may be used but are not included among the drugs making up the standard regimen.

A list of all available drugs is provided in table 1.5.

Table 1.5 Drugs available for the management of tuberculosis including MDR TB

Group 1 (First line)	**Group 2 (FQs*)**	**Group 3 (Injectables)**	**Group 4 (Second line)**	**Group 5 (Limited Data)**
Isoniazid	Ofloxacin	Streptomycin	Ethionamide/ Prothionamide	Clofazamine
Rifampicin	Levofloxacin	Kanamycin	Cycloserine	Amoxicillin/ Clavulanate
Rifabutin	Moxifloxacin	Capreomycin	Para-aminosalicylic acid	Linezolid
Ethambutol		Amikacin		Imipenem
Pyrazinamide				Clarithromycin
				High dose isoniazid
				Thiocetazone
				Bedaquiline
				Delamanid

* FQs = fluoroquinolones; Table adapted from Daley CL et al Management of multidrug resistant tuberculosis. Seminars in Respiratory and Critical Care Medicine, 2013 (178) and updated 2015

Pragmatic issues may play a major part in the treatment of MDR TB and XDR TB owing to the limited availability of some agents, their toxicity and the patient safety monitoring required.

Measuring TB treatment outcomes

Measuring outcomes in TB can be challenging. Therapies are long and the follow up period, traditionally two years after treatment completion, is difficult to achieve in routine practice. Soon after treatment commencement, patients responding to therapy feel well; retention is therefore difficult. Furthermore,

there is no definitive test of cure, as asymptomatic patients may be unable to produce sputum samples for mycobacterial analyses. A successful treatment outcome is usually defined by the absence of relapse without microbiological confirmation. Where sputum samples are collected during treatment, even at the suggested 2 month timepoint, the results are insufficient to predict relapse (179). Samples collected post treatment are difficult to interpret in the absence of clinical or radiological deterioration and Isolated positive cultures have been reported in patients with no evidence of clinical relapse.

At present, the WHO advise the recording and reporting of a variety of different treatment outcomes (table 1.6), with cure defined on the basis of a bacteriologically confirmed absence of failure at the end of treatment. These are markedly different from treatment outcomes in clinical trials which tend to employ more stringent bacteriological methods generally and mandate following patients post treatment to monitor for clinical relapse for 12-18 months. Post treatment samples will often be collected, even in the absence of clinical relapse, to confirm the absence of relapse microbiologically. Any positive cultures may be examined to differentiate relapse from reinfection, with only the former being classified as failure of the regimen under evaluation.

Classification of outcomes for patients on treatment for MDR TB are less well established. At present the WHO advises the same treatment outcome measures as for drugs sensitive disease, but with additional definitions for cure and failure (table 1.7):

Table 1.6. WHO definitions of TB treatment outcomes (3)

Outcome	**Definition**
Cure	Bacteriologically confirmed TB; treatment completed with at least 1 smear- or culture-negative sample collected in the last one month of treatment and on at least 1 previous occasion
Treatment Completion	Completion of treatment, not meeting criteria for cure or failure but without negative testing in the last month of treatment
Died	Any cause
Failed	Remaining smear positive at month 5
Default	Interruption of treatment greater than 2 months
Not evaluated	treatment outcome unknown
Successful outcome	Cured or completed treatment

Table 1.7. Additional WHO outcome definitions for patients being treated for multidrug resistant TB (3)

Cure	• Treatment completed per protocol • Five negative cultures >30 days apart in the last 12 months of treatment • One positive culture with no clinical evidence of disease in the last 12 months of treatment as long as followed by 3 negative cultures > 30 days apart
Failure	• Positive culture in >1 of 5 cultures >30 days apart in the last 12 months of treatment • Clinical decision to withdraw treatment due to poor response, clinical and/or radiological deterioration or adverse events

Some centres will report outcomes based on the best available laboratory outcomes. The advent of typing methodologies allows for defining positive cultures from patients clinically unwell post treatment as either relapse or reinfections. Difficulties arise, however, in interpreting such typing in areas hyper endemic for TB where one type of *M. tuberculosis* may predominate as patients may continue to be exposed to the same TB type during and after treatment. Epidemiological surveillance is ongoing both in the field and in clinical treatment trials to further inform this debate. In future whole genome sequencing is likely to provide additional depth, as has been shown in recent studies in areas of high and low TB incidence (61, 180).

Measuring treatment outcomes can be even more complicated in the context of TB clinical trials. Given the efficacy of current TB treatment in clinical trial settings (>95%), clinical trial design now favours non inferiority trials. In non-inferiority trials, a definite outcome is absolutely necessary to interpreting the results. If not, the wrong conclusion may easily be reached when the intention to treat and per protocol populations outcome analyses do not closely concur.

1.7 Treatment monitoring

Patients commencing TB treatment require at least 6 months of treatment. Following treatment, of the small number of patients who will fail treatment, 91% are likely to do so within 12 months (181). Given that more than 90% of patients with drug susceptible disease will have a favourable outcome on first line therapy, it is neither operationally or financially feasible to monitor all patients for 18 months. Additionally, whilst new drugs are desperately needed to shorten treatment duration and manage drug resistant disease, such a prolonged follow up period does not make TB clinical trials attractive to international pharmaceutical companies. Indeed in a recent article on challenges to the development of new TB drugs, Jindani and Griffin suggested the BMRC trials which have given us the current standard 6 month treatment would be financially unviable in the current era of clinical trial regulation and monitoring (182). If the current goals for TB control are to be met, robust measures able to predict treatment response are required.

Symptom monitoring

Clinical symptoms have been shown to be poorly predictive of a TB diagnosis thus it seems unlikely that such measures will provide valuable data for treatment monitoring. It is common for patients to feel better soon after commencing treatment and indeed isoniazid kills 99% of viable bacteria in the first 5 days of treatment. The subjectivity of this assessment, however, cannot be sufficiently reproducible for use as a robust measure for monitoring treatment. Anecdotally, many clinicians use weight gain as a soft marker of

response and indeed this was shown to be a useful predictor in inpatients, however the benefits were largely lost as patients returned to their preadmission status (183). More recently, it has been shown that changes in sputum volume produced during the first 14 days of TB therapy are reflected in changes in mycobacterial load and may therefore be useful in monitoring response to treatment, but no data are available on long-term treatment outcomes (184).

Radiological monitoring

Chest x-ray are commonly used to diagnose TB and may be the basis of diagnosis in many smear negative cases of TB. It is known that the volume of cavities on x-rays correlates with time to positivity in liquid culture (102). Radiation dosing clearly limits the application of x-ray technology to monitor treatment outcomes, however imaging technology which do involve such exposure, like MRI, may find a role in treatment monitoring.

Immunology

There are insufficient data on the role of IGRAs as a quantitative measure of treatment response. As they are indirect measures of T cells sensitised by previous TB exposure, they are unable to differentiate active TB from latent disease. In healthcare workers and those continuing to be exposed to TB in areas of high endemicity, conversion and reversions are frequent, at least in certain populations (185). As such, they are unlikely to be useful markers of treatment response.

Antibody measurement may also play a role in treatment monitoring. Titres of antibodies are known to increase in response to tuberculosis treatment (186). A

more recent study showed a biphasic antibody response, rising initially with a fall in antibody levels after 2-3 months (187). Those infected with isoniazid resistant strains showed later rises in antibody suggesting that killing of different populations of mycobacteria, deficient in this patient group, may be measured by antibody responses; isoniazid acts only on the continuously growing but not persister populations (71).

Bacteriological monitoring

Sputum smear microscopy results provide semi-quantitative information on mycobacterial load. Smears are commonly graded on the basis of the number of AFBs seen at microscopy by standards defined by the American Thoracic Society (188) or the WHO/IUATLD (189). Such smear gradings have been found to correlate with both the time to detect colonies on LJ media and on the time to a positive result in automated liquid culture prior to treatment. However, sputum smears have a detection limit of 10^4 bacteria/mL, and it is not considered possible to differentiate live from dead *M. tuberculosis*. As ZN stained smear are unable to differentiate the heterogeneous populations of TB present. Changes in proportions of these cells in sputum may reflect treatment response and be useful in predicting outcomes.

Cultures of *M. tuberculosis* are also quantifiable and may have a role in monitoring response to treatment. Such methods are employed in early bactericidal activity (EBA) studies of new drugs. EBA studies show a bimodal fall in sputum viable counts when administering standard TB therapy and may be used as comparison to demonstrate the mycobactericidal and sterilising effect of new drugs. EBA studies of drugs, however, do not measure the effects

of treatment on those persisting mycobacteria beyond 14 days and thus may not adequately predict their impact on long term outcomes. Culture methods of quantification of this sort also take a long time to produce results which limits their use in the clinical setting.

Studies have evaluated whether such limits may be overcome by measuring DNA. Like smear microscopy, however, this may be poor differentiation of live and dead bacilli and has been shown to persist beyond treatment completion (190). The measurement of RNA has been proposed as an improved measure of treatment response as it has a shorter half-life and its labile nature may more accurately reflect changes in viable mycobacteria. Studies of messenger RNA (mRNA), while responsive during the first 7-14 days of treatment, show poor correlation with liquid culture results after 2 months of treatment (191, 192) (193). Studies measuring ribosomal RNA (rRNA) have been more promising with one recent study showing rRNA responds rapidly in response to treatment and that that it's measurement was potentially predictive of relapse (194), (195, 196).

Other molecular methods of TB identification may also be able to provide quantitative or semi-quantitative results which may prove beneficial in monitoring therapy but these have yet to be established. It may also be possible to measure TB antigens, e.g. Ag85, LAM which have been shown to be higher in active TB patients but such studies are far from any clinically translational stage at present.

1.8 An overview of TB biomarker research

A biomarker, as defined by the US NIH Biomarkers Definitions Working Group, is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (9). In the context of TB such markers may be clinical, radiological, serological, immunological or bacteriological. Biomarkers may be of great benefit to early phase drug testing and can drastically reduce the numbers of patients required to evaluate best drugs or combinations. In turn, this speeds up the process of go-no-go decisions and will allow only those promising combinations to progress to phase III studies. The methodology for phase III TB trials is currently being redefined to streamline such decisions (197).

Developing useful biomarkers for TB is particularly challenging due to limitations inherent in using non-specific markers like symptoms or radiological findings, which may persist despite adequate treatment, and our limited understanding of bacteriological and immunological changes occurring during latent and active disease and recovery. Most studies have concentrated on differentiating latent from active disease and identifying factors predicting progression from latent to active TB disease. Innate and adaptive immune responses are required for *M.tb* processing, persistence within the granuloma and maintaining the balance of the immune system required to contain the infection (198). Measuring the inflammatory and immune responses leading to this equilibrium may therefore be useful in determining disease status and predicting progression to active disease. However, it does not follow that augmentation of these responses will

correlate with the response to TB treatment, the goal of which is to eradicate, rather than contain, *M.tb*.

The dynamics of heterogeneous populations of mycobacteria present in sites of disease have not been fully elucidated. Whether such populations are adequately represented in patient samples and identifiable using current laboratory methodologies is not known. Furthermore, the immune responses to these different populations and their variability during adequate or inadequate treatment is not fully understood, although the evidence is growing (199). Such studies may be complicated by innate non-specific immune responses to dead mycobacteria; these dead mycobacteria are likely to increase in proportion to TB persister populations during treatment and it may be impossible to differentiate the immune response to dead mycobacteria from that directed towards persister populations.

Immunological markers also vary markedly in their time to extinction from human samples and may not be reflected in an adequate time-scale for use biomarkers of treatment response. A study comparing patients with a remote history of spontaneously cured TB with those recently treated found high rates of persistent positive results in both with no significant difference in quantifiable T cell responses (200, 201). However, a study of T cell subsets identified opposing patterns of T helper and T-regulatory cells associated with treatment response (201). Clearly biomarkers are best assessed early in treatment when any evidence based interventions could be implemented to improve outcome.

Potential biomarkers

Radiological monitoring

The only radiological test routinely used in TB is chest x-ray and indeed the extent of disease and cavitation has been shown to reflect mycobacterial load (102). Unfortunately inter-rater discrepancy is a significant issue interpreting x-rays; specific training schemes for medical staff have had some success in reducing this problem (202). More recent attention has been given to the potential role of CT scanning as the density and volume of lesions has been shown to correlate with mycobacterial load (102). These changes were relatively unchanged by treatment, however, and the reduction in mycobacterial loads did not correlate with cavitation.

A recent study has employed PET scanning with some success. Of 21 patients receiving treatment, 19 had reduced 18F-FDG uptake (203). Of the 2 remaining patients, 1 had delayed cultured conversion and the other was later diagnosed with lymphoma. A great deal more evidence would be required to assess the potential value in such radiological methods and at present any potential benefit would apply only to high income countries as such testing is outside the reach of low and middle income countries.

Serology

The inaccuracy of commercial serological testing for the diagnosis of *M. tuberculosis* have been highlighted by the WHO edict warning against their use, although they continue to be used in many countries with a high burden of TB disease (105, 204). Some combinations of serological antigens, namely serum amyloid, transthyretin, neopterin and CRP, have, however, shown some

promise as diagnostics (103). These were not tested on serial samples during TB treatment and so no inference may be made on their role in treatment monitoring.

Lipoarabinomannan (LAM), the TB cell wall component with diagnostic value for TB in HIV positive patients with a low CD4 count (205), has been shown to fall in response to treatment. Serum LAM decreased after the first 2 weeks of treatment and continued to decline over 24 weeks (206). Lower detection assays, including of that which is bound to high density lipoprotein, have been developed and may prove useful in future (207, 208).

In another serum study of 168 TB patients, healthy household contacts and community in Guinea, 10 serological antigens were studied and their response to treatment measured. Reductions in ESAT-6, Rv2626c an increases in antibodies to 38kDa protein and LAM were identified (209).

A study showing a biphasic antibody response to treatment supports a role for serological monitoring (187). Differential responses in isoniazid resistant populations and delays in antibody responses to the heat shock protein (hsp) 65 antigen associated with persister populations raise hope that such markers may not only reflect overall bacterial populations, but also reflect the impact of existing and future therapies on the different populations of mycobacteria which may be crucial to reducing treatment durations and effecting long term cure.

Currently there is insufficient data to support the use of serum biomarkers of TB treatment response, however there is some promise in this area, and

identification a serum marker or markers is highly attractive given the ease of sample availability and processing, but more studies are required.

Interferon-gamma release assays (IGRA)

Interferon gamma release assays (IGRA) which evaluate the *ex-vivo* response of peripheral whole blood to stimulation by *M. tuberculosis*-specific antigens not contained within the *M. bovis* BCG including ESAT-6 and CFP10, have proven useful in the diagnosis of latent but not active TB (98, 99, 109, 110, 210). At present, IGRA have no role in TB treatment monitoring. Their potential role, if any, in monitoring treatment responses is unclear and differences between commercial and in-house testing have been significant (211) (212, 213). The platform on which they are based, however, is adaptable and the inclusion of new antigens may yield useful biomarkers in future.

Gene expression profiling

Host gene expression profiling has also been explored as a biomarker of treatment response in TB patients (214-216). One study identified >2-fold change in around 4000 genes during TB treatment, 1200 of which were rapidly down regulated within the first week of treatment; these included inflammatory genes (217). In another study, a transcriptional signature using 144 differentially expressed transcripts was able to distinguish TB from sarcoidosis, pneumonia and lung cancer with a sensitivity and specificity of 80% and >90% (218). Similar studies have shown poorer sensitivity using smaller number of transcripts (219, 220). Both TB and sarcoidosis showed an interferon-inducible

gene signature with pneumonia and lung cancer showing an inflammatory signature (221).

Another study of TB transcriptional signatures evaluated a 393-transcript signature in intermediate and high burden settings showing an IFN-inducible gene profile involving both IFNγ and IFNαβ (221). This signature correlated with the radiological extent of disease. A specific 86 transcript signature was able to discriminate TB from other diagnoses. Importantly, the transcript as measured by the molecular distance to health identified in healthy controls, showed treatment response at 2 months with reversion to that of healthy controls by 12 months. A treatment response may be detected as early as 2 week using a specific 320-transcript signature (222). A study of Indonesian patients confirmed a signature involving interferon alpha-beta (IFNαβ) signalling which was response to treatment and identified 3 specific molecules involved in IFN response which they plan to evaluate further with protein expression studies (223). That these transcriptional signatures are maintained in geographically diverse populations, correlate with radiological extent of disease and are responsive treatment raises hope they may have a role in predicting treatment responses in future. Further research is required to identify whether there are gene expression profiles reflecting different proportions of mycobacterial sub-populations harboured by TB patient prior to and during treatment.

TB culture

TB culture after two month of intensive phase treatment has long been quoted as “probably the best available surrogate marker for the relapse rate” (224). Growing evidence, however, highlight the deficiencies of this measure. A re-analysis of the results of the 12 studies conducted by the BMRC in the 1970s and 1980s, and which provided much of the evidence for current short course chemotherapy, which, despite considerable heterogeneity in treatment arms, had largely uniform clinical and bacteriological methodology, showed 2 month culture result to be a poor surrogate of outcome. Only 6 months regimens were included in the analyses which incorporated 49 treatment arms. Samples were collected monthly and cultured on solid media. Treatment failure was defined by a positive month 5 or 6 culture and relapse by 2/3 positives over 3/4 months following treatment completion, depending on the number of colonies.

Overall, the analyses showed 2 month culture conversion to be a poor surrogate of a combined endpoint of treatment failure and relapse with an explained variation of just 0.36. Interestingly the 3 month culture conversion was a much better marker overall with an explained variation of 0.69. Results differed considerably between geographical locations, with 2 month culture conversion in Hong Kong and a 3 month culture conversion in East Africa explaining variations in treatment outcome much better than overall results, at 0.86 and 0.81 respectively. Potential explanations for geographical variations include differing presentations of disease, with East Africans showing greater cavitation and high bacillary loads than patients in Hong Kong, and different regimens and dosing frequencies, with patients in Hong Kong more often

receiving a regimen containing rifampicin throughout and dosing intermittently than those in East Africa.

This geographical variation was not seen in a different study of biomarkers (225) which analysed results from 30 pairs of treatment regimens selected from all randomised controlled trials conducted by BMRC as published by Fox *et al* in 1999 (171). All regimens were included which reported 2 month culture conversion and treatment failure and relapse at 18-24 months after completing treatment regimens which were different only in the first 2 months of treatment or consistently throughout. A metaregression analysis showed that the natural log relapse rate ratio and natural log 2-month sputum-culture conversion rate ratio correlated with a highly statistically significant slope of −3·3596 (95% CI −4·4420 to −2·2771; p<0·00001), which remained highly statistically significant when analysed by region.

The TBTC study 22 trial reported a relationship between 2 month culture conversion using liquid media and treatment outcome (226). These results came from a randomised control study comparing once weekly rifapentine and isoniazid and twice weekly rifampicin and isoniazid in a North American/ Canadian study of HIV-negative TB patients who received an intensive phase of rifampicin, isoniazid, pyrazinamide and either ethambutol or streptomycin. The intensive phase treatment was given daily for 2 weeks after which intermittent therapy could be prescribed locally as the discretion of the managing clinician. Multivariate analysis of factors associated with failure or relapse calculated the hazard ratio of positive culture at 2 months at 2.8 (95% CI 1.7-4.7).

A systematic review and meta-analysis sputum smear and culture monitoring during TB treatment concluded these had low sensitivity and only modest sensitivity for a combined treatment outcome of failure and relapse. This review included 15 papers describing 28 studies and found the sensitivity and specificity of 2 month culture to be 40 and 85% respectively with a positive predictive value of just 18. Of those 4 studies included in the 2 month culture assessment of relapse (225, 227-229), 2 used LJ culture, 1 used both methods, and 1 used 'local procedures'. This meta-analysis also reported on a group of studies evaluating smear as predictive of failure (226, 230-233) and relapse (226, 228, 234-237) and found similarly poor sensitivity of 57% and 24% with specificity of 81% and 85%. The wide array of studies included in the analyses were considered to have pooled validity owing to the fact that individual studies had similar performance characteristics and provides powerful evidence supporting the potential value of sputum monitoring during TB treatment.

The widespread introduction of liquid media may however have a significant impact on the use of microbiological monitoring. A study of 263 South African TB patients using MGIT has found that TTP at baseline was predictive of 2 month culture (odds ratio 1.35) and relapse (hazard ratio 3.74; 95% CI 1.04-13.41) (238). A post hoc analysis of result of the TBTC study 27 comparing moxifloxacin and ethambutol during the first 8 weeks of treatment (239), evaluated MGIT TTP as predictive of the clinical trial endpoint. There was a clear difference in gradient of MGIT TTP over time, reflecting differing reductions in mycobacterial loads, between patients who responded to treatment and those who failed. A cut-off of less than 21 days for sputum cultures at 2, 4, 6, and 8 weeks after the beginning of study treatment had a

sensitivity of 100%, specificity 74%, and accuracy of 75%. The negative predictive value was excellent at 100% but the PPV was poor at 12% which suggests clinicians maybe reassured by a negative result. Taking action on a positive result on this basis, however, may commit a great many patients to an intervention when it might be required for very few.

One potentially plausible cause for the inconsistent predictive value of culture are the proportions of viable but non-culturable mycobacteria which are present in patient sputum samples (72, 240). In one study, these organisms were able to be resuscitated revealing these cells to be the dominant mycobacterial population prior to treatment (73). A recent study employing this method to serial patient sputum samples during treatment found that the addition of resuscitation-promotion factors (Rpf) was able to reduce the MGIT TTP (241). The change in TTP between Rpf-containing and control samples was positively correlated with the duration of anti-tuberculosis treatment suggesting a changing relationship in heterogeneous mycobacterial populations which importantly were not detected using solid media.

A significant problem with current culture methods of predicting outcome is the requirement for a significant delay prior to assessing treatment response, e.g. 2 months. Moreover, samples taken at 8 weeks require a further 6/8 weeks to be declared negative thereby delaying any intervention required. Potential efficacy studies of new drugs or combinations have been evaluated by the enumeration of mycobacteria during the first 14 days of treatment in so called early bactericidal activity (EBA) studies (242). EBA studies involve the collection of overnight sputum samples at regular intervals during treatment with a novel

agent or combination. Previous studies have identified a bi-exponential curve as best describing changes in mycobacterial load with a turning point at 2-3 days.

While EBA studies have been successful in the evaluation of new drugs or combinations, their role in predicting outcome has not been shown. In part this is due to the limited follow up of such patients who will routinely commence standard anti-tuberculous chemotherapy immediately after their involvement in the EBA study in the NTP. They have not, however, when evaluated been able to differentiate long term treatment outcomes as successful or not (243). EBA studies evaluating linezolid for the treatment of XDR TB highlight this problem; EBA studies demonstrated fall in viable mycobacterial counts during days 0-2 as less than half that of isoniazid with only further minimal reduction between days 2-7. In a study of 40 patients who had failed to respond to previous treatment, however, the immediate addition of linezolid to the regimen, as compared to delaying its introduction for 2 months, resulted in much greater culture conversions, 79% compared to 35%.

At present therefore EBA studies do not have any proven role as a surrogate biomarker of treatment outcome. This may change as further evidence becomes available. Studies to elucidate the activity of different populations of *M. tuberculosis* over time may identify correlations with the bi-exponential decay in mycobacterial load seen in EBA studies; it is proposed that the rapid fall in the first 2 days may reflect the bactericidal effect of drugs with the remainder reflecting the sterilisation of dormant cells in patient sputum samples. Longer follow ups may be required to fully understand how the TB population changes over time.

Molecular measures of bacterial load

In 2010, the WHO endorsed the introduction of Gene Xpert (Cepheid) for TB diagnosis. As a biomarker, Gene Xpert has been studied in a retrospective study of 221 patients in Tanzania (154). After 8 weeks of treatment, 29%, 26% and 42% of patients were positive on smear microscopy, LJ media and MGIT compared to 86% who remained positive by Xpert largely due to the persistence of detectable DNA in sputum.

Employing the Gene Xpert cycle threshold (Ct) as a measure of mycobacterial quantification in this study, there was moderate correlation with culture until week 8, but thereafter this relationship broke down. This was further confirmed in an EBA study which included quantification by Gene Xpert; the Ct value was the least discriminatory method, with quantification on both solid and in liquid culture performing significantly better (155). At present, there appears to be no role in Xpert to monitor treatment and its role in predicting outcomes therefore seems unlikely, but this has yet to be evaluated in a clinical trial.

Any future impact of Xpert would need to be significant given the relatively large costs of the testing kits, even at the current negotiated and charitable funding schemes in place, which put the test out of the reach of many national TB programmes. Modifications to the testing kit to include a DNA binding agent to prevent the amplification of dead or damaged mycobacteria may prove fruitful in improving this as a method of quantification and potential treatment monitoring.

A more recent molecular testing methodology has been identified which measures mycobacterial RNA using a unique internal control step which normalises for RNA loss and inhibition (196). Named the Molecular Bacterial Load (MBL) assay, this technique has shown excellent enumeration of mycobacteria from 10^2 to 10^7 to within 0.5 log with sensitivity for low numbers of organisms approaching that of liquid culture. In a study of 111 patients, using a nonlinear model that differed by relapse status provided a better fit for the data and there was a clear correlation between pre-treatment mycobacterial load and relapse; the odds increased by 3.62 for every 1 $\log_{10}$ increase in day 0 mycobacterial load.

Importantly the MBL assay responded quickly to the rapidly declining bacterial load during the first 3 days of treatment currently used in EBA studies. The rate of decline was directly related to the mycobacterial load prior to treatment. The MBL assay was recently tested in a EBA study in Tanzania and was successful as a replacement to culture with greater precision, less missing data as inhibition affects <1% samples compared to 4-8% culture contaminations, and a shorter time to result availability (244). The MBL assay has also been noted to detect rRNA in culture negative samples collected after 8 weeks of treatment. It is proposed that this may be due to the presence of viable non-culturable mycobacteria and has been described in other papers (195). The ability of MBL to detect these dormant organisms may prove beneficial in predicting treatment outcomes which may plausibly be related to the ability of a regimen to kill these organisms in addition to those readily enumerated by culture.

Genomic analysis of the different populations of mycobacteria and their dynamics during treatment is an attractive prospect as it is postulated that mycobacterial persistence has significant impact on treatment durations. Such analyses are complicated by a potential lack of sufficient bacteria for PCR. Culturing the different populations to increase their bacterial number can only be achieved by shifting these populations from one growth state to another, thereby defeating the purpose of their isolation in this regard. A recent study documenting the rapid and accurate whole genome sequencing of *M. tuberculosis* direct from clinical samples gives cause for hope that technological advances may provide solutions for genomic analyses of different populations of mycobacteria, revealing their individual characteristics and help unravel factors influencing their relative dynamics during TB treatment (245).

1.9 Thesis aims

Despite extensive clinical trials in TB treatment, there remains a paucity of longitudinal data on mycobacteriological responses throughout the duration of TB treatment. The greatest amount of data on mycobacterial response comes from early bactericidal action (EBA) studies of new drugs, but these are limited to the first 14 days of treatment.

Previous clinical studies of TB have generally collected insufficient mycobacteriological data of variable quality. Moreover they have employed variable methodologies which are poorly comparable, even when they appear to utilise similar basic laboratory procedures. This is especially true of trials conducted in resource-constrained settings. Previous trials have therefore failed to identify robust predictors of clinical outcome.

This thesis aims to investigate current standard microbiological methods used in TB clinical practice and clinical trials and to consider their value in predicting mycobacteriological responses to TB treatment.

The following hypotheses will be evaluated in patients diagnosed with TB and receiving treatment as part of the REMoxTB study:

1. Early morning sputum samples are not superior to spot samples for diagnosing TB and monitoring response to treatment

2. Smear microscopy is poorly predictive of TB culture on solid LJ or in liquid MGIT media during treatment

3. The relationship between LJ and MGIT culture, in terms of positive yield, sensitivity, and mycobacterial quantification, varies during treatment

4. Pre-treatment mycobacterial load predicts mycobacteriological responses to treatment

5. The duration of incubation in liquid MGIT culture can be reduced without loss of sensitivity and reduce the time to report a negative culture.

Chapter 2 General Methodology

The analyses contained in this thesis are based on patients, sputum samples and data collected as part of the REMoxTB study [Clinicaltrials.gov NCT00864383] (246).

2.1 REMoxTB study

The REMoxTB study was the Rapid Evaluation of moxifloxacin in the treatment of sputum smear positive tuberculosis which aimed to evaluate treatment shortening regimens in patients with drug-sensitive TB.

The study was a randomised, double blind, placebo-controlled trial of two treatment-shortening regimens of four months containing moxifloxacin, each compared to standard six month treatment for the treatment of smear positive pulmonary TB in treatment naïve patients. Patients were HIV negative, or HIV positive with a CD4 count equal to or greater than 250 cells/μL. The clinical trial enrolled patients internationally at multiple sites in South Africa, Tanzania, Zambia, India, China, Malaysia, Thailand, Kenya and Mexico.

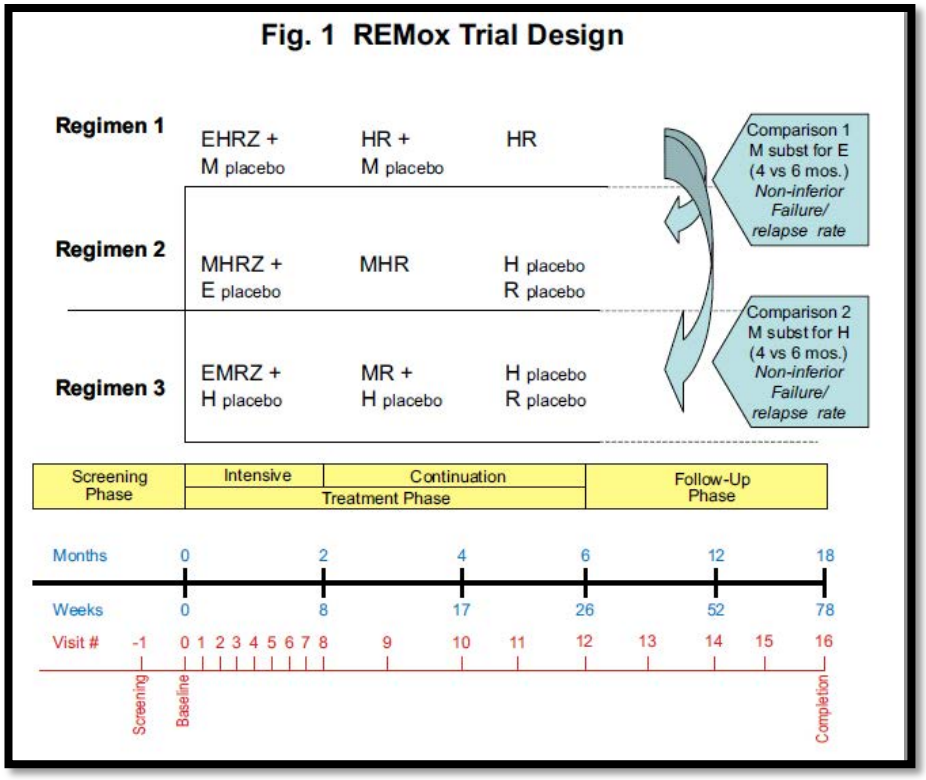
REMoxTB: Hypothesis

The central hypothesis upon which the REMoxTB study was based was that the outcome of antituberculous chemotherapy, in terms of both efficacy and safety, in treatment naïve adults, HIV negative or HIV positive with a CD4 count of 250 cells/μL or greater, with sputum smear positive pulmonary TB receiving either of two four months regimens, one in which moxifloxacin replaces isoniazid and the

other in which moxifloxacin replaces ethambutol, is not inferior to that achieved by patients receiving standard 6 month treatment.

The entire study is summarised in figure 2.1.

Figure 2.1 REMoxTB study design



Study Design

The REMoxTB study was a randomised, placebo controlled, double blind, non-inferiority study of two treatment regimens of four months containing moxifloxacin as compared to standard six month treatment. This was a parallel intervention study conducted at multiple centres internationally.

Patient enrolment

Patients presenting to healthcare service local to the study sites found to be sputum smear positive for AFB were eligible to be invited to be screened for participation in the REMoxTB study. Signed written consent or witness oral consent in the case of insufficient literacy was taken before any trial related procedures were undertaken. The patient information sheet and consent forms are contained in appendix 1.

The study enrolled TB treatment-naïve adults aged 18 years or over with two sputum specimens positive for tubercle bacilli, at least of which had to have been processed at the study-laboratory using study-specific laboratory procedures. Patients were tested for HIV and were able to enrol if HIV negative or, if HIV positive, provided that there CD4 cell count was equal to or greater than 250 cells/μL and they were not already receiving antiretroviral medication.

Laboratory testing was performed to ensure patients had adequate renal and liver function for inclusion. A medical history was also taken to ensure no pre-existing conditions which would have excluded them from the trial were present. Patients found to have MDR TB were excluded either at the time of enrolment using rapid testing, or thereafter. Patients with severe TB disease with a high risk of death were excluded. Also excluded were patients with contraindications to any medicines contained within the study drug. Pre-menopausal women not surgically sterilised or using an intrauterine coil device had to agree to use barrier contraception to be included. Those patients not eligible for enrolment were referred to the national TB programme operating locally for management appropriate to their TB disease.

A full list of inclusion and exclusion criteria is provided in appendix 2.

Study drug

Patients eligible to enrol in the study after screening were randomised to receive one of three treatment regimens below dosed appropriate to their weight. Placebo drugs replaced moxifloxacin (M), ethambutol (E), isoniazid (H) and rifampicin (R) where these were not contained within the allocated study regimen. There was no placebo pyrazinamide (Z) as all patients received this during the intensive phase of treatment. All study staff locally and centrally remained blind to the treatment allocation. Block randomisation was employed to ensure equal numbers of patients in each study arms in each weight category at each of the study sites. A summary table of study drug is shown in table 2.1.

Standard regimen (Control) - 2EHRZ/4HR (Regimen 1)

- Eight weeks of chemotherapy with ethambutol, isoniazid, rifampicin and pyrazinamide plus the moxifloxacin placebo, followed by
- Nine weeks of isoniazid and rifampicin plus the moxifloxacin placebo, followed by
- Nine weeks of isoniazid and rifampicin only.

Experimental - 2MHRZ/2MHR (Regimen 2)

- Eight weeks of chemotherapy with moxifloxacin, isoniazid, rifampicin and pyrazinamide plus the ethambutol placebo, followed by
- Nine weeks of moxifloxacin, isoniazid and rifampicin, followed by
- Nine weeks of the isoniazid placebo and the rifampicin placebo.

Experimental - 2EMRZ/2MR (Regimen 3)

- Eight weeks of chemotherapy with ethambutol, moxifloxacin, rifampicin and pyrazinamide plus the isoniazid placebo, followed by
- Nine weeks of moxifloxacin and rifampicin plus the isoniazid placebo, followed by
- Nine weeks of the isoniazid placebo and the rifampicin placebo

Table 2.1 REMoxTB study drug regimens

Regimen	Week 1-8	Week 9-17	Weeks 17-26
Regimen 1: Standard treatment	HREZ	HR	HR
Regimen 2: Moxifloxacin replaces ethambutol	MHRZ	MHR	Placebo
Regimen 3: Moxifloxacin replaces isoniazid	MREZ	MR	Placebo

Study drug dosing

The prescribed doses per weight are provided in table 2.2. All treatment was taken as a single dose daily, for a duration of up to 26 weeks depending on treatment arm. Study drug dosing was supervised. The method of supervision employed was site specific and included directly observed therapy, therapy supervised by a community engagement worker or a family member or patient nominated treatment supervisor. At each visit, adherence to study drug was recorded from data provided by patients on questioning and reconciled with study drug returned to clinic.

Table 2.2 Study drug doses as per patient weight

Drug	Weight range	Dose
Moxifloxacin	All weight ranges	400 mg daily
Rifampicin	<45 kg	450 mg daily
	45 kg or more	600 mg daily
Isoniazid	All weights ranges	300 mg daily
Pyrazinamide	<40 kg	25 mg/kg rounded to nearest 500mg *dosing in patients < 40 kg, 1000 mg used instead of 500 mg
	45- ≤55kg	1000 mg daily
	>55 kg - ≤75 kg	1500 mg daily
	>75 kg	2000 mg daily
Ethambutol	<40 kg	15 mg/kg daily rounded to nearest 100 mg
	40 - ≤55kg	800 mg daily
	>55kg - ≤75 kg	1200 mg daily
	>75 kg	1600 mg daily

Patient follow up

Patients were reviewed every week during the first eight weeks of treatment, monthly until the completion of study drug at week 26 and 3 monthly for a further 12 months. The total involvement from enrolment was 18 months. To provide flexibility, visit windows allowed for patients to attend for scheduled visits within ±3 days of weekly visit, ±14 days for monthly visits and ±6 weeks for 3 monthly visits; samples collected were allocated to the study visit according to the visit windows which did not overlap. For the purposes of this thesis, samples are analysed as per time on treatment according to the study visit, and not the date of the sample. Patients were able to be reviewed at unscheduled visits at any time where clinically indicated.

At each study visit, patients provided sputum samples for smear microscopy and grading, and culture on solid and in liquid media. For safety analyses, blood samples for full blood count, clotting studies, liver function tests and kidney function tests were collected from patients as per protocol at the baseline visit, then week 2, week 4, week 8, week 12 and week 17.

Full details of the study procedures undertaken in the REMoxTB study at each individual visit is shown in table 2.3.

Table 2.3 Summary chart of REMoxTB study assessments and procedures

Table 2.3 Summary Chart of Assessments and Procedures

			Active Treatment Phase														Follow-Up Phase	
Visit Windows		Up to 14 days after screening	+/- 3 days									- 3 days +1 week after	+/- 2 weeks (except week 26 to +6 weeks after)				+/-6 weeks	
Activity and week of visit	**Screen**	**Base-line**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**	**Week 8[ijk]**	**Week 12**	**Week 17**	**Week 22**	**Week 26[i]**	**Mnth 9**	**Mnth 12**	**Mnth 15**	**Mnth 18**
Visit Number	**-1**	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Inclusion/Exclusion	X	X																
Demographics	X																	
Smoking/Drug History	X																	
Randomisation		X																
Study Drug		X	X	X	X	X	X	X	X	X	X	X	X					
Physical exam/vital signs		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Visual Tests		X								X								
Urinalysis		X																
Sputum smear/culture	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X[a]
Liver function tests	X			X		X				X	X	X						
FBC, clotting, creatinine	X			X						X	X	X						
Pregnancy test	X													X				
Susceptibility testing		X																
Mycobacterial speciation and typing[f]		X																
Adverse event screen	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HIV testing	X																	
CD4 Count[g]	X																	
Chest X-ray		X																

REMoxTB Study outcomes

There were 3 possible study outcomes for patient enrolled in the REMoxTB study; unfavourable, favourable and not assessable.

Unfavourable outcome

1. Patients who required an extension of their treatment beyond that permitted by the protocol, a restart or a change of treatment for any reason except reinfection or pregnancy.
2. Patients who had a positive culture when last seen (with the exception of patients found to have been re-infected) whether confirmed by a second sample or not.
3. Patients dying from any cause during treatment.
4. Patients failing to complete treatment and not assessable at 18 months

Favourable outcome

1. Patients with a negative culture result at 18 months who had not already been classified as having an unfavourable outcome.

Not assessable

1. Patients who, having completed active treatment, default from follow-up their last culture result being negative.
2. Women who became pregnant during the treatment phase and stopped their allocated treatment, unless their last culture was positive in which case they were classified as having an unfavourable outcome.

3. Patients who died during the follow-up phase with no evidence of failure or relapse of their TB.
4. Patients re-infected with a new strain different from that with which they were originally infected.

These study outcomes describe the outcome measures for the REMoxTB study and were not used for the studies presented in this thesis.

2.2 Laboratory methodology

Standardised laboratory procedures were employed across all study sites. Site specific variations were agreed where necessary to improve operational processes while minimising any potential compromise to the quality of the study data. Laboratory data was collected locally using centrally provided laboratory case report forms which can be found in appendix 3.

Sputum Collection

The primary endpoint of the REMoxTB Study was microbiological and therefore care was taken to ensure that quality samples, adequate for microbiological analysis, were collected.

At each patient visit, the patient was given one, or, later in the study, 2, sterile screw cap containers in which to collect their sputum at home as early morning samples were preferred. An early morning sputum sample was collected from the first sputum produced by the patient on the first urge to cough after waking on the day of their clinic visit. Patients failing to provide an early morning sample were encouraged to record the time the sample was produced, or asked to provide a spot sample at the study clinic. Where a time period of greater than 1 hour was to elapse prior to attendance at the study clinic, patients were advised to refrigerate the sample or store in a cool dark place.

During the study visit, study staff collected the samples which a patient brought, and recorded at what time of day the sample was collected and document as either early morning or ‘other’ sample. Patients not providing a sputum sample,

or providing a salivary sample or one of volume <2ml were asked to provide a 'spot' sample in an appropriate environment in or around the study clinic to minimise risk of TB transmission. For the purposes of this thesis, samples not identified as early morning samples were considered spot samples.

Study staff advised patients unable to spontaneously expectorate samples to take a deep breath, hold their breath for a moment and cough deeply and vigorously at the same time the breath is coming out. If the patient remained unable to cough spontaneously, they were instructed to take several deep breaths and hold the breath momentarily, repeating this several times until coughing induced. Where a patient was unable to produce a sputum sample, this was recorded.

Samples collected at the study clinic were stored in a suitable container at a temperature monitored between 2 and 8^{o}C ready for dispatch to the laboratory. Owing to wide variations in temperature and travel conditions, each participating study site devised individual methods by which the temperature of the sample container was maintained between 2 and 8 ^{o}C until arrival at the study laboratory. After arriving at the laboratory, samples not able to be processed within 30 minutes were refrigerated at 2-8^{o}C until processing. Samples received at the laboratory outside the appropriate temperature range prompted site staff to request an additional spot sample from the patient if at all possible. When this was not available, the original sample received outside the temperature range was processed. All samples were processed no later than 48 hours after sample collection.

Sample processing

Each sample was decontaminated before being split for processing for smear microscopy, LJ culture and MGIT culture.

Samples were processed to liquefy organic material and decontaminate bacteria other than mycobacteria while harming as few mycobacteria as possible. N-acetyl-cysteine (NALC), a mucolytic agent, and sodium citrate were used to liquefy the sample and thereby allow for lower concentrations of the decontamination agent, sodium hydroxide (NaOH), to be used. Contamination rates of 3-8% were considered acceptable.

Preparation of decontamination solution

500ml of 4% NaOH was added to 10g NALC and mixed gently until dissolved. 500mls of 2.9% sodium citrate was added to the mixture. Where smaller volumes were required, the amount of each agent was reduced in proportion. The resulting working decontamination solution contained 2% NaOH, 1% NALC and 1.45% sodium citrate. Owing to the rapid loss of activity of NALC in solution, the decontamination solution was stored at 2-8^{o}C and a fresh working solution of decontamination solution was prepared with sufficient frequency to ensure that no more than 24 hours elapsed between preparation of the working solution and sample processing. Working solution was aliquoted to smaller volumes prior to use for sample processing to minimise potential contamination of the working decontamination solution. Changes in the relative concentrations of the decontaminating solution were permitted by the central study laboratory team after consultation in order to maintain the contamination rate within the acceptable range of 3-8% of all samples.

Decontamination

Patient samples were processed in a Class I Biological Safety Cabinet (BSC) preferentially with BSC Class II acceptable where these conformed to British Standard (BS) EN12469:2000; Performance Criteria for microbiological safety cabinets. Samples and reagents were brought to room temperature prior to processing.

The sputum sample was transferred to a 50 mL centrifuge tube. Decontamination solution was added in a volume equal in quantity to the specimen. Where a noticeably bloody sample was provided, an equal volume of a decontamination solution contained only 4% NaOH was added to the sample as NALC does not work in the presence of blood. After vortexing for 15-30 seconds, the tube was inverted to expose the entire sample to the decontamination solution. Additional small quantities of decontamination solution were added to samples which were not sufficiently liquefied and remained mucoid.

Once suitably liquefied, samples were exposed to decontamination solution for 20 minutes before adding phosphate saline (PBS), pH 6.8, to a volume of 50mL. Samples were centrifuged in a refrigerated centrifuge pre-cooled to 4^{o}C at 3000g or more for 15 minutes. After centrifuging the samples were transferred to the BSC and the supernatant discarded into a disinfecting solution of sufficient quantity to ensure mycobacterial killing after dilution with the expected volume of supernatant. The sample pellet was resuspended in a small volume of 1-2mL PBS using a vortex or pipette as required. No more than 8 samples were processed at any one time to minimise errors, ensure

adherence to procedure timing and reduce potential cross-contamination. After processing for smear microscopy and culture, the remaining sediment deposit was refrigerated and stored at 4-8^{o}C for 10 days in case of contamination when the sample was reprocessed.

Smear preparation and staining

Mycobacterium tuberculosis may be detected in patient sputum samples as acid-alcohol fast bacilli (AFB). For the purposes of the study, all patient samples were concentrated and processed for smear microscopy using the Ziehl-Neelsen (ZN) method employing carbol fuchsin as the stain, 3% acid-alcohol for decolourisation and either methylene blue or malachite green as the counter-stain. In addition, some study sites performed an initial staining procedure employing the more sensitive fluorescent stain auramine, with 1% acid-alcohol used for decolourisation and potassium permanganate as counter-stain. Samples initially processed in this way were also processed using the ZN method. The results of the ZN smear were recorded for the purposes of the study database.

Working within the BSC, after labelling a slide, a micropipette with sterilised aerosol resistant tips was used to transfer 30µL of the decontaminated and concentrated sample onto the slide and spread to cover a diameter of approximately 2cm. The slide was placed on a hotplate set at 65-75^{o}C for at least 15 minutes after which time the slide was able to be removed from the BSC. A positive control culture of *M. tuberculosis* H37Rv and a negative control containing only decontamination solution were processed alongside each batch of slides.

Ziehl-Neelsen staining

Heat fixed slides, which may or may not already have been auramine stained, were flooded with carbol fuchsin and heated until steaming with a flame and allowed to stand for 5 minutes before repeating. After 10 minutes exposure to heated carbol fuchsin, slides were flooded with distilled, chlorine-free water, and flooded with 3% acid-alcohol and left to stand for 9 minutes. More acid-alcohol was added during the time where it was noted that the liquid had become heavily stained. After 9 minutes, the acid-alcohol was washed away with distilled, chlorine-free water, and counter-stained with malachite green, or methylene blue, and let to stand for 1 minute before washing away the counter-stain and draining.

ZN stained slides were examined using the 100X objective immersed in oil. For each slide, 100 fields were examined. AFB were assessed in terms of morphology to differentiate them from debris in terms of size, colour, shape, pattern, distribution and uniformity. The presence or absence of AFB was recorded. The amount of AFB present was enumerated and graded as 1+, 2+, 3+ or 4+ using the semi-quantitative method described by the American Thoracic Society (247) (188). The grading used in the study is shown in table 2.4; the grading scale used by the WHO /IUATLD is also provided for reference (248).

Table 2.4 Smear grading reported as per AFB enumeration seen on sputum smear microscopy of ZN stained samples

AFB enumeration	**ATS grading (188)**	**For reference: WHO/IUATLD grading (189)**
1-9 per 100 fields	1+	Scanty
1-9 per 10 fields	2+	1+
1-9 per field	3+	2+
≥9 per field	4+	3+

Where less than 3 AFB were seen on the entire slide, other slides in the batch including the controls were rechecked to ensure quality of processing and smear preparation repeated where appropriate.

Mycobacterial Culture

Each decontaminated and concentrated sample, in addition to being processed for smear microscopy, was split for culture on both solid and liquid media. Culture media provide a nutrient rich environment to encourage growth of the *M. tuberculosis* and a variety of antimicrobials agents to prevent growth of more rapidly growing bacteria and/or fungi. The time taken for mycobacterial growth to occur provided a semi-quantitative method of mycobacterial load.

Solid culture

Decontaminated sputum samples and baseline positive MGIT cultures for archiving were cultured on solid egg-based Lowenstein-Jensen (LJ) media. Each study site arranged their own supply of pre-prepared LJ slopes and had their choice of supplier approved by the UCL laboratory team.

LJ inoculation and incubation

100-200µL of decontaminated sputum or positive MGIT pellet were inoculated on to an LJ slope using a graduated pastette, taking care to minimise aerosol generation particularly with regard to the MGIT tube which is likely to contain larger numbers of mycobacteria. Slopes were incubated at 35-37^{o}C for eight weeks or until growth was detected, whichever was sooner.

LJ slopes were monitored visually each week to detect growth. *M. tuberculosis* colonies were identified by their specific morphological features; dry, buff-coloured colonies. (figure 1.6). Other mycobacteria were identified by rapidity of growth, and their varying morphology, e.g. fine colourless growth on the surface of the slope may represent *M. avium* or *M. malmoense*, coliform-like growth may indicate *M. fortuitum*.

Rapidly growing bacteria over-grow LJ slopes and mycobacteria cannot compete. Slopes contaminated thus or liquefying within 10 days were considered contaminated. In such circumstances, the decontaminated sediment of the sputum sample was retreated and a new LJ slope inoculated. Slopes changing colour but with no visible growth were not considered contaminated unless confirmed by further testing.

Sampling positive LJ slopes for further analyses

LJ slopes with visible growth suggestive of *M. tuberculosis* were further processed by ZN staining and blood agar to determine whether true positive or contaminated.

Blood agar

A 10µL loop of a colony or growth visible on LJ slope was suspended in 1 small volume of sterile saline. Using a fresh loop, 10 µL of suspension was spread onto a blood agar plate and incubated at 37^{o}C for 48 hours. Where growth was detected on blood agar, and the LJ slope was positive within 10 days of inoculation, the decontaminated and concentrated deposit was retreated and LJ culture was repeated as above.

ZN staining

Using a sterile loop, 10 µL of formol saline was placed on a slide. Using a separate sterile loop, part of a colony was picked off of the LJ slope and emulsified in the formol saline on the glass slide. A ZN stained sputum smear was heat fixed and prepared as described above.

Interpreting the results of LJ culture

A sample demonstrating growth on the LJ slope with a negative blood agar and AFB detected on ZN staining was declared a true positive. If the blood agar was positive, with AFB detected or not detected on ZN staining, the sample was reported as contaminated.

The number of weeks before a true positive was detected on an LJ slope was recorded as a measure of mycobacterial load. In addition, growth on the LJ slope was measures semi-quantitatively on the basis of the number of colonies detected. Where no colonies were detected this was recorded as negative and the slope re-incubated. Positive LJ slopes were reported as +, ++ and +++ where 20-100 colonies, innumerable discreet colonies and confluent colonies were detected respectively. Where less than 20 colonies were detected, the number of colonies was recorded.

When a sample was interpreted as contaminated, the time to detect growth was not considered valid. LJ cultures were recorded as negative if no growth detected after 8 weeks incubation.

Table 2.5 LJ result interpretation to obtain overall LJ result

LJ	**ZN**	**Blood Agar**	**Week of growth**	**Overall LJ result**
Positive	+	-	≤8	POSITIVE
Positive	+	+	NA	Contaminated
Negative	NA	NA	>8	NEGATIVE

Liquid culture

Sputum samples were processed for culture in the fully automated BACTEC Mycobacterial Growth Indicator Tube system (BBL™ MGIT™ 960, Becton Dickinson (BD) Microbiology Systems, Sparks, MD, USA). Pre-prepared liquid culture medium was provided in individual 7ml MGIT tubes. The antibiotic supplement PANTA, containing polymixin B, amphotericin, nalidixic acid, trimethoprim, and azlocillin, was added to the medium immediately prior to use.

Preparation of antibiotic supplement for liquid culture

15 mL of MGIT growth supplement was added to a quantity of MGIT PANTA powder pre-provided specifically for this purpose. The resulting mixture was inverted until the powder had dissolved completely and a suspension was produced. The PANTA antibiotic solution was stable for 5 days if stored at 2-8^{o}C.

MGIT Inoculation

Decontaminated and concentrated sputum samples were used to inoculate MGIT tubes; 0.5mL was added to a MGIT tube to which 0.8mLs of antibiotic solution had been added.

MGIT Incubation

MGIT tubes were inserted into station in the Bactec MGIT 960 and incubated until positive or for 42 days when a sample was declared negative, whichever was shorter. Each MGIT tube contains a fluorescent compound in the base of the tube which is sensitive to the presence of oxygen dissolved in the broth. The large amount of oxygen present initially quenches the emission thus

fluorescence is not detected. Micro-organisms present in the tube metabolise oxygen in the culture medium allowing for fluorescence to be detected. A row of LEDs beneath the stations illuminates, activating the fluorescence detectors. The instrument automatically tests all tubes continuously.

Photo detectors take readings of fluorescence measured as growth units. A growth unit (GU) is an algorithmic measure of sensor fluorescence derived from the raw fluorescence voltage signal produced by the optical integration of a MGIT tube in the Bactec 960 instrument. A tests cycle of all tubes is completed every 60 mins. A positive result is flagged when the GU reaches or exceeds the cut-off value of 75 units. If a tube flags positive with a GU of 0 or higher score before 5 hours, this signifies that growth has occurred very rapidly and exploded past the 75 unit cut-off. Such rapid growth is not comparable to the gradual curve generated by a true positive and is likely to represent contamination. Any positive result is indicated on the front of the machine on drawer status indicators, which also indicate negative when a sample has not flagged positive within 42 days or when there is a station error.

Sampling positive MGIT tubes for further analyses

MGIT tubes which flag positive require further processing by ZN staining and blood agar to determine whether true positive, contaminated or false positive.

Blood agar

10µL of MGIT tube solution inoculated with patient sample was spread on blood agar and incubated along with the tube at 37^{o}C for 48 hours. Where growth was detected on blood agar, and the sample flagged positive within 10 days of MGIT

inoculation, the decontaminated and concentrated deposit was retreated and MGIT culture was repeated as above.

ZN staining

There were two possible methods to perform ZN from positive MGIT tubes, the choice of which was employed was at the discretion of the laboratory operator. Direct sampling; a small amount of sediment from the positive MGIT tube was placed onto a slide, with the optional addition of albumin, using a sterile pastette. A smear was made and ZN stained as described above.

Concentrating the sample prior to staining; Either 1 mL of well mixed fluid from the MGIT tube was put into an eppendorf tube and spun in a microfuge to deposit sample prior to removing most of the supernatant and resuspending the pellet in approximately 250µL PBS, Or the whole MGIT tube, or its contents decanted into a universal container, was centrifuged at 300g for 15 mins prior to decanting most of the supernatant into a container containing appropriate disinfectant leaving 2mL broth to resuspend the pellet. However prepared, a small drop of the concentrated sample was placed onto a slide. A smear was prepared and ZN stained as described above.

Interpreting the results of MGIT culture

A sample flagging positive with a negative blood agar and AFB detected on ZN staining was declared a true positive. If the blood agar was positive, with AFB detected or not detected on ZN staining, the sample was declared contaminated.

A false positive sample was declared when the MGIT tube flagged positive but was ZN negative on smear microscopy and no growth was detected on blood agar after 48 hours. In such circumstance, further investigation was undertaken to look for turbidity or evidence of microbial growth in the MGIT tube. In addition, a repeat smear and ZN staining was undertaken using a concentrated sample. If AFBs were still not detected on ZN staining, the MGIT tube was re-incubated for a further 3 days to allow for further growth of *M. tuberculosis* and a repeat ZN smear was prepared. Where this was still negative, the sample was able to be inoculated onto solid LJ media and incubated for 3-4 weeks to identify growth; this was at the discretion of the laboratory personnel at the study site.

Time to positivity (TTP) in the MGIT was recorded as a measure of mycobacterial load. When a sample was interpreted as contaminated, the TTP was not considered valid. Negative MGIT cultures were recorded as having a TTP of 42 days.

Table 2.6 MGIT result interpretation to obtain overall MGIT result

MGIT	**ZN**	**Blood Agar**	**TTP**	**Overall MGIT result**
Positive	+	-	<42	POSITIVE
Positive	+	+	NA	Contaminated
Positive	-	-	NA	False Positive
Negative	NA	NA	≥42	NEGATIVE

Confirmation of Mycobacterium tuberculosis complex species from positive TB cultures

Baseline positive culture samples, or the first positive culture before or during the early part of treatment, and samples with positive cultures at or after 17 weeks were confirmed as *M. tuberculosis* complex by a rapid DNA probe test, Accuprobe *Mycobacterium tuberculosis* culture complex identification test (Gen-Probe, San Diego, USA) according to the manufacturer's instructions as shown in appendix 4. The Accuprobe system uses a single stranded DNA probe with chemiluminescent label complementary to the ribosomal RNA of the target organisms; *M. tuberculosis*, and the closely related mycobacteria *M. bovis*, *M. bovis BCG*, *M. africanum*, *M. microti* and *M. canetti*.

Positive cultures were processed to lyse the organisms releasing their ribosomal RNA. The lysed organisms were exposed to the labelled DNA probe; when complementary strands combine they form a stable DNA:RNA hybrid. A selection reagent was added which differentiated hybridised and non-hybridised probe. The chemiluminescent hybrids were measured in a GEN-PROBE luminometer which generates a numerical result. A numerical result equal to or greater than the cut-off value was considered positive. The test was unable to differentiate between the organisms comprising the *M. tuberculosis* complex. A positive control, *M. tuberculosis* H37Rv, and a negative control organism, e.g. *M. avium*, was included in every run. The probe reagent will not react with mycobacteria other than tuberculous mycobacteria.

Interpretation of results

The results of the negative and positive control were checked to ensure they satisfied the cut-off values provided. Where the control samples failed to achieve the expected results, the sample values were considered invalid and the procedure repeated. Where the control samples satisfied the expected results, the study sample results were read. A sample result equal to or greater than the cut-off value was considered positive. Sample results less than the cut-off values were considered negative. This results falling within approximately one third lower than the cut-off value were repeated and re-interpreted as described.

Positive results confirmed the presence of an organism of the *M. tuberculosis* complex. For the purposes of this thesis, confirmation of an organism of the *M. tuberculosis* complex was considered confirmation of culture confirmed *M. tuberculosis*.

Samples were further processed using IS6110 Restriction Fragment Length Polymorphism Length (RFLP) typing and Mycobacterial Interspersed Repeating Unit (MIRU) / Variable Number Tandem Repeat (VNTR) typing methodologies to confirm the presence of *M. tuberculosis* and as a research tool to establish epidemiological links between strains and patients, and to monitor contamination.

2.3 Statistical analysis

For the purposes of the REMoxTB study, it was determined that a sample size of 633 patients per group would provide a power of 85% to show noninferiority of the two moxifloxacin interventions to the control regimen with a margin of 6 percentage points, assuming a one-sided type I error of 0.0125 (Bonferroni correction) allowing for 10% of the patients in each study group having an unfavourable outcome and 15% with outcomes that could not be evaluated (246).

Analyses presented in this thesis were carried out as sub-studies to the REMoxTB study using available data collected as part of the trial, therefore it was not possible to carry out power calculations for the individual studies presented in each individual chapter. Samples included in the studies presented are all those samples collected prior to week 17 available as described in the methodology section of each individual chapter and range from around 8,500-20,000 samples.

Microbiological results were available for sputum smear microscopy, culture on solid LJ media and culture in liquid MGIT media.

Smear results were recorded as both binary results, positive or negative, and also stratified by semi-quantitative smear grading as; negative, 1+, 2+, 3+ and 4+, using the ATS method as shown in table 2.4 (188).

LJ culture results were recorded categorically as positive, negative or contaminated. Additionally, time to detect (TTD) a positive result in culture was measured for LJ samples as number of weeks (weeks 1-8) until growth detected and provided a continuous measure for analyses. A negative LJ culture was recorded as having a week of growth of 9.

MGIT cultures were recorded categorically as positive, negative, false positive or contaminated. Time to detect a positive result (TTP) in days and hours <42 days was recorded as a continuous measure reflecting mycobacterial load. A negative MGIT culture was recorded as having a time to positivity of 42 days.

Cultures in either media were considered contaminated where growth was detected in primary culture and contaminating bacteria were detected on sub-culture of the primary positive isolate on blood agar incubated for up to 48 hours (tables 2.5 and 2.6). MGIT cultures were categorised as false positive when the machine flagged a positive culture but no acid fast bacilli were detected on smear microscopy and no contaminating growth was detected on subculture of the primary positive isolate on blood agar (table 2.6).

Additional measures used to measure response to treatment include time to first negative and time to sustained negative cultures in either LJ or MGIT.

- Time to first negative (TFN) is defined as the study visit at which the first culture negative sputum sample was collected.
- Time to sustained negative (TSN) is defined as the study visit at which the first of two consecutive culture negative sputum samples was collected, with no positive culture in between

As samples collected after week 17 are not included in these sub-studies of the REMoxTB study, in some patients it is not possible to determine the TSN and/or TFN where these may have occurred after week 17 of treatment. Having attained TSN, some patient reverted to having positive cultures and the time to reversion was recorded as the study visit at which this positive sample was collected.

Proportions and 95% confidence intervals are presented throughout the thesis and significance of differences in proportions are determined using the Chi-square test or Fisher's exact test where the value of any cell was less than 5.

A number of sensitivity and specificity analyses have been performed. For the purposes of these analyses, data have been analysed using binary classification positive/negative, excluding samples with other results, e.g. contaminated, and positive/non-positive where all samples have been included. Sensitivity measures the proportion of positives, as defined by a predetermined gold standard, accurately identified as such, i.e. the true positive rate, by dividing the number of true positives (a) by the total number of positives, i.e. the number of true positives (a) and the number of false negatives (c). Specificity measures the proportion of true negatives, as defined by a predetermined gold standard, which are identified as such, i.e. the true negative rare. This specificity is calculated by diving the number of true negatives (d) over the total number of negatives, i.e. the number of true negatives (d) and the number of false positives (b). (table 2.7)

Table 2.7 Illustration of sensitivity and specificity calculations

		Method 2	
		Positive	Negative/ Non-positive
Method 1	Positive	A	B
	Negative/ Non-positive	C	D
		Sensitivity = a / (a+c)	**Specificity** = b / (b+d)

Continuous data on time to positivity in both LJ and MGIT were positively skewed and non-Gaussian throughout treatment. Histograms are provided in several sections to graphically depict the distribution of data. For the purposes of statistical analysis, normalisation was attempted using logarithmic transformation using the natural log and log base 10, squaring, and square-rooting; none of these methods achieved normality according to Kolmogorov-Smirnov test of normality. When unsuccessful, medians and interquartile ranges are provided and statistical significance of differences determined using the non-parametric Mann Whitney *U* test for unpaired data and Wilcoxon signed-rank test for paired data.

Logarithmic transformation using the natural log (log_n) was used as an approximation of normality where appropriate and means and standard deviations described. Once transformed, the parametric student's t-test was used to determine statistical significance of paired data; for unpaired data, the parametric unpaired t-test was used, with Welch's correction for data with unequal variance.

Regression analyses were used to estimate relationships between dependent and independent variables. Linear regression was used when the dependent variable was continuous, e.g. time to detection.

$$Y = \beta_0 + \beta_1 X_1 + \cdots + \beta_n X_n +$$

Logistic regression was used when the dependent variable was categorical, e.g. positive or negative. Odds ratios were calculated using the following equation:

$$logit(E[Yij|bi]) = \beta_1 X_{ij1} + \cdots + \beta_p X_{ijp} + bi$$

Patients provided multiple samples at serial timepoints giving repeated measures. At the individual patient-level, repeated measures cannot be assumed to be independent, as the result at any timepoint is likely to be dependent on previous and future results in that patient. Mixed effects models with random intercept were therefore used where appropriate to account for repeated measures in individual patients and also allowing for missing values due to variable follow up.

In chapter 4, odds ratios and predicted probabilities of positive cultures compared to negative cultures at each visit given the smear result at the same visit were calculated using a mixed effect logistic regression. Models included a patient-level random intercept which was assumed to follow a normal distribution. Smear result and visit week were included as fixed effects in addition to the smear-visit interaction term. Week of growth of culture on LJ and MGIT TTP in days were recorded to the nearest week and day respectively and log transformed for the analyses since the distributions were positively skewed.

A week of growth of 9 and a TTP of 42 was used for negative cultures on LJ and MGIT respectively.

Mixed effects linear regression was used to estimate mean week of growth on LJ and TTP on MGIT by smear grading, again accounting for between-patent variation, as above. Models included a patient-level random intercept which was assumed to follow a normal distribution. Smear result and visit week were included as fixed effects in addition to the smear-visit interaction term.

In chapter 5, the proportion agreement between different culture methodologies was determined overall and as per time on treatment/study visit. Agreement was further measured using Cohen's kappa scoring. The statistical significance of variability in agreement between different study visits was determined using analysis of variance (ANOVA) as performing multiple t-tests would increase the likelihood of a statistical type I error.

Mixed effects logistic regression analysis was used to measure univariable associations between patient demographics, age, sex, HIV status, used as independent variables, and samples negative in MGIT which are positive on LJ as the dependent variable; these sample results are of particular interest for laboratories replacing LJ culture with MGIT. Models included a patient-level random intercept which was assumed to follow a normal distribution and patient demographics, age, sex, HIV status, were used as fixed effects.

Kaplan Meier plots were generated for time to event analyses comparing TFN and TSN between culture methodologies. Hazard ratios (HR) were calculated

using the log rank test where the ratio of observed events (O) and expected events assuming a null hypothesis (E) in LJ is divided by the same ratio for MGIT

$$HR = \frac{(O_{LJ} / E_{LJ})}{(O_{MGIT} / E_{MGIT})}$$

The Spearman's rank correlation coefficient (R) was calculated as a non-parametric measure of statistical correlation between time to detect positive cultures using the two different culture methodologies MGIT and LJ overall and at each timepoint on treatment. The changes in time to detection over time in each culture method were analysed using a linear regression model and by a non-linear two phase decay and the coefficient of determination (R^2) calculated to compare which model best fit the data. The ratio of time to positivity between MGIT and LJ was calculated for each timepoint.

In chapter 6, Spearman's rank correlation coefficient was calculated for the time to positive culture result at baseline in both culture methods and the time to a first negative (TFN) and time to a sustained negative (TSN) and also for time to positivity at baseline and time to positivity at week 8.

Receiver operating curves (ROC) were constructed for chapter 7 by plotting the sensitivity against 1-specificity as the threshold for MGIT positivity was varied using two different gold standards: LJ culture results, positive or negative; and results classified as positive or negative by maximum positive yield (MPY), where positive is defined as positive in either or both culture media and

negative defined as those samples negative in both media. The area under the receiver operating curve (AUROC) was calculated to determine the strength of the discrimination using MGIT TTP to discriminate positive and negatives. Inflection points for cut-offs with high sensitivity values were considered crucial and reflecting the clinical need to minimise the loss of any positive TB cultures.

All sub-studies in this thesis have a clear selection bias as only smear-positive TB patients were enrolled in the REMoxTB study. These results may not therefore be generalisable to unselected patients presenting for investigation for TB; further research is required. Analyses in the sub-studies were also conducted without knowledge of the treatment allocation which may represent a potential bias. Prior studies, however, have confirmed moxifloxacin to have considerable bactericidal activity with similar results achieved between arms when used during the intensive phase of treatment (239, 249, 250). Furthermore, by excluding samples collected after week 17 when patients in the moxifloxacin-containing regimens were receiving placebo, all samples analysed in this thesis were from patients receiving active treatment. Therefore it is unlikely that treatment allocation had a significant impact on the results.

Results presented were also not routinely analysed per HIV status. As HIV is known to be specific risk factor for TB and may impact the microbiology results, this also represents a potential bias in these studies. However, less than 10% of patients included in any of the sub-studies presented were HIV positive and all had a relatively preserved immune system with CD4>250 cell/μL as a condition of enrolment so it thought any effect would be minimal.

Data storage and analysis

For the purposes of the analyses in this thesis, an extract of the REMoxTB study database, which had been double data entered into a database designed by the Medical Research Council (MRC) and Pharmanet, was performed by the MRC. The data included demographics including age, gender, and HIV status.

The database was extracted and interrogated separately for each specific sub-study on the dates described in the methodology section of the each chapter. All data was stored in Microsoft Excel 2013 and transferred for statistical analyses as defined in each section.

Data were analysed in Microsoft Excel (2013), GraphPad Prism version 6 for Windows (GraphPad Software, California, USA) and Stata 13.0 (Stat Corp, 09). Statistical significance was determined by $p<0.05$.

Ethical approval

The studies in this thesis were included as ancillary studies to the main REMoxTB study in the study protocol for which ethical approval was granted by the UCL ethics committee in addition to the local and national ethics committees at each of the study sites (South Africa: Medicines Control Council; Zambia: Pharmaceutical Regulatory Authority; Tanzania: FDA; Kenya: Pharmacy & Poisons Board; China: State FDA; Malaysia National Pharmaceutical Control Bureau; Thailand: FDA; India: Central Drug Standard Control Organization; Mexico: The Federal Commission for the Protection against Sanitary Risk.)

Chapter 3 Comparing early morning and spot sputum samples in the isolation of Mycobacterium tuberculosis

3.1 Introduction

The diagnosis of TB is largely based on smear microscopy of spontaneously expectorated sputum samples. Early morning sputum samples (EMS) are generally considered to yield a greater number of positive results than spot samples, and have greater sensitivity and specificity for culture, but there is little published data to support this assumption. The most recent edition of Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, however, states that "the best diagnostic sputum specimen is an early morning sample" (251) and the request for an early morning sample is included in the UK National Institute for Health and Care Excellence (NICE) tuberculosis guidelines (252). The World Health Organisation guidelines have historically included an early morning sample in their diagnostic guidelines and this practice has therefore been adopted in the majority of National TB Programmes (NTPs) in developing countries (253-255).

Routine practice in most NTPs involves collecting 3 serial sputum samples; a spot sample collected at the first clinic visit, an EMS early morning sample which the patient brings to their second visit, when a third, spot sample is collected; spot morning spot (SMS). The SMS sampling method is based on a 1959 study which concluded this method has the highest positive yield at the lowest number of patient visits (256). The WHO guidelines changed to advise direct smear microscopy of two samples in 2007 on the basis that 95-98% of

positive cultures were detected using the first two smears, however, the requirement for an early morning sample was unchanged (257, 258). Either practice commits the patient to attend at least two clinic visits before a diagnosis of TB can be made.

The requirement for patients to provide an EMS may prolong the diagnostic pathway and risks losing patients to follow up, owing at least in part to the cost barrier. A significant number of patients are 'lost' during the current diagnostic pathway and fail to commence TB treatment (259) (260-262). The number lost was 15% in rural Malawi during a 6 month period, and 26% were lost during the diagnostic process in Cape Town. Costs of TB as a percentage of annual household income range from 2.8% in Zambia (4) to 10-35% in Ethiopia (263, 264); healthcare costs greater than 10% annual income may be considered 'catastrophic' (265, 266). Moreover such measures may not reflect the additional burden on the poor patients, as compared to 'non-poor', who proportionately spend almost twice as much of their annual income on healthcare costs associated with TB (5).

Given that 50 million patients are investigated for TB with sputum smear microscopy each year globally, and that these patient commonly fall within the age groups of the most economically active, these costs may have considerable implications on a macroeconomic and societal level (267). Families are frequently also affected. In one study in Malawi, 70% of female activities and 30% of male activities were conducted by someone else due to ill health associated with tuberculosis (5). In another study in India, 11% of patient's children ceased attending school indirectly due to TB in a parent and a further

8% were compelled to take up paid employment to support their family (268). The high costs associated with TB may prevent patients from seeking treatment, or lead them to abandon treatment, even when the medical treatment is provided free of charge, as the non-direct costs, such as travel and food, may be prohibitive (269-271).

Awaiting EMS therefore burdens patients, their families and health services in resource limited settings and may contribute to the considerable drop-out of patients in the diagnostic pathway, up to 26% (259-262, 272-274). Sampling guidelines requiring patients to provide an EMS sample may therefore risk the individual patient's treatment response; patients who delay to treatment initiation are known to have poorer outcomes. (275) (276). Patients delaying treatment also represent a continuing reservoir for transmission of TB in the community. (277, 278).

Changes to the requirement for EMS have been reflected in the most recent advice by the WHO in a 2011 policy statement recommending that two 'spot-spot' samples may be collected on the same day (279). The study upon which this is based quotes a 2.8% [95% CI –5.2% – +0.3%] reduction in sensitivity using spot samples. This advice has yet to be implemented widely and only applies to specific settings, emphasizing a responsibility to assure the external quality assurance scheme. Patients providing spot-spot samples rather than awaiting EMS were shown to be less likely to be lost in the diagnostic pathway (2% vs 5.8%).

In addition to their diagnostic value, qualitative and quantitative results of sputum smears and cultures are often used as a biomarker of treatment response and in clinical trials assessing new antituberculous drugs, but there are no published studies on the effect of using either EMS or spot samples for these purposes. Importantly, studies which may provide evidence on which to consider these questions at present tends to predate the introduction of fully automated liquid culture systems which are being rolled out globally and it is unclear what differences, if any, may be identified.

This study aims to compare the qualitative and quantitative value of EMS and spot sputum samples prior to and during TB treatment.

3.2 Hypothesis

Early morning sputum samples are not superior to spot samples for diagnosing TB and monitoring response to treatment

3.3 Methodology

We undertook an analysis of all mycobacteriological results of sputum samples submitted by patients enrolled in the REMoxTB study from January 2008 available on 2 August 2011. We excluded data from those patients who did not enrol in the trial and whose age and/or HIV status and CD4 cell count could not be determined. Samples were excluded where a corresponding smear result and grading, MGIT result and TTP and/or LJ culture result and week of growth were not known. Samples collected after 17 weeks of treatment were excluded

as data were analysed blind to treatment allocation and after this time-point patients on the treatment shortening arms were receiving placebo. Additionally, samples collected at unscheduled visits were excluded.

Microbiology

Patients provided two pre-treatment sputum samples; the type of sample was recorded, usually one EMS and one spot. One weekly sample was collected at each treatment visit for the first 8 weeks. Monthly samples were collected thereafter as described in table 2.3. Early morning samples were requested, with spot samples being collected where this was not provided. Some patients were not able to provide requested sputum samples at every visit. Sputum samples were processed for smear and culture as described in chapter 2.

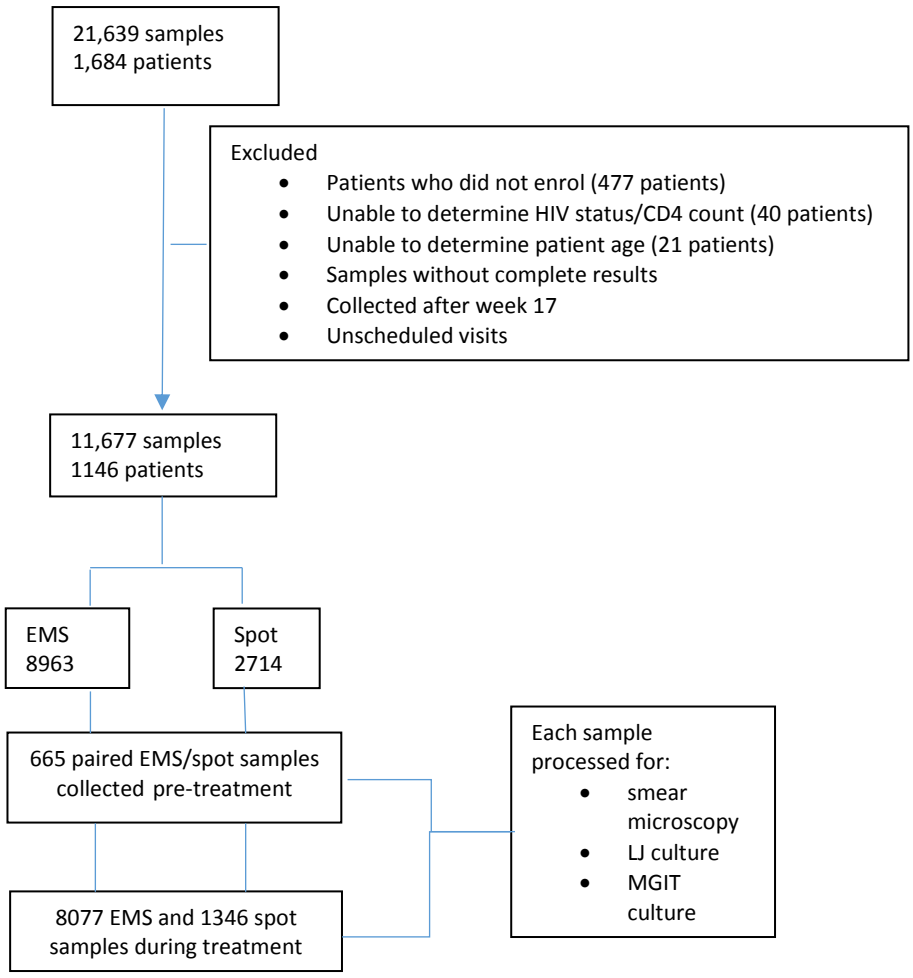
Statistical analysis

Proportions comparing EMS and spot samples are calculated and significance of differences in proportions are determined using the Chi-squared test or Fisher's exact test where the value of any cell was less than 5. Sensitivity and specificity of EMS and spot samples for culture on LJ and in liquid media have been calculated. Differences in time to detection in LJ and MGIT are compared using Wilcoxon matched-pairs signed rank test for paired pre-treatment samples and using Mann Whitney *U* test for unpaired samples collected during treatment. Results with a *p* value of <0.05 were considered statistically significant.

3.4 Results

After exclusions, as shown in figure 3.1, we analysed data on 11,677 samples from 1,146 patients; 8,963 were EMS and 2,714 spot as expected as patient were preferentially requested to provide EMS. Patients were from South Africa (61%), Tanzania (15%), Kenya (8%), Thailand (5%), and Zambia, Malaysia, Mexico (<5%) and India (<1%). 70% were male. The median age was 31 years (IQR 24-41). Ten percent were HIV positive; median CD4 cell count 397 cells/μL (IQR 312-504).

Figure 3.1 Flow chart of samples



3.4.1 Smear results

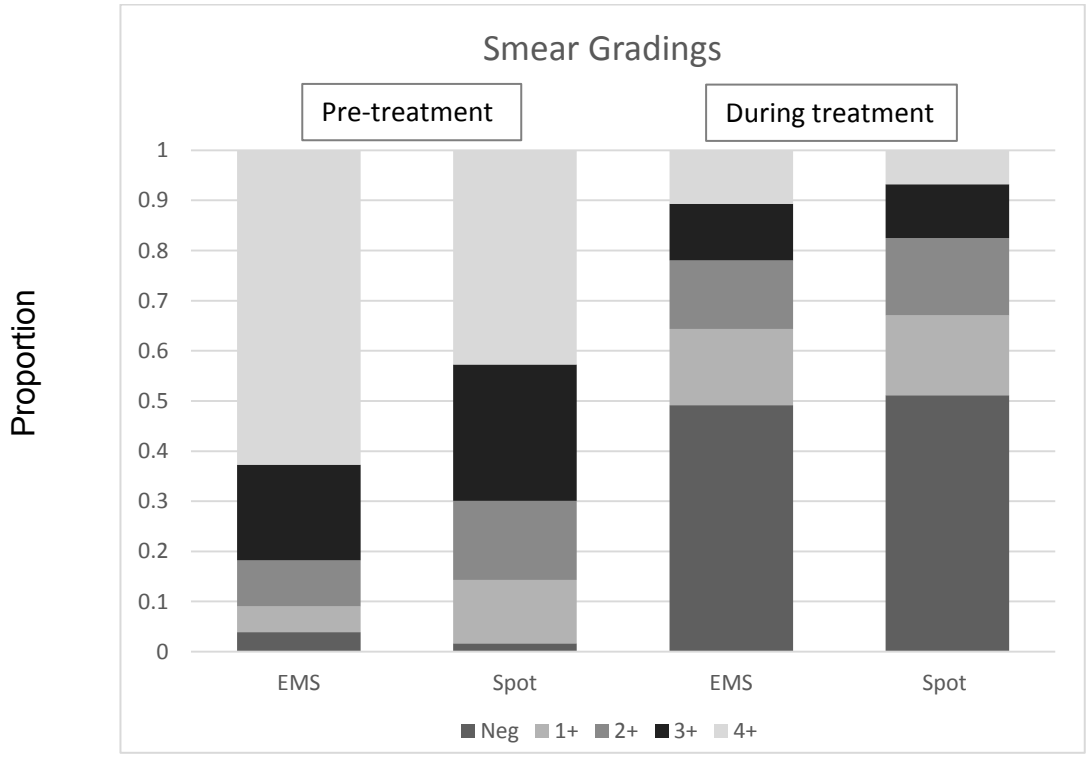
Pre-treatment smear results

Prior to treatment, of 665 diagnostic paired EMS and spot samples, spot samples were significantly more often smear positive than EMS ($p<0.05$) (table 3.1). However, EMS samples were significantly more likely to be graded 4+ than spot samples pre-treatment (63% vs 43%; $p<0.05$) (figure 3.2).

Table 3.1 Proportions of smear positive samples obtained from early morning (EMS) or spot sputum samples as per time on TB treatment

Sample collection timepoint	**EMS**		**Spot**		
	N	**% pos**	**n**	**% pos**	***p* value**
Paired Pre-treatment	665	96	665	98	0.02
Week 2	924	78	141	74	0.27
Week 4	868	62	128	58	0.37
Week 8	779	29	116	32	0.52
Week 12	675	16	136	22	0.09
Week 17	602	8	125	12	0.14
During Treatment	8077	51	1346	49	0.18

Figure 3.2. Distribution of smear gradings for EMS and spot samples for paired pre-treatment samples and samples collected during treatment

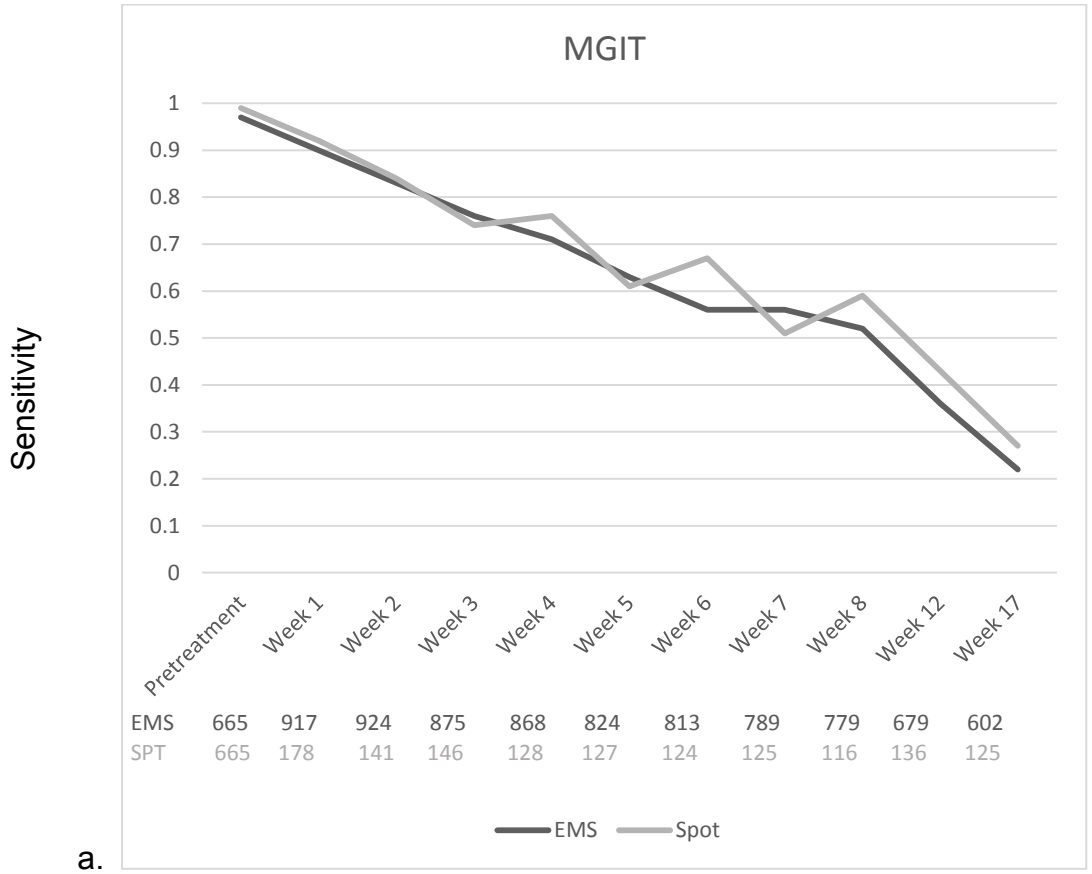


The sensitivity of pre-treatment spot smear samples to predict culture was higher than EMS in both MGIT (0.99 vs 0.97) and LJ (0.98 vs 0.97), (figures 3.3a and 3.3b).

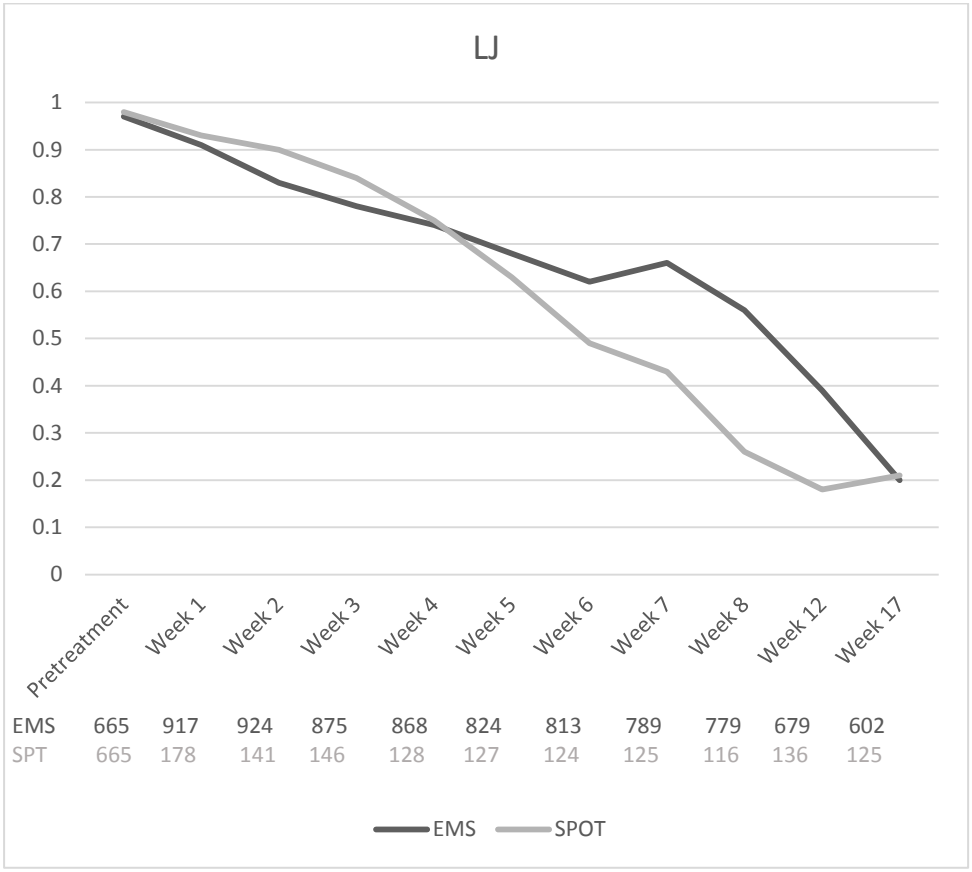
Smear results during treatment

EMS and spot samples had comparable smear positive rates during treatment as shown in table 3.1. Again EMS had higher proportions of smears graded 4+ results compared to spot samples (11% vs 7%; $p<0.05$) (figure 3.2). The sensitivity of smear for culture fell as time on treatment increased and were comparable for both MGIT and LJ as shown in figures 3.3a and 3.3b.

Figure 3.3. Sensitivity of early morning and spot sputum smears to predict MGIT and LJ culture pre-treatment and at study visits during visits during treatment



a.



b.

3.4.2 Culture results

Pre-treatment culture results

Of 665 paired spot-EMS pre-treatment samples, spot samples were significantly more often positive in LJ culture (89% vs 82%; $p<0.05$) and in MGIT culture (94% vs 91%; $p<0.05$) than EMS (table 3.2). EMS had shorter median time to detection than spot samples in MGIT (median 4.2 vs 5.3 days; $p<0.05$) and comparable results in LJ (median 14 days for both EMS and spot; $p=0.05$) (figures 3.4a and 3.4b).

Culture results during treatment

During treatment, EMS were more often MGIT and LJ culture positive (56% and 49%; $p<0.05$) than spot samples (48% and 40%; $p<0.05$) (table 3.2). Median time to detect positive cultures were also faster in EMS than spot samples by around 1 day in MGIT (median 13.6 vs 14.5; $p<0.05$), but there was no difference in the median time to detection on LJ (both 28 days; $p=0.03$ (figures 3.4a and 3.4b).

Table 3.2. TB culture results for early morning and spot sputum samples prior and during treatment: a. liquid MGIT media and; b. solid LJ media

	MGIT results				
Study Visit	***N***	**EMS pos(%)**	***N***	**Spot pos(%)**	***p* value**
Pre-treatment	665	90	665	94	<0.05
2	924	82	141	75	0.05
4	868	71	128	65	0.16
8	779	32	116	28	0.34
12	679	12	136	10	0.59
17	602	8	125	9	0.76
During	8077	56	1346	49	<0.05

a.

	LJ results				
Study Visit	***N***	**EMS pos(%)**	***N***	**Spot pos(%)**	***p* value**
Pre-treatment	665	82	665	89	<0.05
2	924	80	141	69	<0.05
4	868	64	128	47	<0.05
8	779	16	116	14	0.53
12	679	4	136	9	<0.05
17	602	5	125	6	0.53
During	8077	48	1346	40	<0.05

b.

Figure 3.4a. Time to positivity in MGIT culture for EMS vs spot sputum samples collected pre-treatment (paired) and during treatment (unpaired)

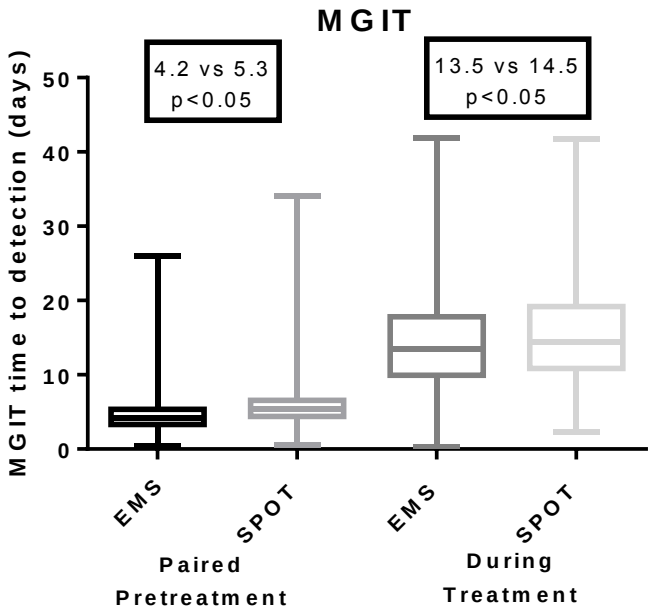
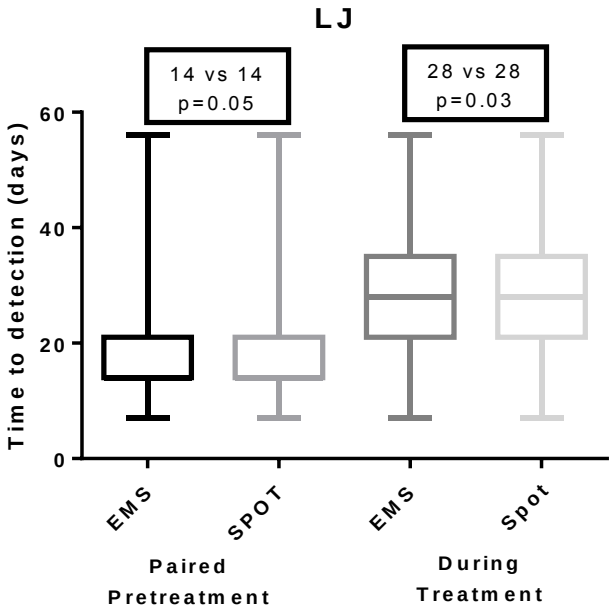


Figure 3.4b. Time to positivity on LJ culture for EMS vs spot sputum samples collected pre-treatment (paired) and during treatment (unpaired)



3.4.3 Contamination and MGIT False Positive rates

Pre-treatment contamination/MGIT false positives

Pre-treatment, contamination rates were higher for EMS than spot samples in both LJ (9% vs 7%) and MGIT (6% vs 4%), but these differences failed to reach statistical significance. The numbers of MGIT false positives was low for either EMS or spot samples pre-treatment with no significant difference. (table 3.3)

During treatment contamination/MGIT false positives

During treatment, contamination rates remained higher in EMS than spot samples in LJ culture (12% vs 9%) but were comparable in MGIT culture (9%). MGIT false positives were higher in EMS than spot samples collected during treatment (7% vs 5%). (table 3.3)

Table 3.3. Culture contamination and MGIT false positive rates for sputum samples from TB patients pre- and during treatment

		Paired Pre-treatment				**During Treatment**			
		Yes	No	pos	*p**	Yes	No	pos	*p**
LJ contamination	**EMS**	60	605	9%	0.19	969	7108	12%	<0.05
	SPOT	47	618	7%		121	1225	9%	
MGIT contamination	**EMS**	40	625	6%	0.1	727	7350	9%	0.96
	SPOT	27	638	4%		121	1225	9%	
MGIT false positive	**EMS**	7	658	1%	0.34	565	7512	7%	<0.05
	SPOT	3	662	0.4%		67	1279	5%	

**p* value – chi-square, or Fishers exact test where <5 in any cell

3.5 Discussion

In this study, early morning sputum samples were not superior to spot samples for diagnosing TB and monitoring response to treatment in terms of positive yield, sensitivity, specificity, contamination and false positives rates, although they had higher proportions positive during treatment. However, EMS had faster time to positive culture in liquid media, of around one day, for both pre-treatment and during treatment samples.

Diagnostic samples collected prior to treatment comprise the greatest proportion of the 50 million smear samples processed globally for TB and have been the focus of most research in efforts to improve early case detection using smear microscopy; this is often the only diagnostic test performed in settings with limited access to mycobacterial culture.

In our data, paired pre-treatment spot samples had higher yield of smear positives and greater sensitivity and specificity for culture in either media, although EMS were more likely to be graded 4+. Given all patients were smear positive at their local laboratory prior to screening for the study, these data are valuable in supporting spot samples over EMS in terms of positive diagnostic smears, although it is important to note that decontaminated samples were used in this study as opposed to direct smears on which the WHO guidance is based. Importantly, all samples in this study were processed in a uniform manner according the REMoxTB specific laboratory manual allowing comparison across sites. Laboratory staff in the trial were trained in the laboratory procedures and externally monitored for adherence to laboratory

methodology to ensure reliability of data, a problem which has hampered interpretation of microbiological data from other TB trials.

It is not possible to comment on value of EMS compared to spot samples in smear negative patients from these data as smear negative patients were not considered for inclusion in the trial. Some recent studies did include patient of unknown smear status. In a study of 101 adult asylum seekers conducted in Switzerland, morning samples has sensitivity of 62% and 42% to detect smear and culture positive cases of tuberculosis compared to 54% and 39% for spot samples (280). A recent study in India evaluating TB case detection using two samples rather than three included more than 7000 symptomatic chest patients who had provided 3 sputum samples for analysis. The study concluded that 2 specimens including an EMS had the highest yield and supported the inclusion of an EMS (120). Another study of 5000 adolescents in Uganda under investigation for TB identified 6 and 21 LJ culture positive cases. This study found EMS more sensitive than spot samples, identifying twice as many LJ and MGIT positive cases. The increase in the incremental yield of the EMS was 9.5% and 42.9% for LJ and MGIT positive cultures respectively.

In a study to define the most efficient laboratory diagnostic strategy in 50 smear microscopy centres in Bangladesh, of around 15,000 smear results stratified by sampling method (SMS, SMM, MMM), it was noted that 3 morning sputum samples were most efficient with 94.2% positives identified on the first smear. Although 10% of the patients dropped out, they conclude that examining 2 morning sputum samples was best, and not necessarily inconvenient for patients given treatment could be initiated at the first positive smear.

That such a strategy is not inconvenient to patients is not supported in other studies. Providing an EMS commits a patient to attend clinic on at least two occasions. In resource limited and rural settings, healthcare facilities may be at a considerable distance from patients' homes and accessing healthcare facilities may present considerable burden to patients being investigated for tuberculosis (TB) and their families (4-6). The costs can equal several month's salary and may therefore exacerbate or push people into poverty (7). A significant proportions of the overall costs are incurred in the pre-diagnosis pathway and therefore apply not only to the small numbers of patients found to be smear positive and requiring TB treatment, but equally to the vast majority who are not diagnosed with TB.

Reducing delays to treatment and removing any barrier to patient care are paramount in TB efforts in developing countries. Arguably the largest study previously conducted was the multicentre study of spot-spot-EMS vs spot-EMS-spot samples processed in solid media (either LJ medium or Ogawa medium); more than 6000 patients were enrolled and found a spot-spot sample collection alone was not inferior to spot-morning samples and resulted in higher numbers of patients actually providing the requested samples (267). Given the incremental yield of the third sample is generally lowest, this strategy would allow most patients to be diagnosed, and potentially commenced on treatment, on day one. The study further concluded that a two sample spot-spot strategy was non-inferior to the spot-morning strategy, as the difference was within the predetermined acceptable non-inferiority margin of -5%.

The new WHO policy statement supporting spot-spot was based on this evidence (279). It should be noted however that this previous study was insufficiently powered as the expected culture positivity of screened participants was 24% instead of the 50% projected at the study outset and thus this strategy warrants further operational testing in routine settings.

The value of smear and culture monitoring during treatment is not clear and there are no studies of EMS or spot sputum samples in this regard. No treatment monitoring guidelines mandate EMS or spot sputum samples. Any evidence which may inform this discussion tends to be based on data which predates the introduction of fully automated liquid culture systems which are being rolled out across the world in line with WHO laboratory strengthening policy (281). We have previously shown that smear has an increasingly poor correlation with both LJ and MGIT culture as treatment progresses (282). A meta-analysis published has shown that culture results during treatment are not suitably predictive of treatment outcomes, but the studies analysed did not take into account the timing of sample collection (179).

In the data presented in this chapter, during treatment, EMS and spot samples had comparable yields of positive smears and comparable sensitivity and specificity for culture positivity both on LJ and in MGIT culture. EMS, however, had significantly higher rates of culture positivity during treatment. Long-term follow up of patients in the REMoxTB study in future may allow comment on the comparative predictive value of EMS or spot smears for clinical outcome.

In addition to smear and culture, this sub-study provides data on the time to detect a positive culture. The time to detect a positive culture in MGIT has shown to correlate well with colony forming units and may therefore be considered as a proxy for mycobacterial load (283-286). Mycobacterial loads have long been used to assess the bactericidal effects of antituberculous drugs (287) and have been investigated as a biomarkers to predict treatment response and guide treatment decisions, but the impact of using either EMS or spot samples has not been considered (238, 288). In this study, we found EMS had faster times to detection in MGIT by around one day for samples collected both prior to and during TB treatment. Clinically such small differences seem unlikely to be considered significant, certainly from a practical perspective, but the predictive value of monitoring the dynamics of mycobacterial quantification during treatment have not yet been fully elucidated.

Much of the analyses presented in this chapter compare EMS and spot sputum samples for their sensitivity for culture. However, mycobacterial culture is not without problems and data is routinely lost through culture contamination and MGIT false positives. Clearly there is a balance to be struck between TB isolation and culture contamination; stringent efforts at decontamination risk also removing the TB bacilli and may reduce the sensitivity of sputum culture by any method. The REMoxTB laboratory quality monitoring guidelines attempted to maintain contamination within an acceptable range of 3-8%, but both MGIT and LJ culture contamination rates were higher in this study. Little is known for the reasons for MGIT false positives and thus practical advice to reduce these is unavailable.

In this study, EMS and spot samples collected prior to treatment had comparable contamination in both MGIT and LJ and comparable MGIT false positives. During treatment, MGIT contamination was comparable for EMS and spot samples, but MGIT false positives were greater in EMS, and EMS had higher contamination rates than spot samples on LJ culture. Few other studies of EMS and spot samples compare contamination rates other than the study of Ugandan adolescents which also reported greater contamination of EMS compared to spot samples (289).

One potential limitation of this study was that patients themselves largely determined whether a sample was an EMS or spot and thus some samples may have been categorised incorrectly, although clear instructions were provided. Severity of disease may be a potential confounder if this impacts the provision of an EMS compared to a spot sample, e.g. patients living far from clinic delaying presentation to healthcare or those with more severe disease admitted to hospital being more likely to provide early morning samples under supervision than patients at home. However inpatient treatment was only used routinely at a single study site, which enrolled 54 patients to the study, and only then during the first 2 weeks, so this is unlikely to have had a significant impact on the results in this chapter which included up to 1146 patients. Moreover, only a very small number of patients suffering grade 3 or 4 adverse events, equal in all study arms, were admitted to hospital during the study at any time.

The analyses presented are also limited by being blind to treatment arm of the REMoxTB study. Whilst we do not think this is likely to have impact our results given similarities in bacteriological responses in moxifloxacin containing

regimens compared to standard treatment in previous studies (239, 249, 250), we cannot exclude a treatment effect. We have attempted to minimise this by only including those samples collected during the first 17 weeks of the study when all patients were receiving active treatment as discussed in chapter 2. The effect of treatment allocation on the results should be considered when the treatment allocation data is available.

3.6 Summary

These data do not support the superiority of EMS over spot samples. In this population with smear positive TB, spot samples had higher positive yields and greater sensitivity for LJ and MGIT culture than EMS, and small differences in mycobacterial load as determined by time to culture positivity were clinically insignificant. However, further studies are required in smear negative populations to assess whether these findings may be generalisable to unselected patients presenting for investigation of possible TB and during treatment, and whether they may reduce delays to treatment and the number of patients lost in the diagnostic pathway.

Chapter 4: Evaluating smear microscopy as a predictor of culture on solid and in liquid media

4.1 Introduction

In the majority of settings, the diagnosis of TB is still based on the identification of acid fast bacilli (AFB) on sputum smear microscopy using auramine or Ziehl-Neelsen (ZN) stains (290), remarkably unchanged from the days when Robert Koch first discovered *Mycobacterium tuberculosis* (*M. tuberculosis*) in 1882 (24). The quantity of AFB present are graded by standards described by the American Thoracic Society (ATS) (247) or the World Health Organisation (WHO) (189). Identification of AFB using sputum smear microscopy has a sensitivity and specificity ranging from 20-80% for the diagnosis of culture positive TB (121), and lower rates are reported in patients co-infected with the human immunodeficiency virus (HIV) (125, 291). Culture is required to confirm the acid fast bacilli as *M. tuberculosis* using standard in vitro testing or molecular techniques, which may be employed directly on patient samples or positive cultures.

Traditionally, culture has been performed on solid media, however, in 2007 the WHO released a policy statement on the implementation of liquid culture systems in low and middle income countries, mainly to improve diagnosis of smear negative TB and in settings with significant incidence of MDR TB (292). As culture is often beyond the budget of many health care facilities in high-burden countries, in practice it is often reserved for those cases where patients

have not adequately responded to first line treatment to allow for assessment of drug resistance (26).

The value of smear and smear grading for predicting culture results and mycobacterial quantification has been established at the time of diagnosis (293) but few studies to date have assessed the validity of using smear status to predict mycobacterial culture during treatment. Despite this, smear results during treatment are often used as a measure of treatment response and many National Treatment Programmes (NTPs), including those in the high burden settings of South Africa (201), Zambia (203) and India (202), advise that they be used to guide treatment decisions.

It is often assumed that the relationship between smear and culture changes over time, but data describing this relationship comprehensively is not available in the literature.

4.2 Hypothesis

Smear microscopy is poorly predictive of TB culture on solid LJ or in liquid MGIT media during treatment

4.3 Methodology

We undertook an analysis of all mycobacteriological results of sputum samples submitted by patients enrolled in the REMoxTB study from January 2008 available on 20 April 2010. Samples without corresponding smear, LJ and MGIT

culture results were excluded as were samples collected after week 17 of study drug treatment as after this timepoint, patients on the treatment shortening arms were receiving placebo. Additionally, samples collected at unscheduled visits were excluded.

Microbiology

Sputum samples were collected at every study visit as per table 2.3. Sputum samples were processed as described in chapter 2. All samples were processed for smear microscopy, culture on solid LJ medium and culture in liquid MGIT medium.

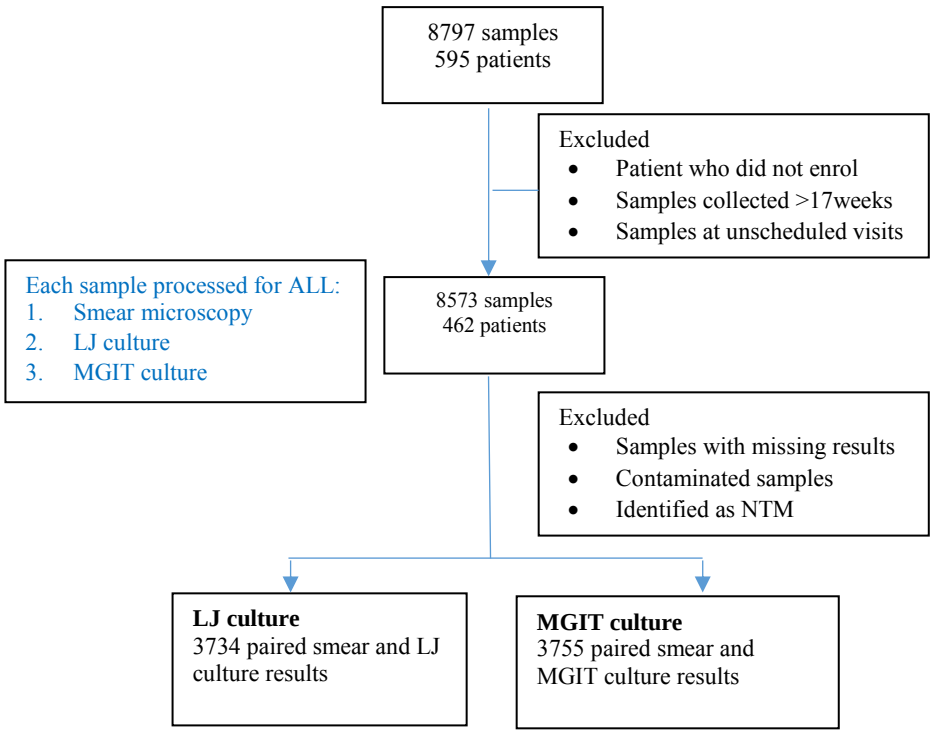
Statistical analysis

Odds ratios and predictive probabilities of positive cultures at each visit given the smear result at the same visit were calculated using mixed effect logistic regression to account for between-patient variation. Mixed effects linear regression was used to estimate mean week of growth on LJ and TTP on MGIT by smear grading. Week of growth of culture on LJ and MGIT TTP in days were log transformed for the analyses since the distributions were positively skewed. A week of growth of 9 and a TTP of 42 was used for negative cultures on LJ and MGIT respectively. Data were analysed blind to the treatment allocation since the trial was ongoing at the time of these analyses.

4.4 Results

Data were available in the study database on 20 April 2010 on 8797 samples from 595 patients. Of those, 462 patients enrolled in the REMoxTB study with 8573 sputum samples provided from a total of 4608 study visits from the baseline visit immediately prior to randomisation and administration of study drug until the week 17 visit inclusive . A flow chart of samples included in this study are shown in figure 4.1. Patients were from study sites in South Africa (76%), Zambia (14%), Tanzania (9%), and Mexico (<1%); 324 (70%) were male, the median age was 30 years. Of those randomised, 46 (10%) patients were co-infected with HIV; the median CD4 cell count was 357 cells/μL.

Figure 4.1 Flow chart of samples



4.4.1 Solid LJ Culture

Excluding missing, contaminated or cultures identified as non-tuberculosis mycobacteria, paired smear and LJ culture results were available at 3734 visits for analysis (figure 4.1). Of 1681 negative smears, 1257 (75%) were also LJ culture negative. Of 2053 positive smears, 1647 (80%) were also LJ culture positive.

The odds ratios and probability of a positive culture in solid LJ media given the sputum smear microscopy result (the predictive probability, PP), stratified by number of weeks of treatment a patient has received, are given in table 4.1.

As time on treatment increased, the predictive probability of a positive culture decreases and both the positive and negative smear results become less useful for predicting culture positivity. The corresponding odds ratios also decrease over time reflecting this weakening relationship.

The end of the intensive phase at eight weeks is considered crucial for making treatment decisions to extend the duration of intensive phase treatment. Although the odds of a positive culture on LJ given for a sample with a positive smear are double those with a negative smear (odds ratio 2.06, 95% CI 1.43-2.68), the predictive probability of culture positivity on LJ at week 8 is only 0.32 (95% CI 0.22-0.45) for a sample positive on smear and 0.10 (95% CI 0.06-0.15) for a sample with negative smear.

Table 4.1. The predictive probabilities (PP) and odds ratios (OR) with 95% confidence intervals of: a. a positive TB culture on LJ at weeks 0 to 17 visits given the smear microscopy result at that visit.

	LJ culture						
Visit Week			LJ result		Smear Negative PP (95% CI)	Smear Positive PP (95% CI)	Odds Ratio (95% CI)
			Pos	Neg			
4	ZN	Pos	186	34	0.58 (0.47, 0.68)	0.89 (0.83, 0.92)	2.28 (1.54, 3.01)
		Neg	61	63			
8	ZN	Pos	34	58	0.10 (0.06, 0.15)	0.32 (0.22, 0.45)	2.06 (1.43, 2.68)
		Neg	30	194			
12	ZN	Pos	8	44	0.02 (0.01, 0.04)	0.08 (0.03, 0.17)	1.70 (0.89, 2.51)
		Neg	8	247			
17	ZN	Pos	5	23	0.04 (0.02, 0.06)	0.11 (0.04, 0.27)	1.15 (0.13, 2.16)
		Neg	17	266			

4.4.2 Liquid MGIT Culture

Excluding missing, contaminated or cultures identified as non-tuberculosis mycobacteria, paired smear and MGIT culture results were available at 3755 visits for analyses (figure 4.1). Of 1691 smear negative samples, 1106 (65%) were also MGIT culture negative. Of 2064 smear positive samples, 1859 (90%) were also MGIT culture positive.

The odds ratios and probability of a positive culture in liquid MGIT media given the sputum smear microscopy result (the predictive probability, PP), stratified by number of weeks of treatment a patient has received, are given in table 4.2.

Table 4.2. The predictive probabilities (PP) and odds ratios (OR) with 95% confidence intervals of a positive TB culture in liquid MGIT media at weeks 0 to 17 visits given the smear microscopy result at that visit.

	MGIT culture						
Visit Week			LJ result		Smear Negative PP 95% CI	Smear Positive PP 95% CI	Odds Ratio 95% CI
			Pos	Neg			
4	ZN	Pos	200	13	0.68 (0.58, 0.77)	0.95 (0.92, 0.97)	1.73 (1.14, 2.32)
		Neg	49	73			
8	ZN	Pos	67	25	0.25 (0.19, 0.32)	0.72 (0.60, 0.81)	1.48 (0.80, 2.15)
		Neg	63	175			
12	ZN	Pos	19	38	0.05 (0.03, 0.09)	0.24 (0.14, 0.38)	1.48 (0.34, 2.62)
		Neg	20	243			
17	ZN	Pos	8	27	0.06 (0.04, 0.09)	0.16 (0.07, 0.32)	1.12 (-.011, 2.36)
		Neg	23	260			

At week 8, the predictive probability of a positive MGIT culture was 0·25 (95% CI 0·19-0·32) when the smear result was negative and 0·72 (95% CI 0·60-0·81) when the smear result was positive. At the same visit, the ratio of the odds of a positive culture on MGIT given a positive smear and those given a negative smear were 1·48 (95% CI 0·80-2·15). This confidence interval is wide and includes an odds ratio of 1. As with the results on LJ, table 4.2 shows decreasing odds ratios and predicted probabilities of smear for culture in liquid media over time on treatment.

The smear result is increasingly less likely to accurately predict the culture result as treatment progresses. For all visits a larger proportion of patients are

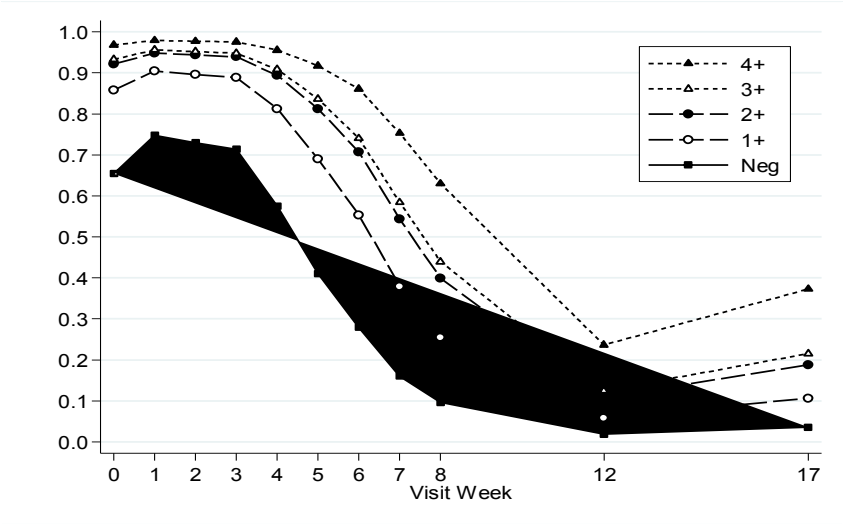
likely to be culture positive on MGIT than on LJ and this is reflected in the greater predicted probabilities (tales 4.1 and 4.2). There is, however, a closer association between the smear and the LJ result than the smear and the MGIT result, as reflected in the higher odds ratio.

4.4.3 Predicted probabilities conditional on smear grading

Figures 4.1a and 4.1b show plots of predicted probability of a positive culture on LJ and MGIT respectively are conditional on the smear grading. In each, the predicted probability increases with grading of smear at every visit. There is a clear separation between predicted probabilities given a negative smear and given a positive smear. Predicted probabilities decrease over time with a sharper decrease seen in LJ culture (figure 4.2).

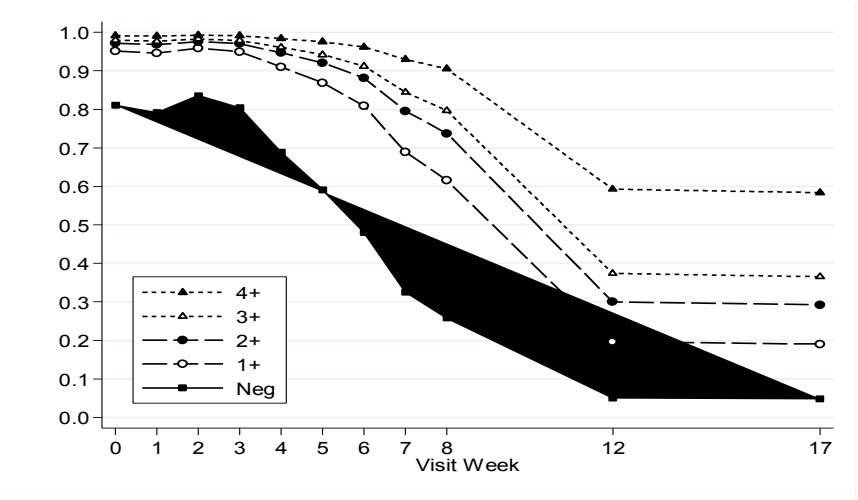
Patients are referred for enrolment into the REMoxTB trial on the basis of a positive smear and therefore very few patients have a negative smear at baseline; 59% had a smear grading of 4+ and a further 19% with a smear grading of 3+. Comparing mixed effect logistic regression models, the baseline smear grading does not predict the LJ or MGIT positivity at week 8 (p=0·33 and p=0·36). Furthermore, the smear grading at baseline does not significantly predict the MGIT TPP (p=0·12) or week of growth on LJ at week 8 (p=0·77).

Figure 4.2. Graphs of the relationship between sputum smear microscopy and culture in a. solid LJ media and b. liquid MGIT media during TB treatment showing the predictive probability of a positive culture, from the week 0 to week 17 visits, given the smear grading at that visit.



a.

LJ POS	ZN	Wk0	Wk2	Wk4	Wk8	Wk12	Wk17
	Neg	9	50	61	30	8	17
	1+	23	57	46	15	3	1
	2+	35	60	52	8	1	2
	3+	64	60	58	6	2	2
	4+	209	95	30	5	2	0



b.

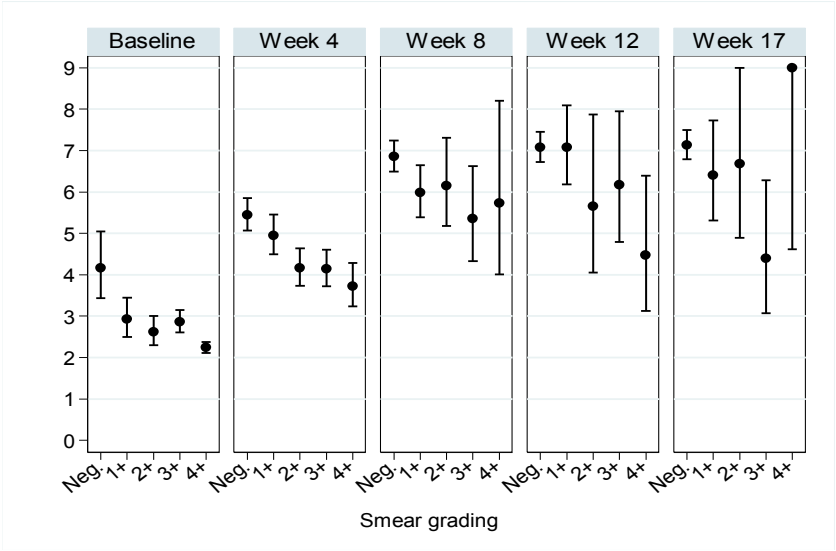
LJ POS	ZN	Wk0	Wk2	Wk4	Wk8	Wk12	Wk17
	Neg	13	49	73	63	20	23
	1+	27	62	51	31	7	4
	2+	42	63	56	17	4	2
	3+	77	74	59	14	5	2
	4+	241	86	34	5	3	0

Smear grading and bacterial load

There was an apparent association between smear grading and mycobacterial quantification in liquid and solid media as shown by an inverse relationship between smear grading and each of MGIT TTP and week growth seen on LJ.

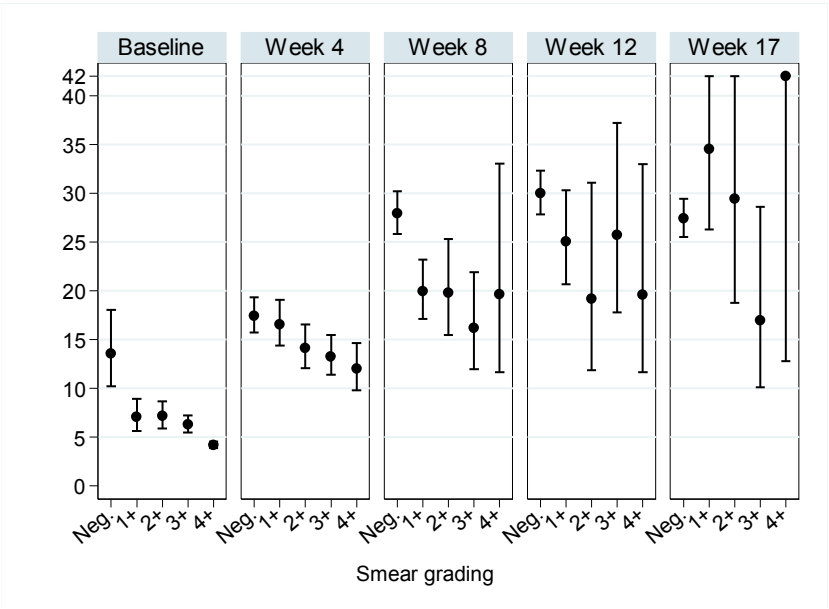
Pooling the data across all visits to week 17, the time to detect growth in culture and give a positive result was less in MGIT than in LJ (median MGIT TTP 16.5 days, IQR 9.4-42; median LJ TTD 35 days, IQR 21-56; $p<0.05$). The median week of growth on LJ and median TTP on MGIT decrease with increasing smear grading. This same relationship was also seen at individual visits, however the median time to detection of a positive culture in both solid and liquid media increased with increasing time from start of treatment (Figures 4.3a and 4.3b).

Figure 4.3a and 4.3b. Graphs of stratified smear grading (Neg, Pos, 1+-4+) and corresponding TTD positive as estimated by; a. LJ culture (weeks) and; b. MGIT culture (days) with 95% confidence interval, at baseline, weeks 4, 8, 12 and 17



a.

ZN	Wk0	Wk4	Wk8	Wk12	Wk17
Neg	21	155	279	314	340
1+	30	83	72	43	22
2+	43	67	26	7	8
3+	87	70	17	12	6
4+	259	41	6	6	1



b.

ZN	Wk0	Wk4	Wk8	Wk12	Wk17
Neg	20	157	282	311	342
1+	31	83	71	45	22
2+	45	67	27	7	8
3+	89	71	18	12	6
4+	261	41	6	6	1

4.5 Discussion

This study demonstrates that smear microscopy is an increasingly unreliable predictor of culture results in solid or liquid media as treatment progresses. In addition, the data show that, during treatment, while time to culture positivity is stratified by smear grading at all time-points, smear grading fails to accurately reflect changes in corresponding viable bacterial load in solid and/or liquid culture over time. As the 2 and 5 month smear results are often used as a proxy for culture as markers of treatment response and cure, this data has significant implications for NTPs globally.

Smear and Culture

As culture facilities are rarely available in resource limited settings and results may take up to eight weeks to become available, many NTPs advise basing treatment decisions on smear results collected at specified time-points during treatment. After eight weeks of standard treatment, many advise patients with a positive smear be prescribed a further four weeks of intensive active phase drugs (202, 203). From these data, whilst a positive smear at week eight has twice the odds of yielding a positive culture in solid LJ media when compared to a negative smear, the predictive probability is low at just 32%. Following NTP guidelines, these data suggest that two thirds of patients with a positive week eight smear will receive an unnecessary extension of intensive active phase treatment given they are in fact already culture negative. It is known that 2 months culture conversion is likely to identify potential relapses when assessing the efficacy of new treatments, however it is acknowledged that this is suboptimal for directing individual patient treatment decisions (294).

In liquid media, the probability of a positive culture is higher at 72% than on solid media. Using smear to define an extension to treatment, based on a supposed prediction of MGIT culture, just over a quarter of patients will be treated unnecessarily. The predictive value of a negative smear at week eight is somewhat better, but 10% and 25% will still be positive on culture in solid and liquid media respectively and will therefore not receive the NTP-intended treatment extension and commence standard two-drug continuation phase therapy which might be considered inadequate. The evidence, however, for treatment extension in reducing failure or relapse is poor to moderate, and the positive predictive value is low (237, 295) such that this guidance has been phased out in more recent WHO guidelines, particularly in patients receiving recommended treatment containing rifampicin throughout (296).

In the standard six-month regimen, a positive smear at month five is commonly regarded as a treatment failure by many NTPs. Patients with a positive smear at five months are often retreated, receiving a further 8 months medication, including injectable antituberculous agents, e.g. streptomycin. Due to the blinding of the REMoxTB study, it was not possible distinguish those patients on four-month regimens from those on six-month standard treatment, therefore month five results were not considered as two-thirds of patient will no longer be receiving active drug. These data do show, however, that a positive smear result at week 17, after four months of treatment, has no convincingly greater odds of a positive culture than a negative smear. The probability a positive smear predicting a positive culture is just 11% and 16% in LJ and MGIT with the probability given a negative smear is only slightly lower at 4% and 6%.

Given the trend to decreasing odds ratios and predictive probabilities over time, it is likely that the data would show further deterioration in the ability of the smear result to predict culture results at month five. Thus most patients being retreated on the basis of a positive month five smear will have a negative culture in both solid and liquid media and such retreatment might be considered unnecessary.

4.4.4 Smear grading, culture and mycobacterial quantification

In addition to positive or negative results, smears are also graded on the assumption that smear grading reflects bacterial load (293) and clinicians often assess patients' response to treatment on this basis. Overall, the sputum smear gradings in our study seemed to correlate well with bacterial quantification in liquid and solid media and, for any given visit, the predicted probabilities of positive cultures on LJ and MGIT were ordered by smear grading. As treatment progressed, however, this relationship changed; all smear gradings correlated with increasing trend in the time taken to detect a positive culture in both solid and liquid media, although there was considerable overlap in their confidence intervals increasingly as time on treatment increased. This idea is supported by data from another study evaluating recommendations for duration of isolation in TB patients; patients in the 4+ smear group, on the basis of their highest pre-treatment smear grading, had decreasing viable bacterial loads as treatment duration increased and the average TTP had a stronger correlation with duration of treatment than with smear gradings (297).

These data are important for clinicians managing patients with tuberculosis. For example, if we consider how one might assess the patient who presents at baseline with a positive smear graded 2+; when culture positive, growth will be detected on average in 2.5 weeks on LJ and 7 days in MGIT. If the same patient has a 2+ smear positive at week eight or week 17, this is now less likely to yield positive in culture and, when positive, this is now not detected until after 6 and 7 weeks incubation on LJ and 20 and 30 days in MGIT. So, although pre-treatment, smear gradings correlate well with viable bacterial load in solid and liquid culture, the changing relationship during treatment limits their clinical application in assessing treatment response and they are not sufficiently robust to guide management decisions.

These data raise questions as to what is being identified as AFB positive on smear that fails to grow on culture. Whilst some dead or dying mycobacteria will retain sufficient cell wall properties to resist decolourisation and be identified as AFB positive on smear microscopy, some of these organisms may be those in a viable but non-culturable or dormant state. The proposal that increasing proportions of fat laden cells identified in patients' sputum samples may be so-called non-replicating persistent mycobacteria challenge our existing understanding of TB pathogenesis (72, 73, 240). These recent studies have identified large populations of dormant organisms in patients' sputum samples prior to treatment which failed to grow in standard culture. The addition of resuscitation promoting factors, first discovered in *Micrococcus luteus*, resuscitated these organisms and increased the bacterial load of *M. tuberculosis* recovered from culture by 90%. These cells have been identified as lipid-body positive on sputum smear stained with a combined auramine-nile red

stain (240). It has been shown that triacylglycerol (lipid) accumulates in *M. tuberculosis* in a dormant/non replicating state during hypoxia and other stresses. Studies showing the gene encoding triacylglycerol-synthase is a member of the DosR regulon encoding genes linked to survival in *M. tuberculosis* support the hypothesis that these lipid-body positive cells have a role in mycobacterial persistence and may show much greater tolerance to drugs currently used in antituberculous chemotherapy (298).

Implications for TB programmes

The data presented have significant implications for national TB programmes which, with limited access to sputum culture, use smear and smear grading as a proxy for culture results and mycobacterial quantification upon which to base treatment decisions. As shown, this strategy will lead to patients having the intensive phase of TB treatment extended unnecessarily due to a positive smear at week eight. Similarly at month five, basing treatment decision on smear results, most patients retreated will turn out to have negative cultures. Extended and retreatment regimens pose unnecessary additional hazards to the patient through adverse drug reactions, increasingly poor compliance and may increase the risk of developing drug resistance (299, 300). For this reason it is critical that additional resources are provided to improve laboratory capacity for NTPs in developing countries.

At present, a great deal of effort is being made to improve microbiology laboratory standards and reduce the burden on limited staff resources. The roll-out of MGIT in resource-poor settings has been a key component of these efforts due to its automation and faster time to positivity than culture in solid

media, overall 39 compared to 22 days in our dataset. Whilst we cannot draw conclusions on the use of MGIT results to direct therapy, we can say that the probability of a positive MGIT is greater than a positive culture in LJ media at all timepoints during treatment; the significance of this increased sensitivity during treatment is unclear. A recent paper evaluating the cost-benefit of MGIT culture in the field concluded that the costs for the increased yield and speed of liquid MGIT culture were relatively high (47). The role of liquid culture in programmatic management of TB in resource-limited settings remains controversial, but it may be wise to expand current efforts to consider more robust and reliable biomarkers of microbiological and clinical response to TB treatment.

Limitations of this study

Only a handful of patients in this study were smear-negative at baseline since a positive smear was one of the inclusion criteria on which patients were invited to participate in the REMoxTB study. As such, these data therefore cannot be used to comment on patients with initially smear negative TB. These patients may be more likely to be co-infected with HIV and it would be important to consider their microbiology similarly during treatment given their increased risk of morbidity and mortality (301).

Patient demographics and HIV status were not considered in these analyses and may represent potential bias. However, all adults who consented to treatment were included and match those of TB in resource limited settings. Furthermore, patients with HIV comprised just 10% of those included and all have relatively preserved immune system with CD4 >250 cells/μL so this is unlikely to have had a significant impact on the results.

Only data from samples submitted prior to week 17 were included in these analyses owing to the blinding of the REMoxTB study. The effects of treatment arm on the mycobacteriological results have not been considered which may represent a potential bias in this study if treatment arm impacts on the findings. This seems unlikely to have had an important impact on the results of the study as all patients were on active anti-tuberculosis treatment for the duration and early bactericidal activity (EBA) studies of moxifloxacin in TB conducted by our group found it to have similar activity to rifampicin but less than isoniazid (302, 303) and similar outcomes in phase II clinical trials (239, 249, 250).

As the REMoxTB trial was ongoing at the time of these analyses, it was not possible to correlate results with long term clinical outcomes. Smear and culture are therefore being considered as surrogate markers for clinical endpoint of failure or relapse 12 months post-treatment. Further analysis is needed to evaluate their true biomarker potential when outcome data is available.

4.6 Conclusion

These data challenge the validity of using smears results to guide TB treatment as they are insufficiently predictive of culture positivity or mycobacterial quantification during treatment. The study provides an analysis of an extensive data set, aiding our understanding of the relationship between sputum smear microscopy and TB culture results in patients on anti-tuberculosis chemotherapy. The findings have significant implications for NTPs that advise that treatment decisions are made on the basis of sputum smear results during treatment.

Chapter 5 Comparing LJ and MGIT culture results

5.1 Introduction

Lowenstein-Jensen (LJ) medium is the traditional growth medium for the isolation of *Mycobacterium tuberculosis*. This is an egg-based solid medium containing nutrients to support mycobacterial growth and inhibit gram positive and gram negative bacterial growth, and includes malachite green which gives the medium its characteristic green colour. Without these inhibitors, LJ culture may easily become contaminated with faster growing bacteria; *M. tuberculosis* has a slow division cycle time of 15-20 hours compared to, for example, 20 minutes for *Escherichia coli* or 2 hours for the rapidly growing mycobacteria *Mycobacterium fortuitum*.

Growth of *M. tuberculosis* is normally detected on LJ between 2-8 weeks after incubation at 37^{o}C. Colonies appear as colourless-yellow with a rough surface (figure 1.6). When growth is detected, it is standard practice to perform a ZN stain to confirm the presence of acid-fast bacilli as an indicator of mycobacterial growth, and to subculture the isolate onto blood agar to exclude contamination. Positive cultures should be confirmed as *M. tuberculosis* complex using biochemical or molecular methods. Phenotypic antimicrobial sensitivity testing should be performed by conventional means on solid media or, as per more recent WHO guidelines, using liquid media (281).

Due to the prolonged time to detect a positive result, or indeed to report a negative result, liquid culture systems have been developed which detect *M.*

tuberculosis more quickly and have been endorsed by the WHO (292). The fully automated BACTEC Mycobacterial Growth Indicator Tube system (BBL™ MGIT™ 960, Becton Dickinson (BD) Microbiology Systems, Sparks, MD, USA) has been introduced most widely into clinical practice. MGIT tubes containing liquid Middlebrook 7H9 broth are nutritionally supplemented with OADC (Oleic Acid, Albumin, Dextrose, Catalase) for nutritional supplementation and PANTA antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) to suppress the growth of other bacteria. Within the silicone at the bottom of the tube is a fluorescent compound which is sensitive to the oxygen levels within the tube. Early on, there is adequate oxygen present to quench the emissions and no fluorescence is detected. As the culture grows, the organisms present respire consuming the oxygen releasing carbon dioxide. Once insufficient oxygen remains to quench the fluorescent emissions, a fluorescent signal will be emitted and detected by the machine sensor. Samples are monitored every 60 minutes and flag positive, normally when 10^4 to 10^7 colony forming units per millimetre (CFU/ml) are present in the medium.

The faster time to detection and improved sensitivity in vitro and in clinical studies for diagnostics have led to the implementation of BACTEC MGIT in most developed countries (132, 133, 135-137, 304-313). In developing countries with the highest burden of clinical TB, solid media has been the most commonly used method for culture and there are limited published data on the evaluation of liquid culture system implementation (314). The Foundation for Innovative New Diagnostics (FIND) has partnered with BD, the maker of the BACTEC MGIT, to support its roll-out in resource-limited settings and have been able to negotiate price reductions. Concerns have however been raised

about the cost of implementation, which may be particularly sensitive to contamination rates (47, 315).

Using culture to quantify concentrations of mycobacteria present *in vitro* and *ex vivo* has a long history (316-322). Smear microscopy, the first method by which *M. tuberculosis* was identified, was able to provide a semi-quantitative measure of the amount of TB present and remains an important part of the TB diagnostic process in the American Thoracic Society and the WHO guidelines (188, 323). Smear microscopy has sensitivity to reliably identify 10,000 acid fast bacilli (AFB) per millilitre (ml) and identifies the most infectious patients (324). Enumeration using smear microscopy is frequently operator dependent, particularly in areas of low incidence of positive results. Clearly, however, more robust methods to detect concentrations lower than this were required.

Culturing sputum on LJ medium can isolate *M. tuberculosis* growth from as little as 10 organisms (324) (133). MGIT culture is more sensitive with a limit of detection as low as 1 organism (Personal communication; Dr Isobella Honeyborne). The time to detect a positive culture can be considered as a measure of mycobacterial growth. MGIT has recently been shown to correlate strongly with traditional CFU counts on solid agar-based media (283-286, 325). Utilising the time to detect a positive culture as a direct measure of mycobacterial load is of great benefit; enumerating mycobacteria by colony counting as per the Miles Misra method is time consuming, requiring perhaps another 3-4 weeks, and is operator dependent. In low and middle income countries, where qualified scientific staff may be the most limited resource,

quantification using less laborious methods with less subjective measures is of particular benefit.

EBA studies have been at the forefront of new drug discovery, drug efficacy, dosing and safety studies since 1970s (326-329). Such studies measure the quantity of TB present in serially collected patient sputum samples during treatment with a single or multiple agents with potential anti-tuberculous activity. These studies are able to test the bactericidal and sterilising action of antituberculous drugs during the first two weeks of antituberculous chemotherapy. Given in combination, these studies are also able to measure the contribution, or indeed the potential antagonism, of new drugs to established regimens or regimens determined in preclinical testing mouse models.

Monitoring mycobacterial load in EBA studies is performed over the first 14 days of treatment. In clinical trials, monitoring is extended in phase II safety and efficacy studies to week 8, long considered a marker of outcome, although this is not supported by a recent meta-analysis concluding poor predictive value of culture to detect treatment failure or relapse (179).

Mycobacterial quantification at baseline has been shown to correlate with symptoms, radiographic features, cavitation and treatment outcomes in a number of studies (102, 238, 285, 330, 331). During treatment, increasing time to detect a positive culture may be considered as a measure of sterilisation. Previous studies have identified a biphasic decay in mycobacterial load in response to antituberculous chemotherapy (332, 333) which may reflect

heterogeneous populations of mycobacteria present in patient samples including viable but non culturable organisms (73). These studies demonstrate the importance of mycobacterial monitoring during treatment in informing not only potential treatment outcomes but also highlighting the impact on our understanding of population dynamics *in vivo*.

The current TB therapy was arrived at through a series of trials conducted by the British Medical Research Council (171). All mycobacteriology employed in these trials used LJ as a growth medium providing the ability to make cross trial comparisons as the methodology remained unchanged from 1956-1985. Clearly with the introduction of MGIT as a potential replacement, it is important to consider the relationship to LJ culture results not just for diagnosis, but throughout treatment to ensure valuable data is not lost. Moreover, the impact of MGIT implementation in the clinical trial setting are required alongside comparisons to other measures of bacteriological responses and clinical outcomes.

5.2 Hypothesis

The relationship between LJ and MGIT culture, in terms of positive yield, sensitivity, and mycobacterial quantification, varies during treatment in patients enrolled in the REMoxTB study.

5.3 Methodology

All results contained in the REMoxTB study database on 29 August 2012 were extracted for the purposes of these analyses. We excluded data from those patients who did not enrol in the trial and whose age and/or HIV status and CD4 cell count could not be determined. Samples without corresponding smear, LJ and MGIT culture results were excluded. As the REMoxTB study was ongoing at the time of these analyses, they were conducted blind to treatment allocation. Those samples collected after week 17 of study drug, when patients in the moxifloxacin containing arms would have been receiving placebo, have been excluded. Samples collected at unscheduled visits were also excluded.

Microbiology

Sputum samples were collected at every study visit as described in table 2.3 and processed for smear and culture on both solid LJ and liquid MGIT media. Microbiological procedures were performed as described in chapter 2.

Statistical analysis

Proportions have been calculated and differences compared using the Chi-square test. Agreement between culture methods are provided and statistically analysed using Cohen's kappa scoring. The statistical significance of variability in agreement between different study visits was determined using analysis of variance (ANOVA). Mixed effects logistic regression analysis was used to measure univariable associations between patient demographics for samples negative in MGIT which are positive on LJ as these are of particular interest for laboratories replacing LJ culture with MGIT. Sensitivity and specificity of LJ and

MGIT have been calculated against 3 gold standards: LJ culture, MGIT culture and using the maximum positive yield in which positive in either or both culture methods is considered positive and a sample only considered negative where not positive using either media. Sensitivity and specificity of contaminated MGIT ZN result for LJ culture results are also calculated.

Time to first negative culture (TFN; the study visit at which a patient converted to a negative culture), time to a sustained negative (TSN; the study visit where the patient provided the first of 2 consecutive negative culture results), and time to reversion (the difference in time between the study visits at which a patient had a positive culture having previously had a sustained negative culture) are described. Histograms show the frequency of TSN and TFN over time on treatment for both LJ and MGIT culture. Differences in TFN and TSN between LJ and MGIT are compared with Kaplan-Meier analysis curves and hazard ratios determined using the log rank test. Median time differences between MGIT TTP and LJ TTD, TFN and TSN, and TSN and time to a reversion after having sustained a negative result are compared between LJ and MGIT using Mann Whitney *U* test. Data were logarithmically transformed using the natural log to give a better approximation of normality and means and standard deviations described; the parametric students paired t-test was used to determine statistical significance of paired data; for unpaired data, the parametric unpaired t-test was used, with Welch's correction for data with unequal variance.

A lasagna plot provides a visual depiction of the variability in culture results in both media in individual patients, represented by a single horizontal line, during

time on treatment and stratified by baseline smear grading. The Spearman's rank correlation coefficient (R) was calculated as a nonparametric measure of statistical correlation between time to detect positive cultures using the two different culture methodologies MGIT and LJ overall and at each timepoint on treatment. The changes in time to detection over time in each culture method were analysed using a linear regression model and by a non-linear two phase decay and the coefficient of determination (R^2) calculated to compare which model best fit the data. Ratio of time to positivity between methods was calculated at each timepoint.

5.4 Results

5.4.1 Patient demographics

The database contained data on 40235 samples from 2625 patients. Patients who did not enrol in the study were excluded. HIV co-infection was identified in 380 patients, of whom 246 had CD4 cell counts less than 250 cells/μL.

After exclusions, data were analysed on 20,654 samples from 1928 patients. A flow chart of samples analysed is shown in figure 5.1. Patient demographics are shown in table 5.1. Patients were from study sites in South Africa (46%), East Africa (20%), India (18%), East Asia (11%) and South America (1%).

Figure 5.1 Flow chart of samples

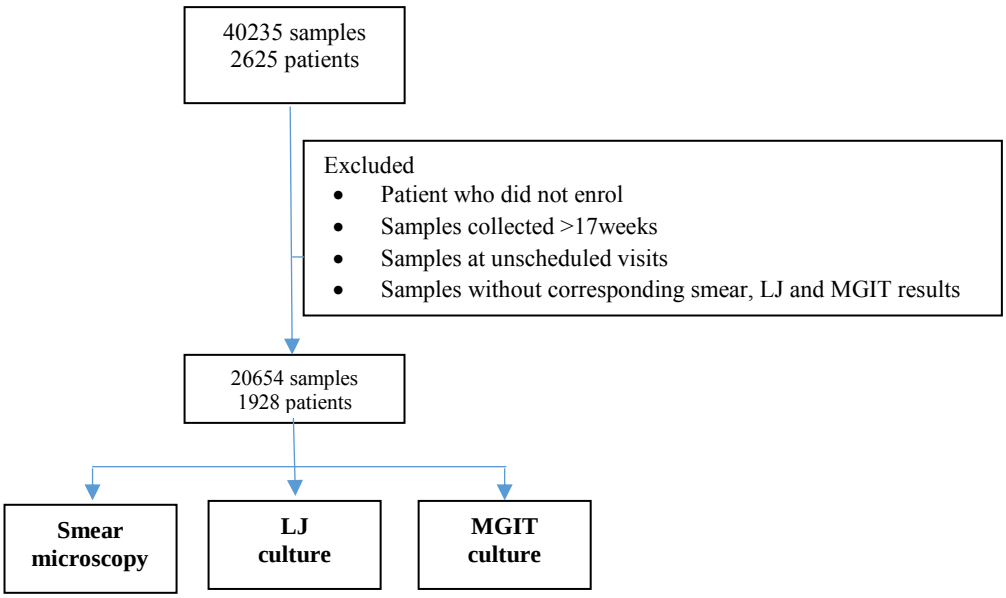


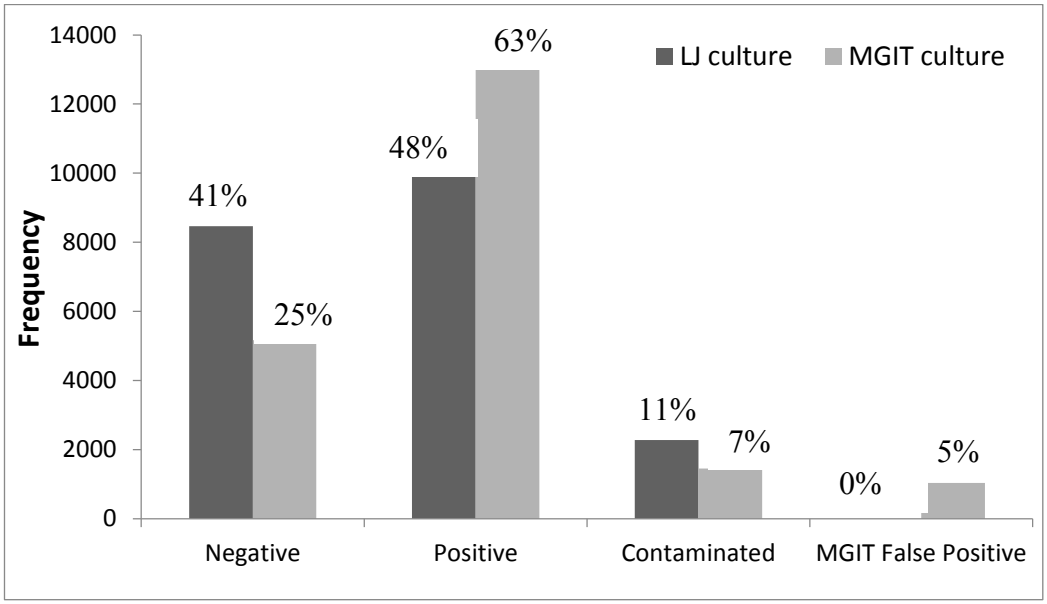
Table 5.1. Patient demographics

Patients	N=1928		
Sex	Male	1288 (67%)	
	Female	568 (29%)	
	Not defined	72 (4%)	
Median age (years) [interquartile range]	30 [23-41.25]	**Male**	31 [24-42]
		Female	28 [22-40]
HIV status	Positive	134 (7 %)	
	Negative	1722 (89%)	
	Unknown	72 (4%)	
CD4 count (cells/µL; IQR)	404 [319-530]	**Male**	395 [307-476]
		Female	437 [350-558]

5.4.2 Proportions of culture positive and negatives in LJ and MGIT

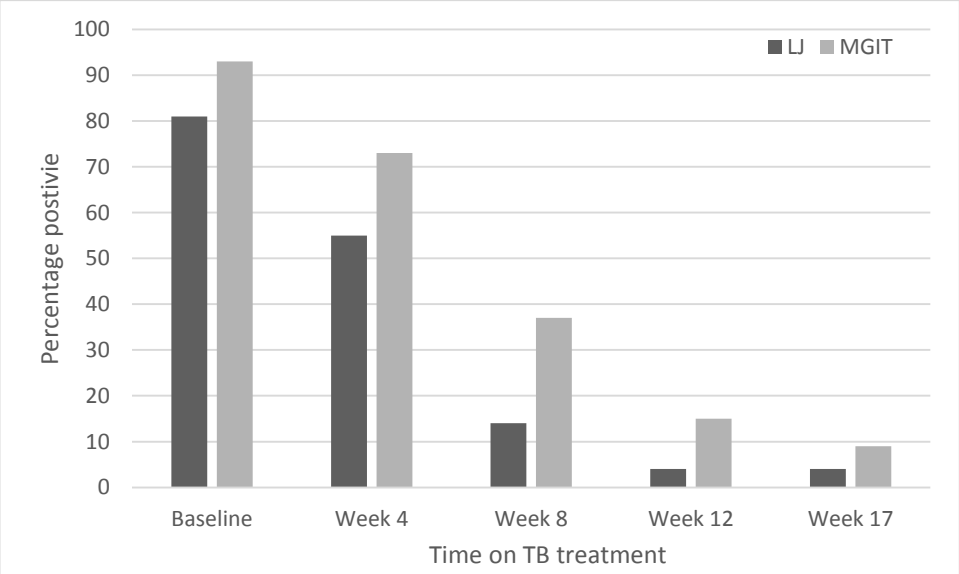
The proportion of LJ culture positive results was 48% compared to 63% for MGIT culture. The overall proportions of results in MGIT and on LJ are shown in figures 5.2.

Figure 5.2. Histogram showing frequency and percentage of culture results for LJ and MGIT culture for all samples collected at all timepoints



The proportions of culture positive using either methodology fell as time on treatment increased. A greater proportion of samples were positive in MGIT than on LJ at all time points (figure 5.3).

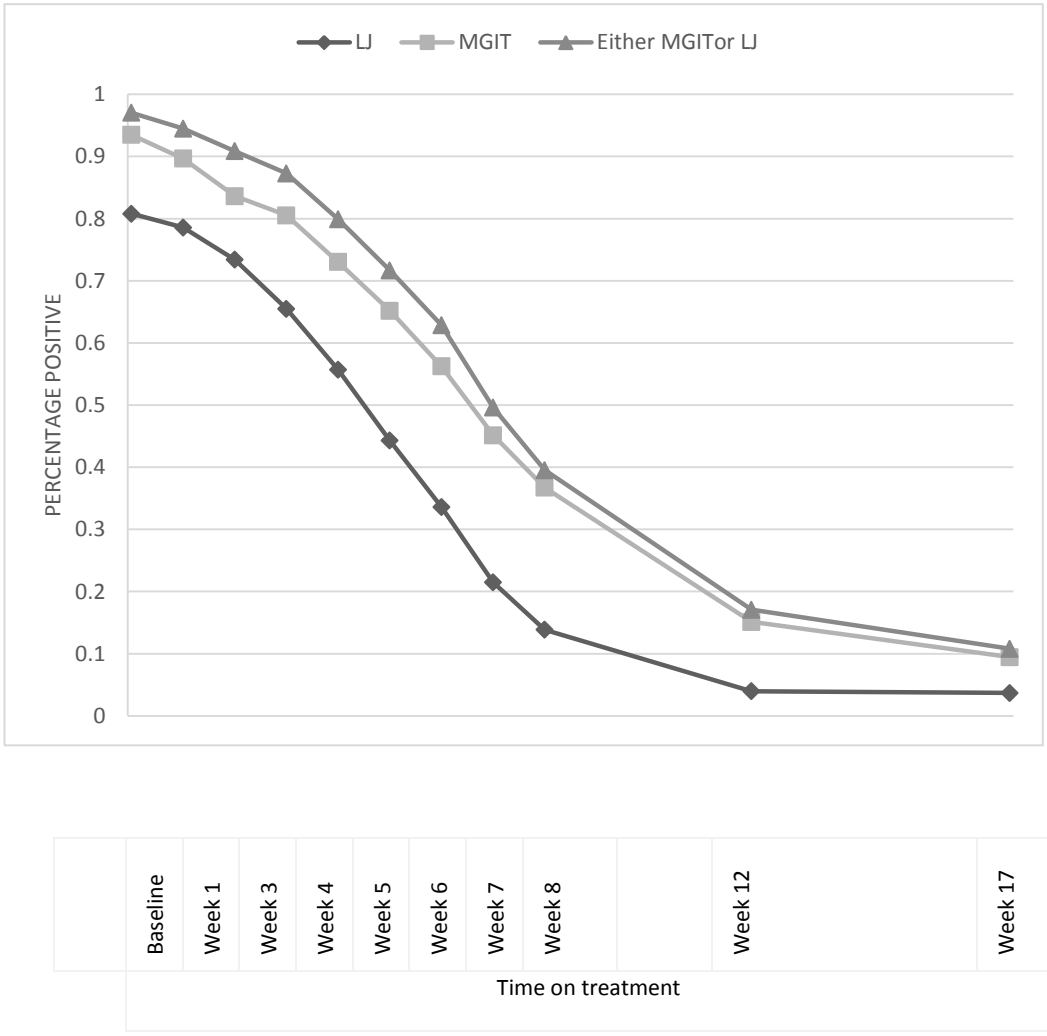
Figure 5.3 Percentage of positive cultures in LJ and MGIT as per time on TB treatment with data table showing number of positives from number of samples



LJ pos	1501	957	231	65	60
MGIT pos	1737	1255	612	248	154
Total	1858	1718	1667	1640	1630

The decreasing proportions of LJ and MGIT culture positives at over time are shown in figure 5.4 which additionally includes the line of maximum positive yield showing the proportion of positive cultures in either or both LJ and MGIT.

Figure 5.4 Percentage culture positive prior to and during TB treatment in LJ or MGIT culture and the maximum positive yield (MPY) of positive culture when either or both MGIT and LJ positive



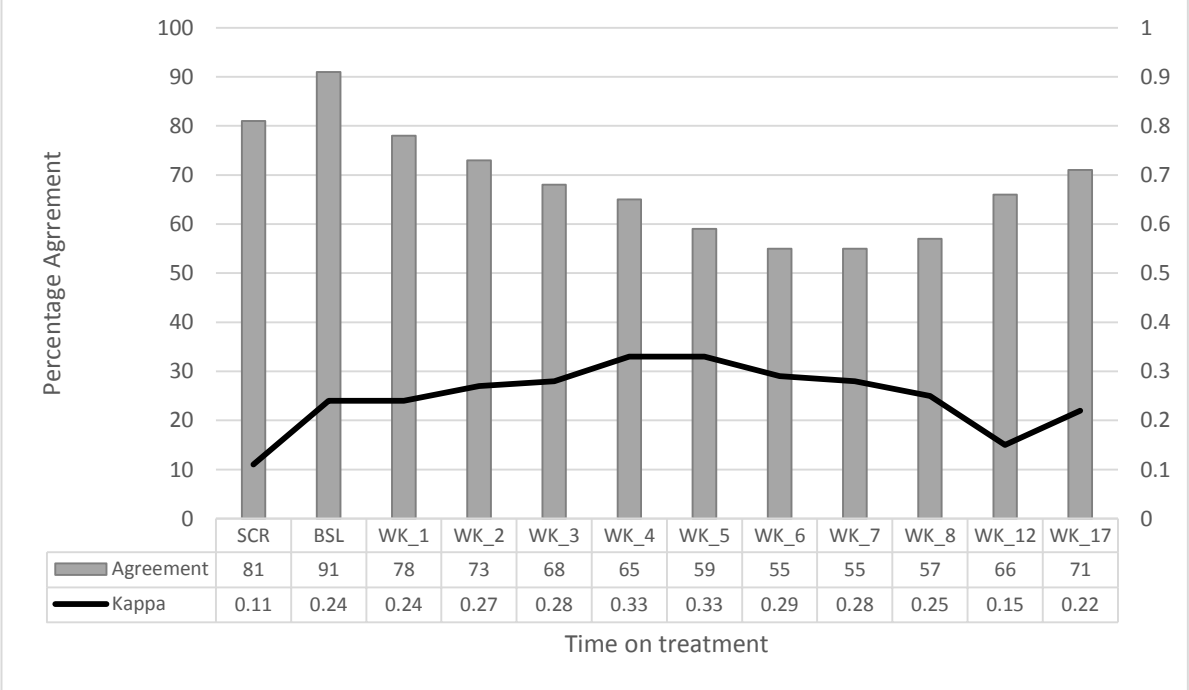
5.4.3 Agreement between LJ and MGIT culture results

LJ and MGIT culture can be positive, negative or contaminated. In addition, MGIT culture results can be MGIT false positive. MGIT false positives and contaminated results are combined for the purposes of the agreement analyses.

LJ and MGIT cultures results agreed as both positive, both negative, or both contaminated/MGIT false positive in 13984 of 20654 (68%) samples tested; Cohens kappa score 0.45 (95% confidence interval 0.44-0.46). The agreement between the two methods was 86% prior to treatment; Cohens kappa score

0.16 (95% CI 0.12-0.20). The paradox of low kappa scores despite high levels of agreement throughout treatment likely reflect the imbalance of marginal totals owing to the low frequency of contaminated/false positive samples (334). The percentage agreement changes significantly over time on treatment with an average per week drop in agreement of 2.16 % (95% CI -3.73--0.58%; ANOVA $p<0.05$). The percentage agreement of LJ and MGIT culture and the kappa score over time on treatment is shown in figure 5.5.

Figure 5.5 Agreement (%) of LJ and MGIT culture results during TB treatment, with secondary axis showing kappa scores



	SCR	BSL	WK_1	WK_2	WK_3	WK_4	WK_5	WK_6	WK_7	WK_8	WK_12	WK_17
Agreement	81	91	78	73	68	65	59	55	55	57	66	71
Kappa	0.11	0.24	0.24	0.27	0.28	0.33	0.33	0.29	0.28	0.25	0.15	0.22

During treatment, the number of samples in which culture is positive in both LJ and MGIT falls over time, with a concomitant increase in samples with negative cultures in both media (figure 5.6). The proportions where both results are contaminated or contaminated and/or false positive increases over time as shown in figure 5.7.

Figure 5.6 Percentage of samples where both LJ and MGIT cultures are positive or negative during TB treatment

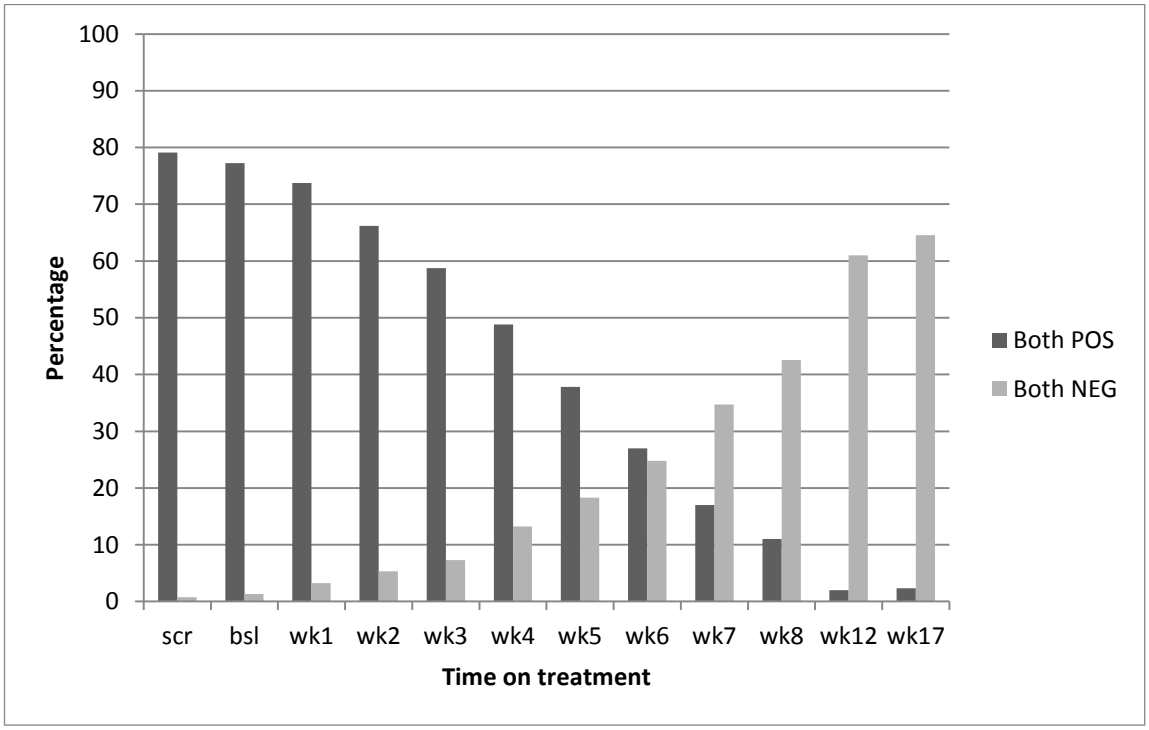
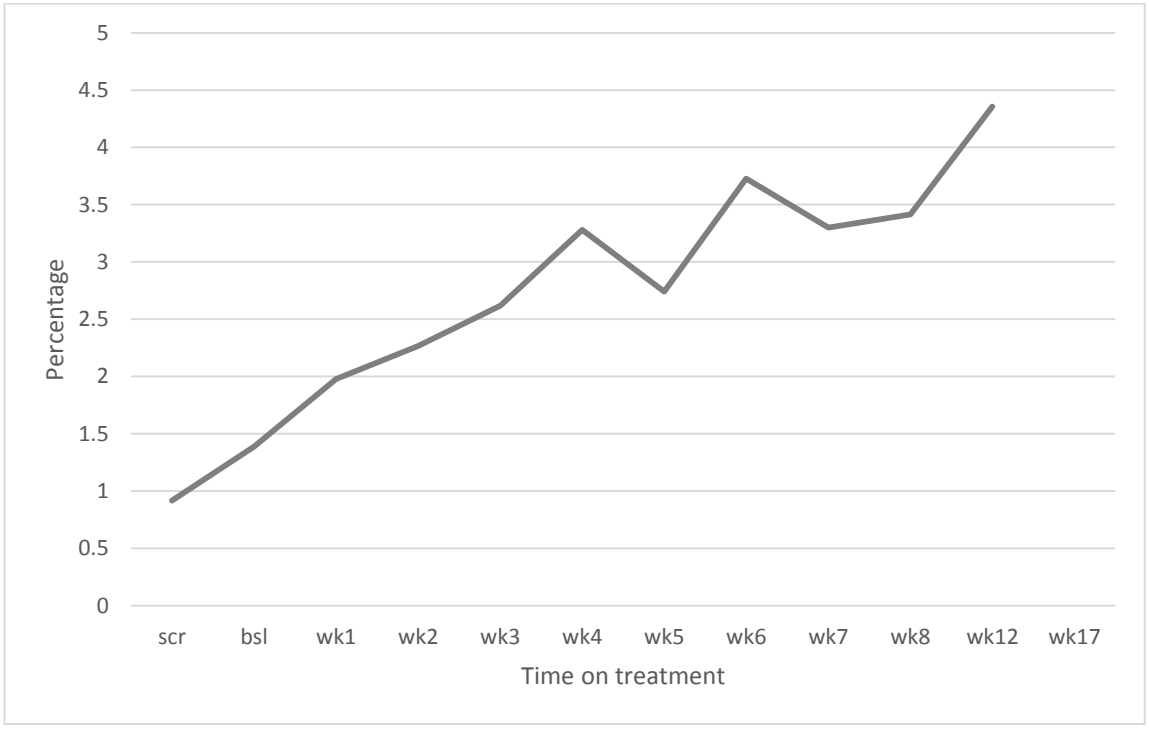


Figure 5.7 Percentage of samples where both LJ and MGIT cultures are contaminated or MGIT false positive during TB treatment



5.4.4 Sensitivity and specificity analyses

The overall sensitivity and specificity of MGIT culture using LJ culture as the gold standard are 98% and 61% respectively. The results over time are shown in table 5.2; sensitivity trends downwards over time.

Table 5.2 Contingency tables for MGIT and LJ culture results at selected timepoints during treatment and sensitivity and specificity of MGIT culture for LJ gold standard

Time on treatment			**LJ culture results**		**LJ gold standard**	
			Pos	Neg	Sens	Spec
Paired Pre treatment	**MGIT results**	Pos	2883	248	99%	92%
		Neg	21	38		
Week 4	**MGIT results**	Pos	839	296	98%	74%
		Neg	16	227		
Week 8	**MGIT results**	Pos	184	364	95%	34%
		Neg	10	709		
Week 12	**MGIT results**	Pos	33	194	73%	15%
		Neg	12	1000		
Week 17	**MGIT results**	Pos	38	100	81%	28%
		Neg	9	1052		

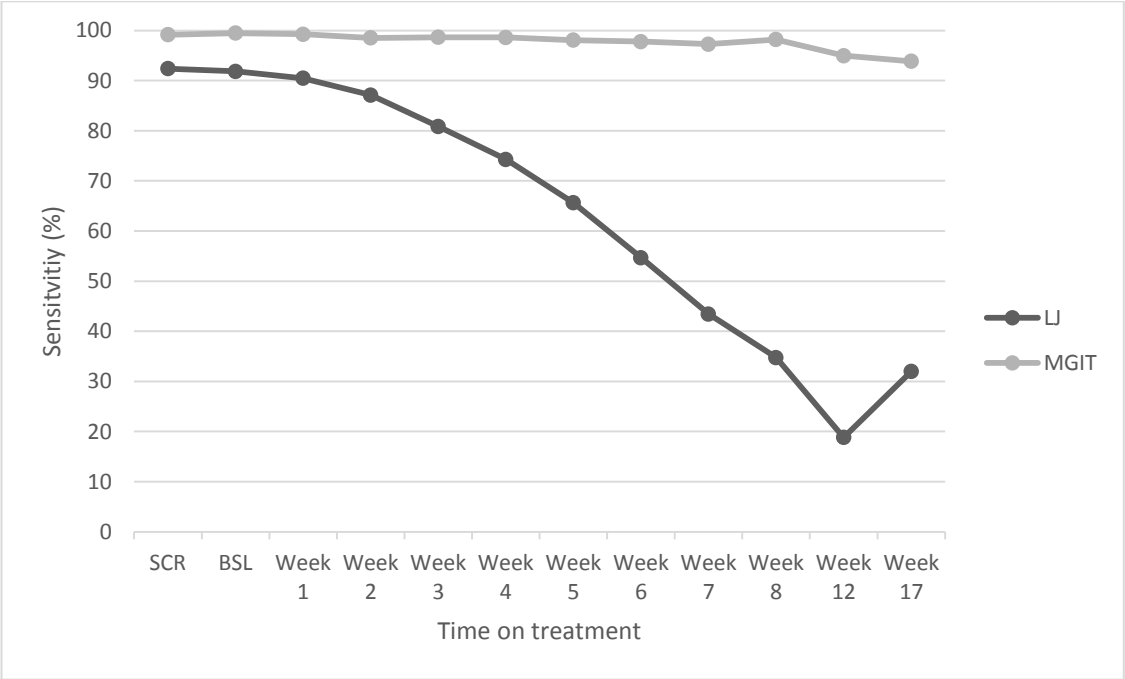
Using MGIT as the gold standard, the overall sensitivity of LJ culture to detect MGIT positives is lower at 75%, with greater specificity of 96%.The results over time are shown in table 5.3; again, sensitivity trends downwards over time.

Table 5.3 Contingency tables of LJ and MGIT culture results at selected timepoints during treatment and sensitivity and specificity of LJ culture for MGIT gold standard

Time on treatment			**MGIT result**		**MGIT gold standard**	
			Pos	Neg	Sens	Spec
Paired Pre treatment	**LJ results**	Pos	2883	21	92%	75%
		Neg	248	38		
Week 4	**LJ results**	Pos	839	16	74%	93%
		Neg	296	227		
Week 8	**LJ results**	Pos	184	10	34%	99%
		Neg	364	709		
Week 12	**LJ results**	Pos	33	12	15%	99%
		Neg	194	1000		
Week 17	**LJ results**	Pos	38	9	28%	99%
		Neg	100	1052		

Using the maximum positive yield (MPY) of results from a single sample as the gold standard, i.e. positive either or both media as the gold standard, MGIT culture is more sensitive overall than LJ; 99% compared to 76%. MGIT sensitivity for MPY of any positive culture remains high throughout treatment reflecting the greater proportion of MGIT cultures positive at all timepoints (figure 5.3, 5.4). Figure 5.8 shows the sensitivity for MGIT and LJ for any positive culture throughout the duration of treatment.

Figure 5.8 Sensitivity of LJ and MGIT for culture with maximum positive yield (either or both cultures positive) as gold standard from screening until week 17



LJ negative MGIT positive / LJ positive MGIT negative

The increased sensitivity of MGIT over LJ results in large proportions of negative LJ cultures yielding a positive result in MGIT culture; 39% of all samples negative on LJ are positive in MGIT. If we include samples which are negative or contaminated on LJ, 38% are MGIT positive. These figures are even higher, 87% and 86%, if we include only pre-treatment samples as shown in table 5.4.

MGIT negatives which are positive in LJ are most concerning in laboratories to replace LJ culture with MGIT. These are fewer; overall 4%. If we include all MGIT contaminations and false positives, this rises to 13%. Higher rates are seen in pre-treatment samples of 35% and 57% respectively (table 5.4). MGIT

negatives positive in LJ are not associated with age (OR 0.99, 95% CI 0.97-1.01; p=0.53), sex (OR 1.2, 95% CI 0.75-1.92; p=0.44), or HIV status (OR 0.85, 95% CI 0.37-1.96; p=0.7) in univariable analysis.

Table 5.4 Negative ± contaminated/false positive MGIT and LJ culture results which are positive using the other culture methodology

ALL SAMPLES		**LJ results**		**LJ pos/ of MGIT neg (%)**	**MGIT pos/ of LJ neg (%)**
		Pos	Neg		
MGIT result	Pos	8857	2888	173/4777 (4%)	2888/7492 (39%)
	Neg	173	4604		
ALL SAMPLES		**LJ results**		**LJ pos/ of MGIT neg (%)**	**MGIT pos/ of LJ neg (%)**
		Pos	Not pos		
MGIT results	Pos	8857	4152	968/7645 (13%)	4152/10829 (38%)
	Not pos	968	6677		
PRETREATMENT SAMPLES		**LJ results**		**LJ pos/ of MGIT neg (%)**	**MGIT pos/ of LJ neg (%)**
		Pos	Neg		
MGIT result	Pos	2883	248	21/59 (36%)	248/286 (87%)
	Neg	21	38		
PRETREATMENT SAMPLES		**LJ results**		**LJ pos/ of MGIT neg (%)**	**MGIT pos/ of LJ neg (%)**
		Pos	Not pos		
MGIT result	Pos	2883	588	124/218 (57%)	588/682 (86%)
	Not pos	124	94		

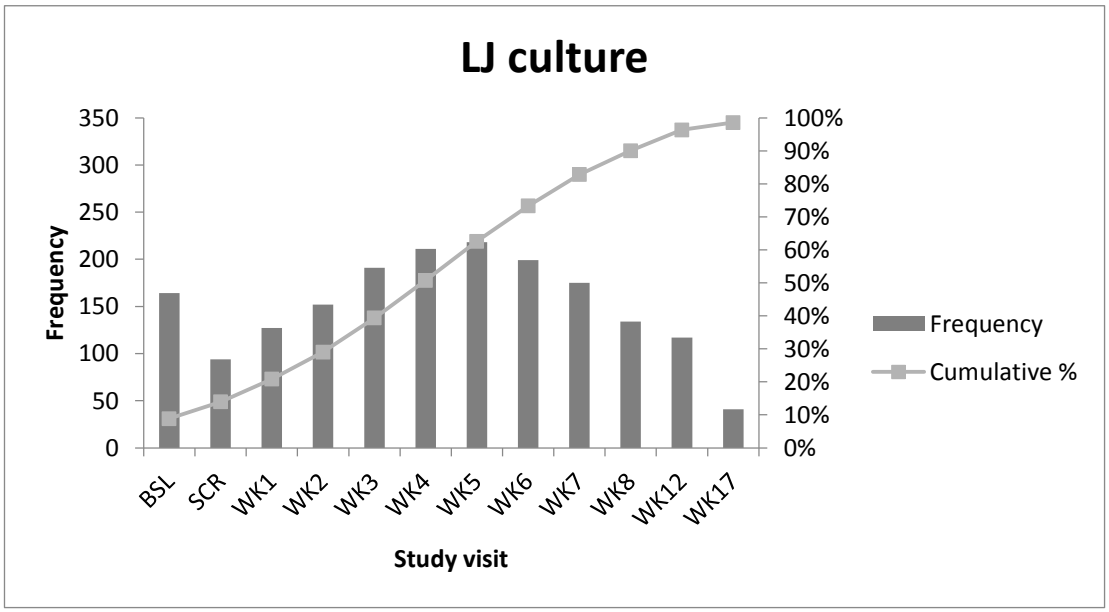
5.4.5 Time to First Negative

Of 1881 included in these analyses, a time to first negative did not occur before week 17 or could not be determined in 9% of patients using LJ culture and 24% of patients using MGIT culture. By the week 8 study visit, 83% of patients have already provided their first LJ culture negative sample, compared to 67% who will have provided a first MGIT culture negative sample. A histogram of the TFN for LJ and MGIT are shown in figures 5.9a and 5.9b.

A greater proportion of samples have the first negative LJ at earlier study visits than those in MGIT. The median time to first negative (TFN) using LJ media was week 5, i.e. the sample collected at the week 5 [IQR 3-7] visit. In MGIT the median TFN was at week 7 [IQR 5-12]. At any given timepoint, samples on LJ media have 1.734 times the probability of having converted from positive to negative for the first time (TFN) by the next visit than samples cultured in MGIT media (HR_1.734; $p<0.0001$). This is more easily visualised by the Kaplan-Meier plot shown in figure 5.10.

Figure 5.9 Histogram showing the frequency of study visits at which the first culture-negative sputum sample was collected in solid and liquid media

a.



b.

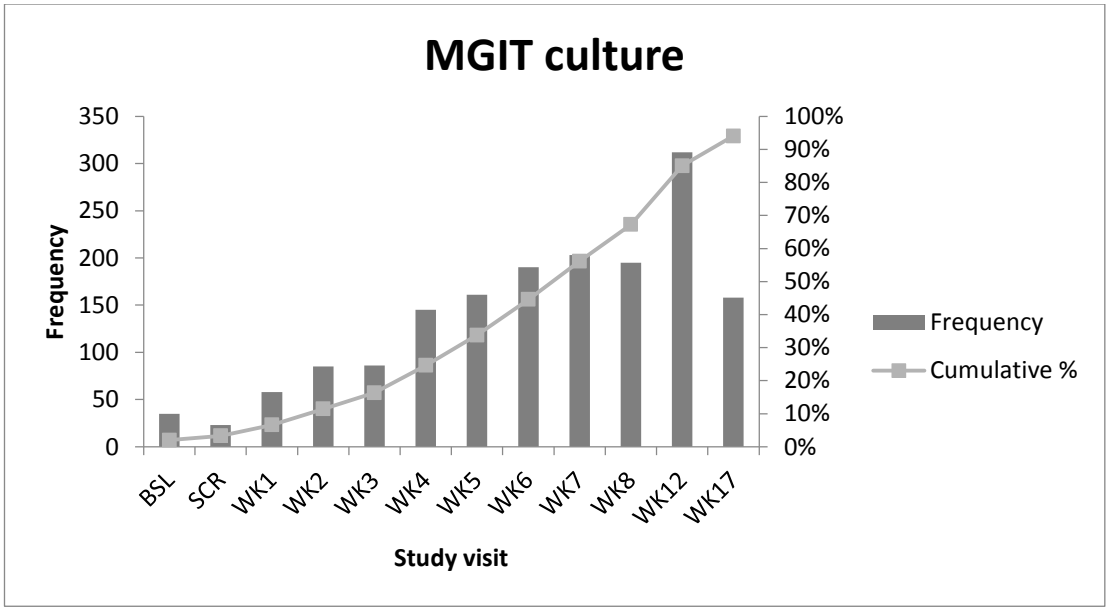
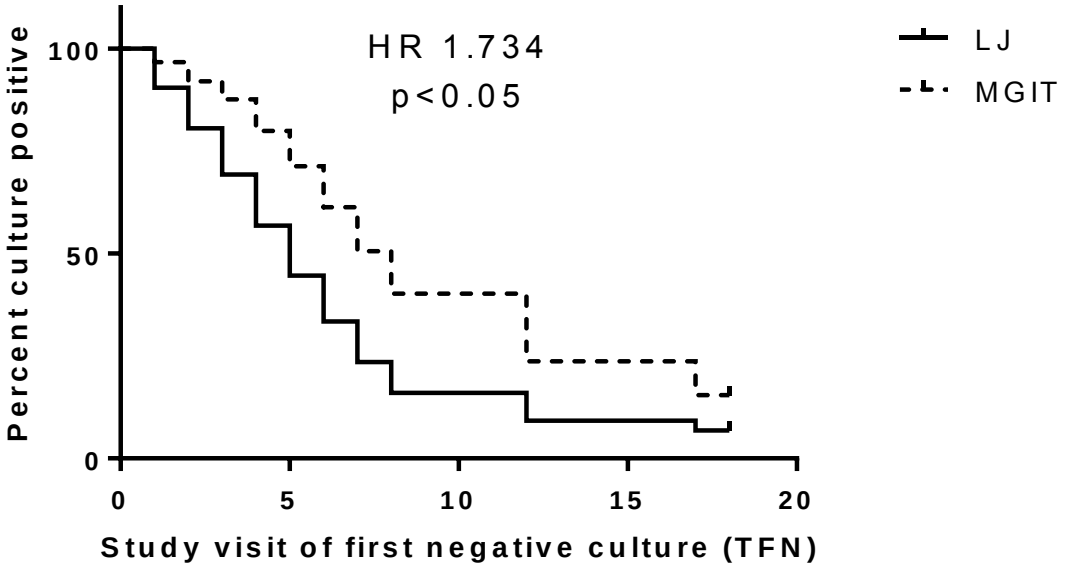


Figure 5.10. Kaplan Meier estimates of study visit at which first culture negative sample collected (TFN) on LJ and in MGIT culture, with table showing numbers at risk at selected timepoints



No. at risk

Timepoint	BSL	Wk4	Wk8	Wk12	Wk17
LJ	1881	1302	443	301	173
MGIT	1881	1648	951	756	446

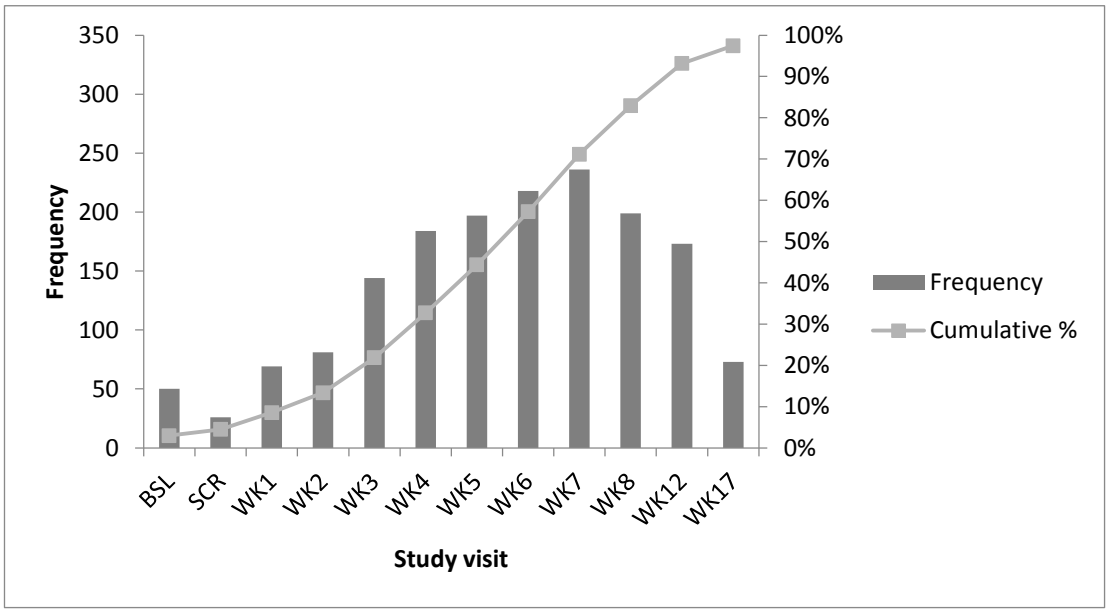
5.4.6 Time to sustained negative

Of 1881 included in these analyses, a time to sustained negative (TSN), defined as two consecutive negative cultures, did not occur before week 17 or could not be determined in 19% of patients using LJ culture and 40% of patients using MGIT culture. By the week 8 study visit, 82% of patients already have achieved a sustained LJ culture conversion from positive to negative, compared to 60% who will have achieved MGIT culture conversion. A histogram of the TFN for LJ and MGIT are shown in figures 5.11a and 5.11b

A sustained culture negative was reached at an earlier study visit using LJ culture than using MGIT. The study visit at which a sustained negative was achieved was week 6 [IQR 4-8] when using LJ culture results and week 8 [IQR 6-12] when using MGIT culture. At any given timepoint, a patient who has not yet achieved a sustained culture negative will be 1.909 times more likely to have achieved this by the next visit using LJ culture than MGIT culture. (HR_1.909; $p<0.0001$). A Kaplan-Meier plot of time to sustained negative is shown in figure 5.12.

Figure 5.11 Histogram showing the frequency of study visits at which a sustained; a. LJ and; b. MGIT culture-negative was achieved (TSN)

a



b.

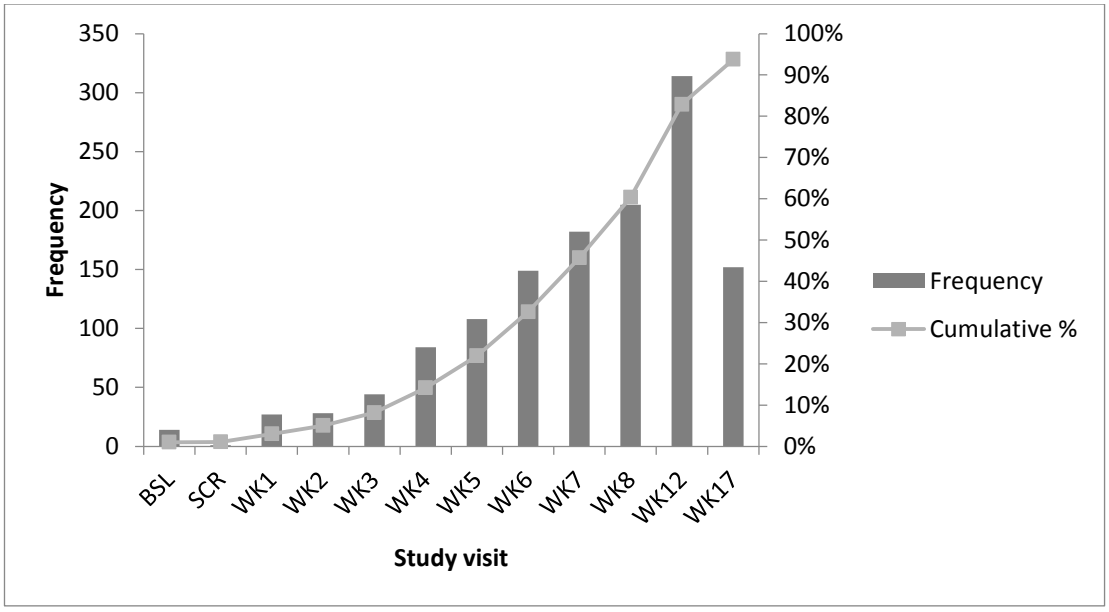
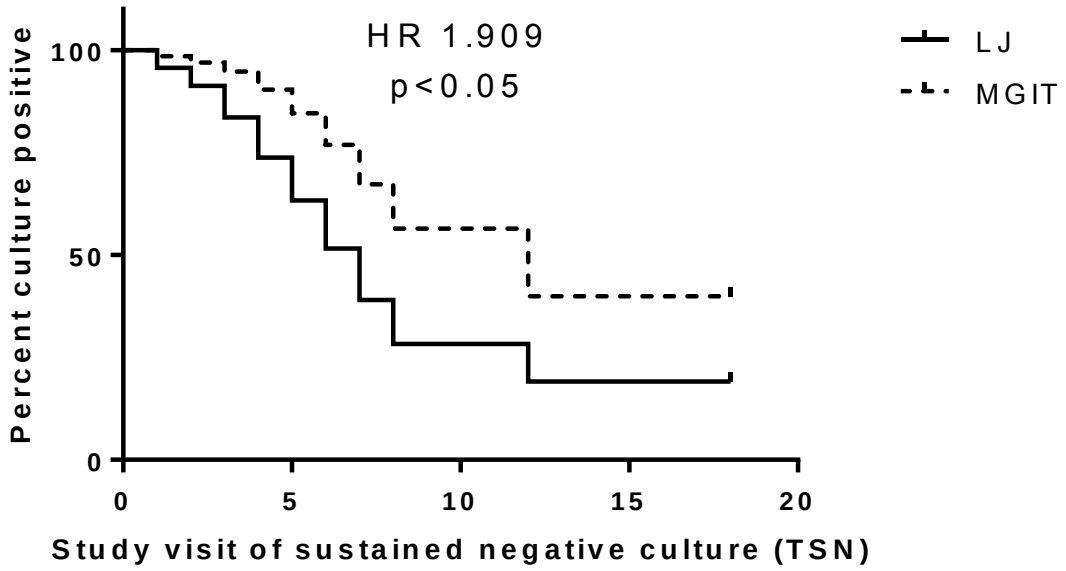


Figure 5.12. Kaplan Meier estimates of study visit at which a sustained culture negative (TSN) was achieved on LJ and in MGIT culture, with table showing numbers at risk at selected timepoints



No. at risk

Timepoint	BSL	Wk4	Wk8	Wk12	Wk17
LJ	1881	1572	970	532	359
MGIT	1881	1782	1445	1062	751

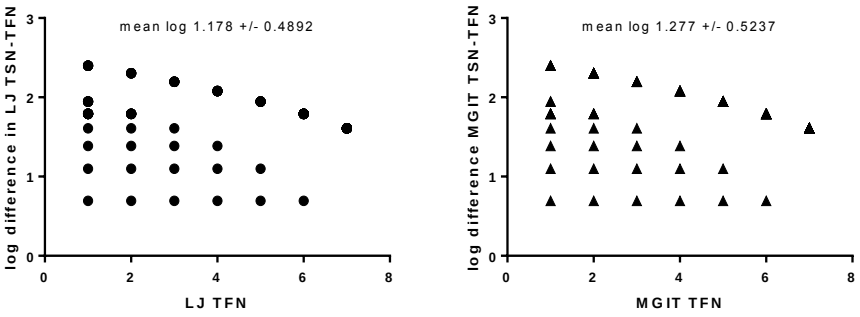
5.4.7 Variability

Proportions and frequencies do not inform the variability of positive and negative samples over a period of time in a single patient. Lasagna plots allow visualisation of the repeated measures on LJ and MGIT. These plots also depict those data points which are missing due to contamination false positives or simply missing data. Two lasagna plots are shown in figures 5.13a and 5.13b depicting variability of LJ and MGIT culture results in each single patient over the first 17 weeks of the REMoxTB study stratified by baseline smear grading; MGIT has later time to sustained culture conversion, greater positive negative variability and more missing data.

Figure 5.13 Lasagna plot of; a. LJ and; b. MGIT culture results for all patients in the REMoxTB study over time on treatment, stratified by baseline smear grading; each patient is represented by a single horizontal row (red=culture positive; green=culture negative; white=contaminated/MGIT false positive/missing data

One possible numerical measure of variability may be to compare the week at which a patient has their first negative culture (TFN) and the week at which this culture conversion becomes sustained (TSN). Where culture conversion occurs and is maintained, TFN and TSN will be in agreement. Of 1881 patients, TFN and TSN were the same in 1066 patients (57 %) in LJ culture and 839 patients (45%) in MGIT media. Comparing the differences in those patients in whom TFN and TSN did not agree, the median difference TSN-TFN for LJ was 3 weeks [IQR 2-5] and for MGIT was also 3 weeks [IQR 2-5] ($p<0.05$). The data have been logarithmically transformed and the variability is shown in figure 5.14.

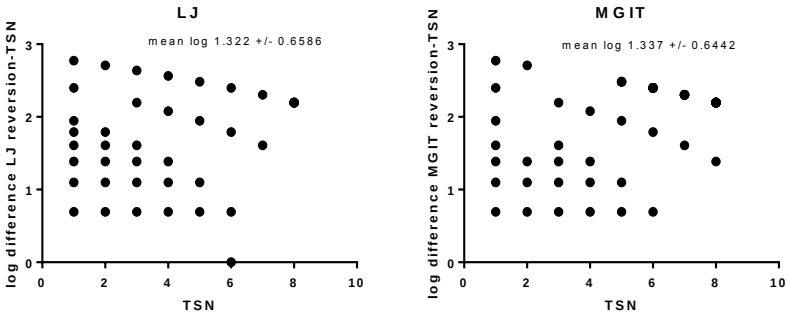
Figure 5.14. Variability in the natural logarithm of difference between time to a first negative (TFN) and a sustained negative (TSN) culture on LJ and in MGIT



5.4.8 Reversion to culture positive after having attained sustained culture negative

Of 1522 patients in whom a TSN was determined in LJ, 180 (12%) later reverted to LJ culture positive. Of those 1130 patients in whom a TSN was determined in MGIT culture, 184 (16%) later reverted to MGIT positive. The median time to reversion after TSN was the same for both LJ and MGIT; 3 weeks [2-6 weeks] and 3 weeks [2-7 weeks] respectively (p=0.85). Graphs showing the comparable variability in times to revision in LJ and MGIT are shown in figures 5.15.

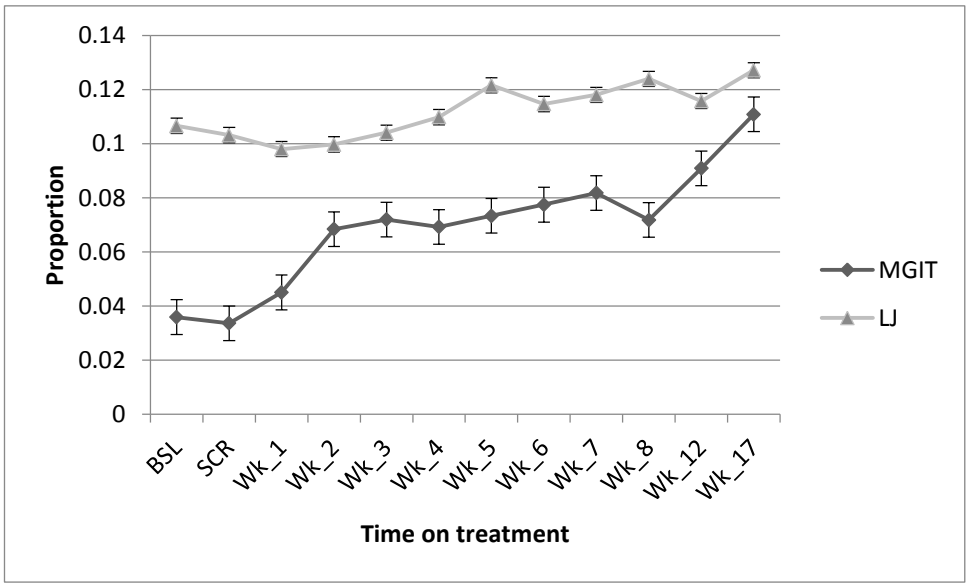
Figure 5.15. Variability of the natural logarithm of the difference from time to reversion to culture positivity in LJ and MGIT after having achieved sustained culture negativity



5.4.9 Contamination

The overall mean contamination rate was higher on LJ media at 11% compared to 7% in MGIT culture. There was a slight increase in LJ culture contamination as time on study drug increased. Contamination in MGIT culture was considerably lower than LJ at earlier time points and increased markedly increase over time as shown in figure 5.16.

Figure 5.16 Proportion of contaminated culture results in LJ and MGIT during TB treatment

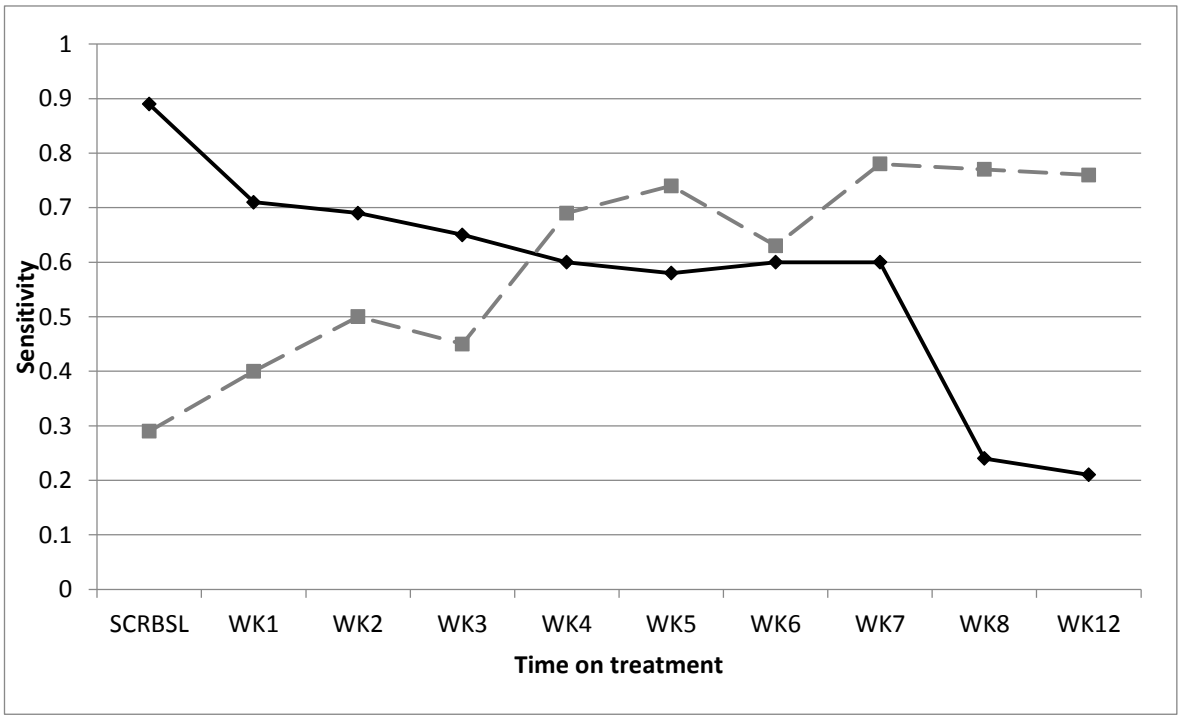


In addition to being contaminated, MGIT cultures can also falsely flag positive. Comparing overall LJ culture contamination rates with the combined MGIT contamination and false positive rates overall, the results are comparable at 11%. Again, the rates of the combined MGIT contamination and false positive results were lower at earlier time points and increased markedly as treatment progresses. By week 17, contaminated and false positive MGIT cultures (18%) were significantly higher than on LJ culture (13%).

MGIT contamination

While MGIT contamination is determined by the growth of contaminating bacteria on blood agar after 48 hours incubation, all such samples are also simultaneously processed for the presence of AFB by ZN staining. There were 1061 contaminated MGIT samples which had a corresponding LJ result that was positive or negative, i.e. not contaminated. Of these 1061 sample, 482 (45%) were MGIT ZN positive; 325 (67%) of corresponding LJ culture results were also positive. The overall sensitivity and specificity of the ZN result of a contaminated MGIT culture for LJ culture results was 65% and 71% respectively. This relationship changed over time on treatment as shown in figure 5.17.

Figure 5.17 Sensitivity (black) and specificity (grey) of the ZN result of a contaminated MGIT for LJ culture results during TB treatment

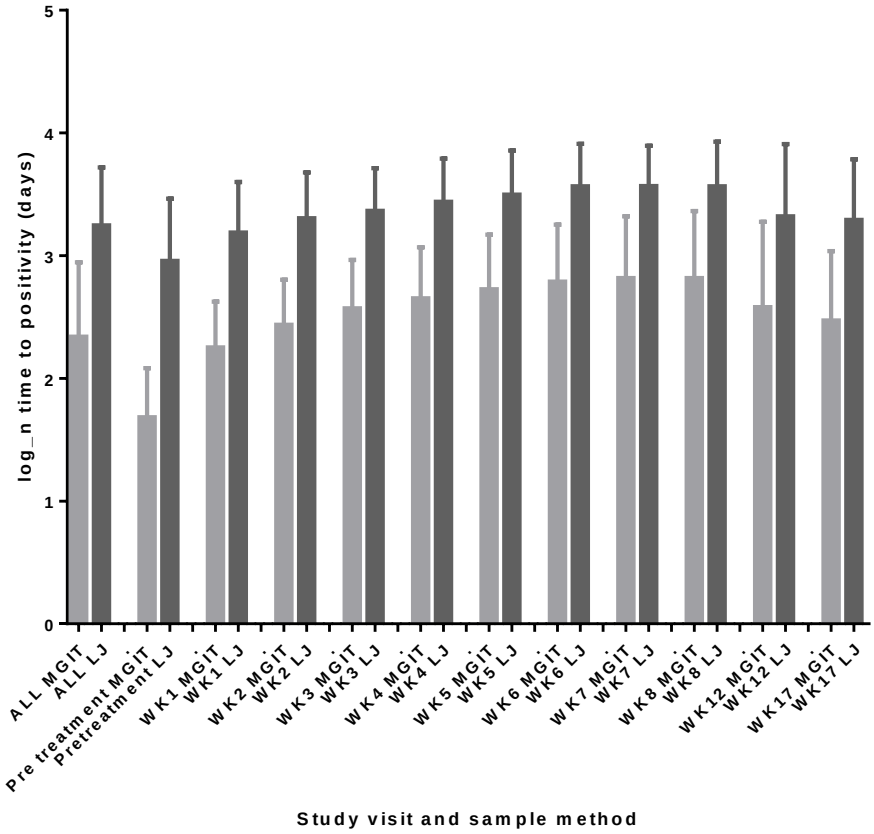


5.4.10 Mycobacterial quantification

Comparing time to detection on LJ and in MGIT media

The median time to detect a positive culture was faster in MGIT than on LJ; 11 days [IQR 7-16] compared to 28 days [IQR 21-35]. Median times to detection were lower prior to treatment in both media, and again faster in MGIT than in LJ; 5 days [IQR 4-7] and 14 days [IQR 14-21] respectively. Owing to the skewing of the data, and unequal variances, these data were log transformed and compared using unpaired T test with Welch's correction; faster times to detection in MGIT than on LJ continued throughout treatment as shown in figure 5.18 ($p<0.05$ for all analyses).

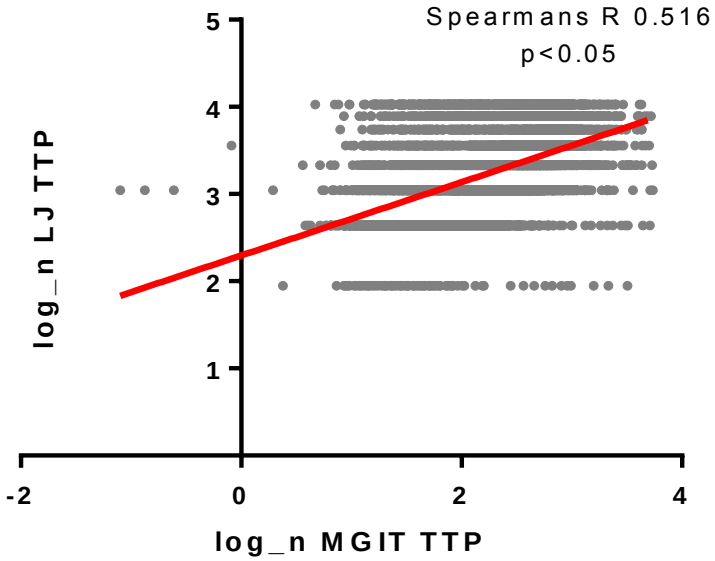
Figure 5.18 Mean and standard deviation of the natural logarithm of times to positivity in days on LJ and in liquid MGIT media overall and per study visit



Correlations between LJ and MGIT times to detect a positive results

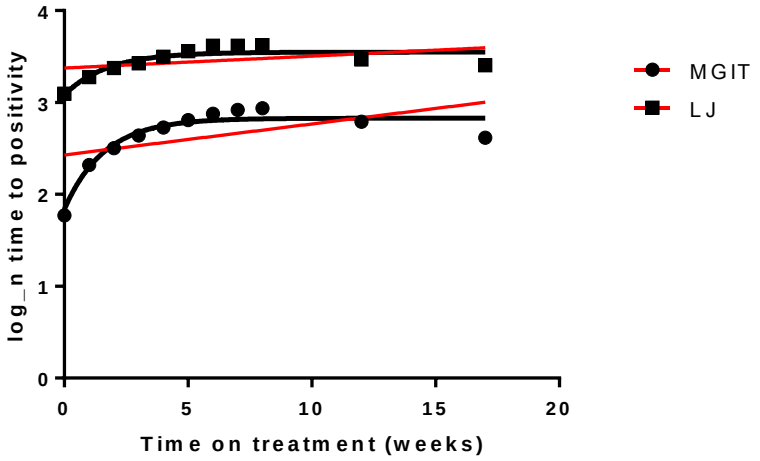
There is a clear correlation between MGIT TTP and LJ TTD; as the data are positively skewed these have been logarithmically transformed using the natural logs and, as shown in figure 5.19, both variables increase together (R 0.519; $p<0.05$).

Figure 5.19 Correlation between MGIT TTP and LJ TTD in paired positive samples at all time points combined



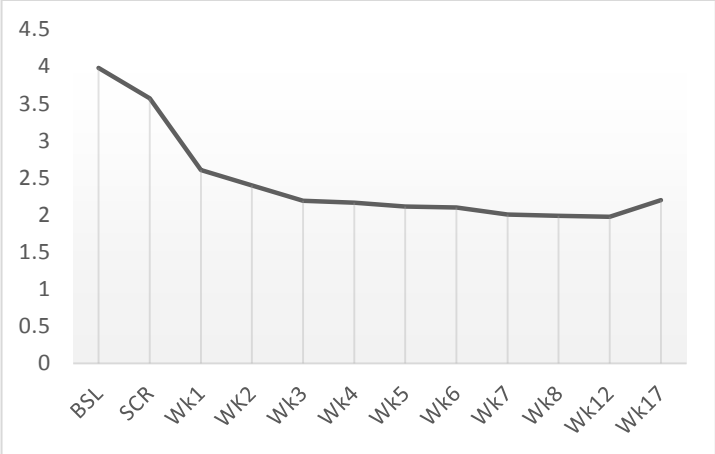
The relationship is not fixed during treatment. The decline in mycobacterial loads, represented by increasing natural log of times to detect a positive culture on LJ and in MGIT over time on treatment, are shown in figure 5.20. The change in times to detection over time on treatment were better described by coefficient of determination for a non-linear two-phase decay model (R^2 0.9308 and 0.8099 for LJ and MGIT respectively) than for a linear regression model (R^2 0.2486 and 0.161 for LJ and MGIT respectively) (figure 5.20).

Figure 5.20. Increasing natural logarithm of time to detect a positive culture in LJ and MGIT media over time on treatment with linear (red) and nonlinear regression lines (black)



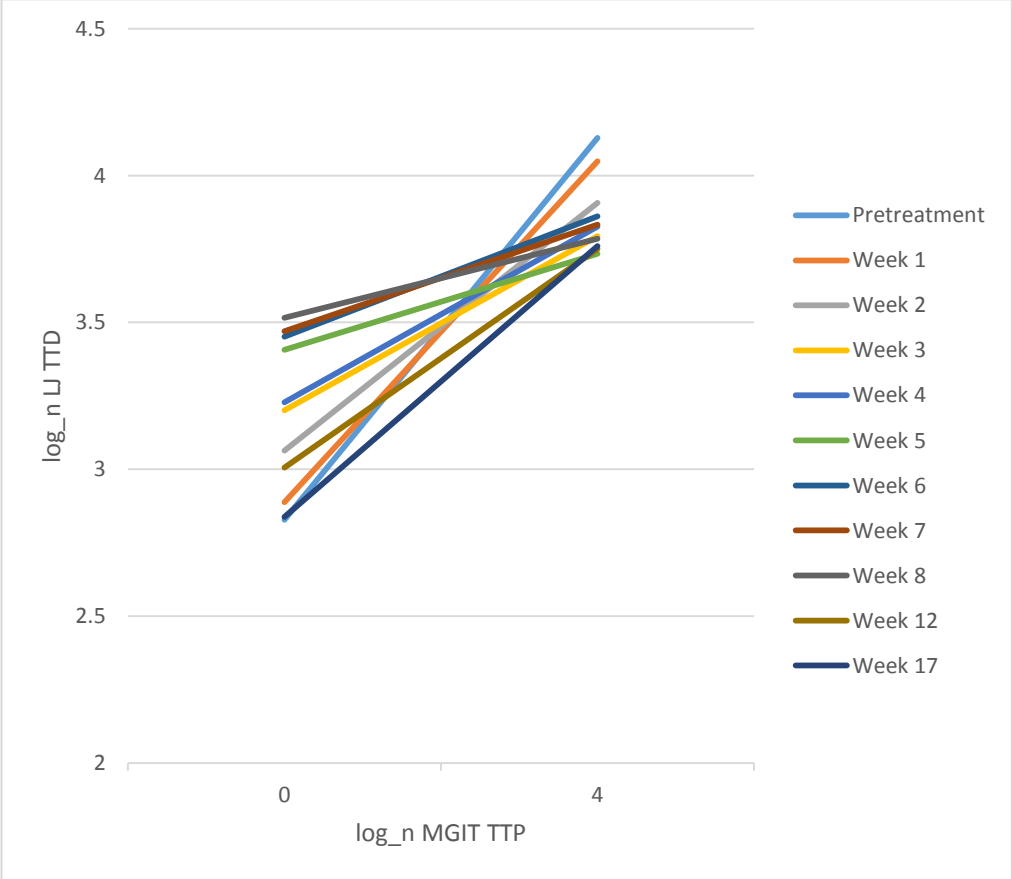
The ratio between MGIT TTP and LJ TTD is higher at earlier time points, after which the ratio is relatively constant as shown in figure 5.21 suggesting MGIT is able to detect greater proportions of the heterogeneous mycobacterial populations present at earlier time points and that the proportions of these populations present change rapidly during early time points and explaining the different ratio during treatment.

Figure 5.21. Ratio of MGIT TTP and LJ TTD as per time on treatment



The correlation between LJ TTD and MGIT TTP is therefore evident at all time points, but the slope of correlation changes over time on treatment with stronger correlation at early time points falling over time until week 8 after which the strength of correlation improves. Figure 5.22 outlines the changing nature of correlation between the natural logarithm of the times to detect a positive culture on LJ and in MGIT at each study visit. The slope of the linear regression line becomes less steep and the y-intercept tends to increase as treatment progresses.

Figure 5.22 Graph of linear correlation lines of natural logarithm of MGIT TTP and LJ TTD at each study visit



Graphs of the linear correlation between the natural log of MGIT TTP and LJ TTD at each study visit are shown in Appendix 5. A table of the numbers of paired samples and their correlation coefficients are shown in table 5.5.

Table 5.5 Correlation coefficients of the natural logarithm of MGIT TTP and LJ TTD as per study visit

Study visit	N	Correlation coefficient	p value
SCRBSL	2888	0.301	<0.05
Wk1	1328	0.337	<0.05
Wk2	1172	0.310	<0.05
Wk 3	1012	0.276	<0.05
Wk 4	839	0.310	<0.05
Wk 5	634	0.287	<0.05
Wk 6	453	0.198	<0.05
Wk 7	283	0.188	<0.05
Wk 8	184	0.233	<0.05
Wk 12	33	0.497	<0.05
Wk 17	38	0.475	<0.05

5.5 Discussion

This study shows that the relationship between MGIT and LJ culture varies significantly during treatment. As expected, MGIT culture had a higher positive yield and greater sensitivity to detect *M. tuberculosis* than LJ culture, with faster time to detection, but these changed during treatment. Combined contamination and false positive rates, were however higher in MGIT culture than on LJ and increased as treatment progressed. Culture conversion occurred earlier in the course of treatment using LJ than MGIT and was more sustained. Changes in times to positivity between culture methods, are likely due to MGIT and LJ supporting different populations whose relative proportions change early on during treatment.

Current recommendations from the WHO to implement liquid culture systems are based on vast amounts of data showing the improved sensitivity of liquid MGIT over culture on LJ media has been published in many studies (133, 134, 304, 308, 310, 335-340). Some studies, however, have concluded that the implementation of MGIT should complement rather than replace solid media owing to the additional sensitivity offered by combining methods and to avoid losing any positive yield (341, 342). This matches the WHO advice which does not advocate abandoning LJ culture in LMIC at present and which recommends liquid culture implementation as part of a comprehensive package of laboratory strengthening (292) . The Foundation for New and Innovative Diagnostics (FIND) are backing the use of the MGIT 960 and are currently working with BD to generate much needed operational and costing data. The move to fully automated liquid culture systems as the sole mycobacterial medium has,

however, been implemented in many laboratories in developed countries. There are fewer studies assessing the use of MGIT in resource limited settings (311, 315, 343-345).

The data upon which MGIT and LJ comparisons are based do not discuss the comparative value of solid and liquid culture during treatment for monitoring patients' response to antituberculous chemotherapy. Where defined, most of the studies using clinical samples were from patients suspected of having TB prior to treatment and contained many samples with non-tuberculous mycobacteria. Some studies included samples from patients at varying stages of treatment but without analysing as per time on treatment and therefore cannot inform this discussion. There are to date no published studies comparing the value of LJ and MGIT during treatment. In the REMoxTB study all samples are simultaneously processed for culture on both LJ media and liquid MGIT media allowing detailed descriptions of the relationship between these methodologies.

These data show a 15% increased yield in positive cultures using MGIT compared to LJ across all samples. At each time-point MGIT yields considerably higher positive results than LJ. What this means in terms of patient outcomes remains unclear. The use of culture conversion has been proposed as a biomarker of treatment response, particularly after 2 months of intensive phase treatments. Using MGIT culture results, the proportions of patients with a positive culture at this time-point was 37% compared to 14% in LJ. This represents a huge increase in positive cultures at a time-point when treatment is being downgraded to continuation phase. The impact of this increase in patient

outcomes has yet to be evaluated in the REMoxTB study. The sensitivity of culture to predict relapse has been found to be low in a systematic review (179), but the considerable reduction in culture conversion has potential implications for the roll out of MGIT culture, not least operationally in terms of capacity.

Such a large increase in positive cultures may generate considerable increased workload; samples flagging positive in MGIT require processing to confirm organisms are *M. tuberculosis* or of the *M. tuberculosis* complex, especially in areas with high level of non-tuberculous mycobacteria or in patients at risk of colonisation or clinically significant infection with non-tuberculous mycobacteria. Moreover, when monitoring treatment, such samples may require to be processed for drug susceptibility testing to evaluate whether drug resistance has developed in patients which may explain their continuing positive culture results or ongoing symptoms.

The reasons for such discrepant results between MGIT and LJ are unclear from this data. In the REMoxTB study all patient samples are processed simultaneously for LJ and MGIT from the same sputum preparation, although 0.5ml of suspension is inoculated into MGIT compared to 0.2ml inoculated onto LJ slope. The poor agreement between results is therefore concerning as discrepancies exist in around one third of culture results in LJ and MGIT. Agreement is poor even at early time-points in these patients who are have already been diagnosed as having microbiologically confirmed TB. This certainly poses a challenge in considering replacing one culture methodology with another as this may impact on the sensitivity. This has been shown in other studies with up to 10% samples positive only in solid culture (134, 342, 346).

Of all the MGIT negative samples in our study, 3% were positive in LJ culture and would have been missed when using only a single culture method. Looking only at the pre-treatment samples, of 96 MGIT negatives samples, 25 (26%) were LJ positive. The patient population is of course biased as all patients clinically have TB and may not apply to an unselected populations presenting with suspected TB, but these data indicate the potential magnitude of the problem. Negative cultures on LJ are much more commonly MGIT positive at one third overall, and over 80% for pre-treatment samples.

The sensitivity of the MGIT for LJ culture is high at 98% and improves even further when the combined MGIT and LJ culture results are used as the gold standard. LJ sensitivity for the combined MGIT/LJ culture result falls dramatically as time on treatment increases reflecting the increasing contribution of MGIT positives to the combined total. The high number of false negatives in LJ is potentially cause for concern if monitoring samples are to influence treatment decisions.

The difference between the two culture methods in terms of contamination is significant with LJ samples more often contaminated than MGIT. Early studies of MGIT found similar results (134, 304, 336) however many more recent studies have identified lower contamination rates in LJ culture (47, 310, 338, 340, 347). A recent health technology assessment of fully automated liquid culture systems failed to find any difference in liquid and solid culture contaminations (348). However, the review included studies of both respiratory and non-respiratory samples and the authors noted that some data on culture

contamination was likely to be outdated given advances in culture methodologies, including the use of antibiotics to reduce contamination. The inclusion of such methodological advances in the REMoxTB laboratory procedures may therefore account for the differences in the results presented here.

While LJ cultures are more commonly contaminated, interestingly from the REMoxTB data, we have identified the additional contribution of MGIT false-positives to the number of samples upon which a positive or negative result cannot be determined in MGIT; taken together, the overall LJ contamination and combined MGIT contamination-false positive rates are comparable. While the LJ contamination rate is relatively stable during treatment, the combined MGIT contamination rate increases markedly over the first 17 weeks of treatment, by which point 18% of samples have indeterminable results. This is probably most important for clinical trials of TB drugs which evaluate interim treatment response and may in future inform treatment alterations or intensifications; the loss of this data will impact on the power and therefore the sample size for clinical trials which is known to be a barrier in TB studies in terms of both patient recruitment and costs. Efforts to improve TB study design to reduce costs and assist in interim go-no go decisions (197) must consider what measures are being assessed and whether these results can be compared to historical studies using LJ or other solid media alone.

If TB sites are to abandon LJ culture, the additional information provided by the ZN smear of samples contaminated in MGIT should not be ignored. Two thirds of those samples contaminated in MGIT culture found to be ZN positive had

positive cultures on LJ media. Although the sensitivity and specificity of the ZN smear of a contaminated MGIT for LJ culture is low at 65% and 71%, clearly such information must be factored into algorithms where LJ is no longer being performed. This creates an operational challenge should these MGIT samples be crucial for monitoring, as in most NTPs, and may place an additional burden on patients.

Culture conversion

The potential value in TB culture conversion in predicting outcomes is not clear. A meta-analysis concluded that sputum monitoring was insufficiently robust and that on-treatment cultures at 2 months have a poor sensitivity for relapse at 40% [95% CI 25-56%]. Newer measures which maybe predictive of outcome need to be adopted. In this study, culture conversion and reversion have been explored as potential such measures and compared across culture methods. This matches somewhat with EBA study methodology which evaluates the decline in bacterial load as evidence of a drug's potential bactericidal and sterilising activity. By extrapolation, culture conversion measures the declining bacterial load over a longer period of time on treatment until the patient is rendered culture negative.

This paradigm of TB treatment is rather based in the 'perfect world' scenario; a patient presents with a positive culture, starts treatment, provides frequent samples for monitoring, converts to negative at a given week and remains negative for the remainder of treatment OR by not converting by a crucial time-point, for which evidence exists of a poor clinical outcome, receives an evidence based intervention to reduce treatment failure or relapse. It seems unlikely that

such a clean mycobacteriological storyline is present in the majority of patients as shown clearly in the Lasagna plots.

In our study, the first negative culture was achieved earlier in the treatment course when using LJ culture compared with MGIT culture and reflects the increased sensitivity of MGIT over LJ at all time-points. It is important to also consider the variability in sputum results, especially when considering set time-points for monitoring maybe operationally easier to implement, e.g. all patients provide a 2 month sample. In order to measure this variability we have defined the time to what we consider a sustained negative, i.e. the study visit after which two consecutive negative cultures are collected.

The TSN was also achieved at an earlier study visit if using LJ culture rather than MGIT. We have attempted to make some measure of this variability by comparing the time from first negative sample to time until this conversion may be considered sustained. This should take account of the variability in subtle differences in declining mycobacterial loads if there is in fact a uniformly downward trajectory in viable mycobacteria as treatment progresses. LJ had a higher number of patient in whom culture conversion was sustained after the first culture negative than in MGIT. However, there was no difference in time between the first and sustained cultures in LJ and MGIT. The implications this may have on follow up culture monitoring are significant as these data would seem to support repeated sputum examination.

These results also raise the possibility of continual variability in MGIT culture result on a day-to-day or week-to-week basis. There were considerably greater

numbers of patients who reverted to MGIT culture positive after having achieved a sustained culture conversion than was seen using LJ. The time taken until a reversion was, however, no different using either media.

Whether or not these measures of treatment response will ultimately be sufficiently robust to predict patient outcomes remains to be seen, but there are clear differences in bacteriological monitoring between LJ and MGIT culture which must be considered when replacing solid culture with MGIT. The massive increase in sensitivity will also need to be considered in workload planning and equipment purchasing.

Time to detection

As expected, times to detect a positive culture were significant faster in MGIT than on LJ. This relationship has been confirmed in many studies to date both *in vitro* and (308, 310, 339, 340)*in vivo* (47, 133, 134, 304, 336, 337). This is one of the main reasons, in addition to increased sensitivity, for the roll-out of MGIT globally for the diagnosis and management of tuberculosis. Our data show also that the relationship between times to detect a positive in MGIT and LJ, however, was not fixed throughout treatment. Pre-treatment, and at early time points, the ratio of MGIT TTP to LJ TTD was higher than at later time points during treatment. As TTD has been shown to reflect mycobacterial load, this raises important questions as to what may be being cultured in MGIT that is not being captured in LJ culture. Clearly this may relate to the relative efficiencies if the culture media, however given the theories surrounding the different populations of *M. tuberculosis* which may be present in patients, further work is required to elucidate more robust answers.

Recent research investigating mycobacterial populations in patient samples and found that 90% of the bacilli in sputum are persisters that can grow in liquid media but not on solid plates (349). A similar proportion of cells identified as lipid body positive in another study were only culturable by the addition of resuscitation promoting factors (73). An evaluation of data from a rifampicin dosing study found similar changes in the correlation between culture in liquid and solid media (350), but only ran to the first 14 days of treatment. Interestingly this study found that increasing doses of rifampicin had an impact on the correlation providing further support for the presence of heterogeneous populations of mycobacteria and their differential growth on solid and in liquid media.

The variability in proportions of these heterogeneous mycobacteria during treatment has not been fully elucidated. These data, however, support the increasing evidence that MGIT may be culturing populations of mycobacteria which may be non-culturable on LJ media. If culture dynamics have any role in predicting patient outcomes, it will be important to consider how the roll-out of MGIT to replace culture might diminish the information which may be available where both culture methods are employed.

The data in this thesis go some way to identifying the relationship between MGIT and LJ culture results, however, correlation co-efficients of the MGIT TTP and LJ TTD were poor overall and at all time-points. This may reflect a genuinely poor correlation; if indeed different populations are being cultured using the different methods, poor correlation may be plausible, however such

poor correlation may reflect inter-patient variability. However, we cannot however ignore the effect that the categorical nature of the LJ TTD data (week 1, 2, 3..) compared to continuous data for MGIT TTP may have on the interpretation of these results and further research producing a continuous measure of growth for analysis may be beneficial.

Limitations of this study

As smear positivity was an inclusion criterion for the REMoxTB study, these data cannot be generalised to smear negative patients. Furthermore, the analyses were conducted blind to the REMoxTB treatment allocation which is a potential confounder; the data suggest that different mycobacterial populations are supported by different culture methods and these populations may also be differentially affected by different drugs and thus treatment allocation may have an impact on these results which will require further consideration on unblinding of the REMoxTB trial data.

5.6 Conclusion

MGIT culture has an increased positive yield and sensitivity throughout treatment while the sensitivity and positive yield of LJ culture falls. Contamination rates are lower in MGIT, but contamination increases as treatment progresses and, with the additional MGIT false positives, results in comparable overall loss of definitive sample results. There are poor and variable levels of culture agreement in samples processed using the same methodology for preparation; over a third of LJ negatives are MGIT positive, but 3% of LJ positives are not picked up by the MGIT. To replace LJ with MGIT

culture, the impact of this loss in sensitivity would need to be balanced against potential increased losses to follow up by requiring repeated culture testing and would need to be evaluated in field settings.

MGIT culture is significantly faster than LJ culture; this is particularly marked at early timepoints during treatment which may support a hypothesis that the two culture methods support different mycobacterial populations whose relative proportions are known to change dramatically early on treatment. Measures of culture conversion show shorter times on treatment to a first negative or sustained conversion on LJ reflecting higher sensitivity of MGIT at all points. MGIT cultures seem to be more variable than LJ culture, and culture conversion may be a less fixed concept employing MGIT culture.

These data have implications for sites considering replacing LJ and implementating MGIT in terms of cost and workload which will in future need to be weighed against clinical outcome data; this will be available at the end of the REMoxTB study. Treatment monitoring will additionally need to factor in the increased variability of MGIT cultures when considering fixing time-points for sample collection and processing and methods for following up those patients with positive results. There is however no evidence based intervention which is known to reverse a predicted negative outcome and further work on treatment alterations is required.

Chapter 6 Does pre-treatment time to detect positive culture predict bacteriological responses to antituberculous chemotherapy?

6.1 Introduction

Biomarkers of TB treatment response are highly sought after to inform treatment decisions and act as surrogate endpoints in clinical trials of new drug regimens (9). As the implementation of TB culture is in the process of being scaled up (281), largely in liquid culture, it is useful to consider the information this may provide not only in terms of diagnosis and drug sensitivity, but also any prognostic markers.

Measures of mycobacterial burden have been shown to correlate with cavitation and extent of disease on chest imaging (102, 238) which has been shown to impact on time to culture conversion, 2 month culture conversion and clinical outcomes (239, 288, 351). The rate of decline in mycobacterial load with or without cavities is similar suggesting treatment duration maybe guided by baseline mycobacterial loads, however there is insufficient evidence to use this information to guide patient treatment and prolongations are not advised in international guidelines on this basis or in the presence of cavitation.

Studies of mycobacterial load at baseline have also been shown to predict the speed of decline in mycobacterial load and clinical outcome (196, 238, 330). In one study, a positive culture pre-treatment detected in ≤3 days using the BACTEC 12B liquid radiometric method, and reflecting high mycobacterial burdens, was associated with an increased risk of relapse and recurrence

(238). In this study, the baseline TTD was predictive of 2 month culture conversion, but 2 month culture conversion was not associated with relapse or recurrence in multivariate analyses.

Studies so far have employed single culture methods and do not take account of the different mycobacterial populations which may be cultured on solid and media (349, 352). These different mycobacterial populations may go some way to explaining the increased sensitivity of liquid culture to detect *M. tuberculosis* during treatment and the slower culture conversion compared to culture on solid media. In the REMoxTB study, patients serial sputum samples are monitored using both solid and liquid culture and thus any prognostic markers may be measured in both media.

6.2 Hypothesis

Pre-treatment mycobacterial load as measured by time to positivity on LJ and in MGIT media predicts mycobacteriological responses to treatment in patients being treated for TB as part of the REMoxTB study during the first 17 weeks of treatment.

6.3 Methodology

All results contained in the REMoxTB study database on 29 August 2012 were extracted for the purposes of these analyses. We excluded data from those patients who did not enrol in the trial and whose age and/or HIV status and CD4 cell count could not be determined. Samples without corresponding smear, LJ

and MGIT culture results were excluded. As the REMoxTB study was ongoing at the time of these analyses, they were conducted blind to treatment allocation. Those samples collected after week 17 of study drug, when patients in the moxifloxacin containing arms would have been receiving placebo, have been excluded. Samples collected at unscheduled visits were also excluded.

Microbiological analyses

Sputum samples were collected prior to TB treatment and at every scheduled study visit as described in table 2.3 and processed for culture on LJ and in liquid MGIT media. Microbiological analyses were performed described in chapter 2.

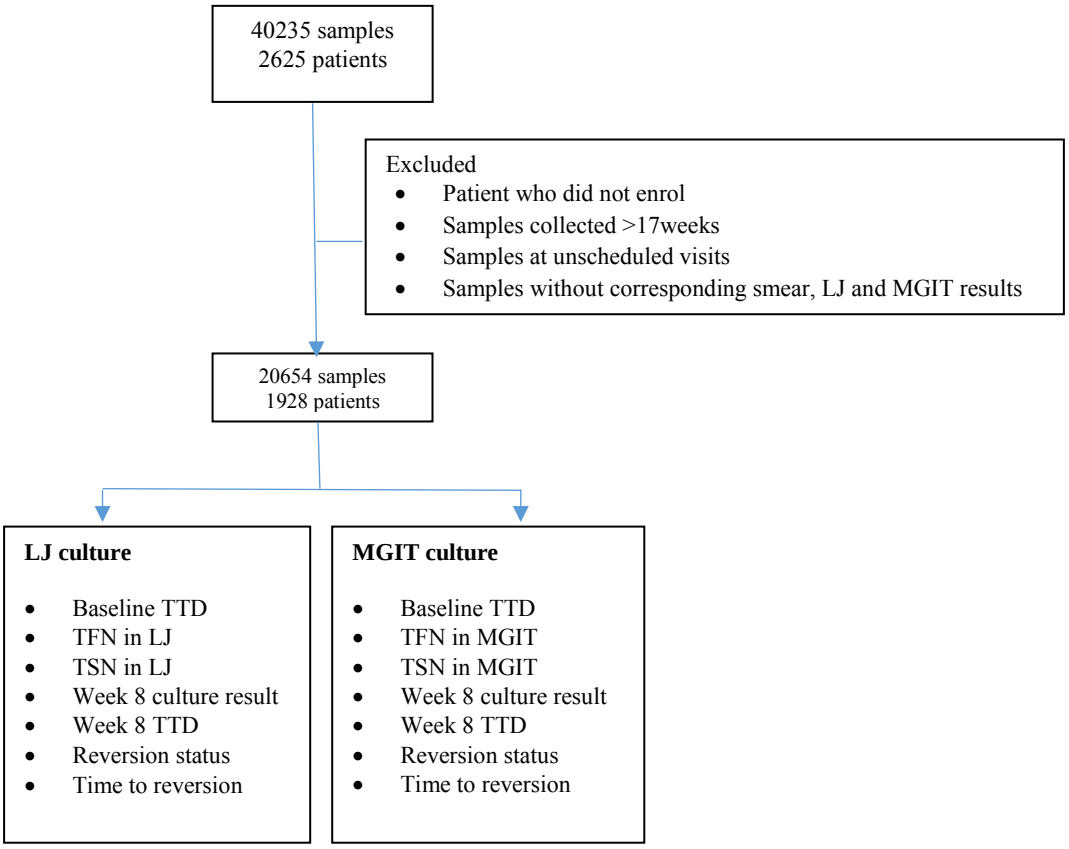
Statistical analyses

The Spearman's rank correlation coefficient (R) was calculated as a nonparametric measure of statistical correlation between baseline mycobacterial load and TFN, TSN and week 8 TTD. Linear regression was used to determine the coefficient of determination (R^2). The difference in baseline median TTD as per week 8 culture status and reversion status were compared using the non-parametric Mann Whitney U test. Data were also logarithmically transformed using the natural log as best approximation of normality and differences compared using unpaired t test, with Welch's correction where data were found to have unequal variance.

6.4 Results

Patient demographics were as described in table 5.1. A flow chart of samples included for this chapter is given in figure 6.1.

Figure 6.1 Flow chart of samples



6.4.1 Mycobacterial load as a predictor of TFN

There is an inverse correlation between baseline time to detect a positive culture in both LJ and MGIT and time to the first culture negative sample being collected (both $p<0.05$). The correlation coefficients are low for both LJ TTD ($R= -0.279$; $p<0.05$) and MGIT TTP ($R= -0.339$; $p<0.05$). The variation in TFN is poorly explained by baseline time to detection in either media; 5% in LJ culture and 6% in MGIT culture (R^2 0.049, $p<0.05$ for LJ; R^2 0.056, $p<0.05$ for MGIT).

6.4.2 Mycobacterial load predictor of TSN

Patients with higher mycobacterial loads prior to treatment took longer to achieve sustained negative culture as shown by the inverse relationship between baseline time to detection and time to sustained negative using both MGIT and LJ cultures ($p<0.05$). The correlation, however, is poor using either LJ ($R=-0.238$) or MGIT ($R=-0.339$), with just 4% and 6% of variation in TSN being explained by baseline time to detection in LJ and MGIT respectively (R^2 0.037, $p<0.05$ for LJ; R^2 0.056, $p<0.05$ for MGIT).

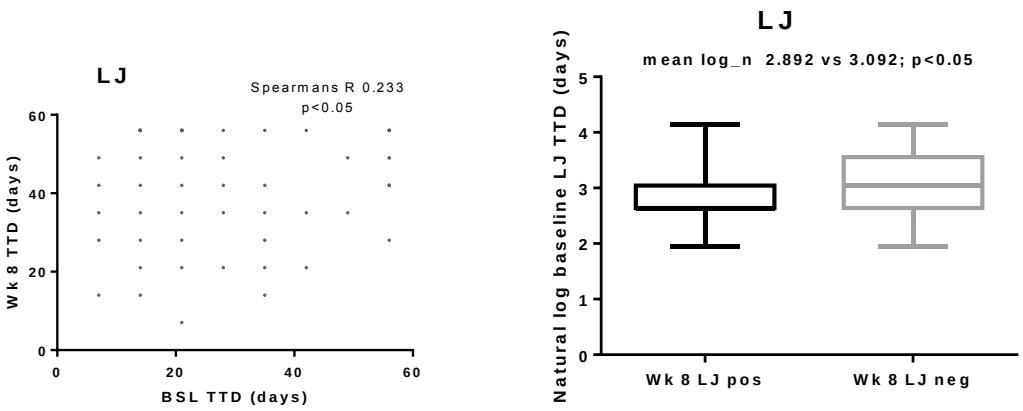
6.4.3 Mycobacterial load predictor of week 8 culture conversion

LJ

Mycobacterial load in LJ at baseline correlates significant with that at week 8 culture (R=0.233; p<0.05; Figure 6.2a), but the time on treatment explains only 5% of the variability in TTD (R^2=0.054; p<0.05). There is a significant difference in median LJ TTD of 7 days between baseline LJ TTD in patients who have positive LJ cultures at week 8 and those who have negative LJ cultures at week 8 (14 days vs 21 days; p<0.05), with a shorter TTD reflecting a higher baseline mycobacterial load in those patients who are LJ culture positive at week 8. The mean difference remains significant after natural logarithmic transformation (figure 6.2b).

Figure 6.2 a. Correlation of baseline and week 8 culture TTD on LJ culture and: b. baseline LJ TTD of patients with a positive and negative week 8 LJ culture

a b

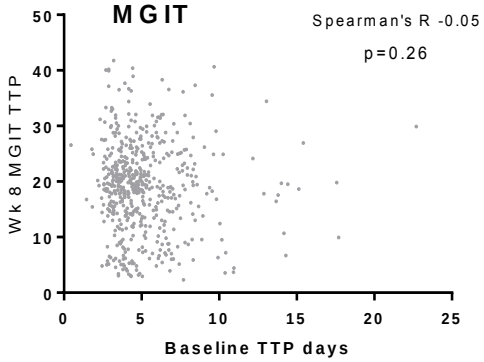


MGIT

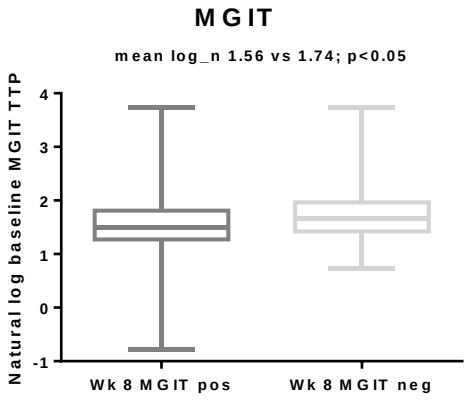
There is no meaningful correlation between pre-treatment MGIT TTP and TTP of sample collected at week 8 (R -0.05; p=0.26) as shown in figure 6.3a. There is, however, a small but statistically significant difference in median baseline MGIT TTP of around 1 day between patients who are culture negative at the week 8 study visit and those who are culture positive (4.5 vs 5.3; p<0.05), with higher baseline mycobacterial loads in those patients who remain MGIT culture positive at week 8. The mean difference remains significant after natural logarithmic transformation (figure 6.3b).

Figure 6.3. a. Correlation of baseline and week 8 culture TTP in liquid MGIT culture: b. baseline MGIT TTP comparing patients with a positive and negative week 8 MGIT culture

6.4a



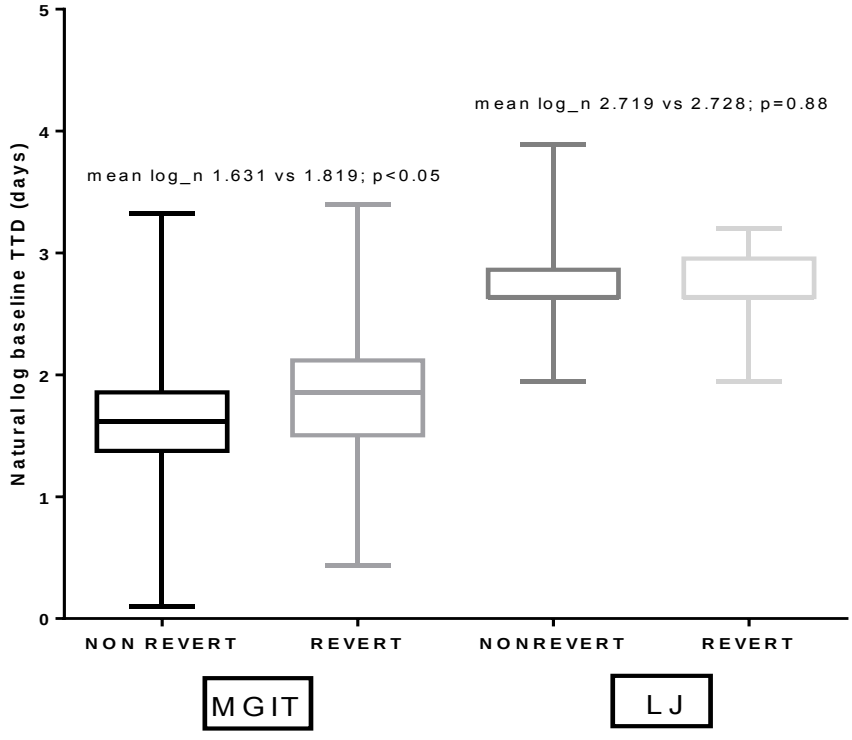
6.4b



6.4.4 Mycobacterial load predictor of reversion

Higher pre-treatment mycobacterial load did not predict reversion after a sustained culture negative in either media; patients who reverted had longer median times to detection at baseline, reflecting lower mycobacterial loads, in both LJ and MGIT cultures as shown (median 4.96 and 6.22 days in MGIT; p <0.05 and median 18 and 21 days on LJ; p<0.05). These results remained statistically significant when the data were log transformed (figure 6.5) likely owing to the large number of samples analysed, but don't appear plausible or clinically applicable.

Figure 6.4 Mean natural logarithm of times to detection a positive culture in MGIT (left) or LJ (right) at baseline stratified by reversion status using the same media



6.5 Discussion

In this study, pre-treatment mycobacterial load in MGIT or LJ was poorly predictive of mycobacteriological response to treatment during the first 17 weeks of treatment.

There is considerable interest in using pre-treatment mycobacterial load to predict treatment responses. From our data we have been able to identify an inverse correlation between time to detect a positive culture at baseline, as a measure of mycobacterial load, on both solid and liquid culture, and time to first negative culture and time to sustained negative culture. The correlation, however, is poor overall and only a small proportion of the variability in either TFN or TSN is explained by mycobacterial load at baseline.

Although a recent systematic review and meta-analysis has highlighted the low sensitivity and moderate specificity of 2 month culture conversion to predict long term outcomes (179), many studies to date have used the week 8 culture as a predictor of patient outcome. From REMoxTB data, the mycobacterial load at baseline is not predictive of the week 8 mycobacterial load. Perhaps given Mitchison's description of the heterogeneous populations of *M. tuberculosis* this is unsurprising given the baseline TTD reflects mainly a continuously growing population 97% of which will be killed by day 14; this timepoint may have been more appropriately considered as a predictor of the persister population (71).

There is, however, a significant difference of just over 1 day in MGIT and 7 days in LJ in the pre-treatment TTD between patients who have converted to culture

negative at week 8 and those who remain culture positive. From data in other studies in our group, a 40 hour difference in pre-treatment time to detection in MGIT reflected a 1 log difference in mycobacterial load (Personal Communication: Dr Isobella Honeyborne) which may well be significant both clinically and detectable using molecular testing methodologies (196). There are limited data to determine the clinical and laboratory significance of the 7 day difference in mycobacterial load LJ TTD represents. The spread of results is wide for both MGIT and LJ with considerable overlap, and such it is difficult to imagine what clinically useful cut-offs could be applied to impact patient treatment and outcomes. As culture conversion at week 8 has limitations in predicting long term outcomes, measures to predict the week 8 culture or other parameters may have limited clinical relevance even were those predictions perfect, which they are far from in this data set.

Analysing data on those patients who revert to a positive culture having sustained at least two consecutive negative cultures shows pre-treatment mycobacterial load did not predict those patients likely to revert. Indeed, patients who reverted had lower pre-treatment mycobacterial loads, as measured by TTD, than those who did not revert. These results were statistically significant, even after logarithmic transformation. Scientifically, however, this result seems implausible and likely clinically meaningless. On a wider level, this highlights the limitations of the ubiquitous '*p* value' in analysing large datasets; statistically significant results are commonly obtained but their clinical significance or scientific relevance must be interrogated to ensure incorrect conclusions are not reached. These data do not support a role for pre-

treatment mycobacterial load to predict culture reversions in either culture media.

The predictive value of identifying reversions routinely during treatment is unknown. We were able to identify considerable variability in culture results during treatment (figure 5.11), particularly in MGIT culture, which may reflect variations in sample quality and/or inter-patient variability. Such reversion may however be plausibly related to disease and the intermittent expectoration of sputum from sites where cells in a state of dormancy may transform from a non-culturable to a culturable state. Clearly in patients failing treatment clinically, culture reversions may provide evidence to inform patient management, as such failure may reflect deteriorating adherence to treatment in the face of clinical improvement and/or the development of drug resistance.

Reversions in this study may have been influenced by the treatment allocation. Previous trials of moxifloxacin containing regimens have only considered the initial 8 weeks of treatment and so the impact of the continuation phase of treatment has not been considered. The data on reversion presented may therefore reflect differences in the impact of the continuation phase of treatment on the final treatment outcome in each arm of the REMoxTB study, which we have been unable to consider due to the blinding of the study data.

The potential predictors identified in these analyses have disappointingly limited value in predicting mycobacterial responses to treatment. It remains to be seen what value these measures may have in predicting long terms treatment outcomes. The search for a clinically significant biomarker of treatment

response, however, remains a priority in TB research and has been the subject of numerous reviews (9, 353, 354). Being able to robustly predict outcomes at baseline would have immeasurable impact in patient treatment and follow up and therefore human and laboratory resource management. Such a measure has the potential to limit the spiralling costs associated with lengthy clinical trials. Furthermore, it may be possible to identify those patients in whom shorter durations of treatment may be successful.

Shorter treatments have thus far been unsuccessful, despite promising data from animal models which may not therefore adequately reflect the disease process in humans. It is notable, however, that, historically, many patients receiving 4 months of regimens containing isoniazid, rifampicin and pyrazinamide similar to current standard treatment do not fail or relapse on cessation; 11% relapse at 2 years in East and Central Africa (355), and 8% 2 year relapse in Hong Kong and Singapore rising to 14% after 5 years (356-358). In a recent study which stratified patients without cavitatory disease who converted to culture negativity at 2 months, only 7% of patients in the 4 month arm had unfavourable outcomes (288). This compared to just 1.6% in patients receiving standard treatment and thus this study was halted early. Given this reduction in treatment duration would offer considerable benefits both to individual patients, whose burdensome treatment may be limited, and to global health, by reducing transmission and thereby the incidence of disease, predictors of response must be pursued.

Predictors of response applicable to routine treatment programmes in resource limited settings are urgently required. The US CDC has recently produce a

roadmap for biomarker discovery (9); many examples of these have been discussed in chapter 1.

In other branches of medicine, specifically cancer studies, scientific advance are being employed to guide individualised treatment based on host and tumour genetics and taking into account pharmacogenomics. In future, such advances promise personalised treatment. Perhaps such technologies may be employed in the fight against TB, with treatment decisions made on the basis of the individual patient and mycobacterial factors and the changing nature of the latter during treatment.

Clearly any research must be realistically available for implementation in resource-limited settings where TB disease is concentrated, however innovations in technology in these regions have had considerable success, most notable in Gene Xpert in TB. In developed countries, benchtop whole-genome sequencing is not far from the horizon of routine microbiology practice and advances in equipment and bioinformatics are likely to bring such technology to developing countries. Coupled with the connectability of the internet in even the remotest places, such advances are not out-with the realms of possibility and may be available sooner rather than later.

Limitations of this study

The analyses were conducted blind to the REMoxTB treatment allocation which is a potential confounder; different mycobacterial populations may be supported by different culture methods and these populations may also be differentially affected by different drugs and thus treatment allocation may have an impact on

the mycobacterial burden during treatment and may alter their predictive value. Further consideration is required on unblinding of the REMoxTB trial data.

6.6 Conclusion

Pre-treatment mycobacterial loads in the REMoxTB study were correlated with both time to sputum conversion and time to sustained negative cultures, but only very weakly explained the variation in these measures. Differences between the baseline LJ TTD stratified by week 8 LJ culture response were statistically significant but there was no clear correlation on linear regression and there was no meaningful value in baseline MGIT TTP predicting week 8 MGIT culture. Further work is required to determine what patient and/or external factors may influence these outcomes.

Chapter 7 Can we reduce the MGIT incubation time?

7.1 Introduction

The slow growth of mycobacterial cultures has a major impact on TB laboratory capacity. Liquid culture systems like MGIT have significantly reduced the time to detect a positive culture compared to traditional LJ medium, as shown in figure 5.18. However, the turnaround time to declare a negative result still requires 6 weeks incubation. Such a long duration has potential clinical implications for patients in terms of TB diagnosis, drug susceptibility testing and monitoring for treatment response, relapse and reinfection.

In vitro, MGIT has sensitivity to detect <10 organisms in maximum 28 days (359, 360). The vast majority of patients commencing TB treatment on the basis of smear microscopy, with a limit of detection around 10,000, have a significantly greater mycobacterial burden. In patient samples, there are many factors which might affect the mycobacterial burden detected in culture, including the large proportion of resuscitation-factor dependent cells present in sputum prior to treatment (73). It is proposed however that persister populations of *M. tuberculosis* may grow preferentially in liquid culture, but not solid LJ culture (349). In the REMoxTB study, liquid cultures remained positive for considerably longer than LJ culture (figure 5.3), as elsewhere.

The incubation time required for MGIT culture has not been extensively described in clinical trials outside of comparative reductions associated with liquid culture. No study of the time to report a negative culture are reported in

the literature. This is an important consideration for the roll-out of liquid culture systems and will have significant impact on the operational capacity required and/or available in resource limited settings and may inform the centralisation/decentralisation debate.

7.2 Hypothesis

The duration of incubation in liquid MGIT culture can be reduced without loss of sensitivity and reduce the time to report a negative culture in patients being treated for TB as part of the REMoxTB study.

7.3 Methodology

All results contained in the REMoxTB study database on 29 August 2012 were extracted for the purposes of these analyses. Samples without corresponding smear, LJ and MGIT culture results were excluded. Those samples collected after week 17 of study drug, when patients in the moxifloxacin containing arms would have been receiving placebo, and those collected at unscheduled visits were also excluded.

Microbiological analyses

Sputum samples were collected prior to TB treatment and at every scheduled study visit and processed for culture on LJ and in liquid MGIT media. Microbiological analyses were performed described in chapter 2.

Statistical analyses

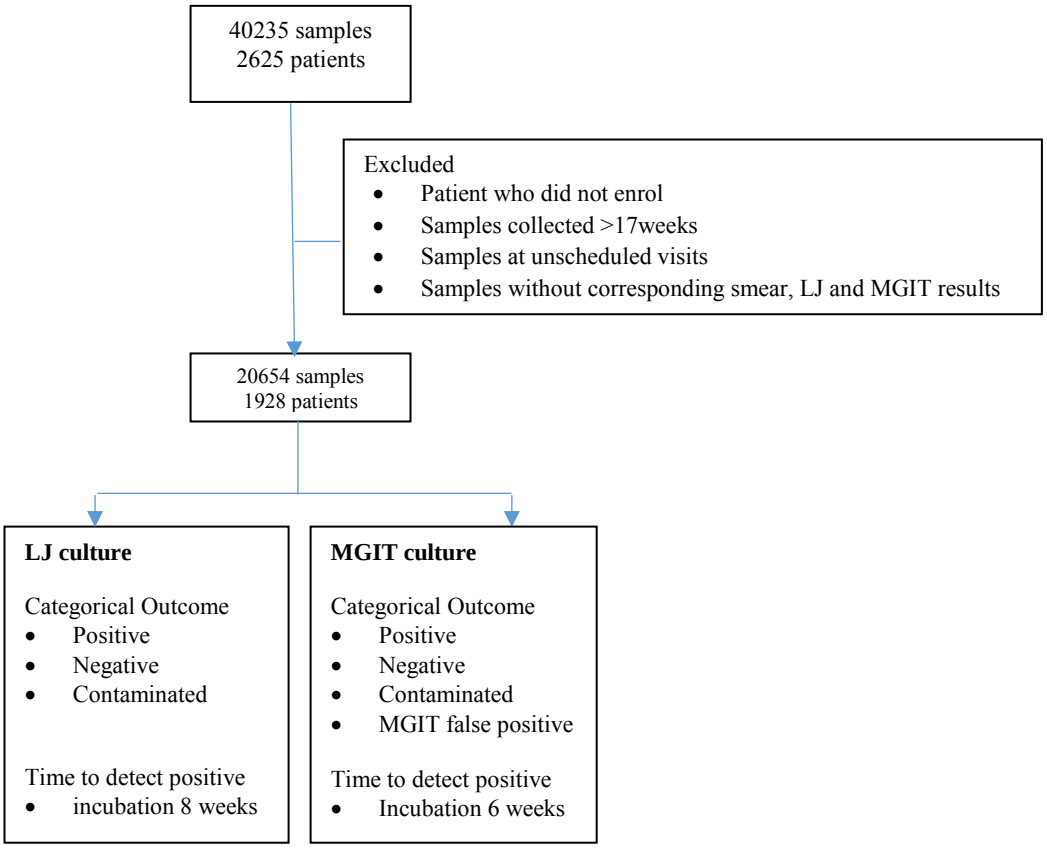
The time to detect a positive culture in both media was recorded in the REMoxTB study database and are considered here as continuous quantitative measures of mycobacterial load. Histograms describing the time to positivity have been created. Using different cut-offs, sensitivity and specificity of MGIT to discriminate LJ culture results and the maximum positive yield of results, that is positive by either or both culture methods, were calculated. Receiver operating curves have been created and the area under the receiver operating curve used to determine the strength of the measure to discriminate positive and negative results.

Data extracted from the main trial database were stored in Excel. Statistical analyses were carried out in Microsoft Excel and GraphPad PRISM. Statistical significance was determined by a $p<0.05$.

7.4 Results

Patient demographics were as described in table 5.1. A flow chart of samples included for this study are given in Figure 7.1.

Figure 7.1 Flow chart of samples



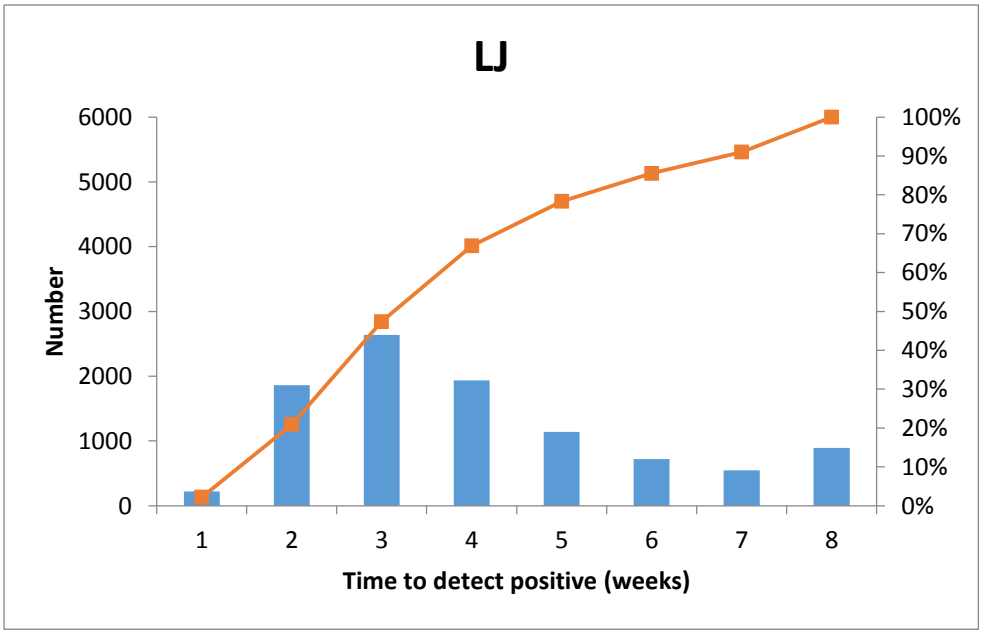
7.4.1 Varying MGIT TTP as discriminator between LJ negative and positive

The median TTP for MGIT is 11.33 days [IQR 6.63-16.46] and 28 days [IQR 21-5] for LJ. Histograms of time to detect positives in LJ and MGIT are showing in figure 7.2a and 7.2b. In MGIT culture, 90% samples have TTP less than 21 days in MGIT compared to 47% of LJ cultures with a TTD less than 21 days. An incubation period of 7 weeks is required before 90% of LJ culture positives are

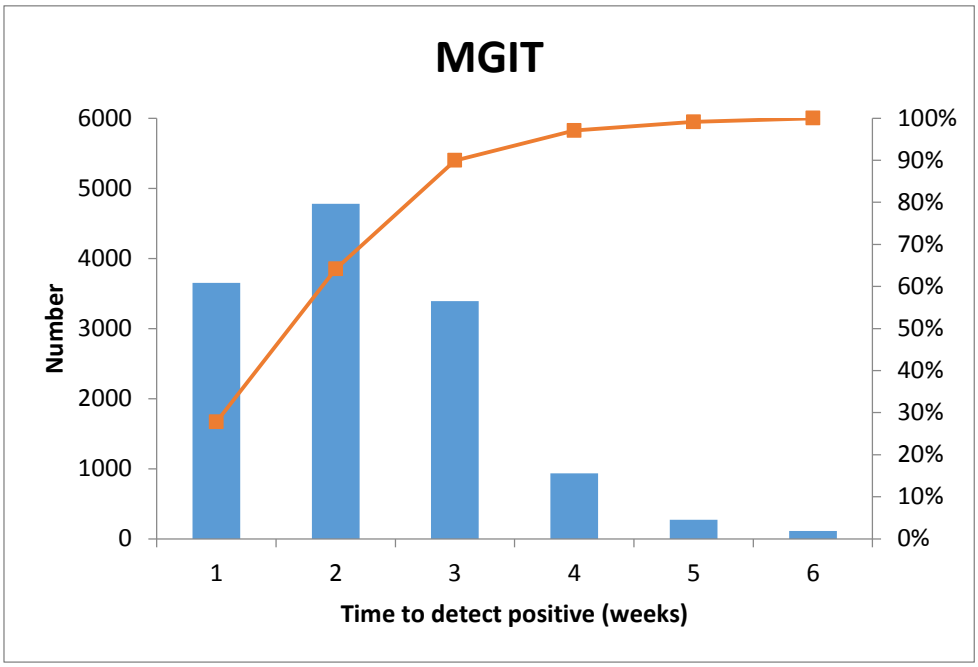
detected. Overall, less than 10% of MGIT culture positives were detected after 3 weeks incubation and less than 2% after 4 weeks.

Figure 7.2 Histogram of time to detect (TTD) a positive result in a. LJ and b.MGIT culture

a.



b.



In order to assess the optimal cut-off for MGIT TTP, receiver operating curves (ROC) were generated; the area under the ROC (AUROC) was 0.8813 for MGIT to discriminate between LJ negatives and positives over all time-points combined (figure 7.3). The sensitivity and specificity for a cut-off at 4 weeks was 97% and 65% respectively. Table 7.1 shows the sensitivity and specificity of varying MGIT cut-off times to detect LJ culture results. Given the clinical need to identify all patients with TB, maintaining high levels of sensitivity is more crucial than specificity.

Figure 7.3 ROC curves and AUC for MGIT TTP to discriminate positive LJ culture including all samples collected at all time-points

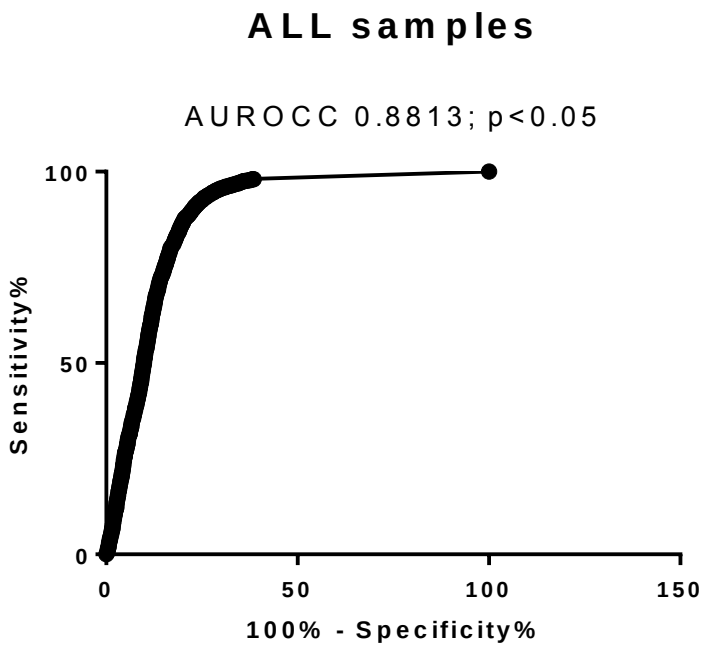


Table 7.1 Sensitivity and specificity for weekly cut-off MGIT TTP for LJ culture results comparing all samples at all time-points combined

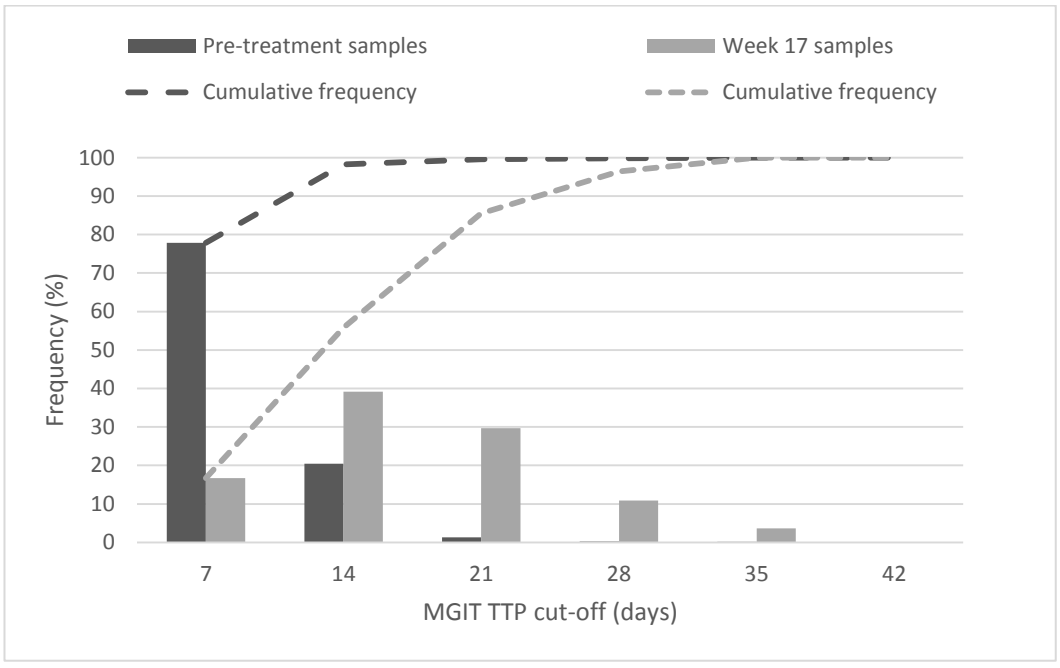
Cut-off			**LJ result**		**Sensitivity**	**Specificity**
			Pos	**Neg**		
7 days / <168 hrs	**MGIT result**	**Pos**	2714	426	30	94
		Neg	6316	7066		
14 days / <336 hrs	**MGIT result**	**Pos**	6387	1035	71	87
		Neg	2643	6457		
21 days / <504 hrs	**MGIT result**	**Pos**	8498	2033	94	73
		Neg	532	5459		
28 days / <672 hrs	**MGIT result**	**Pos**	8772	2617	97	65
		Neg	258	4875		
35 days / <840 hrs	**MGIT result**	**Pos**	8827	2782	98	63
		Neg	203	4710		
42 days / <1008 hrs	**MGIT result**	**Pos**	8857	2888	98	61
		Neg	173	4604		

Looking at individual study visit time-points, the AUROC changed over time as did the sensitivity using different cut-offs (table 7.2). Pre-treatment, the AUROC was lower but acceptable at 0.6458 ($p<0.05$) with a sensitivity of 98% using a 14 days cut-off. As time on treatment increased, the AUROC improved and the sensitivity for all cut-offs fell. At weeks 8 and 17, using a 28 day cut-off, the sensitivity was 89% and 81% respectively (table 7.2). A histograms of MGIT TTP for pre-treatment diagnostic samples and week 17 samples are shown in figure 7.4 for comparison. Appendix 6 contains the individual ROC graphs for all time-points.

Table 7.2 Area under ROC (AUROC) of MGIT TTP to discriminate LJ negative and positive and sensitivities of MGIT for LJ at selected visits using MGIT TTP cut-offs of 14, 21, 28 and 42 days, as per study visit

Study visit	AUROC	Sensitivity of 14-day cut-off	Sensitivity of 21-day cut-off	Sensitivity of 28-day cut-off	Sensitivity of 42-day cut-off
Pre-treatment	0.6458	98	99	99	99
Week 2	0.7218	75	96	98	99
Week 4	0.7896	45	92	97	98
Week 8	0.8343	22	72	89	95
Week 12	0.8077	56	73	73	73
Week 17	0.8698	60	77	81	81

Figure 7.4 Histogram of the distribution of MGIT TTP for pre-treatment and week 17 samples



	7 days	14 days	21 days	28 days	35 days	42 days
Pretreatment	2483	652	42	8	4	1
Week 17	200	469	356	130	43	0

7.4.2 Varying MGIT TTP to determine maximum positive yield

The AUROC is higher at 0.99 if we use the MGIT TTP to discriminate results determined by the maximum positive yield, i.e. a positive result is declared if positive in either or both media, as expected given that MGIT culture contribute large numbers of positives during treatment which are not detected in LJ. The overall sensitivity is lower using earlier cut-offs than when discriminating LJ negative and positive, as shown in table 7.3, but results are comparable using the 4 week cut-off.

Table 7.3 Sensitivity and specificity for weekly cut-off MGIT TTP for negative and positives results using maximum positive yield comparing all samples

Cut-off			**LJ result**		**Sensitivity**	**Specificity**
			Pos	**Neg**		
7 days / <168 hrs	**MGIT result**	**Pos**	3140	0	27	100
		Neg	8778	4604		
14 days / <336 hrs	**MGIT result**	**Pos**	7422	0	63	100
		Neg	4496	4604		
21 days / <504 hrs	**MGIT result**	**Pos**	10531	0	88	100
		Neg	1387	4604		
28 days / <672 hrs	**MGIT result**	**Pos**	11389	0	96	100
		Neg	529	4604		
35 days / <840 hrs	**MGIT result**	**Pos**	11641	0	98	100
		Neg	277	4604		
42 days / <1008 hrs	**MGIT result**	**Pos**	11745	0	99	100
		Neg	173	4604		

For pre-treatment samples, the AUROC was also 0.99 with sensitivity at 98% using a 14-day cut-off (table 7.4). The AUROC remained high at all time-points. Appendix 7 contains the invidual ROC graphs for all time-points for MPY. Sensitivity fell as time on treatment increased and, employing a 28 day cut-off, by week 8 and 17, had fallen to 87% and 90% respectively. The numbers of samples which are MGIT negative and MPY positives using 14, 21, 28 and 42 days cut-offs are shown in table 7.4 and represent the positives results which would be lost if MGIT culture replaces LJ culture. For pre-treatment samples, employing a 14 days cut-off, 2% of results positive by MPY would be missed by MGIT alone, but, at week 8, 34% of positive results would be missed. A 28 day cut-off at weeks 8 and 17 would miss 6% and 1% of results positive by MPY if using MGIT culture only.

Table 7.4 Samples negative on MGIT culture but positive by MPY using 14, 21, 28 and 42 day cut-offs as study visit/time on treatment

Study visit	N	MGIT negative / MPY positive n (%)			
		14-day cut off	21 day cut off	28 day cut off	42 day cut off
Pre-treatment	3190	77 (2)	34 (1)	27 (1)	21 (1)
Week 2	1461	383 (26)	66 (5)	33 (2)	20 (1)
Week 4	1378	680 (49)	147 (11)	46 (3)	16 (1)
Week 8	1267	430 (34)	215 (17)	73 (6)	10 (1)
Week 12	1239	134 (11)	76 (6)	47 (4)	12 (1)
Week 17	1199	71 (6)	29 (2)	14 (1)	9 (1)

7.5 Discussion

This study shows that prolonging MGIT incubation beyond 28 days does not improve sensitivity which is greater than 95% for all samples collected at all time-points. This varies considerably depending on time on treatment. For pre-treatment samples, the duration of incubation of MGIT cultures may be reduced to 14 days without loss of sensitivity, which remains greater than 98%, and with minimal loss of positive results if using MGIT as sole culture methodology. Given the pressing clinical need to identify all patients with positive TB cultures, reducing MGIT incubation times will increase laboratory capacity and potentially remove barriers to implementing culture more widely. During treatment, at week 8, however, a significant number of MGIT negative LJ positive samples would be missed by reducing MGIT incubation from 42 to 28 days and the impact of these results on treatment monitoring requires further consideration.

Pre-treatment

Given current efforts to increase the confirmation of TB in patients with positive smears using culture are directed towards the implementation of liquid cultures for TB diagnosis and drug susceptibility testing, the sensitivity of variable MGIT incubation times to detect results in solid LJ culture are crucial to these analyses. The inflexion point for sensitivity of the ROC curve must remain high, even if this is at the expense of specificity, to ensure no positive TB diagnosis is missed.

For pre-treatment samples, using an incubation time of 14 days, sensitivity to predict LJ culture results was 98%. These results were further improved when

sensitivity and specificity were calculated against a gold standard of the maximum positive yield achieved from combining both culture methods, which is perhaps the more valid comparator given MGIT culture misses 3% of LJ positives (chapter 5). Using this gold standard, the AUROC was much greater reflecting greater strength in the MGIT TPP to differentiate positive and negative results as MGIT comprises a greater proportion of positive results.

These findings have significant clinical application. The majority of patients presenting with symptoms which may be attributable to TB and have samples submitted for analysis will be TB culture negative. In a large study multicentre international study conducted in four low and middle income countries in Africa and Asia, a quarter of patient presenting with a cough lasting longer than 2 weeks were TB culture positive on solid LJ or Ogawa medium (267). Culturing all such samples for 6 weeks places a considerable burden on diagnostic capacity. Moreover, given the increased sensitivity of the MGIT producing greater numbers of positives, this may be an even greater burden than at present, especially in resource-poor areas where staff and space may be limited. If all samples being processed were pre-treatment diagnostic samples, the incubation time for smear positive samples might feasibly be reduced to 14 days thereby increasing laboratory processing capacity by 200%.

At present, liquid culture is being scaled up in low and middle income countries on the advice of the WHO and supported by FIND. It remains, however, outside the reach of many national health budgets which may struggle to meet the increased costs, even at negotiated price (47). A quicker turnaround time may increase laboratory capacity and significantly impact on costs. Moreover,

clinically and operationally, laboratory testing nearer the patient has significant advantages which have been explored for molecular testing kits like Xpert (143). The most commonly used liquid culture system, the MGIT 960, is a large floor standing piece of equipment with 3 incubating drawers which is costly to purchase, requires continuous electricity supply and has a large capacity to process 8000 samples per year. It is, therefore, not likely to be feasibly implemented in the large numbers of smear microscopy laboratories where samples are being processed.

Laboratories with limited space and finances may look to the smaller table top MGIT 320 which works on the same principle as the MGIT 960 but at half its physical size and containing only one drawer. Benchtop installation is feasible. Implementing this smaller machinery may become feasible if capacity could be increased by the reduced turnaround time, although the cost of MGIT tubes and reagents would remain unchanged. Healthcare economic analyses would be required to consider all the costs involved and estimate the potential savings of reducing the incubation time.

There are also potential clinical benefits in reducing incubation times in guiding treatment decisions. TB is often considered in the differential diagnosis for a wide range of patients. Clinicians often start empirical TB therapy on the basis that the microbiological diagnosis cannot be excluded for 6 weeks. Once started, TB treatment may be less likely to be stopped for fear of under-treating a patient in whom the diagnosis remains elusive, and exposes patients to the hazards of antituberculous drugs which may be severe or fatal in rare cases. With shortened incubation times, clinicians may be reassured by having to wait

for a much shorter time for culture results in cases where immediate treatment is not indicated. Moreover patients treated presumptively for TB may have other diagnoses missed which may delay the treatment for those conditions. In this way a shorter incubation time may improve clinical decision making.

In terms of clinical trial design, reducing incubation times may inform the operational capacity of participating sites to recruit patients. Limited laboratory capacity is a recognised problem for clinical trials in resource limited settings where the scale up from routine diagnostic laboratory to an accredited laboratory capable of delivering data for clinical trials to international regulatory standards is a considerable and costly challenge. There are limited finances available to fund TB research and the value of capacity development to improve the infrastructure and expertise in healthcare settings globally must be balanced against the urgent need for successfully conducted clinical trials of new TB drugs and regimens.

During treatment

During TB treatment, prolonged incubation times had considerably greater sensitivity than shorter cut-offs and these result do not support a uniform reduction in incubation time during treatment monitoring. Even with excellent AUROC scores for a gold standard of MGIT and LJ culture results combined, after 8 weeks of treatment, the sensitivity for positive in either media is significantly reduced from 98% using the current cut-off to 87% and 24% using 4 and 2 week cut-off respectively. Clearly reducing the cut-off would result in a potentially unacceptable loss of data, although the acceptable point of inflexion for sensitivity during treatment is unclear.

Using only MGIT culture and using the current 42 days cut off, 1% of samples which are positive using MGIT/LJ combined would be considered negative. Decreasing the cut-off to 28 or 14 days, 4% and 33% respectively of combined positive results would be considered negative. Such lost information is potentially concerning for monitoring patients during treatment.

Although the role of sputum culture monitoring during treatment is unclear, data on the dynamics of heterogeneous populations of mycobacteria is gaining currency as a factor influencing treatment outcomes and may explain, at least in part, the prolonged times to incubation at later treatment time-points. In fact, some studies have proposed longer incubation times. In a study comparing liquid and solid media for TB isolation, the maximum positive bacterial yield was not detected until 12 weeks incubation (349). By contrast, a separate study showed accelerated growth in liquid culture by addition of the supernatant from *M. tuberculosis* in early stationery phase growth, postulated to provide resuscitation-promoting factors (241). Further research into the ideal culture conditions for heterogeneous populations is required and may provide insights in mycobacterial responses to treatment (241, 361).

Limitations of this study

The major limitation of these data is that only smear positive patients were included in the REMoxTB study and as such these results are not generalisable to smear negative patients. In fact, it is perhaps most crucial to determine these results in this population as most patients are treated on the basis of a compatible clinical history and a positive smear. Smear negative TB suspects

present the greatest diagnostic challenge and reduced incubation times may reduce empirical treatment of this population.

The REMoxTB treatment allocation is a potential confounder as different mycobacterial populations may be supported by different culture methods; these populations may also be differentially affected by different drugs and thus treatment allocation may have an impact on these findings and it would be important to consider how incubation times may reflect these populations during treatment. Further consideration is required on unblinding of the study data.

7.6 Conclusion

The MGIT incubation time for pre-treatment smear positive sputum samples may be reduced to 14 days without loss of sensitivity. Given pre-treatment samples are the most abundant in TB programmes, a reduction in the required incubation time may significantly reduce turnaround times and increase laboratory capacity. During treatment, however, the MGIT incubation time cannot be reduced without significant reduction in sensitivity and with potential consequences for treatment monitoring. As only smear positive patients were included in the REMoxTB study, these results may not be generalisable to smear negative patients and further research is required.

Chapter 8 Thesis Conclusions

The REMoxTB study has provided a robust dataset on which to investigate the mycobacteriological responses to tuberculosis treatment. Such comprehensive pre-treatment and on-treatment mycobacteriological data on such a large number of patients is unprecedented in the history of TB research. Access to this dataset has allowed a thorough evaluation of a number of significant areas of uncertainty.

EMS vs spot

Early morning sputum samples have long been considered the optimal sample for the isolation of *Mycobacterium tuberculosis*. This practice has been based on studies conducted in the mid-twentieth century (256) and has been endorsed by the WHO until 2011 (279). An operational analysis of a large dataset concluded that the loss of patients in the diagnostic pathway outweighed the reduced sensitivity of collecting 2 sputum samples on a single day (267). Given the cost burden of TB diagnosis on patients, this change is expected to have considerable impact. Moreover, there are over 50 million smear samples performed annually for suspected TB, the majority of which are negative, and this change in the diagnostic algorithm can also be expected to significantly reduce the burden on limited healthcare resources.

From the REMoxTB data, we can show that contrary to a reduction in sensitivity, pre-treatment spot samples have a higher yield of positives and greater sensitivity for culture than EMS in both solid and liquid media.

The increase positive yield of pre-treatment spot over EMS sputum samples is marginal (98% vs 96%; $p<0.05$), but given the sheer volume of samples processed for diagnostics, this marginal difference may have operational impact. The same can be said for the slightly greater sensitivity of spot samples over EMS for culture in MGIT (99% vs 97%) and on LJ (98% vs 87%), perhaps of even greater importance given the increasing need to confirm mycobacterial identification and perform drug sensitivity testing.

During treatment, the positive yield and sensitivities were variable but overall higher in spot samples than EMS. The value of monitoring TB treatment using smear is not clear and treatment extensions on this basis are no longer included in the WHO guidelines (296), but smears are commonly used in this way in NTPs (201-203), especially those without access to culture. The variability between EMS and spot samples over time emphasizes the inherent limitation examining a single sample at a single time-point during treatment.

In terms of mycobacterial quantification, as measured by the time to detect a positive culture, spot samples overall had higher mycobacterial loads compared to EMS in both MGIT (9.2 vs 14.4 days; $p<0.001$) and LJ culture (21.2 vs 26.9 days; $p< 0.001$). Prior to treatment, however, EMS samples were positive 29 hours faster in MGIT than spot samples. Clinically of uncertain value, however, at the advent of quantifiable molecular diagnostics this difference may prove significant in future where this may also impact on treatment monitoring.

Interestingly, contamination was consistently higher in EMS than LJ samples. MGIT false positives were also higher in EMS. Together, contamination and

false positives contribute to a significant amount of lost data using EMS samples. These results further increase the number of visits a patient must attend before diagnosis and in clinical trials terms will need to factored into 'lost data' and impact on study power and sample size.

Smear and culture correlations

In the analyses in this thesis, the data presented shows that smear is an increasingly poor correlation for culture results during treatment. This is of potentially global significance; the majority of patients are managed without access to culture and NTPs rely on smear results collected at specific time-points to guide treatment decisions.

At week 8, the predictive probability of a positive smear to predict culture is moderate; 0.32 and 0.72 for LJ and MGIT respectively. At this time-point, a positive smear may lead to continuation of intensive phase treatment associated with the greatest risk of adverse events. Around of quarter of patients will have their treatment extended in this way on the basis of a positive smear when the culture is actually negative. Moreover, 10% of patients with a positive culture will not receive this extension, if indeed it is of value, on the basis of an initial smear negative.

After 4 months of treatment, the smear has no value in predicting the LJ (OR 1.15; 95% CI 0.13-2.16) or MGIT (OR 1.12; 95% CI -0.11-2.36) culture result. It is on the basis of the month 5 smear, however, that patients are deemed treatment failures and retreated with regimens including injectable agents

carrying a high risk of adverse events and increasing the burden on already limited resources.

Guidelines in this area are changing and the WHO no longer endorse treatment extension at week 8 (296). Operational change at NTP level takes a considerably greater time to implement and it is important to provide robust data on which to base changes to guidance as the strategy of treatment extension has proven beneficial is some studies (237, 295).

In terms of evaluating mycobacterial load, at each time-point, all our study results are ordered by smear grading; smear is a relatively well validated measure of mycobacterial quantification. The change in the relationship between smear and culture quantification is therefore not random. Rather this discovery contributes further evidence that cells identified on smear microscopy may fail to grow in culture. The presence of viable but non-culturable organisms may be reflective of heterogeneous populations of mycobacteria in patient sputum samples and presents a challenge for current mycobacteriology laboratories based upon culture methodologies. The changing nature of different mycobacterial populations during treatment, and the potential impact this may have on antituberculous chemotherapy, remains poorly understood but may prove crucial to future TB control efforts.

MGIT and LJ comparison

We have been able to confirm an increased yield of positive results in MGIT and the faster times to detection as expected. Around 35% of samples negative in LJ will be positive in MGIT. Despite this, current TB treatment effects cure in

>90% of patients, so what to make of this increased yield is not obvious. What must be considered is the operational impact this will have on sites already working with very constrained budgets. The potential to reduce the time samples are incubated in MGIT could certainly augment capacity at site laboratories to turnover a greater number of samples. Our data show that for diagnostic samples, by far the most abundant, this may be significantly reduced to 14 days with minimal loss of sensitivity.

The benefits of MGIT are plain. Of MGIT negatives, however, some 3% of those which would be positive on LJ will be lost by implementing a system of only fully automated MGIT cultures. Processing a single sample in each of the 8.6 million patients diagnosed with TB in 2012, this 3% equates to a misdiagnosis of 250,000 patients. Further research is required to explain samples positive in LJ and negative in MGIT, but the data show they are not due to age, gender or HIV status.

Comparisons of the MGIT and LJ mycobacterial loads support the idea that different culture media may better support different mycobacterial populations. As data on those samples positive on LJ but negative on MGIT will be instantly lost in laboratories converting to all liquid culture methodologies, it is important to consider what populations fail to be identified and whether this may be informative of bacteriological response and long term treatment outcome. Further characterisation of the dynamics of different mycobacterial populations during treatment is likely to improve our understanding TB disease and may identify better predictors of treatment response. Our intensive follow up has allowed for detailed analysis of continuous data but useful stratifications of

times to detect positive cultures (e.g. <2 weeks, 2-4 weeks etc) and times to culture conversion (conversion within 4 weeks of starting treatment etc) in future may allow for evaluation of these measure as they may be applied in routine practice outside of a clinical trial setting.

Measures of mycobacterial quantification and response to treatment have been considered as potential biomarkers of treatment outcome. While statistically significant correlations were found in solid and liquid media in these data, none of the chosen measures correlated well enough for clinical use. Furthermore, the between media variability of the measures was significant. The effect of these differences in patient is not known and any answers which may lie within these differences and the different populations which may be identified between the culture methods may be lost in efforts to streamline laboratory services and reduce turnaround times.

Cost benefit analyses

While not examined in this thesis, cost benefit analyses are crucial to provide evidence to make efficient use of limited laboratory resources in low and middle income countries where healthcare spending per capita may be as low as $12, in Eritrea, compared to $8362 in the US (362). The government may contribute as little as $2 per person and only ‘pay as you go’ healthcare may be available, unaffordable to the poorest patients at greatest risk of tuberculosis (5). Given that TB is high on the differential diagnosis of respiratory presentations in countries with high incidence of disease, costs of diagnostic tests form the majority of laboratory costs at present and impact all patients including those found to be TB negative.

Current laboratory services are largely based around the staffing and operation of laboratories for diagnostic smear microscopy where reagent costs are minimal. In this context, the additional use of culture adds considerable consumable and other costs. The costs of solid and liquid culture were evaluated in a study in South Africa found LJ culture as costing $12.35 with MGIT culture costing $16.62 (47). The data presented in this thesis suggest there may be additional value in using both methods to support different populations of mycobacteria, which increased costs to $19.29. A separate study evaluating the inclusion of molecular testing, including molecular DST which the WHO advises in settings with high rates of MDR TB, estimated costs of liquid culture using MGIT as $16.88 compared to $14.93 for Xpert which provided additiona data on rifampicin resistance (363).

Such costs are highly variable and are likely to be dependent on country-specific or regional costs of living given the staffing costs of implementing culture comprise two thirds of the cost of LJ culture and half the costs associated with MGIT culture (47). Furthermore, equipment and reagent costs have been negotiated for low and middle income countries, mainly by the FIND organisation, and are considerably higher in high income nations, e.g. Xpert cartridge costs in LMIC $9.98 compared to standard costing of $71.63 (363). Both studies comparing costs are based on the use of reference laboratories and do not include transport costs, which are also subject to considerable geographical variation. Moreover these studies do not consider the costs to patients and their families, which may be considerable (4, 263, 264).

Given the value of using culture in monitoring treatment is unknown, no studies have considered costs associated with microbiological follow up during treatment. This may be appropriate given on-treatment samples are likely to be dwarfed by costs of diagnostic samples. Should further research delineate a role for culture using solid and/or liquid media during treatment, perhaps assessing the change in proportions of different mycobacterial populations, these specific costs would need to be considered further. Moreover, positive cultures during treatment, vastly increased using MGIT culture as we have shown, may reasonably prompt first and second line DST in patients with poor clinical response suspected of drug resistance disease which would considerably impact costs of treatment monitoring.

Microbiological research concentrates mainly on scientific analyses and is often divorced from operational studies considering real-life implementation in LMIC (364). The WHO World Health Report 2013 recommended that 'all countries should become producers as well as consumers of research, and that research capacity should extend beyond academic centres to public health programs—close to the supply and demand for health services' (365). Such research may be less motivated by academic interests and more focused on providing solutions applicable to the setting in which they are conducted. Furthermore, studies such as these are more likely to result in capacity development relevant to specific requirements of public health programmes and therefore contribute to sustainable improvements in health.

Funding of operational studies including cost-benefit analyses are generally poorly funded and there may be limited expertise in conducting such studies in

countries which may enjoy the greatest benefit. The WHO/TDR global 'Structured Operational Research and Training Initiative' (SORT IT) programme aims to fund operational research in LMIC with The International Union Against TB and Lung Diseases funded to provide TB-specific research. So far, the initiative has produced operational research cost-effectively with the majority impacting on public health practice in the setting in which they were conducted (366). Importantly, continued involvement in operational research continued after the course programme providing evidence for long term capacity building (367).

The findings presented in this thesis are based on laboratory capacity provided by a large scale international REMoxTB clinical trial of new drugs, with involvement of academic partners in developed countries and commercial research organisations. Laboratory capacity development was concentrated in smaller number of sites. It is unlikely the follow up and monitoring conducted in this study would be feasible for implementation in routine practice and further operational and cost-benefit analyses are required to ensure best use of limited resources in real-life settings to improve individual patient outcomes and public health.

Summary

This study provides further mycobacteriological data from TB patients prior to and during TB treatment. The data provide robust evidence for spot smears and against the use of smears as a proxy for culture to guide treatment decisions. Together with the comparison between MGIT and LJ, this thesis highlights our limited understanding of *Mycobacterium tuberculosis* and the changes induced by chemotherapy on the heterogeneous populations which may be present at different times during treatment. Outcome data for the REMoxTB study will provide some guidance on the potential biomarkers of clinical response, however, further work is required to fully understand the basic science of mycobacteriology. Through such understanding, we may be able to move forward to better targeted treatment and reduce the treatment duration to that of any other respiratory bacterial infection. Such a reduction would limit transmission and reduce morbidity and mortality. Operational research is required to ensure microbiological findings are feasible for implementation and relevant to public health policy in the countries most burdened by TB.

Chapter 9 Further research arising from this thesis

There are a number of areas of further research arising directly from the data presented in this thesis. All analyses will be repeated with the unblinded treatment allocation to identify any effect on these results. However, the results themselves support further research in several specific areas.

9.1 EMS/spot sputum samples

We have shown that EMS are not superior to spot samples for TB, in fact, overall the opposite is true. Pre-treatment diagnostic samples are most important clinically and operationally and the spot and EMS were comparable in this regard. However, as a requisite of screening for entry into the REMoxTB study, all patients had a least one positive smear at their local laboratory. These results cannot, therefore, be generalised to smear negative populations. As smear negative TB is more common in patients with HIV and immunosuppression who are at the highest risk of developing active TB, it will be important to ascertain what effect sample timing has on the yield of positive cultures, the sensitivity for culture using different media and the mycobacterial load recovered in patients sputum smear negative for AFB on microscopy.

Differences in mycobacterial load may have further impact on the sensitivity and specificity of molecular testing methods which have a limit of detection. Sample timing has not been a feature of these studies to date. It is therefore unclear whether the collection of EMS or spot samples would impact on the validity of molecular methods, like Gene Xpert, to detect organisms of the *Mycobacterium*

tuberculosis complex or of the identification of markers of genotypic drug resistance.

Our data analyse samples submitted only up to week 17 of treatment thus we are also unable to comment on the effect sample timing may have on the sensitivity of samples collected after this time period or post treatment. Identifying treatment failures and relapses are important aspects of patient management and public health aspects of TB control. Furthermore, it is not clear whether the use of EMS or spot samples would prove beneficial in using smear as a predictor of clinical outcomes. It is also not clear whether sample timing may impact on biomarker research outcomes. To further investigate the value in predicting longer terms outcomes, I plan to perform the analyses described using the entire REMoxTB database and consider how this may affect measures of treatment outcome.

Given the impact of awaiting EMS in terms of healthcare economics and the catastrophic financial impact on work-age patients, their families and productivity in the wider society, it is important to clarify these further areas of research to allow already limited resources to be put to best use.

9.2 The value of smear in predicting culture results

The most pressing research arising from this data is whether there is any role for NTPs to advise treatment decisions based on smear microscopy. Additionally, given the much shorter time to a positive culture in MGIT, it might be clinically beneficial to await culture results before making any alterations to patient treatment.

To this end, further analyses will be conducted on the REMoxTB data to consider whether smear microscopy has any value in predicting treatment outcomes, independently of any effect on predictions of culture results. Given the potential ability of smear to identify viable but non-culturable organisms, there is plausibility in these analyses to clarify the role of smear and/or culture conversion in monitoring treatment responses and predicting TB outcomes.

9.3 Comparing LJ and MGIT results during treatment and reducing incubation times

Having access to the unblinded study data and long-term treatment outcomes, I plan to investigate whether LJ or MGIT culture offers any advantage in predicting clinical responses to treatment. This may inform ongoing biomarker studies applicable to both patient management and clinical trials. Additionally, these analyses will inform the clinical and operational debate as to whether it is appropriate to replace LJ with MGIT or whether they might provide complimentary data.

Data presented supporting the reduction of MGIT incubation times, at least for diagnostic samples require repeating for smear negative patients. This will have important implications for HIV positive patients more likely to have smear negative disease, although in other analyses of the REMoxTB data not presented in this thesis, the mycobacterial loads were comparable n HIV infected and uninfected patients and thus the effect would be expected to be similar.

Papers describing the obvious benefits of MGIT are well publicised but a recent paper in Nature has described methods to further optimise TB culture (361). In this study, a new media was employed, samples were incubated in microaerophilic conditions, supplemented with ascorbic acid and growth was detected using autofluorescence. Primary culture and rifampicin susceptibility were available in 72 hours. Such investigations signal a resurgence in culture-based research, including that describing the faster time to detect a positive culture by supplementing MGIT culture with the supernatant of a positive control in log phase growth (241). Whilst such strategies have not been assessed in an operational capacity for the programmatic management of TB, they do present interesting data encouraging further research. Robust data will be required to support any changes to the current laboratory procedures, particularly given the limited resources available and the largely successful treatment available, at least for drug sensitive TB.

9.4 Predicting treatment response using baseline mycobacteriology

Whilst the data presented did show a positive relationship between mycobacterial load and treatment responses, these were insufficient to be useful in clinical practice. Other studies of baseline mycobacterial load using culture and molecular methods have shown a more positive association with treatment responses and clinical outcome so further research is required to explore these conflicting results (196, 238, 330).

Ongoing analyses of the REMoxTB data is currently underway to identify biomarkers of treatment response applicable to both clinical practice. Additionally, crucial investigations of biomarkers to inform go-no-go decisions in

clinical trials of new drugs are being conducted given the experimental arms were found to be not non-inferior to standard treatment (246), despite supporting data from phase II trials (239, 249, 250). The costs of clinical trials in TB are immense and data which might identify failing and successful regimens during clinical trials would be enormous benefit, allowing for prioritisation of drug combinations most likely to succeed.

9.5 Basic mycobacteriology

Interesting data from these analyses and others provides support for my further research into heterogeneous mycobacterial populations present in patient samples and their variability during treatment.

Initial challenges will require accurate visualisation and quantification of different populations of organisms, which have so far been somewhat problematic. Combined auramine-nile red staining of sputum smears to identify lipid body positive cells has significant operational limitations. There is crossover of excitation and emission spectra which can interfere with slide interpretation. Furthermore, reductions in fluorescence on repeated viewing reduces the reproducibility of the results, a problem in common with collaborators in resource limited settings. Non-uniformity of the dispersal of sample on slides presents additional challenges in automated processing of digital images, which, at volumes required to generate clinically meaningful data can create data storage issues.

Exploring different methods of identifying these different populations of *M. tuberculosis* is crucial. Recent success in doing so has been achieved in Prof

Gillespie's group at University of St Andrews by using Raman spectroscopy. In unpublished data, the team have been able to differentiate lipid body positive cells from those organisms rapidly dividing. Preliminary studies have generated interesting data challenging my initial understanding of the field and which generate hypotheses I hope to explore further on my return to full time research in 2015.

Ultimately I am interested in identifying different populations of mycobacteria present in patient samples in order to separate them and investigate their dynamics in liquid and solid culture, gene expression profiles and metabolomics and proteomics. I would also plan to assess the response of these different populations to standard and experimental new antituberculous drugs individually and in combinations and explore notions of drug sensitivity, resistance and tolerance.

Studies of the efficacy of different drugs to control different mycobacterial populations may inform EBA studies which may be improved by considering not only reductions in the viable mycobacteria grown using current standard and future novel culture methodologies, but by also considering the impact of drugs on mycobacterial population structure. Changes in proportions of different populations during treatment may be useful in predicting long term treatment outcomes.

9.6 The bigger picture

Tuberculosis has existed since antiquity and researchers in the field are therefore challenged with impacting on the most successful human pathogen in history. Translating research findings into tangible reductions in morbidity and mortality is a global priority, as are reductions in drug resistance, and to my mind requires a re-examination of the microbiological factors associated with latency, dormancy, persistence and reactivation.

Appendices

Appendix 1 REMoxTB Patient information sheet and informed consent forms

Appendix 2 REMoxTB inclusion and exclusion criteria

Appendix 3 REMoxTB laboratory case report forms (CRFs)

Appendix 4 Procedure for confirming positive cultures as organisms of the *Mycobacterium tuberculosis* complex Accuprobe *Mycobacterium tuberculosis* culture complex identification test

Appendix 5 MGIT TTP and LJ TTD correlations and linear regression lines at each time point during TB treatment

Appendix 6 ROC curves and AUROC for MGIT TTP to discriminate positive and negative LJ culture on samples collected at each timepoint during TB treatment

Appendix 7 ROC curves and AUC for MGIT TTP to discriminate positive and negative results determined by maximum positive yield where a positive is declared when positive in either or both media, as per time on study treatment

Appendix 8 Publications and presentations arising from this thesis

1. Gillespie SH, Crook AM, McHugh TD, Mendel CM, Meredith SK, Murray SR, Pappas F, Phillips PP, Nunn AJ; REMoxTB Consortium (including Murphy ME). Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. N Engl J Med. 2014 Oct 23;371(17):1577-87

2. Murphy ME, Bongard E, McHugh TD, Gillespie SH. Comparing early morning vs. spot sputum samples for the identification of Mycobacterium tuberculosis. Oral presentation, Federation of Infection Societies, Edinburgh 2010

3. Murphy ME, Phillips PP, Honeyborne I, Bateson A, Brown M, McHugh TD, Gillespie SH. Poor correlation of smear microscopy for TB culture on solid and in liquid media during TB treatment. Poster discussion; 41st Union World Lung Conference, International Union of Tuberculosis and Lung Disease, Berlin 2010

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Appendix 1 REMoxTB Patient information sheet and informed consent forms

To be printed on local headed note paper

INFORMATION FOR PATIENTS

Study Title: Controlled comparison of two moxifloxacin containing treatment shortening regimens in pulmonary tuberculosis

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Sir, Madam,

Thank you for taking time to read this information. You are being invited to take part in a research study. Before you decide, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Feel free to ask us if there is anything that is not clear or if you would like more information. You are completely free to choose whether or not you wish to take part. Please take your time to decide.

What is the Purpose of the Study?
The purpose of this study is to compare the effect of two new combinations of drugs for tuberculosis (TB) with a standard treatment combination. The new combinations contain three out of the four drugs that are normally used for the treatment of tuberculosis as well as a new drug, moxifloxacin, which has been shown in other studies to be active against tuberculosis. Moxifloxacin has been in use throughout the world for treatment of other infectious diseases, and there is evidence from several studies that it can be used to treat tuberculosis.

Who can take part?
Patients over the age of 18 who have TB of the lungs and have not been treated for TB in the past can take part in this study. About 1500 patients from 4 different countries in Africa will be asked to take part in this study, and more countries may be added later.

Do I have to take part?
Whether or not you take part in the study is entirely up to you, and you do not have to give a reason if you don't want to be in it. If you decide not to take part you will receive standard TB treatment, which is very effective. If you decide to take part in the study, but change your mind later, you will be free to withdraw from the study at anytime without giving a reason. If you withdraw from the study it will not affect your medical care; you will be given standard TB treatment.

What does the study involve?
The study is in two parts: 1) the screening phase; 2) the treatment phase.

Part 1 – Screening Phase
The first part is called "screening" when we will explain the study to you to see whether you would be happy to be involved and find out whether you are eligible. If you agree to be screened, we will ask you to sign a consent form. You will be given this information sheet to keep and will receive a copy of the signed consent form.

We will ask you about your medical history and do blood and urine tests to see that there are no major abnormalities in your results that would prevent you entering the trial. This will include an HIV test with pre- and post-test counselling. If your HIV test is positive, you will have another blood test to measure your CD4 cell level (these are blood cells involved in fighting infections, which fall in HIV). The level of CD4 cells will help to decide when you should have HIV treatment. If you may need HIV treatment at the same time as TB treatment you will be referred to specialist doctors and you will not be eligible for this study.

The other blood tests will include a blood count to see if you are anaemic (have weak blood), and tests to check that your blood clots and that your kidneys and liver are functioning normally. The blood tests will require approximately 3 or 4 teaspoons of blood in total depending on whether you need a CD4 count.

The urine test will check for diabetes and urine infections. If you are female of childbearing age you will also have a urine pregnancy test.

If there are no major abnormalities and you are happy to proceed, we will invite you to join the main part of the study – the Treatment Phase.

Part 2 – Treatment Phase
If you are eligible for the TB treatment part of the study, we will discuss the study with you again at this stage so that you can choose whether or not you want to continue. If you wish to participate, you will be asked to sign a second consent form.

To find out the best way of treating patients, we need to compare the results of different treatments. To do this, people participating in the trial are divided into groups and each group is given a different treatment; the results are compared to see if one treatment is better. The best way of dividing people into groups to compare treatments fairly is to use what is called random allocation. The process is equivalent to flipping a coin or rolling a dice; however in this study we use a computer to determine which of the three treatments an individual is allocated to. Neither you nor your doctor will be able to choose which treatment you will receive. There are three different treatments being compared in this study, one standard treatment and two new

treatments. You will have an equal chance of receiving any one of the three; neither you nor the people looking after you will know which treatment you are given.

You will be given a general clinical examination and vision test and your blood pressure, pulse and temperature will be measured. You will also have a chest x-ray if this has not already been performed.

You will be given the TB treatment to which you have been allocated and will be seen regularly during treatment and for a year after finishing treatment to see that the TB is cured. You will be asked to come to the clinic weekly for the first 8 weeks of treatment and then monthly until the end of treatment (at month 6) to see the doctor/clinical officer/nurse for the study. He/she will check your clinical condition and check that the treatment is working and monitor side effects from it. After the end of treatment, you will be asked to come to the clinic every 3 months for 1 year, in order to check your clinical condition and to see if you have any signs of the TB coming back. If you are not cured after taking the study treatment or you relapse during follow-up, you will be given the treatment your doctor thinks will be best for you.

After you are first entered into the trial, a member of the study team will come to your home with you, so that they know where you live. If for some reason you do not attend one of the follow-up clinics, they will then be able to visit you in your home, to see that you are well, and arrange further visits.

Samples of your sputum will be taken weekly during the first 8 weeks of the study, monthly to the end of treatment, and then at 3, 6, 9 and 12 months after finishing treatment. These specimens will be tested in the laboratory to see if they contain TB bacteria. Samples of your blood will be taken before you start treatment, and at weeks 2, 4 and 8 and at months 3 and 4 to check that the treatment is not having any adverse effects. Approximately 3 teaspoons of blood will be collected on each occasion.

What are the treatments that are being tested?

There are three different treatments being compared in this study: the standard treatment and two new treatment combinations. The active components of each treatment regimen are as follows:

Treatment A: The standard treatment comprising 2 months of four anti-tuberculosis drugs rifampicin, pyrazinamide, isoniazid and ethambutol followed by 4 months of rifampicin and isoniazid. This is the best treatment combination currently known.

Treatment B: A new treatment that includes the new drug moxifloxacin together with three of the standard drugs, rifampicin, pyrazinamide and isoniazid for 2 months followed by 2 months of moxifloxacin, rifampicin and isoniazid.

Treatment C: A new treatment that includes the new drug moxifloxacin together with three of the standard drugs, rifampicin, pyrazinamide and ethambutol for 2 months followed by 2 months of moxifloxacin and rifampicin.

To compare the treatments fairly, it is important that so far as possible the medical staff working on the study do not know which of the treatments patients are on in case this knowledge influences the care they provide or their judgment of how effective the treatment is. However, if your doctor needs to know what treatment you are on for your medical care, they will be able to

find out from the study organisers. Because each of the treatments is made up of different drugs, each regimen will also include 'placebo' tablets, to make the regimens appear as similar as possible. A placebo is a tablet that is similar in appearance to the real treatment but does not contain any drugs.

All patients in the study will take five different types of tablet by mouth for the first two months, three for the second two months and two for the last two months. The tablets that are additional to the drugs in the combination to which you have been allocated, will be placebos.

What do I have to do?

If you decide to take part, you will agree to take the medication as instructed and to attend the clinic for the follow-up visits, although you may withdraw at any time. This means attending all 18 scheduled visits over the next 18 months. If any adverse side effects appear during the study, the study doctor will prescribe appropriate treatment for them. You are advised not to take any other drugs (including vitamins, herbal medicines and over the counter treatments) except those prescribed to you by the study doctor, as they may lead to dangerous reactions or may interfere with the study. If you need any other treatment, you should inform the doctor at the clinic. You should not currently be involved in any other drug trial or have been involved in one in the previous 3 months.

For women of child bearing potential

Pregnant women cannot be included in this study because the effects of the new drug on babies that are still in the womb are not completely known. It is possible that it could be dangerous to an unborn child. Women of childbearing age must have a pregnancy test before they can be included in the study and they must be using an effective method of barrier contraception, such as a condom. If a woman who is participating in the study falls pregnant she must inform her doctor immediately. She will be withdrawn from the study treatment and given standard TB therapy (which is known to be safe in pregnancy) but will be seen in the study clinic until she delivers.

What are the possible disadvantages and risks of taking part in the study?

Current treatment is 6 months and if the full course of treatment is completed, it is very rare for TB to recur. It is not known whether this will also be true for a 4 month course of treatment. It is possible that patients receiving the shorter treatment combinations (only 4 months of active drugs) may be slightly less likely to be cured of the TB or to have TB again later. Patients in the study will be followed for a year after the end of treatment to look for this, and will receive a further course of standard treatment if the TB recurs.

All TB treatment may be associated with side effects. You may or may not suffer from some of these effects. Common side effects of the current TB treatments are as follows: nausea, vomiting, diarrhoea, skin rashes, a flu like illness, drowsiness, headache, confusion, inflammation and damage to the liver, inflammation of the kidneys, orange discolouration of the urine and tears, joint pains, signs of nerve disturbances, such as pins and needles or vision disturbances. We will give you an extra vitamin tablet that should prevent the nerve disturbances. You will also have eye tests at the beginning of treatment and after 8 weeks to check your vision. You should let the study team know if you notice any change in your eyesight.

Moxifloxacin, the new drug, is generally well tolerated and has been used widely in Europe, North America and South Africa. The most common side effects that can occur include nausea, diarrhoea, headache, dizziness and inflammation of the tendons in your legs. You will be asked if you experience any of these at each appointment. In some people, moxifloxacin may produce a small effect on the heart rhythm. Disturbances of blood sugar, liver function or kidney function might also occur in a few cases and this will be looked for in blood tests.

In the event that you experience any of these or other adverse reactions to the study drugs during the course of this study, you should immediately contact the doctor in charge of the study so that you can be tested for them.

What are the possible benefits of taking part?
There will be no direct benefits to you from this study, but the information that will be obtained from this study will help future TB patients by showing us how to treat patients with your disease in a better way.

What if new information becomes available?
Sometimes during the course of a research project, new information becomes available about the drug that is being studied. If this happens, your study doctor/clinical officer/nurse will tell you about it and discuss with you whether you want to continue in the study. If you decide to withdraw, your study doctor will make arrangements for your care to continue. If you decide to continue in the study you may be asked to sign an updated consent form. New information may also lead your study doctor to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue.

What if something goes wrong?
In the event that something goes wrong and you are harmed as a result of participating in the study, you would be eligible for compensation. Treatment for adverse effects of study treatments will be provided free of charge. If you are harmed due to someone's negligence then you may have grounds for a legal action for compensation against that person but you may have to pay your legal costs.

Will my information be kept confidential?
All personal and medical information collected for this study will be treated as strictly confidential. If you consent to take part in the study, the staff working on the study and the regulatory authorities would be able to see your medical records but only to ensure that the study is being carried out correctly. Details about you that are stored on a computer will be identified by a number and your initials but will not include your name.

Will any samples I give be stored?
The sputum and blood samples that you give will be used to diagnose and monitor your TB on treatment. In addition we would like to have your permission to store your sputum and blood samples taken during the study and the TB bacteria isolated from the sputum to undertake new diagnostic tests as they become available in the future. Genetic tests may be performed on the TB bacterium isolated from your sputum. No genetic tests related to you will be performed on your samples.

What will happen to the results of the research study?
The results of this study will be made available to national and international drug regulatory agencies to help them determine whether the new treatments are useful for tuberculosis. In addition, the results will be published in international medical journals so that doctors and other health workers can learn from this work.

Who is organising and funding this study?
University College London is the sponsor of this study – their role is to oversee that the work is performed to international standards of research and ethics. The money to run the study is provided by the European Developing Country Clinical Trials Partnership, and the Global Alliance for Tuberculosis Drug Development (who promote the discovery and development of new drugs for the treatment of tuberculosis). Some of the drugs used in this study are provided free of charge by Bayer AG, an international pharmaceutical company who developed moxifloxacin, and by Sanofi-Aventis, a drug company, who have supplied the rifampicin drug and placebo.

Who has reviewed the study?
This study has been reviewed and approved by University College London Ethics Committee, in London; the Ethics Committee of insert hospital name, the insert national Ethics body name as well as the insert regulatory authority name. The study has also been reviewed and approved by the ethics committees of the hospitals in the other African studies involved in the trial (South Africa, Zambia, Tanzania and Kenya).

Whom to contact for further information:
Insert local contact name and address.

Thank you very much for having considered participating in this study.

To be printed on local headed note paper

SCREENING CONSENT FORM

Patient Screening Number:

Study Title: Controlled comparison of two moxifloxacin containing treatment shortening regimens in pulmonary tuberculosis

Chief Investigator Prof Stephen Gillespie
Local Principal Investigator

1. I confirm that I have read and understand the information sheet, dated .....(version .....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that participation in the trial will require me to have an HIV test, the result of this will be kept confidential and will not be given to me without my permission. I have been given a chance to think it over and to ask questions. ☐

4. I understand that sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from University College London (the sponsor of the study) and responsible individuals authorised by UCL, from regulatory authorities or from the Hospital, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I agree to be screened to take part in the above study. ☐

All 5 boxes must be initialled or marked for the consent to be valid

_____	_____	_____
Name of Patient	Date	Signature/thumb print
_____	_____	_____
Name of Witness (if appropriate)	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

When completed 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

To be printed on local headed note paper

TREATMENT CONSENT FORM

Patient Screening Number:
Patient Study Number:

Study Title:
Controlled comparison of two moxifloxacin containing treatment shortening regimens in pulmonary tuberculosis

Chief Investigator Prof Stephen Gillespie
Local Principal Investigator

1. I confirm that I have read and understand the information sheet, dated .....(version .....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from University College London (the sponsor of the study) and responsible individuals authorised by UCL, from regulatory authorities or from the Hospital, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

All 4 boxes must be initialled or marked for the consent to be valid

_____	_____	_____
Name of Patient	Date	Signature/thumb print
_____	_____	_____
Name of Witness (if appropriate)	Date	Signature
_____	_____	_____
Name of Person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

When completed 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 2 REMoxTB inclusion and exclusion criteria

Inclusion Criteria:

- Signed written consent or witnessed oral consent in the case of illiteracy, before undertaking any trial related activity. (appendix)
- Two sputum specimens positive for tubercle bacilli on smear microscopy at least one of which must be processed and positive at the study laboratory.
- Aged 18 years or over.
- No previous anti-tuberculosis chemotherapy.
- A firm home address that is readily accessible for visiting and willingness to inform the study team of any change of address during the treatment and follow-up period.
- Agreement to participate in the study and to give a sample of blood for HIV testing (see appendices 1 & 2).
- Pre-menopausal women must be using a barrier form of contraception or be surgically sterilised or have an IUCD in place.
- Laboratory parameters performed up to 14 days before enrolment.
    - Serum aspartate transaminase (AST) and alanine transaminase (ALT) activity less than 3 times the upper limit of normal.
    - Serum total bilirubin level less than 2.5 times upper limit of normal. Creatinine clearance (CrCl) level greater than 30 mls/min.
    - Haemoglobin level of at least 7.0 g/dL.

    - Platelet count of at least 50x109cells/L.
    - Serum potassium greater than 3.5 mmol/L.
- Negative pregnancy test (women of childbearing potential).

2.1.3.2 Exclusion Criteria:

- Unable to take oral medication.
- Previously enrolled in this study.
- Received any investigational drug in the past 3 months.
- Received an antibiotic active against M. tuberculosis in the last 14 days (fluoroquinolones, macrolides, standard anti-tuberculosis drugs).
- Any condition that may prove fatal during the first two months of the study period.
- TB meningitis or other forms of severe tuberculosis with high risk of a poor outcome
- Pre-existing non-tuberculosis disease e.g. diabetes, liver or kidney disease, blood disorders,peripheral neuritis, chronic diarrhoeal disease in which the current clinical condition of the patient is likely to prejudice the response to, or assessment of treatment.
- Pregnant or breast feeding.
- Suffering from a condition likely to lead to uncooperative behaviour e.g. psychiatric illness or alcoholism.

- Contraindications to any medications in the study regimens.
- Known to have congenital or sporadic syndromes of QTc prolongation or receiving concomitant medication reported to increase the QTc interval (e.g. amiodarone, sotalol, disopyramide, quinidine, procainamide, terfenadine).
- Known allergy to any fluoroquinolone antibiotic or history of tendinopathy associated with quinolones.
- Patients already receiving anti-retroviral therapy.
- Patients whose initial isolate is shown to be multiple drug resistant (i.e. resistant to rifampicin and isoniazid) or monoresistant to rifampicin, or resistant to any fluoroquinolone). (see section 5.9.2)
- Weight less than 35kg
- HIV infection with CD4 count less than 250 cells/µL.
- End stage liver failure (class Child-Pugh C).

LABORATORY DOCKET

ESSENTIAL LABORATORY FORMS

Patient Number:

Country ID		Site ID		Randomization Number				

SITE NUMBER

PATIENT NUMBER

Patient's Initials:

Sputum Smear Form

Essential Lab Forms

Visit:_____

REMoxTB

Patient's Initials | | |

Patient Number: Country ID | Site ID | Randomization Number | | | | | | |

SITE NUMBER

PATIENT NUMBER

1. SPUTUM SMEAR

1.1 Sputum collection: Sputum collected ☐ No sputum produced ☐

Date of collection: D D M M M 2 0 Y Y Time(24 hr clock): H H : M M

Laboratory accession number *Attach label*

Type of Sputum: Early Morning ☐ Other ☐

1.2 Laboratory receipt:

Date of receipt: D D M M M 2 0 Y Y Time(24 hr clock): H H : M M

1.3 Specimen volume:

Estimated volume (mL): <2 ☐ 2-<5 ☐ 5-<10 ☐ 10-15 ☐ >15 ☐

1.4 Date processed: D D M M M 2 0 Y Y Time(24 hr clock): H H : M M

1.5 Date of microscopy: D D M M M 2 0 Y Y

1.6 Ziehl-Neelsen smear:

Smear result: No AFB seen ☐ + ☐ ++ ☐ +++ ☐ ++++ ☐ Not done ☐

If not done, give reason _____

Form completed by: _____

_____ D D M M M 2 0 Y Y

Signature

QC completed by: _____

_____ D D M M M 2 0 Y Y

Signature

MGIT Culture Form

Essential Lab Forms
Visit:_____

REMoxTB

Patient's Initials | | | |

Patient Number: Country ID | Site ID | Randomization Number
SITE NUMBER
PATIENT NUMBER

2. MGIT

2.1 Laboratory accession number *Attach label*

2.2 Was the sputum specimen retreated? Y N

2.3 Date started (MGIT inoculated) D D M M M 2 0 Y Y

2.4 MGIT tube number _____

2.5 Date positive D D M M M 2 0 Y Y Time to positivity ___ days : ___ hours

OR

2.6 MGIT result negative (if MGIT has not flagged positive at 42 days the result is negative) ☐

3. MYCOBACTERIAL CONFIRMATION (IF MGIT IS POSITIVE)

3.1 Blood agar culture - conclusion

3.1.1 Date of reading D D M M M 2 0 Y Y

3.1.2 Result: Positive (contaminated) ☐ Negative (MGIT result valid) ☐ Other ☐

If other, describe _____

_____

3.2 Ziehl-Neelsen smear AFB: Positive ☐ Negative ☐ Not done ☐

If not done, give reason: _____

Date of test D D M M M 2 0 Y Y

Form completed by: _____

_____ D D M M M 2 0 Y Y
Signature

QC completed by: _____

_____ D D M M M 2 0 Y Y
Signature

LJ Culture Form

Essential Lab Forms

Visit:_____

REMoxTB

Patient's Initials | | | |

Patient Number: Country ID | Site ID | Randomization Number

SITE NUMBER

PATIENT NUMBER

4. LJ SLOPE CULTURE

4.1 Laboratory accession number *Attach label*

4.2 Was the sputum specimen re-treated? Y N

4.3 Inoculation date D D M M M 2 0 Y Y

4.4 Result date D D M M M 2 0 Y Y

Positive

Week growth seen	Amount of Growth (complete one column only)				
	Number of Colonies	+	++	+++	Contaminated
		☐	☐	☐	☐

OR

Negative ☐ (Specimen considered negative if **no** growth seen 8 weeks after inoculation date)

Other ☐ Describe_____

_____

5. MYCOBACTERIAL CONFIRMATION (IF LJ CULTURE IS POSITIVE)

5.1 Ziehl-Neelsen smear AFB: Positive ☐ Negative ☐ Not done ☐

If not done, give reason:_____

Result date D D M M M 2 0 Y Y

Form completed by: _____

_____ D D M M M 2 0 Y Y

Signature

QC completed by: _____

_____ D D M M M 2 0 Y Y

Signature

MGIT Susceptibility Form

Essential Lab Forms
Visit:_____

REMoxTB

Patient's Initials | | | |

Patient Number: Country ID | Site ID | Randomization Number
SITE NUMBER
PATIENT NUMBER

7. MYCOBACTERIAL SUSCEPTIBILITY TESTING

7.1 SIRE set

Date started D D M M M 2 0 Y Y

Laboratory accession number *Attach label*

	Sensitive	Resistant	Contaminated	Other	If 'other' describe
Streptomycin	☐	☐	☐	☐	
Isoniazid	☐	☐	☐	☐	
Rifampicin	☐	☐	☐	☐	
Ethambutol	☐	☐	☐	☐	

7.2 Pyrazinamide set

Date started D D M M M 2 0 Y Y

Laboratory accession number if different from above *Attach label*

	Sensitive	Resistant	Contaminated	Other	If 'other' describe
Pyrazinamide	☐	☐	☐	☐	

7.3 Moxifloxacin set

Date started D D M M M 2 0 Y Y

Laboratory accession number if different from above *Attach label*

	Sensitive	Resistant	Contaminated	Other	If 'other' describe
Moxifloxacin	☐	☐	☐	☐	

Form completed by: _____

_____
Signature

D D M M M 2 0 Y Y

QC completed by: _____

_____
Signature

D D M M M 2 0 Y Y

Molecular Speciation Form

Essential Lab Forms
Visit:_____

Patient's Initials | | | |

Patient Number: Country ID | Site ID | Randomization Number | | | | | | | |
SITE NUMBER
PATIENT NUMBER

6. *M. TUBERCULOSIS* MOLECULAR SPECIATION

6.1 Laboratory accession number *Attach label*

6.2 *M. tuberculosis* complex confirmed Y N

Result date D D M M M 2 0 Y Y

Form completed by: _____

_____ D D M M M 2 0 Y Y
Signature

QC completed by: _____

_____ D D M M M 2 0 Y Y
Signature

Molecular Typing Form

Essential Lab Forms

REMoxTB

Patient's Initials | | | |

Patient Number: Country ID | Site ID | Randomization Number | | | | | | | |

SITE NUMBER

PATIENT NUMBER

8. DNA PREPARATION AND DISPATCH

8.1 Has a DNA isolate specimen been prepared and dispatched for molecular typing? Y N

If No, reason _____

9. MOLECULAR TYPING

9.1 Date and laboratory accession number of specimen

Initial specimen:

Laboratory accession number *Attach label*

Date of test: D D | M M M | 2 0 Y Y

Subsequent specimen (If applicable):

Laboratory accession number *Attach label*

Date of test: D D | M M M | 2 0 Y Y

9.2 Are the two strains indistinguishable? Y N

The form must be checked by a designated senior member of lab. to ensure it has been filled in correctly, who must then sign and date the form.

Form completed by: _____

_____ D D | M M M | 2 0 Y Y

Signature

QC completed by: _____

_____ D D | M M M | 2 0 Y Y

Signature

Appendix 4 Procedure for confirming positive cultures as organisms of the *Mycobacterium tuberculosis* complex Accuprobe *Mycobacterium tuberculosis* culture complex identification test

Preparing samples from positive cultures on LJ media

As soon as growth was visible on the LJ slope, or during 60 subsequent days of incubation, a small quantity of the isolate, without disrupting the media, was removed using a disposable plastic loop, a wire loop or a disposable plastic needle. These cells were added to a lysing reagent tube containing 100µL of lysis reagent and hybridisation buffer supplied with the test kit and mixed until resuspended. The lysing reagent tube was capped and briefly vortexed

Preparing samples from positive culture in liquid MGIT media

Growth in the MGIT with turbidity equal to or greater than McFarland 1 Nepholometer was suitable for testing. After mixing the MGIT tube and allowing the large clumps to settle, 100µL of the broth was added to a lysing reagent tube containing 100µL of hybridisation buffer supplied with the test kit. The lysing reagent tube was capped and briefly vortexed.

Sample lysis

The lysing reagent tubes were placed in the sonicator rack to submerge the reagents in water while ensuring the caps were kept above water. The sonicator rack was placed thus in the water bath sonicator taking care to ensure that the tubes did not

contact with the sides or bottom of the bath. The samples were sonicated for 15 minutes. After sonication, the lysing reagent tubes containing the sonicated organisms were placed in a heating bath or water block at 95^{o}C ± 5 ^{o}C for 10 minutes.

Hybridisation

Sufficient probe reagent tubes to were removed from a resealable pouch which had been stored at 2-8^{o}C, opened for the first time less than two months previously and which remained within the expiration date. To the tubes, 100µL of the sonicated lysed sample was added. The probe reagent tubes were recapped and incubated for 15 mins at 59.5-61 ^{o}C.

Selection

The probe reagents tubes were removed from the heating block or water bath and their caps removed. 300µL of selection reagent supplied with the test kit was added to the probe reagent tube and recapped. The tubes were vortexed until completely mixed and incubated for 10 mins at 59.5-61 ^{o}C. After incubation, the probe reagent tubes were removed from the wter bath or heat block and left at room temperature for 5 minutes.

Detection

Within 1 hour of selection, the results of the sample were read in the luminometer. After selecting the appropriate protocol on the software interface, the tubes, with

their caps were removed and wiped to remove any residue, were inserted into the luminometer as per the instrument instructions. After analysis is complete the tubes were removed and the results recorded.

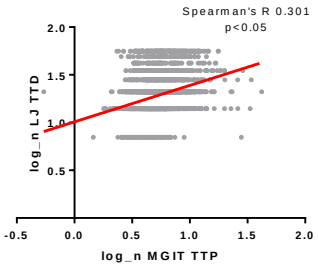
Interpretation of results

The results of the negative and positive control were checked to ensure they satisfied the cut-off values provided. Where the control samples failed to achieve the expected results, the sample values were considered invalid and the procedure repeated. Where the control samples satisfied the expected results, the study sample results were read. A sample result equal to or greater than the cut-off value was considered positive. Sample results less than the cut-off values were considered negative. This results falling within approximately one third lower than the cut-off value were repeated and re-interpreted as described.

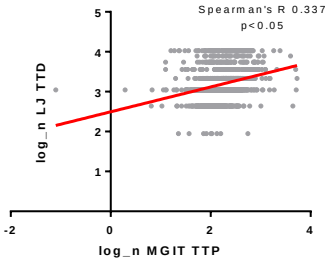
APPENDIX 5

Correlations of natural log of MGIT TTP and LJ TTD and linear regression lines at each timepoint during TB treatment

Pretreatment



Week 1



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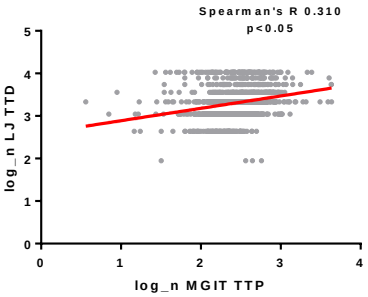
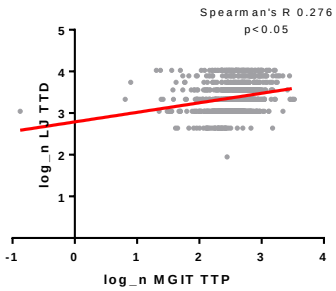
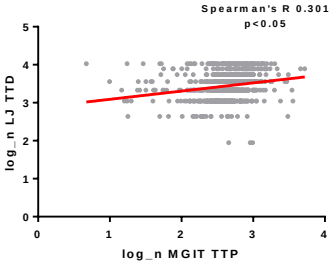


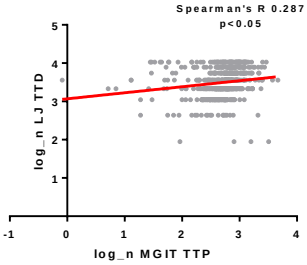
Week 3



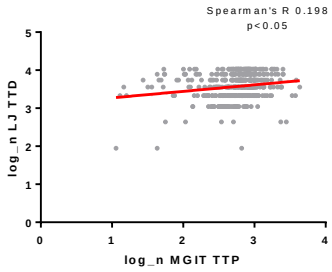
Week 4



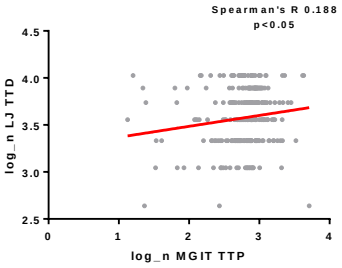
Week 5



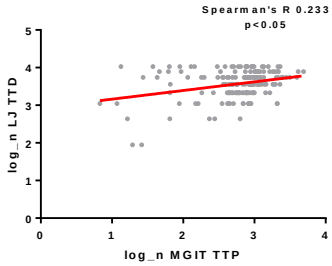
Week 6



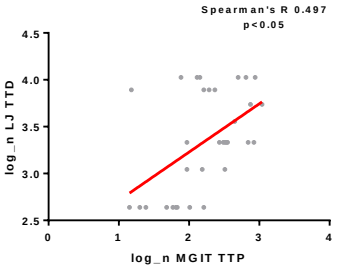
Week 7



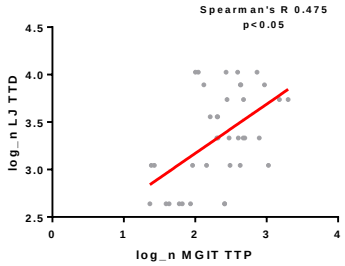
Week 8



Week 12

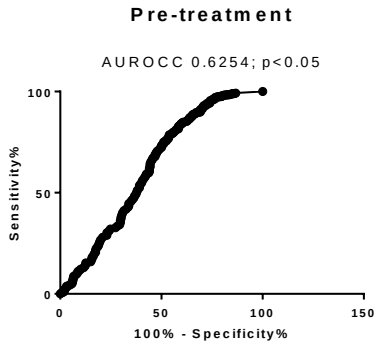


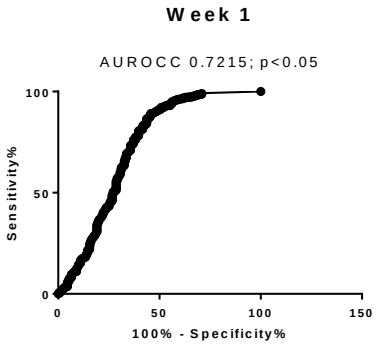
Week 17

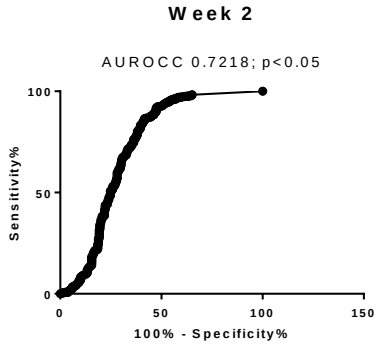


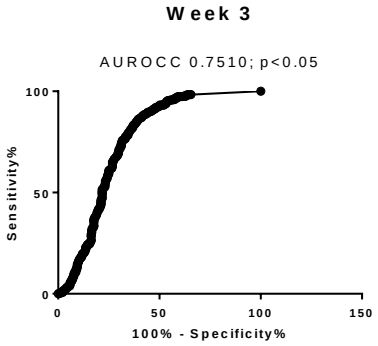
APPENDIX 6

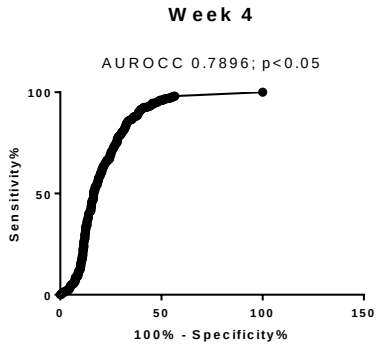
ROC curves and AUC for MGIT TTP to discriminate positive and negative LJ culture on samples collected at varying timepoints during treatment

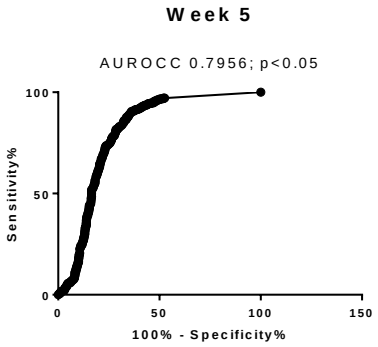


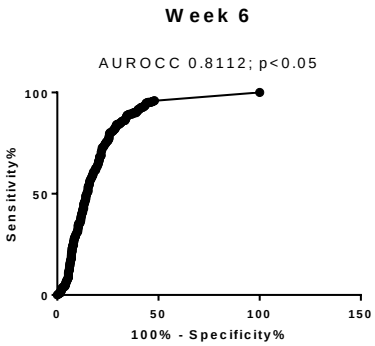


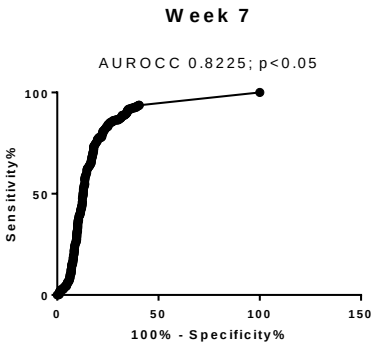


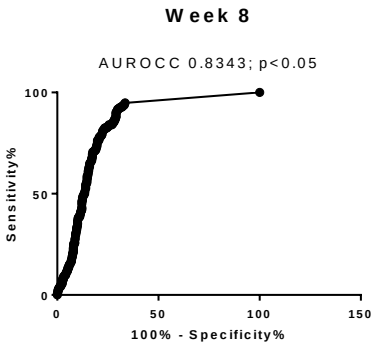


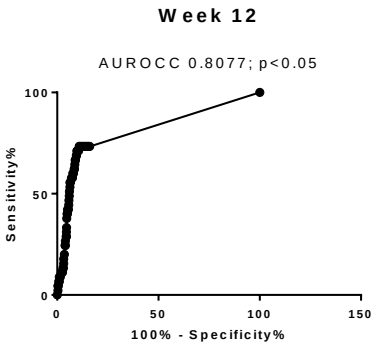


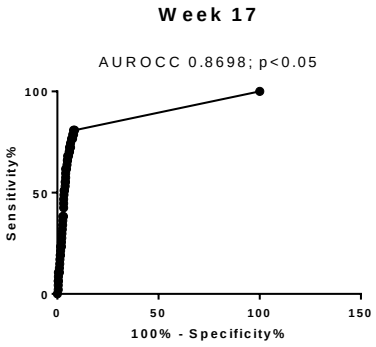










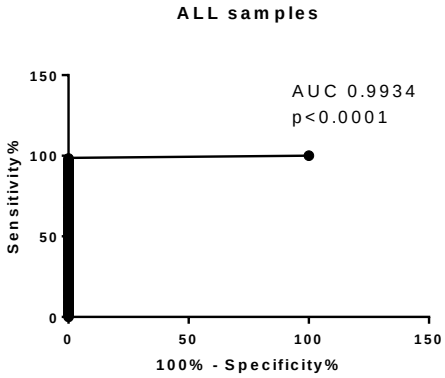


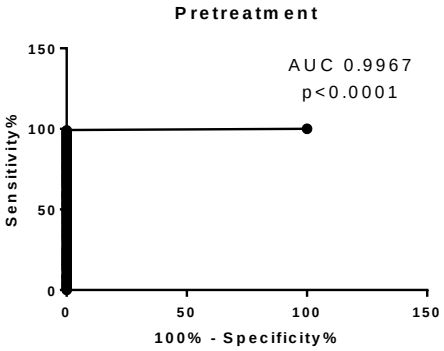
ROC receiver operating curve

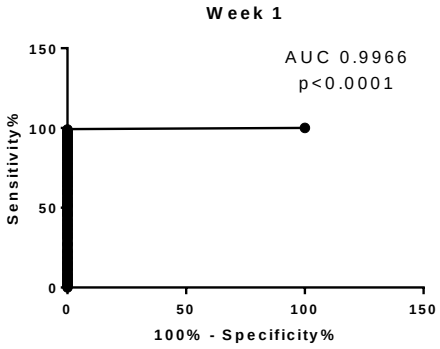
AUC area under receiver operating curve

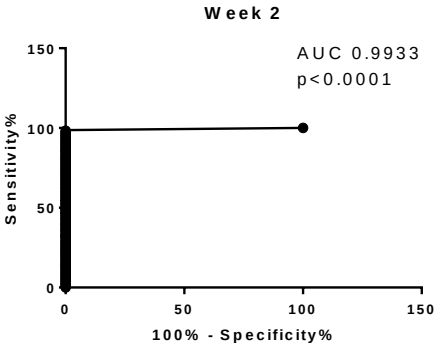
APPENDIX 7

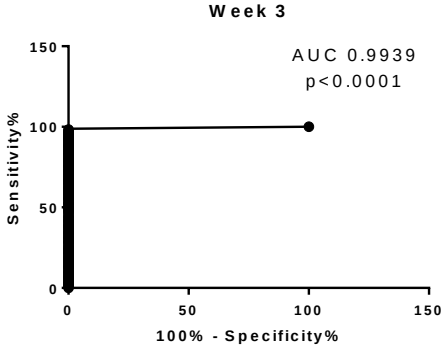
ROC curves and AUC for MGIT TTP to discriminate positive and negative results determined by maximum positive yield where a positive is declared when positive in either or both media, as per time on study treatment

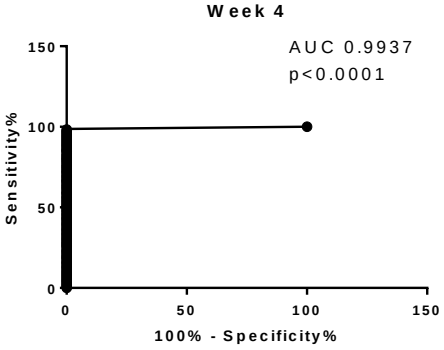


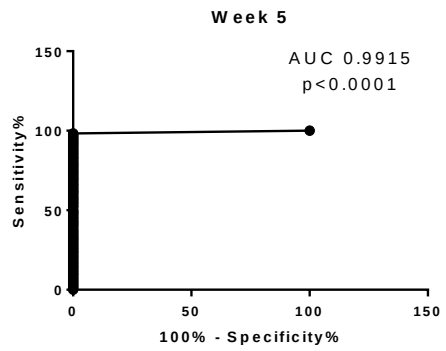


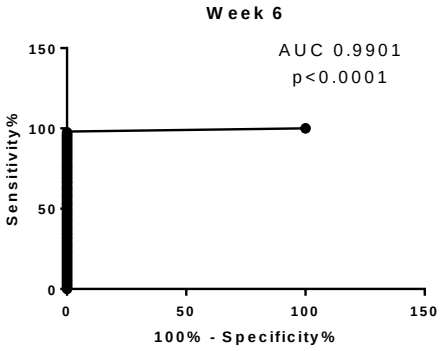


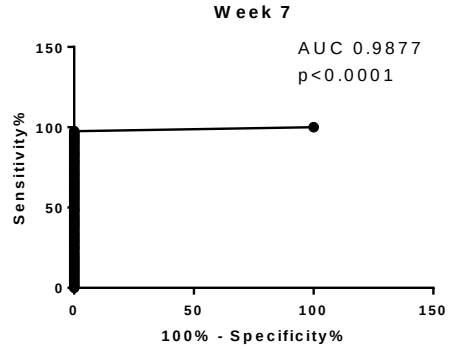


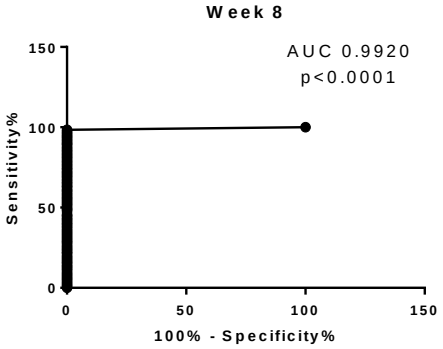


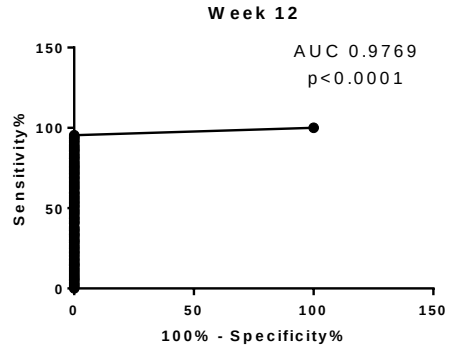


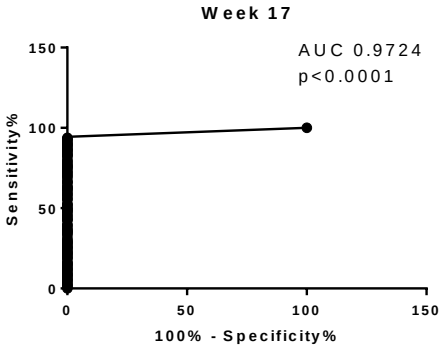












ROC receiver operating curve

AUC area under receiver operating curve

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Four-Month Moxifloxacin-Based Regimens for Drug-Sensitive Tuberculosis

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ABSTRACT

BACKGROUND

Early-phase and preclinical studies suggest that moxifloxacin-containing regimens could allow for effective 4-month treatment of uncomplicated, smear-positive pulmonary tuberculosis.

METHODS

We conducted a randomized, double-blind, placebo-controlled, phase 3 trial to test the noninferiority of two moxifloxacin-containing regimens as compared with a control regimen. One group of patients received isoniazid, rifampin, pyrazinamide, and ethambutol for 8 weeks, followed by 18 weeks of isoniazid and rifampin (control group). In the second group, we replaced ethambutol with moxifloxacin for 17 weeks, followed by 9 weeks of placebo (isoniazid group), and in the third group, we replaced isoniazid with moxifloxacin for 17 weeks, followed by 9 weeks of placebo (ethambutol group). The primary end point was treatment failure or relapse within 18 months after randomization.

RESULTS

Of the 1931 patients who underwent randomization, in the per-protocol analysis, a favorable outcome was reported in fewer patients in the isoniazid group (85%) and the ethambutol group (80%) than in the control group (92%), for a difference favoring the control group of 6.1 percentage points (97.5% confidence interval [CI], 1.7 to 10.5) versus the isoniazid group and 11.4 percentage points (97.5% CI, 6.7 to 16.1) versus the ethambutol group. Results were consistent in the modified intention-to-treat analysis and all sensitivity analyses. The hazard ratios for the time to culture negativity in both solid and liquid mediums for the isoniazid and ethambutol groups, as compared with the control group, ranged from 1.17 to 1.25, indicating a shorter duration, with the lower bounds of the 95% confidence intervals exceeding 1.00 in all cases. There was no significant difference in the incidence of grade 3 or 4 adverse events, with events reported in 127 patients (19%) in the isoniazid group, 111 (17%) in the ethambutol group, and 123 (19%) in the control group.

CONCLUSIONS

The two moxifloxacin-containing regimens produced a more rapid initial decline in bacterial load, as compared with the control group. However, noninferiority for these regimens was not shown, which indicates that shortening treatment to 4 months was not effective in this setting. (Funded by the Global Alliance for TB Drug Development and others; REMoxTB ClinicalTrials.gov number, NCT00864383.)

From the University of St. Andrews Medical School, St. Andrews (S.H.G.), and the Medical Research Council Clinical Trials Unit at University College London (A.M.C., S.K.M., P.P.J.P., A.J.N.) and the Division of Infection and Immunity, University College London (T.D.M.), London — both in the United Kingdom; and the TB Alliance, New York (C.M.M., S.R.M., F.P.). Address reprint requests to Dr. Gillespie at the School of Medicine, University of St. Andrews, North Haugh, St. Andrews KY16 9TF, United Kingdom, or at shg3@st-andrews.ac.uk.

*A complete list of investigators and committee members in the Rapid Evaluation of Moxifloxacin in Tuberculosis (REMoxTB) study is provided in the Supplementary Appendix, available at NEJM.org.

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A short-term tuberculosis treatment regimen could improve rates of adherence, reduce rates of adverse events, and lower costs. Fluoroquinolones have shown promising activity against mycobacteria[1] and are established as a critical component of the treatment of multidrug-resistant tuberculosis,[2,3] with later fluoroquinolones recognized as having a more potent effect. It has been proposed that these drugs may have a role in reducing the duration of tuberculosis treatment.[4]

Moxifloxacin has been approved for a range of indications globally.[5] It has favorable pharmacokinetics, a large volume of distribution, and penetration into epithelial-lining fluid and macrophages.[6-8] The activity of moxifloxacin in vitro against *Mycobacterium tuberculosis*, which has been confirmed in murine models[9] and in clinical monotherapy studies,[10,11] has raised the prospect that the drug could be used as part of an improved regimen.[1] Subsequent studies in mice showed that combination regimens that included moxifloxacin had greater bactericidal activity than standard treatment and could produce cure without relapse after a shorter treatment duration.[12,13]

When different fluoroquinolones were substituted for ethambutol in a clinical trial, the moxifloxacin-containing regimen produced the most rapid decline in bacterial load and in the proportion of patients with culture negativity at 8 weeks.[14] These findings were confirmed by investigators in Brazil.[15] In contrast, substituting moxifloxacin for isoniazid in an 8-week study resulted in a nonsignificant enhancement in bactericidal effect.[16]

On the basis of supportive evidence from phase 2 studies and the uncertain relationships between 8-week bacteriologic data and the duration of effective therapy, we designed the Rapid Evaluation of Moxifloxacin in Tuberculosis (REMoxTB) study to determine whether the replacement of either isoniazid or ethambutol with moxifloxacin would provide effective tuberculosis treatment in 4 months, as compared with the standard 6-month regimen.

Methods

Study Design and Oversight

REMoxTB was a placebo-controlled, randomized, double-blind, phase 3 trial to test the noninferiority of two moxifloxacin-containing 4-month regimens, as compared with the standard 6-month regimen (Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). The full trial protocol and statistical analysis plan are also available at NEJM.org.

A trial steering committee with an independent chair supervised the conduct of the trial. An independent data and safety monitoring committee with access to unblinded data oversaw the safety of the study patients. The ethics committee at University College London and all national and local ethics committees approved the study. The Food and Drug Administration, the Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte), and the national regulatory authorities of the countries in which the trial was conducted reviewed and approved the protocol.

Bayer Healthcare donated moxifloxacin, and Sanofi donated rifampin. Neither company had any role in the study design, data accrual, data analysis, or manuscript preparation. Representatives of Bayer Healthcare reviewed the manuscript but did not suggest revisions. All the authors vouch for the completeness and accuracy of the data and analyses presented.

Study Patients

Patients were adults (≥18 years of age) who had newly diagnosed, previously untreated *M. tuberculosis* infection, as determined by positive results on sputum smears on two occasions, with culture-confirmed susceptibility to rifampin and fluoroquinolones. Patients who were coinfected with the human immunodeficiency virus (HIV) were eligible to participate in the study if the CD4+ count was at least 250 cells per cubic millimeter and they were not already receiving antiretroviral therapy. Detailed inclusion and exclusion criteria are provided in the Supplementary Appendix. All patients provided written or witnessed oral informed consent.

Randomization and Study Treatments

Randomization was performed with the use of lists with blocks of variable sizes that were stratified according to the patient weight group and study center. During randomization, patients were assigned a unique study number selected sequentially from the appropriate randomization list that corresponded to the treatment pack allocated. Eligible patients were assigned in a 1:1:1 ratio to one of the following daily regimens: a control regimen,

which consisted of isoniazid, rifampin, pyrazinamide, and ethambutol for 8 weeks, followed by 18 weeks of isoniazid and rifampin (control group); a regimen in which we replaced ethambutol with moxifloxacin for 17 weeks, followed by 9 weeks of placebo (isoniazid group); and a regimen in which we replaced isoniazid with moxifloxacin for 17 weeks, followed by 9 weeks of placebo (ethambutol group). Details about the regimens are provided in Figure S1 in the Supplementary Appendix.

In all three groups, drug doses were adjusted according to patient weight, as described in Table S2 in the Supplementary Appendix. Only statisticians who were responsible for preparing the reports for the independent data and safety monitoring committee and essential manufacturing and distribution staff members had access to the list of identifiers matched to the intervention.

STUDY PROCEDURES

After initial screening and baseline visits, patients were scheduled for eight weekly visits, which were followed by eight visits until 18 months after randomization (Fig. S1 in the Supplementary Appendix). All patients underwent a baseline clinical examination that included posteroanterior chest radiography, pregnancy testing if relevant, collection of two sputum specimens for microbiologic examination, physical examination, tests of visual acuity (Ishihara and Snellen), and urinalysis. Safety monitoring — which included testing of hepatic function (aspartate aminotransferase, alanine aminotransferase, and bilirubin), vitamin K, prothrombin time, partial thromboplastin time, blood count (hemoglobin and platelet count), urea, electrolytes, and creatinine — was performed at screening and at weeks 2, 8, 12, and 17, with additional liver-function testing at week 4.

Sputum was decontaminated with acetylcysteine–sodium hydroxide, examined microscopically, and cultured on Lowenstein–Jensen solid medium and in liquid medium in a Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson). All analyses were performed according to the REMoxTB laboratory and quality manuals (available on request). We performed mycobacterial speciation using the AccuProbe assay (GenProbe), and determined the susceptibility of strains to streptomycin, isoniazid, rifampin, and pyrazinamide using the MGIT manufacturer's instructions. We tested the susceptibility to moxifloxacin using a breakpoint of 0.125 mg per liter. In countries with a high rate of multidrug-resistant tuberculosis or quinolone resistance (>5%), initial sputum samples were tested for rifampin resistance with the use of the GenoType MTBDRplus assay and GenoType MTBDRsl assay, respectively (Hain Lifescience). We used 24-locus mycobacterial-interspersed-repetitive-unit (MIRU) analysis to compare the initial strains with the recurrence strains.[17]

STUDY OUTCOMES

The primary efficacy outcome was the proportion of patients who had bacteriologically or clinically defined failure or relapse within 18 months after randomization (a composite unfavorable outcome). Culture-negative status was defined as two negative-culture results at different visits without an intervening positive result. The date of culture-negative status was defined as the date of the first negative-culture result. This status continued until there were two positive cultures, without an intervening negative culture, or until there was a single positive culture that was not followed by two negative cultures. Relapse strains were those shown to be identical on 24-locus MIRU analysis.

The primary safety outcome was the proportion of patients with grade 3 or 4 adverse events that were graded according to a modified version of the toxicity criteria of the Division of AIDS of the National Institute of Allergy and Infectious Diseases.

STATISTICAL ANALYSIS

We determined that a sample size of 633 patients per group would provide a power of 85% to show noninferiority of the two moxifloxacin interventions to the control regimen with a margin of 6 percentage points, assuming a one-sided type I error of 0.0125 (Bonferroni correction). We estimated that 10% of the patients in each study group would have a unfavorable outcome and that 15% would have outcomes that could not be evaluated. (All definitions are provided in the Supplementary Appendix.) This margin of 6 percentage points reflected consultation with clinicians in high-burden countries and reanalysis of previous trials showing the effect of shortening treatment to 4 months without substituting a new drug.

Noninferiority was defined as a between-group

difference of less than 6 percentage points in the upper boundary of the two-sided 97.5% Wald confidence interval for the proportion of patients with an unfavorable outcome. We used a generalized linear model with identity-link function with adjustment for stratification variables (weight group and study center). We performed both modified intention-to-treat and per-protocol analyses, with the latter considered to be the primary analysis. In the modified intention-to-treat analysis, we excluded patients with resistance to moxifloxacin or rifampin at baseline and those in whom the outcome could not be assessed (e.g., patients who had reinfection). (Detailed definitions are provided in Section 2 in the Supplementary Appendix.) We also performed a number of sensitivity and secondary analyses of the primary outcome to test the robustness of the results (Tables S3A and S3B in the Supplementary Appendix).

We used the chi-square test to compare the patients' sputum-culture status at the end of 8 weeks (intensive phase) across treatment groups and the log-rank test to compare the time to culture-negative status. We used similar methods to analyze other secondary outcomes, including the time to an unfavorable outcome, the status at the end of treatment, the status at 12 and 18 months among patients with a favorable outcome at end of treatment, and the status at 18 months according to a blinded clinical review of the data.

All patients who received at least one dose of a study medication were included in the safety analysis. The proportions of patients who had at least one grade 3 or 4 adverse event were compared across treatment groups with the use of the chi-square test.

RESULTS

STUDY PATIENTS

A total of 2763 patients were screened and 1931 underwent randomization: 909 in South Africa, 376 in India, 212 in Tanzania, 136 in Kenya, 119 in Thailand, 69 in Malaysia, 66 in Zambia, 22 in China, and 22 in Mexico (Table S4 in the Supplementary Appendix). The principal reasons for ineligibility were a lack of confirmation of smear positivity in the study laboratory, a CD4+ count of less than 250 cells per cubic millimeter, or multidrug-resistant disease, as detected by means of the Hain test (Fig. 1). The demographic and clinical characteristics of the patients were similar in the three study groups (Table 1, and Tables S5 and S6 in the Supplementary Appendix).

The most common reason that patients were excluded from the modified intention-to-treat analysis was that they were found to be ineligible on the basis of data that were collected before randomization (e.g., lack of confirmation of the diagnosis of tuberculosis or confirmed multidrug-resistant tuberculosis). The most common reasons for exclusion from the per-protocol analysis were a change of treatment for reasons other than treatment failure and a loss to follow-up (Fig. 1). Of the 1931 patients who underwent randomization, 89% in the isoniazid group, 92% in the ethambutol group, and 89% in the control group met the requirements for treatment adherence, which was based on receipt of approximately 80% of the assigned regimen (see the Supplementary Appendix for details).

PRIMARY OUTCOME

In the per-protocol analysis, a favorable outcome was reported in 436 patients (85%) in the isoniazid group, as compared with 467 patients (92%) in the control group, for an adjusted absolute difference of 6.1 percentage points (97.5% confidence interval [CI], 1.7 to 10.5) favoring the control group (Table 2, and Fig. S2 in the Supplementary Appendix). A favorable outcome was reported in 419 patients (80%) in the ethambutol group, for an adjusted absolute difference of 11.4 percentage points (97.5% CI, 6.7 to 16.1), as compared with the control group.

In the modified intention-to-treat analysis, the corresponding values also favored the control group, with a favorable outcome reported in 436 patients (77%) in the isoniazid group, as compared with 468 (84%) in the control group, for an adjusted absolute difference of 7.8 percentage points (97.5% CI, 2.7 to 13.0), and in 419 patients (76%) in the ethambutol group, for an adjusted absolute difference of 9.0 percentage points (97.5% CI, 3.8 to 14.2) (Table 2, and Fig. S2 in the Supplementary Appendix). Results of all sensitivity analyses were consistent with those in the per-protocol and modified intention-to-treat analyses (Table S3A in the Supplementary Appendix).

The most common reason for an unfavorable outcome was relapse after conversion to culture-negative status after the end of active treatment (in 46 patients in the isoniazid group, 64 in the ethambutol group, and 12 patients in the control

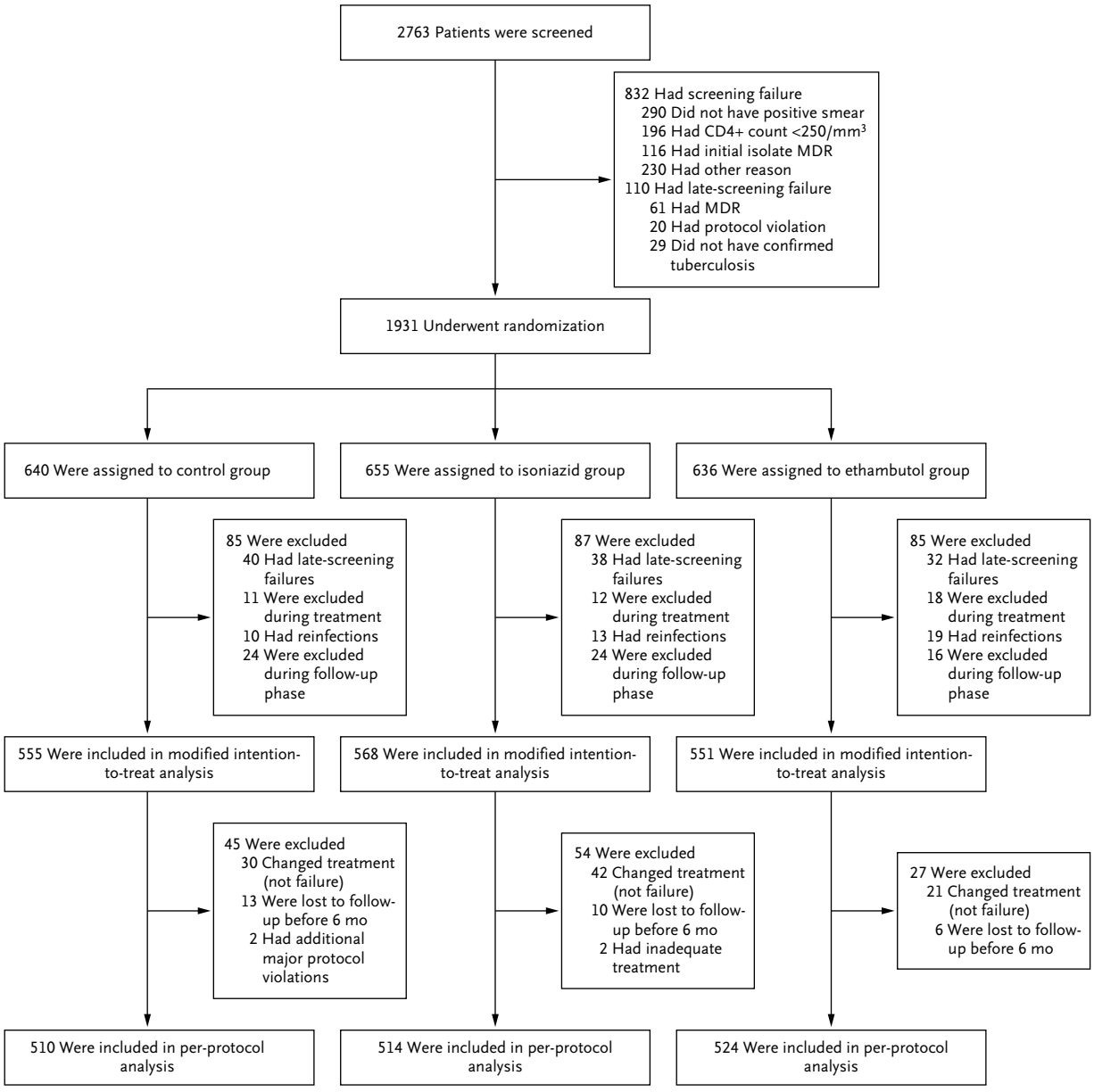


Figure 1. Enrollment and Outcomes.
MDR denotes multidrug resistance.

group). A similar pattern of results was seen in the modified intention-to-treat analysis (Table 2). There were no unequivocal cases of acquired resistance, but there were four cases of possible resistance — one in the ethambutol group (for moxifloxacin) and three in the control group (two for rifampin and one for isoniazid) — which require future whole-genome sequencing for interpretation.

SUBGROUP ANALYSES

There was no evidence that between-group differences in the primary outcome varied according to HIV status, region, recruitment site, age group, isoniazid susceptibility, or cavitation. The proportion of unfavorable outcomes among female patients, as compared with male patients, was similar in the three study groups (test of interaction,

Table 1. Baseline Characteristics of Patients in the Per-Protocol Population.*

Characteristic	Control Group (N=510)	Isoniazid Group (N=514)	Ethambutol Group (N=524)	All Patients (N=1548)
	number of patients (percent)			
Male sex	356 (70)	351 (68)	369 (70)	1076 (70)
Weight group†				
<40 kg	50 (10)	44 (9)	58 (11)	152 (10)
40–45 kg	80 (16)	90 (18)	82 (16)	252 (16)
>45–55 kg	206 (40)	210 (41)	204 (39)	620 (40)
>55–75 kg	161 (32)	158 (31)	174 (33)	493 (32)
>75 kg	13 (3)	12 (2)	6 (1)	31 (2)
Age group				
<25 yr	160 (31)	162 (32)	146 (28)	468 (30)
25–35 yr	145 (28)	162 (32)	175 (33)	482 (31)
>35 yr	205 (40)	190 (37)	203 (39)	598 (39)
Race or ethnic group‡				
Black	238 (47)	210 (41)	237 (45)	685 (44)
Asian	160 (31)	154 (30)	161 (31)	475 (31)
Mixed race	111 (22)	148 (29)	126 (24)	385 (25)
Other	1 (<1)	2 (<1)	0	3 (<1)
Smoking status				
Never	246 (48)	231 (45)	230 (44)	707 (46)
Past	119 (23)	111 (22)	134 (26)	364 (24)
Current	145 (28)	172 (33)	160 (31)	477 (31)
HIV positivity§	38 (7)	37 (7)	35 (7)	110 (7)
Drug resistance¶				
Isoniazid	29 (6)	34 (7)	39 (7)	102 (7)
Pyrazinamide	14 (3)	7 (1)	6 (1)	27 (2)
Cavitation‖	368 (72)	357 (69)	367 (70)	1092 (71)
Time to positivity on MGIT sputum culture				
≥5 days	266 (52)	263 (51)	258 (49)	787 (51)
<5 days	229 (45)	239 (46)	254 (48)	722 (47)
Not available	15 (3)	12 (2)	12 (2)	39 (3)

* There were no significant differences between the study groups. HIV denotes human immunodeficiency virus, and MGIT Mycobacteria Growth Indicator Tube.
† The median body-mass index (the weight in kilograms divided by the square of the height in meters) was 18.4 (range, 12.1 to 50.9) in the control group, 18.3 (range, 12.0 to 33.1) in the isoniazid group, 18.4 (range, 12.2 to 32.6) in the ethambutol group, and 18.3 (range, 12.0 to 50.9) for all patients.
‡ Race or ethnic group was reported by the investigator. Asian category included both South Asians and East Asians.
§ A single patient had missing HIV status.
¶ Resistance results were missing for isoniazid in 24 patients and for pyrazinamide in 27 patients.
‖ Cavitation status was missing for 148 patients.

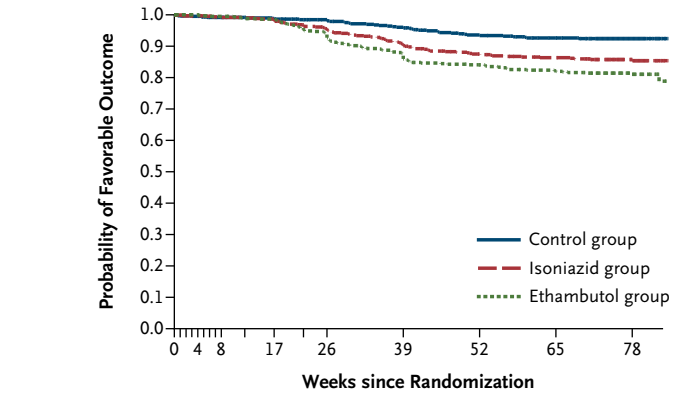
Table 2. Primary Efficacy Analysis in Per-Protocol and Modified Intention-to-Treat Populations.*

Variable	Per-Protocol Analysis				Modified Intention-to-Treat Analysis			
	Control Group (N=510)	Isoniazid Group (N=514)	Ethambutol Group (N=524)	All Patients (N=1548)	Control Group (N=555)	Isoniazid Group (N=568)	Ethambutol Group (N=551)	All Patients (N=1674)
Favorable outcome — no. (%)								
Patients with outcome	467 (92)	436 (85)	419 (80)	1322 (85)	468 (84)	436 (77)	419 (76)	1323 (79)
Culture-negative status at 18 mo	409 (80)	389 (76)	367 (70)	1165 (75)	410 (74)	389 (68)	367 (67)	1166 (70)
Unable to produce sputum	0	2 (<1)	0	2 (<1)	0	2 (<1)	0	2 (<1)
Unable to produce sputum at 18 mo but culture-negative status earlier	49 (10)	31 (6)	35 (7)	115 (7)	49 (9)	31 (5)	35 (6)	115 (7)
Missing data on L–J culture at 18 mo and MGIT negative	9 (2)	14 (3)	17 (3)	40 (3)	9 (2)	14 (2)	17 (3)	40 (2)
Unfavorable outcome — no. (%)†								
Patients with outcome	43 (8)	78 (15)	105 (20)	226 (15)	87 (16)	132 (23)	132 (24)	351 (21)
6-Mo treatment phase								
Nonviolent death	5 (1)	6 (1)	7 (1)	18 (1)	5 (1)	6 (1)	7 (1)	18 (1)
Treatment failure‡								
Culture-confirmed	3 (1)	4 (1)	1 (<1)	8 (1)	3 (1)	4 (1)	1 (<1)	8 (<1)
Not culture-confirmed	4 (1)	1 (<1)	4 (1)	9 (1)	4 (1)	1 (<1)	4 (1)	9 (1)
Adverse reaction	NA	NA	NA	NA	18 (3)	15 (3)	9 (2)	42 (3)
Withdrawal of consent	NA	NA	NA	NA	8 (1)	18 (3)	8 (1)	34 (2)
Relocation	NA	NA	NA	NA	2 (<1)	4 (1)	4 (1)	10 (1)
Other investigator decision	NA	NA	NA	NA	2 (<1)	5 (1)	0	7 (<1)
No completion of treatment	NA	NA	NA	NA	13 (2)	10 (2)	6 (1)	29 (2)
Follow-up								
Relapse after culture-negative status	12 (2)	46 (9)	64 (12)	122 (8)	13 (2)	46 (8)	64 (12)	123 (7)
Retreated for tuberculosis	14 (3)	17 (3)	27 (5)	58 (4)	14 (3)	18 (3)	27 (5)	59 (4)
Death from tuberculosis or respiratory distress	2 (<1)	0	0	2 (<1)	2 (<1)	0	0	2 (<1)
No culture-negative status								
Ever	1 (<1)	1 (<1)	0	2 (<1)	1 (<1)	2 (<1)	0	3 (<1)
At last visit	2 (<1)	3 (1)	2 (<1)	7 (<1)	2 (<1)	3 (1)	2 (<1)	7 (<1)
Adjusted difference from control in rate of unfavorable outcome — percentage points (97.5% CI)	NA	6.1 (1.7–10.5)	11.4 (6.7–16.1)	NA	NA	7.8 (2.7–13.0)	9.0 (3.8–14.2)	NA

* The treatment phase was defined as any time from randomization to 32 weeks after randomization (26 weeks plus 6-week window). L–J denotes Lowenstein–Jensen solid medium, and NA not applicable.

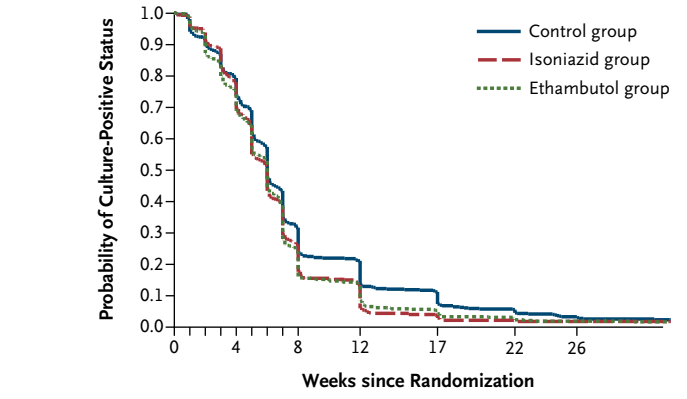
† During follow-up, the relapse and retreatment categories include patients during the scheduled end of active treatment (after month 4 for the moxifloxacin-containing groups and month 6 for the control group). In the per-protocol analysis, data from 24-locus mycobacterial-interspersed-repetitive-unit analysis were missing for 9 of 17 patients with treatment failure, 42 of 122 patients with relapse, and 38 of 58 patients who were retreated for tuberculosis.

‡ Listed are patients who were receiving active treatment in whom treatment failed.



No. at Risk

Control	600	563	533	493	472
Isoniazid	617	570	522	459	439
Ethambutol	604	568	523	445	425



No. at Risk

Control	600	465	183	122	64	19
Isoniazid	617	459	154	76	21	9
Ethambutol	604	449	141	79	30	9

Figure 2. Kaplan–Meier Estimates of the Time to an Unfavorable Outcome and Conversion to Culture-Negative Status.

Panel A shows that the time until patients had an unfavorable outcome was shorter in the isoniazid group than in the control group (hazard ratio, 1.25 [97.5% CI, 1.08 to 1.42]) and was further reduced in the ethambutol group (hazard ratio, 1.21 [97.5% CI, 1.05 to 1.37]). Panel B shows the time until conversion to culture-negative status, which occurred sooner in the isoniazid group and the ethambutol group than in the control group, according to analyses of sputum samples cultured in Lowenstein–Jensen solid medium. Patients who were excluded from the primary per-protocol analysis were included in this analysis, but data were censored at the time of exclusion from the per-protocol analysis.

P=0.004 for the isoniazid group and P=0.02 for the ethambutol group) (Table S3B in the Supplementary Appendix).

TIME TO CULTURE-NEGATIVE STATUS

In Kaplan–Meier analyses, patients in the isoniazid group and the ethambutol group had conversion to culture-negative status sooner than those in the control group in sputum analyses with the use of Lowenstein–Jensen solid medium (Fig. 2B) and MGIT medium (Fig. S3 and Table S7 in the Supplementary Appendix) (P<0.01 for both analyses). More patients receiving the moxifloxacin-containing regimens had culture-negative status at 8 weeks, but the difference was not significant (Table S8 in the Supplementary Appendix).

TIME TO AN UNFAVORABLE OUTCOME

In the per-protocol analyses, the time to an unfavorable outcome was shorter in the isoniazid group than in the control group (hazard ratio, 1.87; 97.5% CI, 1.07 to 2.67) and was further reduced in the ethambutol group (hazard ratio, 2.56; 97.5% CI, 1.51 to 3.60) (Fig. 2A, and Table S9 in the Supplementary Appendix).

ADVERSE EVENTS

There were no significant between-group differences in the incidence of grade 3 or 4 adverse events, with reports of events in 127 patients (19%) in the isoniazid group and 111 patients (17%) in the ethambutol group, as compared with 123 patients (19%) in the control group (Table 3). A total of 349 serious adverse events occurred in 173 patients, with 246 events occurring during the treatment period and 103 during follow-up. There were 43 deaths (16 during the treatment period and 27 during follow-up) during the study, 30 of which were deemed to be tuberculosis-related (Table S10 in the Supplementary Appendix). Overall, the numbers of serious adverse events, types of events, and numbers of patients with events (including the number of deaths) were similar in the three study groups during both the treatment period and the follow-up period.

There were no significant between-group differences in the incidence of adverse events of special interest, including tendinopathy, seizure, clinically significant cardiac toxicity, hypoglycemia or hyperglycemia, and peripheral neuropathy. The proportions of events were similar in the study groups when all adverse events were considered. There were no significant differences in any measures of biochemical, hematologic, or hepatic safety.

Table 3. Safety Analysis.*

Adverse Event	Control Group (N=639)	Isoniazid Group (N=655)	Ethambutol Group (N=636)	All Patients (N=1930)
	number of patients (percent)			
During treatment phase or follow-up				
Any	123 (19)	127 (19)	111 (17)	361 (19)
Grade 3 only	83 (13)	90 (14)	82 (13)	255 (13)
Grade 4	40 (6)	37 (6)	29 (5)	106 (5)
Serious adverse event	59 (9)	62 (9)	52 (8)	173 (9)
Death				
Any	16 (3)	15 (2)	12 (2)	43 (2)
Tuberculosis-related	11 (2)	10 (2)	9 (1)	30 (2)
During treatment phase only				
Any	111 (17)	105 (16)	99 (16)	315 (16)
Grade 3 only	76 (12)	71 (11)	73 (11)	220 (11)
Grade 4	35 (5)	34 (5)	26 (4)	95 (5)
Serious adverse event	46 (7)	40 (6)	35 (6)	121 (6)
Death				
Any	5 (1)	6 (1)	5 (1)	16 (1)
Tuberculosis-related	4 (1)	6 (1)	5 (1)	15 (1)

* Listed are all patients who had at least one grade 3 or 4 adverse event. The safety population includes all patients who underwent randomization and who received at least one dose of a study drug. One patient who underwent randomization but did not receive a study drug was excluded from the safety analysis. A detailed list of serious adverse events is provided in Table S10 in the Supplementary Appendix.

DISCUSSION

In this phase 3 trial, we aimed to determine whether the promising data that were observed for moxifloxacin in studies in animals and phase 2 studies translated into an effective reduction in the duration of the standard tuberculosis treatment regimen. The trial showed that the substitution of moxifloxacin in 4-month regimens based on either isoniazid or ethambutol did not meet the margin for noninferiority, as compared with the 6-month control regimen. The same conclusions were reached when the outcome was determined with the use of MGIT cultures of sputum samples. Among patients receiving the two moxifloxacin-containing regimens, a small number had treatment failures, but a larger number had a relapse after the end of active treatment. The difference between the isoniazid group and the ethambutol group may be due to the bactericidal effect of isoniazid or the presence of three drugs over a 4-month period. The similarity in outcome among women in the isoniazid group and the control group may represent a chance finding but merits further investigation.

It has been previously suggested that Asian patients often have a more chronic form of tuberculosis with a different clinical course than that in African patients,[18,19] but we did not see any evidence of variation in clinical-disease outcome in the different racial groups. Our approach in the conduct of this trial, including standardized laboratory methods and clinical management, has resulted in consistent results across more than 20 sensitivity analyses, with minimal variation among study centers on different continents.

In comparison with other trials that used fluoroquinolones in a 4-month regimen, the rates of an unfavorable outcome in the experimental groups in our study are lower than those in the RIFAQUIN regimen[20] and similar to those found

in the OFLOTUB trial.[21] In trials evaluating 4-month streptomycin-containing regimens that were performed in the 1970s in East Africa and Singapore, rates of relapse ranged from 11 to 40% after 2 years of follow-up.[18,19]

In our study, a daily regimen of moxifloxacin in combination with standard antituberculosis agents for 4 months had an acceptable side-effect profile. We did not find any evidence of either hypoglycemia or hyperglycemia or tendinopathies that have been associated with fluoroquinolones,[22,23] nor did we find evidence of increased hepatic dysfunction, a potential concern in regimens containing moxifloxacin or lacking isoniazid.[24] There was no clinical evidence of cardiac toxicity, although electrocardiography was not performed systematically. These are important findings for future regimens that may use moxifloxacin in combination with other agents in tuberculosis treatment.[25]

Our findings raise questions about progression decisions throughout the development pathway for tuberculosis drugs. Data from studies in mice predicted that the inclusion of moxifloxacin would result in a reduction of 1 to 2 months in the treatment duration, as compared with standard therapy.[12,13] In our study of such treatment shortening, the moxifloxacin-containing regimens did not work adequately, suggesting that the murine model may have overpredicted the sterilizing potency of moxifloxacin in this regimen.

More important is the observed poor predictability of culture conversion for long-term outcomes. Although 2-month culture conversion is associated with relapse-free cure, this observed correlation in populations is not strong enough to reliably predict outcomes for individual patients or definitively guide the selection of regimen in drug development.[26,27] This finding underlines the importance of the content and duration of treatment in the following weeks.[28] Four 2-month studies of the inclusion of moxifloxacin in the standard regimen have been reported, with variable results.[14-16,29] The only study to report a hazard ratio for the time to culture conversion was that of Rustomjee et al.,[14] who, in a study involving approximately 50 patients per group, found that the hazard ratio for the time to culture conversion for the moxifloxacin-containing regimen, as compared with the standard regimen, was 1.73, indicating a shorter duration. This raised the possibility that a 4-month regimen might be effective, although the 95% confidence interval ranged from 1.15 to 2.60. In our study, with more than 600 patients in each group, we found a more precise estimate of the hazard ratio to be 1.25 (95% CI, 1.10 to 1.40), a result that is within the confidence interval found previously[14] but with a smaller effect, which would seem unlikely to merit progression to a phase 3 trial. Thus, such short trials may correlate with long-term outcomes, but the small sample size and resulting wide confidence intervals limit their ability to predict treatment shortening.

This limitation suggests that efficient drug development for tuberculosis may require a different approach. Instead of relying on the results of 2-month phase 2 trials to select candidate regimens for phase 3 studies, investigators might find that the most efficient approach is to conduct phase 3 trials as quickly as possible while establishing more feasible and less costly approaches to performing these studies. Possible improvements could include larger noninferiority margins, permitting smaller sample sizes, and building multiple treatment durations into each study.

In conclusion, in patients with uncomplicated, smear-positive tuberculosis, the noninferiority of the moxifloxacin-containing regimens was not shown, despite the fact that these regimens had better bactericidal activity than the standard control regimen.[30]

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Comparing early morning vs. spot sputum samples for the identification of Mycobacterium tuberculosis. [Abstract 0194]

Title: Comparing the value of early morning and spot sputum smears in identifying *Mycobacterium tuberculosis* in solid and liquid culture

Authors: Murphy ME, Bongard E, McHugh TD, Gillespie SH.

Introduction: The diagnosis of tuberculosis (TB) continues to be based on the identification of acid-fast bacilli in sputum smear; the sensitivity is between 20-80%, with lower rates found in patients co-infected with the human immunodeficiency virus (HIV). As such, the case detection rate remains low at around 50%.

The World Health Organisation advises the examination of 3 sputum samples, one of which should include an early morning sputum sample (EMS). EMS are thought to increase the diagnostic yield, with one systematic review reporting 86.4% yield compared to 73.9% in a spot sample, have higher mycobacterial loads and reduce contamination rates. Few prospective studies have compared the value of EMS vs. spot sputum samples in patients during treatment.

In this study we compare the value of EMS vs spot sputum samples in patients being treated for TB in a double blind randomised controlled

trial of fluoroquinolone-containing treatment-shortening regimens being compared to standard 6 month treatment. (Clinical Trial.gov NCT00864383). Patients provide two pre-treatment sputum samples, a sample every week during the first 8 weeks of intensive phase treatment, and every month for the remainder of the treatment phase and 3-monthly for 12 months after treatment completion. Patients were asked to provide EMS where possible with a spot sputum sample being collected during clinic visits when an EMS was not provided. Data on all samples in the main trial database as of 23 August 2010 were included in this analysis. Statistical analyses were performed in Microsoft Excel 2003 and Graphpad Prism 5.

Scientific Findings: From data on 10179 samples from 610 patients (68% male,55% HIV positive,average CD4 426) 42% EMS were smear-positive compared to 29% spot samples. The sensitivity and specificity of EMS was 78% and 85% for LJ-culture compared to 88% and 92.5% for spot samples. For MGIT-culture, the sensitivity and specificity was 75% and 91% for EMS and 86% and 89% for spot samples. Contamination rates were 17% and 9.9% in EMS and 16% and 6.6% in spot samples in LJ and MGIT culture respectively. Higher mycobacterial loads were identified in spot compared to EMS samples in both LJ and MGIT culture ($p<0.0001$).

Discussion: This study adds much needed longitudinal data from TB patients enrolled in an international regulatory trial with standardised laboratory

procedures. This data challenges the superiority of EMS over spot sputum samples; EMS are more likely contaminated and less sensitive for culture in both solid-LJ and liquid MGIT-media. Return visits to collect EMS are an additional burden on TB programmes and significant numbers of patients are reported to drop out of the diagnostic pathway before starting treatment; awaiting EMS may not represent 'value-for-money'. The effect of time-on-treatment, however, have not been considered and may well be significant.

Conclusions During TB treatment, early morning sputum samples have lower sensitivity and specificity than spot samples for LJ culture results, and lower sensitivity and similar specificity than spot samples for MGIT culture results. Contamination rates are higher in EMS for both LJ and MGIT culture. Overall, the bacterial load in higher in spot samples; this may be significant as molecular diagnostics become increasingly available. Further analyses must consider how long a patient has been receiving treatment and the effect of treatment regimen in further assessing the value of EMS and spot sputum smear in identifying TB patients and quantifying mycobacterial burden.

Categories Lesson in Microbiology & Infection Control

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Poor correlation of smear microscopy for TB culture on solid and in liquid media during TB treatment

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BACKGROUND: Sputum culture is considered the gold standard for TB (tuberculosis) diagnosis and monitoring but it is often unaffordable in those resource-poor settings bearing the greatest burden of disease. Therefore, many national TB programmes base management decisions on smear microscopy performed during treatment.

OBJECTIVE: This study investigates the correlation between smear microscopy and culture during TB treatment.

METHOD: We analysed mycobacteriological results of serial sputum samples from 462 patients during 4608 visits for the treatment-shortening REMox study during the first 17 weeks of TB treatment.

PATIENTS:

- Adults >18yrs, Median 30 yrs
- Male (70%), Female (30%)
- Smear Positive Pulmonary TB
- Treatment naive
- HIV Neg OR HIV Pos CD4>250
- Sites: South Africa (76%), Zambia(16%) Tanzania (9%), Mexico (<1%)

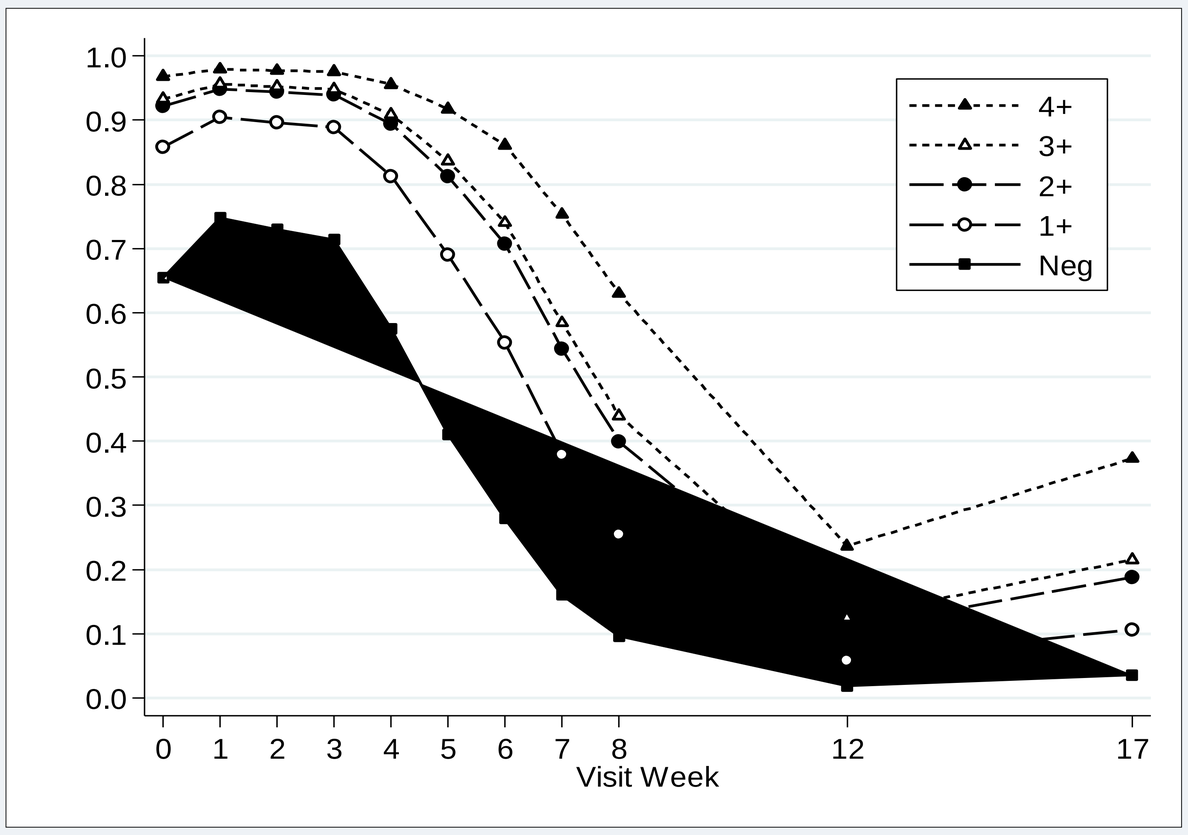
REMoxTB TREATMENT REGIMENS	WEEKS 0-8	WEEKS 9-17	WEEKS 18-26
Regimen 1 (Standard 6/12 Rx)	2 EHRZ M Plc	2 HR	2 HR
Regimen 2 (4/12, M replaces E)	2MHRZ E Plc	2 MHR	H Plc R Plc
Regimen 3 (4/12, M replaces H)	2EMRZ H Plc	2 MR H Plc	H Plc R Plc

* M=moxifloxacin, E=ethambutol , H=isonaizid, R=rifampicin, Z=pyrazinamide, Plc=placebo

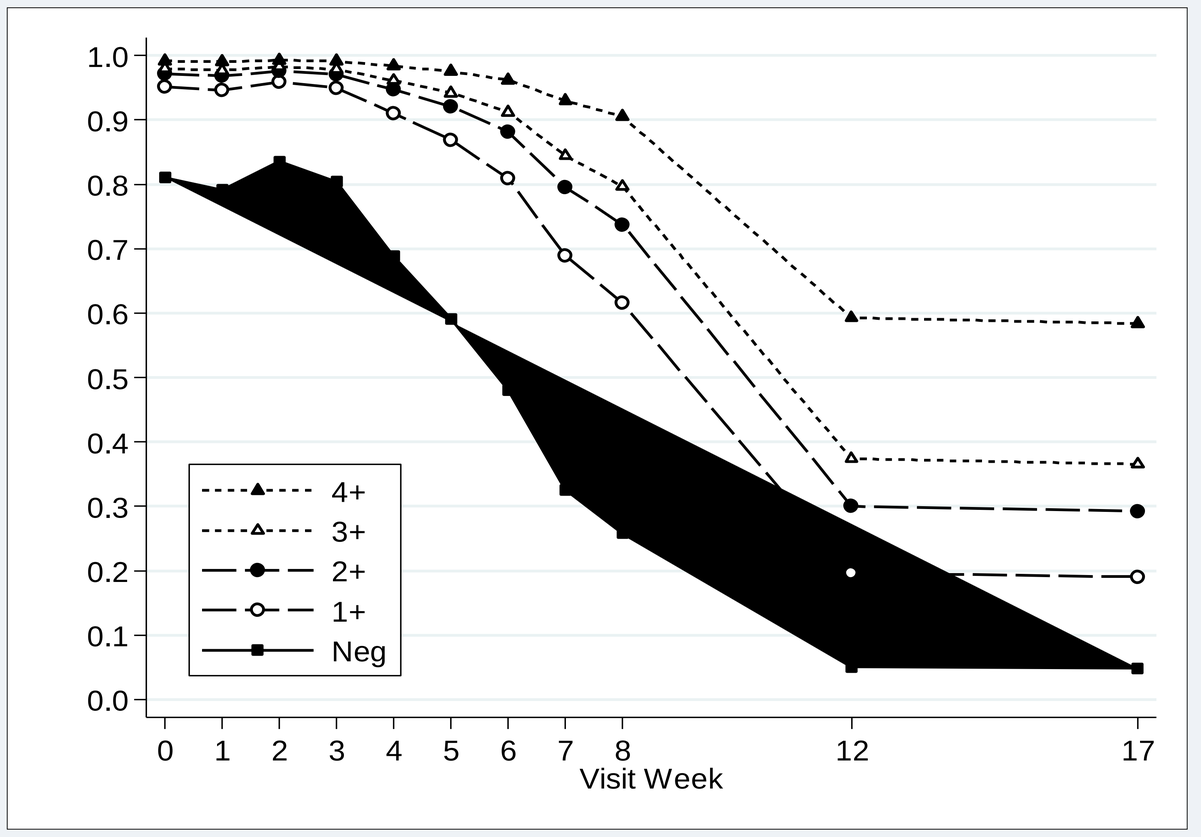
RESULTS: Smear microscopy and culture result on solid LJ and liquid MGIT media

NUMBER OF WEEKS ON TREATMENT	LJ						MGIT					
	Smear Negative		Smear Positive		Odds Ratio	95% CI	Smear Negative		Smear Positive		Odds Ratio	95% CI
	PP	95% CI	PP	95% CI			PP	95% CI	PP	95% CI		
4	0.58	(0.47, 0.68)	0.89	(0.83, 0.92)	2.28	(1.54, 3.01)	0.68	(0.58, 0.77)	0.95	(0.92, 0.97)	1.73	(1.14, 2.32)
8	0.10	(0.06, 0.15)	0.32	(0.22, 0.45)	2.06	(1.43, 2.68)	0.25	(0.19, 0.32)	0.72	(0.60, 0.81)	1.48	(0.80, 2.15)
12	0.02	(0.01, 0.04)	0.08	(0.03, 0.17)	1.70	(0.89, 2.51)	0.05	(0.03, 0.09)	0.24	(0.14, 0.38)	1.48	(0.34, 2.62)
17	0.04	(0.02, 0.06)	0.11	(0.04, 0.27)	1.15	(0.13, 2.16)	0.06	(0.04, 0.09)	0.16	(0.07, 0.32)	1.12	(-.011, 2.36)

Graph of the relationship between sputum smear microscopy and culture in solid LJ media during TB treatment showing the predictive probability of a positive culture on LJ, from the week 0 to week 17 visits, given the smear grading at that visit.



Graph of the relationship between sputum smear microscopy and culture in liquid MGIT media during TB treatment showing the predictive probability of a positive culture on MGIT, from the week 0 to 17 visits, given the smear grading at that visit.

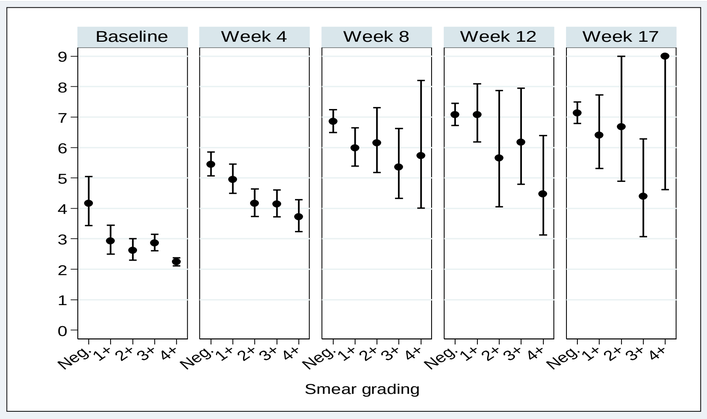


RESULTS: Smear grading and mycobacterial quanitification in solid LJ and liquid MGIT culture

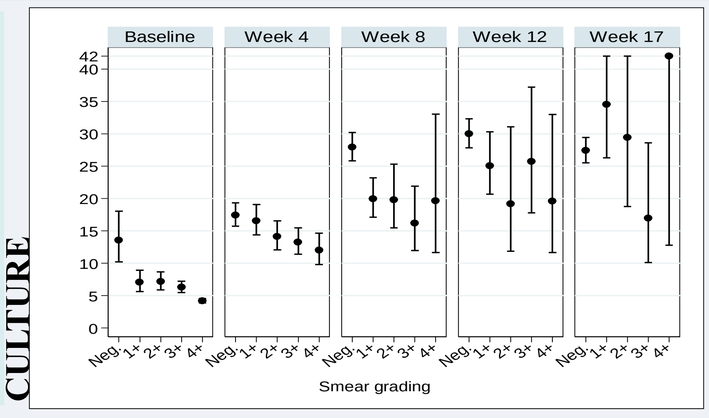
Smear grading	Mean week of growth(SE mean)	Mean TTP in days (se of mean)
Negative	7.12 (0.06)	30.06 (0.31)
1+	5.65 (0.10)	20.06 (0.45)
2+	4.61 (0.11)	15.43 (0.42)
3+	4.21 (0.10)	12.74 (0.37)
4+	3.12 (0.10)	8.73 (0.26)
Overall	5.64 (0.04)	21.51 (0.22)

Graph of smear grading and corresponding mycobacterial load during TB treatment; estimated mean week of growth until positive LJ culture and time-to-positivity in MGIT culture, in days, with 95% confidence interval, stratified by smear grading (Neg or Pos, 1+-4+).

SOLID LJ CULTURE



LIQUID MGIT CULTURE



CONCLUSION: During TB treatment, smear microscopy is increasingly less reliable in predicting culture results and all smear gradings correspond to decreasing mycobacterial loads in both solid LJ and/or liquid MGIT culture.
TB programmes should not base treatment decisions on smear results. Improved biomarkers are urgently required.