

**DEVELOPMENT AND EVALUATION OF A RAPID MOLECULAR
ASSAY AND A MASK SAMPLING METHOD ENABLING THE
STUDY OF TUBERCULOSIS TRANSMISSION**

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ABSTRACT

Development and Evaluation of a Rapid Molecular Assay and a Mask Sampling Method Enabling the Study of Tuberculosis Transmission

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Mycobacterium tuberculosis (Mtb) is transmitted in small aerosol droplets expectorated by individuals with active pulmonary tuberculosis (TB). There is still no established method for sampling these infectious aerosols and measuring the bacterial concentration in them. Little is known about the relationship between tuberculous aerosol output and infectiousness of TB cases; medical practice currently relies on the acid-fast smear status to gauge this and determine when it is safe to discharge TB patients. This study involved the development and evaluation of a rapid molecular assay and a mask sampling approach to increase our knowledge relating to TB transmission. A molecular assay targeting the mycobacterial 16S rDNA and differentiating Mtb complex from non-tuberculous mycobacteria was developed to facilitate recruitment of TB patients for the mask sampling study. Although the assay did not achieve this outcome during the study, we demonstrated its clinical utility on mycobacterial isolates. A second molecular assay targeting the Mtb global lineage-defining and locally prevalent RD750 polymorphism was also developed for preliminary genotyping of Mtb strains to assess for potential recent transmission events. Both the 16S and RD assays were performed in single thermocycler runs but in separate reaction tubes and utilised a combination of TaqMan and SYBR Green technologies to simultaneously differentiate two products. The 16S-RD assay shows promising potential for direct specimen analysis and detection of mixed infections. A novel method of mask aerosol sampling coupled to a mycobacteriophage amplification assay developed in this study was able to detect and to some extent quantify the amount of respiratory-borne Mtb. This approach could potentially be more reliable than smear microscopy in assessing TB infectivity. With further optimisation and improvement, both the 16S-RD assay and the mask sampling method have potential to facilitate better understanding of TB transmission and subsequently contribute to public and clinical management of this disease.

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For my beloved family:
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May you rest in peace, Ma.

X X X

DECLARATION OF JOINT EFFORTS

Although my work was central to the development and the final version of the molecular assay, the preliminary work was done by colleagues, who also co-authored the article reporting its development and evaluation (Appendix 4). Specifically, the 16S and RD primers were designed by R. C. Free and J. Malkin, respectively.

ABBREVIATIONS & UNITS OF MEASUREMENTS

ADC	albumin-dextrose-catalase
AFB	acid-fast bacilli
AIDS	acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
BCG	Bacille Calmette-Guérin
BLASTn	nucleotide-nucleotide Basic Local Alignment Search Tool
BSA	bovine serum albumin
<i>Bst</i>	<i>Bacillus stearothermophilus</i>
CDC	Centers for Disease Control and Prevention
CFU	colony-forming unit(s)
CFP-10	culture filtrate protein-10
Ct	cycle threshold
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DOT	directly observed treatment
DOTS	directly observed treatment, short-course
DR	direct repeat
EA-I	East African-Indian
EDTA	ethylenediaminetetraacetic acid
ELISPOT	enzyme-linked immunospot
ESAT-6	early secretory antigenic target-6
FAS	ferrous ammonium sulphate
FDA	Food and Drug Administration
FPTB	<i>FASTPlaqueTB</i>
<i>g</i>	gravity
GLIP	genome level-informed PCR
HIV	human immunodeficiency virus
IPTG	isopropyl β -D-1-thiogalactopyranoside
IS	insertion sequence
LAMP	loop-mediated isothermal amplification
LB	Luria-Bertani
LJ	Löwenstein-Jensen
LRP	luciferase reporter phage
LSP	large-sequence polymorphism
MDR-TB	multidrug-resistant tuberculosis
MGIT	mycobacterial growth indicator tube
MIRU	mycobacterial interspersed repetitive unit
MOUDI	micro-orifice uniform deposit impactor
MRCA	most recent common ancestor
Mtb	<i>Mycobacterium tuberculosis</i>
MTBC	Mtb complex
NALC	N-acetyl-L-cysteine
NAP	<i>p</i> -nitro-acetylamino-hydroxypropionophenone
NOA	nystatin-oxacillin-aztreonam
NTC	no-template control
NCTC	National Collection of Type Cultures
NTM	non-tuberculous mycobacteria

OADC	oleic acid-albumin-dextrose-catalase
OD	optical density
OGC	OADC-glycerol-calcium
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFU	plaque-forming unit(s)
PGRS	polymorphic GC-rich repetitive sequence
PPD	purified protein derivative
RD	region of difference
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RIDOM	Ribosomal Differentiation of Medical Microorganisms
Rpf	resuscitation-promoting factor
SNP	single-nucleotide polymorphism
TAE	Tris-acetate-EDTA
<i>Taq</i>	<i>Thermus aquaticus</i>
TB	tuberculosis
TbD1	Mtb-specific deletion 1
T _m	melting temperature
UHL	University Hospitals of Leicester
UV	ultraviolet
VNTR	variable-number tandem repeat
WHO	World Health Organization
XDR-TB	extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen

bp	base pair
kbp	kilobase pair
g	gramme
mg	milligramme
µg	microgramme
ng	nanogramme
pg	picogramme
µJ	microjoule
ml	millilitre
µl	microlitre
cm	centimetre
µm	micrometre
nm	nanometre
m/s	metre per second
M	molar
mM	millimolar
µM	micromolar
nM	nanomolar
pmol	picomole
rpm	revolutions per minute
v/v	volume per volume
w/v	weight per volume

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CHAPTER 1

Introduction

1.1 General Introduction

Tuberculosis (TB) is caused by the pathogen *Mycobacterium tuberculosis* (Mtb), one of the most common and successful infectious agents known to mankind. The World Health Organization (WHO) estimates that one-third of the world's population is currently infected with Mtb (WHO, 2010b). The resurgence of TB in the industrialised world has prompted increased global attention and concern on this contagious disease. The emergence of multidrug- and extensively drug-resistant strains of Mtb and the close association with human immunodeficiency virus (HIV) infection complicate this problem further. Despite the large numbers of studies and investigations, it is readily evident that there are multiple gaps in the understanding of TB and its aetiological agent Mtb. Among the areas poorly understood is the transmission and infectiousness of TB.

The first scientific evidence for the airborne spread of TB appeared in the mid 20th century (Riley *et al.*, 1959). Mtb is transmitted in respiratory aerosol droplets expectorated by individuals with pulmonary and laryngeal TB. These droplets are produced during expiratory manoeuvres such as coughing, sneezing, singing, and talking. Inhalation of these infectious particles by another individual can result in infection or disease. WHO estimated that TB is spreading at the rate of one person per second and an untreated active TB case can go on to infect 10-15 people throughout the year (WHO, 2010b). Therefore, identification and clinical management of source cases are crucial in controlling and interrupting disease transmission.

Until now, the most common way of establishing the transmission of TB has been through screening of contacts based on the tuberculin skin test, a test that can be difficult to perform and interpret, although latterly T cell-based assays have made such assessments easier. Molecular fingerprinting of Mtb strains has better specificity but, requires successful culture and isolation of strains, is technically demanding, and the degrees of molecular clustering can vary depending on the typing methods used. The superior performance of strain genotyping relative to conventional epidemiological methods in estimating the level of disease transmission necessitates careful interpretation.

Acid-fast bacilli (AFB) smear status has been used to recognise the infectiousness of TB patients. It is generally known that AFB smear-positive TB cases are more infectious than those who are smear-negative. Apart from this fact, it is still poorly understood why certain cases are more infectious than the others. Possible factors have been proposed but have yet to be verified; examples include duration and proximity of exposure, nature of expiratory manoeuvres, characteristics of tubercle bacilli, and presence of chemotherapy. In medical practice, it is traditionally believed that two weeks of chemotherapy is sufficient to render a TB patient non-infectious (Ormerod *et al.*, 1998). However, this view has been criticised (Farer, 1973; Beck-Sague *et al.*, 1992). In this context, a misjudgement runs the risk of community and nosocomial transmission.

There have been a few studies evaluating methods for the sampling of respiratory-borne Mtb; these range from the use of animal models to molecular- and culture-based methods (Riley *et al.*, 1959; Mastorides *et al.*, 1999; Fennelly *et al.*, 2004). Among these, only a few methods can be used for direct sampling of Mtb aerosols and all suffer from limitations. Therefore, there is still no reliable method for collecting tuberculous aerosols and measuring the concentration of tubercle bacilli in them. As a result, the correlation between Mtb aerosol excretion and infectiousness of TB cases is poorly understood. In this study, the novel use of standard face masks for sampling and quantification of Mtb expectorated by TB patients was proposed and evaluated to address this problem and a variety of methods assessed to support this approach.

1.2 The Genus *Mycobacterium*

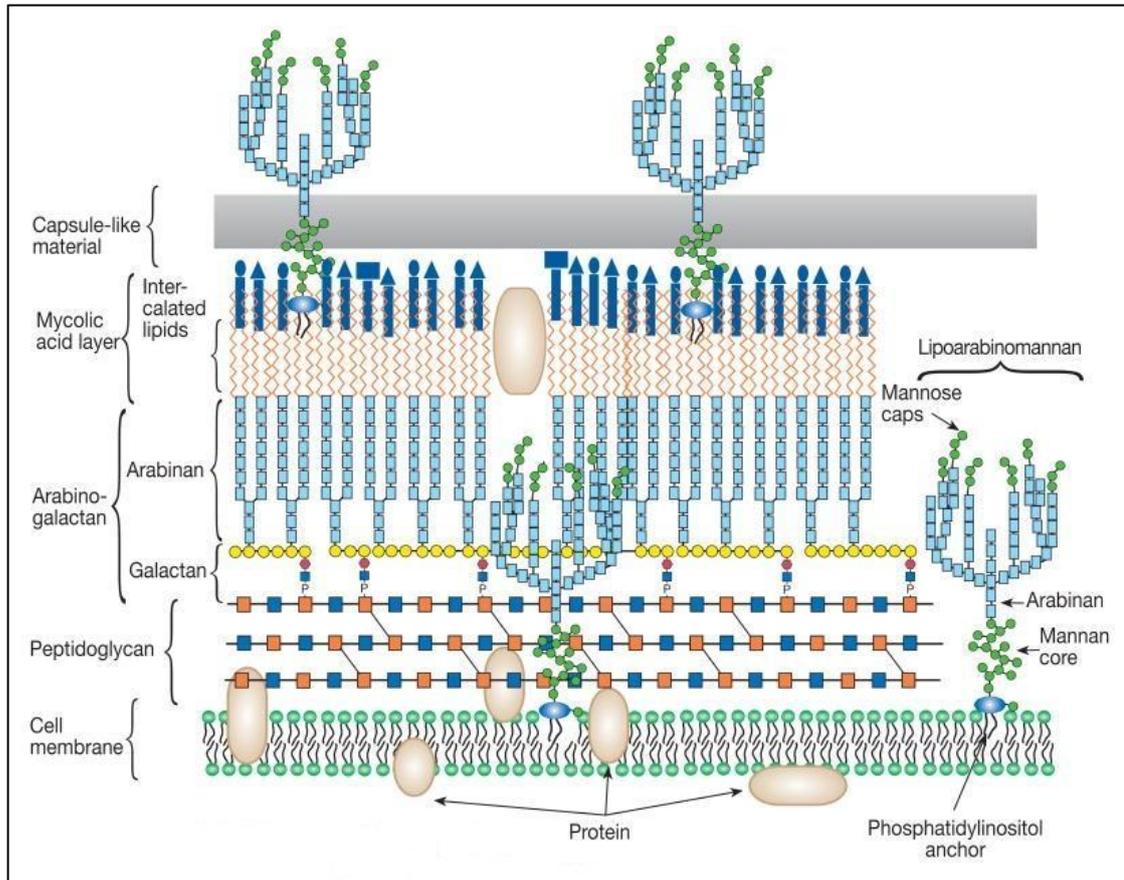
1.2.1 Mycobacteria in general

Mycobacterium is a genus within the actinobacteria, assigned to the family *Mycobacteriaceae*. The Latin prefix "myco-" means both fungus and wax; its use here relates to the waxy compounds in the bacterial cell wall. Mycobacteria are closely related to *Nocardia*, *Rhodococcus*, and *Corynebacterium spp.* They are widespread organisms, typically found in environmental reservoirs. Some, however, including Mtb complex and *M. leprae*, are obligate parasites in mammals which are causative agents of TB and leprosy, respectively.

Mycobacterial cells are slightly curved or straight rods which rarely branch measuring 0.2-0.7 by 1-10 μm in size. They are aerobic, non-motile, and do not have capsules. The ability of mycobacteria to form spores was recently reported (Singh *et al.*, 2010). Mycobacteria have unusually high lipid content relative to other bacteria; this is evident from the large number of genes involved in lipid metabolism in their genomes (Cole, 1999). The lipids form an integral part of the cell envelope, which is rich in high molecular weight 3-hydroxy-2-alkyl branched fatty acids termed mycolic acids (Brennan and Nikaido, 1995; Minnikin *et al.*, 2002). These mycolic acids are 60-90 carbons in length and are a distinguishing feature of the mycobacteria. They are made up of two structural groups, the meromycolate moiety and the α -branch, and are arranged side by side perpendicular to the plane of the cell surface (Minnikin, 1982). The inner section of the cell envelope consists of layers of mycolate and peptidoglycan held together by arabinogalactan, a branched polysaccharide composed of arabinose and galactose (Minnikin, 1982). The outer section is made up of free fatty acids, lipoarabinomannans, and dimycocerosates; the latter is responsible for the waxy nature of the mycobacterial cell wall (Minnikin *et al.*, 2002; Brennan, 2003). Lipoarabinomannans are anchored to phosphatidylinositol mannosides in the plasma membrane and span the whole cell wall, interspersed among the mycolic acids (Brennan and Nikaido, 1995). The mycobacterial peptidoglycan layer is thought to be similar to those of other bacteria (Minnikin *et al.*, 2002); however, the muramic acid residue in its peptidoglycan is N-glycolylated, whereas this residue is N-acetylated in

other bacteria (Brennan and Nikaido, 1995). A model of a typical mycobacterial cell wall is shown in Figure 1.1.

Figure 1.1: Structure of the mycobacterial cell envelope



The mycobacterial cell envelope is divided into the inner and outer sections. The inner section (core of the envelope) consists of the mycolyl arabinogalactan-peptidoglycan complex, which is anchored to the plasma membrane. The outer section is made up of a heterogeneous group of complex free lipids with medium-chain fatty acids. A proportion of these are the lipoarabinomannans which are anchored to the plasma membrane via their base phosphatidylinositol mannosides. Reproduced from the proceedings paper by Brennan and Crick (2007) with permission from Bentham Science Publishers Ltd. Copyright © 2007 Bentham Science Publishers Ltd.

The high proportion of lipids makes the cell envelope waxy and hydrophobic, and this contributes to low permeability to a variety of solutes (Nikaido and Jarlier, 1991). Mycobacterial cells retain the carbolfuchsin dye upon decolourisation with acidified alcohol during the Ziehl-Neelsen staining, and therefore said to be acid-fast. They appear either beaded or almost as negative image against the counterstained background during Gram staining, as the crystal violet in the dye does not readily penetrate their cell envelope. The thick waxy cell envelope also contributes to increased resistance of mycobacteria to acids, alkalis, disinfectants, and antibiotics (Jarlier and Nikaido, 1994).

Most *Mycobacterium* species adapt readily to growth on very simple substrates, using glycerol as a carbon source and ammonia or amino acids as nitrogen sources, in the presence of mineral salts. Optimal temperatures for growth vary among different species; these range from as low as 28°C for *M. chelonae* to as high as 45°C for *M. thermoresistibile*. Mycobacterial species that form visible colonies within seven days on nutritionally rich medium at optimal growth temperature are termed rapid growers, while those requiring longer periods are termed slow growers (Stahl and Urbance, 1990). However, the rapid-growing species still grow slower than most other non-mycobacterial species. Slow growth among mycobacteria might be due to the possession of very low numbers of 16S rRNA cistrons (normally one or two), impermeability of the lipid-laden cell envelope to certain hydrophilic nutrients, and the energy cost for the synthesis of long-chain mycolic acids (Pimm *et al.*, 2004). Some mycobacterial species can be very difficult to culture. Indeed, *M. leprae* is not culturable *in vitro*.

Pathogenic mycobacteria can be classified into several major groups for the purpose of diagnosis and treatment: Mtb complex, which can cause TB; *M. leprae*, which causes leprosy; and non-tuberculous mycobacteria, which can cause lymphadenitis, skin disease, disseminated disease, and pulmonary disease resembling TB.

1.2.2 *M. tuberculosis* complex

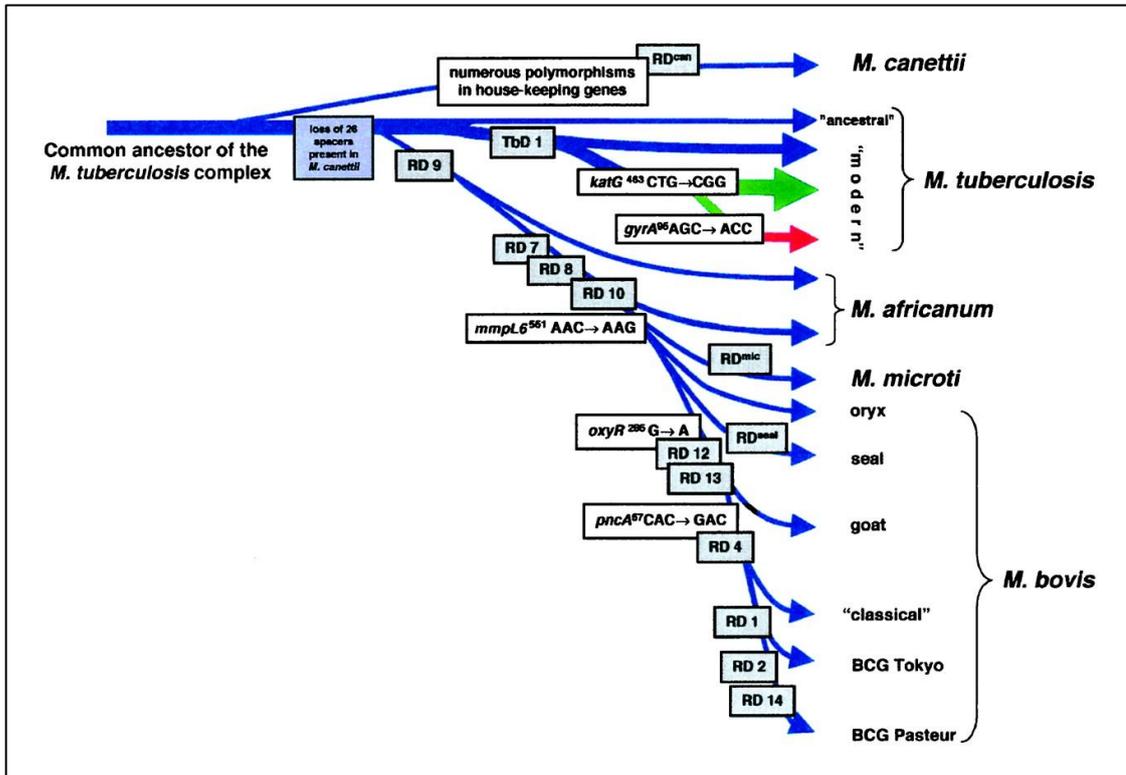
The Mtb complex (MTBC) is a group of closely related mycobacterial species consisting of Mtb, *M. africanum*, *M. bovis*, *M. canetti*, *M. microti*, *M. caprae*, and *M. pinnipedii*. Mtb causes the majority of human TB cases, while *M. africanum* is a major cause of the disease in sub-Saharan Africa. *M. bovis* is the causative agent for bovine TB; however, animal-to-human transmission has long been recognised and includes milk-borne transmission (Baker *et al.*, 2006). *M. canetti* is also capable of causing TB in humans but is relatively uncommon. The other members of the complex primarily infect non-human hosts. *M. microti* is a rarely encountered pathogen of voles and other small mammals. The two recent additions to the complex, *M. pinnipedii* (Cousins *et al.*, 2003) and *M. caprae* (Aranaz *et al.*, 2003), are the causative agents for TB in seals and goats, respectively.

For many years, it was thought that human TB is a zoonosis that derived from the bovine disease due to the domestication of cattle by humans (Stead, 1997). This hypothesis arose based on the property of Mtb to be almost exclusively a human pathogen, as opposed to *M. bovis* which has a much broader host range. However, the idea was dismissed by the first major molecular phylogeny of the MTBC that shows that *M. bovis* has undergone numerous deletions relative to Mtb (Figure 1.2) (Brosch *et al.*, 2002). Modern Mtb strains are segregated from other members of the complex by possessing an intact chromosomal region of difference 9 (RD9) and the Mtb-specific deletion 1 (TbD1). The RD9 deletion gave rise to a separate MTBC lineage consisting of *M. africanum*, *M. microti*, and *M. bovis*; the latter includes *M. pinnipedii*, *M. caprae*, and the vaccine BCG strains. Further successive genomic deletions within this lineage might have led to clonal expansion and the appearance of more successful host-specific tubercle bacilli. *M. canetti* had probably diverged from the common ancestor of modern Mtb strains before other members of the MTBC.

The members of MTBC share 99% identity at the nucleotide level for some loci (Sreevatsan *et al.*, 1997; Cole *et al.*, 1998). However, they can be differentiated by several phenotypic and epidemiological characteristics (Shinnick *et al.*, 1994). Also, various repetitive DNA elements which contribute to strain variation have been

discovered; these include insertion sequences and short repetitive DNA sequences with unknown function (Small *et al.*, 1994b).

Figure 1.2: Proposed molecular phylogeny of the MTBC



The scheme illustrates successive loss of DNA at RDs beginning from the proposed common ancestor of the MTBC. The intact RD9 and TbD1 separated modern Mtb strains from other members of the complex. The RD9 deletion gave rise to a separate MTBC lineage, in which further successive deletions might have led to clonal expansion and adaptation of tubercle bacilli in specific mammalian hosts. Single-nucleotide polymorphisms in five selected genes are also shown. Two of these, *katG* and *gyrA*, were used to assign modern MTBC strains into three principle genetic groups (Sreevatsan *et al.*, 1997) (see Section 1.6.3). The blue arrows represent group 1 strains, the green arrows represent group 2 strains, and the red arrow represents group 3 strains. Reproduced from the article by Brosch *et al.* (2002) with permission from the National Academy of Sciences. Copyright 2002 National Academy of Sciences, U.S.A.

1.2.3 Non-tuberculous mycobacteria

In the late 1950s, species of mycobacteria other than MTBC were being encountered with increasing frequency in medical practice. These mycobacteria are known as non-tuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT), environmental mycobacteria, or atypical mycobacteria. Runyon (1959) proposed the classification of NTM according to their growth rate and pigment production. Group I consisted of photochromogenic slow growers which produced pigmented colonies after exposure to light. Group II was made up of slowly growing scotochromogenic species which produced deep yellow to orange colonies when cultured in the dark. Group III consisted of non-chromogenic slow growers, while the rapidly growing mycobacterial species were members of Group IV. Woods and Washington (1987) have suggested a clinically oriented grouping of NTM (Table 1.1).

Table 1.1: Woods and Washington Classification Scheme for NTM

Species potentially pathogenic in humans
<i>M. avium-intracellulare</i> complex, <i>M. kansasii</i> , <i>M. scrofulaceum</i> , <i>M. xenopi</i> , <i>M. szulgai</i> , <i>M. malmoense</i> , <i>M. simiae</i> , <i>M. genavense</i> , <i>M. marinum</i> , <i>M. ulcerans</i> , <i>M. haemophilum</i> , <i>M. celatum</i> , rapid growers (<i>M. fortuitum</i> , <i>M. chelonae</i> , <i>M. abscessus</i>)
Saprophytic mycobacteria rarely causing disease in humans
<i>M. gordonae</i> , <i>M. asiaticum</i> , <i>M. terrae</i> , <i>M. trivial</i> , <i>M. shimodei</i> , <i>M. gastri</i> , <i>M. nonchromogenicum</i> , <i>M. paratuberculosis</i>
Species with an intermediate growth rate
<i>M. flavescens</i>
Other rapidly growing species
<i>M. thermoresistibile</i> , <i>M. smegmatis</i> , <i>M. vaccae</i> , <i>M. parafortuitum</i> complex, <i>M. phlei</i>

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There are currently more than 130 identified NTM species. Different species vary in relation to growth rates, colony morphology, drug and biocide susceptibility, plasmid carriage, and virulence (Primm *et al.*, 2004). NTM are distinguished from MTBC by the fact that they are not obligate pathogens but are true inhabitants of the environment, and hence the alternative label “environmental mycobacteria”. They are widely distributed in a wide variety of environmental reservoirs such as natural and municipal water, wet soil, aerosols, protozoans, plants, and animals.

The prevalence of many NTM species in potable water is attributed to their high resistance to disinfectants, ability to form biofilms and persist in a flowing system, starvation survival under low-nutrient conditions, and tolerance of temperature extremes (Primm *et al.*, 2004). *M. avium*, *M. kansasii*, *M. goodii*, *M. flavescens*, and *M. mucogenicum* are examples of NTM species isolated from public potable water (Le Dantec *et al.*, 2002). In a study by von Reyn *et al.* (1994), DNA fingerprints of *M. avium* isolates from HIV patients were identical to those of isolates recovered from the patients' drinking water.

Clinically relevant NTM species can also be found in various other water and environmental reservoirs. *M. xenopi* is frequently isolated from recirculating hot water systems. *M. marinum*, which infects through skin abrasions, is commonly found in the water in aquariums. *M. fortuitum*, *M. chelonae*, and *M. abscessus* are all water borne, but can also be found as inhabitants of soil. Food is also a possible source of atypical mycobacteria. Grant and associates (2002) reported from a survey that *M. paratuberculosis* can be isolated from pasteurised retail milk. This NTM species is the causative agent of Johne's disease in cattle, resulting in decreased milk production and fertility in infected animals (Pavlik *et al.*, 2000).

NTM have significant impacts on the morbidity and mortality of humans, especially among immunocompromised individuals and those with pre-existing lung disease. Co-infection with *M. avium* is common among individuals with acquired immune deficiency syndrome (AIDS). NTM, predominantly *M. avium* and *M. scrofulaceum*, can cause cervical lymphadenitis in children. Aerosolisation of mycobacteria from water reservoirs can lead to environment-to-human transmission. Hypersensitivity pneumonitis has been reported in workers exposed to aerosols generated from fluid used in metal

grinding (Kreiss and Cox-Ganser, 1997) and in individuals exposed to aerosols from hot tubs and spas (Embil *et al.*, 1997). In a number of cases, *M. avium* and *M. chelonae* have been cultured from the fluid or water. The possible association between *M. paratuberculosis* and the development of Crohn's disease in humans has also been proposed (Naser *et al.*, 2004). NTM infection can also manifest itself as skin, soft tissue, and disseminated diseases.

The majority of human-NTM interactions are transient, self-curing colonisations as the immune system in most infected cases contains and clears the mycobacteria. However, the incidence of NTM disease is predicted to increase as a consequence of the surge of the HIV epidemics in the global population. Furthermore, novel opportunistic species will continue to be discovered. Therefore, efforts must be focused on control measures that will specifically remove mycobacteria from habitats where there is high risk of human exposure.

1.3 Tuberculosis in General

1.3.1 History of tuberculosis

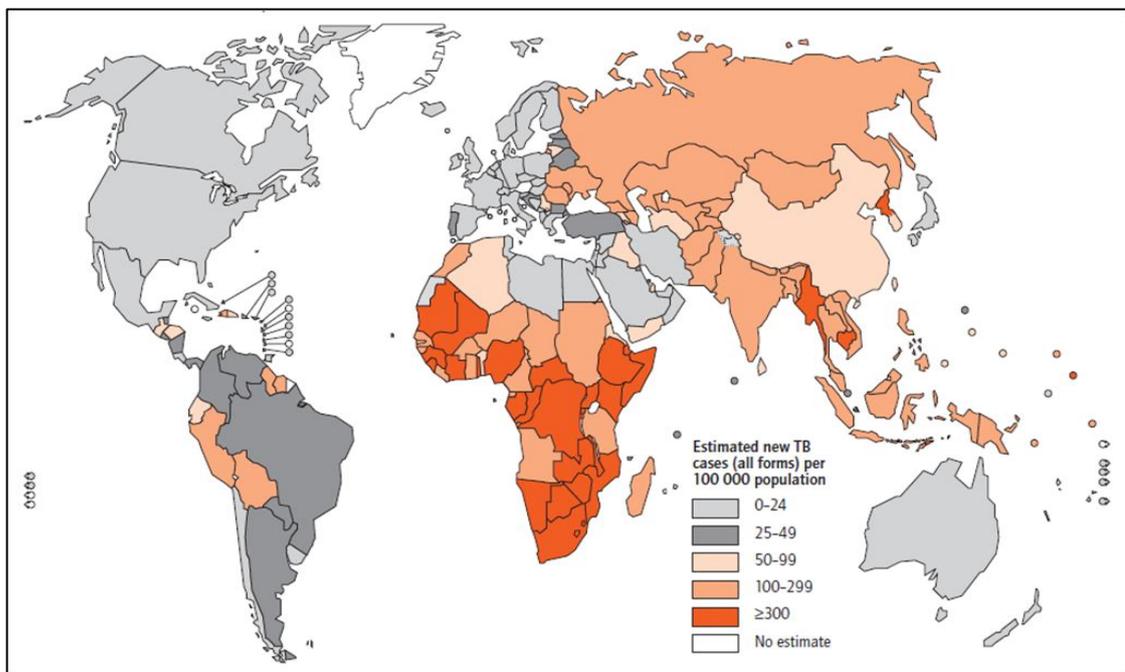
TB has been present in the human population since antiquity. There is evidence of skeletal abnormalities of TB found in Peruvian and Egyptian mummies, also demonstrated by recovery of Mtb DNA from mummified tissues (Arriaza *et al.*, 1995) (Nerlich *et al.*, 1997). Hippocrates noted that TB, known as phthisis (consumption) in ancient Greece, was the most widespread and fatal disease of his time, affecting mostly young adults. Clarissimus Galen, physician to the Roman Emperor Marcus Aurelius in 174, wrote of TB and recommended fresh air, milk, and sea voyages for its treatment. In China and India, TB was described in ancient writings aged more than 1,000 years old.

TB was well established in East Africa by the time the Europeans arrived in the area in the 19th century (Daniel, 1998); early voyages and trades might have contributed to the spread of this disease to the European and Asian continents. There is abundant archaeological evidence that TB occurred throughout the American continent before the arrival of the first European explorers (Daniel, 2000). During the renaissance period, Théophile Laennec elucidated the pathogenesis of TB and described most of the physical presentations of the disease in his 1819 book entitled “D’Auscultation Mediate”; this marks the point when modern understanding of TB began. By Laennec’s era, TB had spread across Europe in an epidemic tsunami. The history of TB was dramatically changed in 1882 when Hermann Heinrich Robert Koch identified the tubercle bacillus as the aetiological agent for the disease.

1.3.2 Global burden of tuberculosis

In the 1970s, the number of global TB cases was decreasing and the disease was on the verge of eradication. However, TB made a comeback in the 1980s, complicated by HIV co-infection, with startlingly high rates of disease and death. At present, WHO (2010b) estimated that more than 2 billion of people are infected with *Mtb* worldwide, of which 10% will develop active disease in their lifetimes. In 2008, there were 9.4 million new TB cases, of which 5.7 million were notified and 1.8 million died (WHO, 2010b). The vast majority of TB cases are in the developing world (Figure 1.3); attributed to high rates of endemicity, declining or chronically poor socioeconomic conditions, and surge of the HIV epidemics. Although sub-Saharan Africa has the highest incidence rate, the densely populated countries of Asia have the largest numbers of cases; India, China, Indonesia, Bangladesh, and Pakistan together account for more than 50% of the global burden. Nevertheless, the re-emergence of TB in the developed world has triggered increased global awareness and solicitude.

Figure 1.3: Estimated incidence rates for TB throughout the world in 2008



Reproduced from “Global Tuberculosis Control: A Short Update to the 2009 Report” (WHO, 2009) with permission from the WHO Press. © World Health Organization 2009. All rights reserved.

If their control plan is successful, WHO envisage that the increasing incidence of TB will be halted and reversed by 2015. This reversal will involve reducing prevalence and death rates of TB by 50% relative to the 1990 levels (WHO, 2009). The Stop TB Strategy is the approach recommended by WHO to achieve these global targets. This strategy becomes the main focus of national and international TB control programmes. The six major components of this strategy are: (1) to pursue high-quality directly observed treatment, short-course (DOTS) expansion and enhancement, (2) to address TB-HIV co-infection, multidrug-resistant TB, and the needs of poor and susceptible populations, (3) to strengthen the health system based on primary care, (4) to engage all care providers, (5) to empower people with knowledge and awareness on TB, and communities through partnership, and (6) to enable and promote research (WHO, 2009). The DOTS strategy has five elements: political commitment, diagnosis primarily by sputum-smear microscopy among patients, short-course treatment with effective case management, regular drug supply, and systematic monitoring to assess outcomes of patients on treatment (WHO, 2002). If the Stop TB Strategy is fully funded and implemented, 14 million lives will be saved and 50 million people will be treated (WHO, 2009).

1.3.3 Pathophysiology of tuberculosis

The disease states observed during TB infection are consequences of host-pathogen interaction; the possible outcomes of Mtb infection are shown in Figure 1.4. When a person with active TB coughs, sneezes, sings, or talks, aerosol droplets loaded with Mtb are generated and propelled into the air. The moisture in these droplets evaporates rapidly to form droplet nuclei which are then inhaled by those exposed to this source case. These droplets enter the lungs and are deposited in the alveoli in the distal airways. The bacilli in these droplets are engulfed by alveolar macrophages, and if not rapidly killed, can invade the host inside these cells. In many cases, it is likely that the innate immune response eliminates the pathogen at the earliest stage. The phagocytosed bacilli are killed by the bactericidal activities of the macrophages which include the release of reactive oxygen or nitrogen intermediates, digestion by lysosomal enzymes, and exposure to low intracellular pH (Parrish *et al.*, 1998). As a result, the infected hosts exhibit no pathology, no symptoms, and no apparent adaptive immune response (Dietrich and Doherty, 2009).

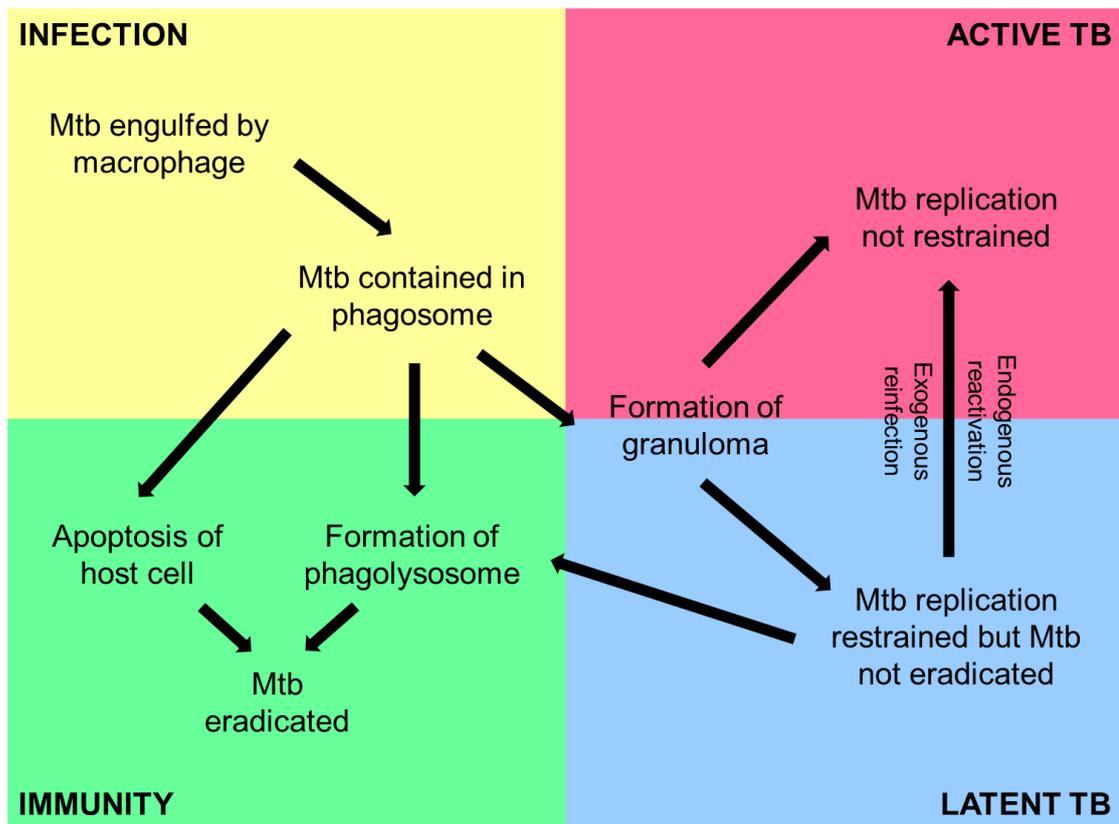
In some infected individuals, a proportion of ingested bacilli survive the innate immune response and replicate within the macrophages. Infected macrophages then spread via the lymphatic system to the hilar lymph nodes (Frieden *et al.*, 2003). A cell-mediated immune response is induced 2-8 weeks post-infection. This involves the release of cytokines, which leads to the recruitment of monocytes, lymphocytes, and neutrophils to the focal site of infection (Russell, 2007). CD8-positive T lymphocytes lyse infected host cells. Infected macrophages release interleukins 12 and 18 which stimulate the CD4-positive T lymphocytes to release interferon- γ , which in turn stimulates the phagocytosis of Mtb and the release of tumour necrosis factor- α by macrophages; the latter is a pro-inflammatory cytokine that seems to have a role in maintaining the organisation of the granuloma (Flynn *et al.*, 1995; Tufariello *et al.*, 2003).

The granuloma limits the replication and spread of Mtb. It is characterised by a relatively small number of infected macrophages, surrounded by lipid-laden macrophages and mononuclear phagocytes (Gonzalez-Juarrero *et al.*, 2001). These are enclosed by lymphocytes, and the periphery of the granuloma consists of a fibrous layer of collagen and other extracellular matrix components (Russell, 2007). The granuloma structure matures and a fibrous sheath develops. The subsequent reduction of blood supply to the centre of the lesion leads to cell death and necrosis. Tubercle bacilli in an unknown physiological state are thought to reside within macrophages at the necrotic centre of the granuloma during latent infection (Russell, 2007). Latently infected individuals show no signs of the disease and most cases do not develop active TB during their lifetimes (Tufariello *et al.*, 2003). They exhibit a delayed type hypersensitivity response to mycobacterial antigens; this forms the basis of the tuberculin skin test. Those who escape active disease may eventually reactivate this latent infection at some point during their lifetimes; this may be triggered by a change in the host immune status such as old age, malnutrition, or HIV infection. Individuals with latent infection can also be reinfected and develop active disease as a result of a new exposure (Figure 1.4).

If the infection is successfully contained, the granuloma will shrink and may eventually disappear, leaving behind a small scar or a calcified lesion (Dietrich and Doherty, 2009). When the host immune response fails to contain the initial replication of Mtb, the granuloma increases in its size and cellular content. If the granuloma is located close to the surface of the lung, the necrosis-induced tissue destruction can breach the mucosal

surface and the granulomatous contents spill into the lumen of the lung. This process, referred to as cavitation, gives rise to the characteristic symptom of persistent cough with blood in the sputum (Dietrich and Doherty, 2009). At this point, Mtb infection has progressed to active pulmonary TB. Individuals with active disease are infectious, spreading bacilli via the aerosol route. It was estimated that a newly infected young child has 10% lifetime risk of developing active TB (Comstock *et al.*, 1974).

Figure 1.4: Possible outcomes of Mtb infection



Ingestion of Mtb by the macrophage results in its killing by the innate immune response in most cases (green box). However, failure of this initial defence system leads to Mtb replication in the infected macrophage. In immunocompetent individuals, an adaptive immune response is mounted and the formation of a granuloma contains the infection. This results in latent infection and hosts are asymptomatic (blue box). Most latently infected cases do not develop active TB during their lifetimes; however, a change in the host immune status can reactivate latent infection. Active disease as a result of exogenous reinfection is also possible. When the immune system fails to contain the initial infection, active pulmonary TB develops and the bacilli can be disseminated via the bloodstream to other organ systems (pink box). Those with active disease are normally infectious, transmitting Mtb in respiratory aerosols.

The phenomenon of TB latency has long been recognised, but the physiological state of the persisting bacilli remains unknown. Some evidence suggests that these organisms have become dormant or do not grow *in vitro* under oxygen limitation (Wayne, 1994). The mechanism by which tubercle bacilli exit from this postulated physiological state remains poorly understood. However, a role for a secreted Mtb protein termed resuscitation-promoting factor (Rpf) has been proposed in recovery from dormancy. Mtb has five *rpf* homologues and mycobacterial Rpf s have been shown to promote the growth of *M. bovis* BCG exposed to a prolonged stationary phase (Mukamolova *et al.*, 2002). The exact mechanism by which Rpf s exert their growth stimulatory effect is not known, but lysosomal-like hydrolysis of bacterial cell wall has been hypothesised (Cohen-Gonsaud *et al.*, 2005; Mukamolova *et al.*, 2006). Mycobacterial cells that have been starved *in vitro* may be in a similar physiological state with bacilli that persist *in vivo* during latent TB (Parrish *et al.*, 1998). Quintuple Rpf mutants of Mtb have been shown to be unable to resuscitate in culture and attenuated in murine infection (Kana *et al.*, 2008). More recently, Mukamolova and colleagues (2010) reported the presence of Rpf-dependent bacilli in sputum samples, which may represent a population of non-replicating cells adapted to a physiological state for infection *in vivo*. The possible existence of dormant Mtb during latent infection has also been linked to the intracellular accumulation of triacylglycerol in the form of lipid bodies (Garton *et al.*, 2008).

1.3.4 Clinical manifestations of tuberculosis

The most common clinical manifestation of primary progressive infection with Mtb is pulmonary TB, which frequently affects the parenchyma of the mid and lower lungs. Primary disease can also manifest itself in extrapulmonary sites, particularly in the hilar lymph nodes or as disseminated lesions. Although more common among those infected with HIV, extrapulmonary TB accounts for about 20% of disease in HIV-negative individuals (Shafer *et al.*, 1996). Lymphatic TB is particularly common in HIV-negative women and young children (Rieder *et al.*, 1990). TB of the central nervous system manifests itself as inflammation of the meninges and tuberculomas of the brain. Both young children and HIV-infected individuals have increased risk of developing tuberculous meningitis (Berenguer *et al.*, 1992), which can be fatal due to serious neurological manifestations. Both pleural and disseminated TB occur as a result of either primary progressive disease or reactivation of latent infection. For the latter,

pulmonary involvement manifests itself in a miliary (millet seed) pattern rather than an infiltrate in most cases, however not all disseminated cases have pulmonary involvement. TB can also affect the bones and joints, but the spine (Pott's disease) is the most common bony structure involved. Genitourinary TB is not common and is difficult to distinguish from other infections of the genitourinary tract. It is an important cause of infertility in women in areas with high endemicity of TB (Goldfarb and Saimn, 1996).

1.3.5 Clinical diagnosis of tuberculosis

1.3.5.1 Primary progressive disease

Patients with persistent cough, lasting longer than two weeks, should be tested for TB. Other common symptoms include fever, night sweats, weight loss, shortness of breath, haemoptysis, and chest pain. HIV patients with TB, especially those late in the course of infection, generally show greater weight loss and fever (Harries *et al.*, 1996). Radiographic findings suggestive of TB include upper-lobe infiltrates, cavitary infiltrates, and hilar or paratracheal adenopathy. Some patients with active disease and those with HIV co-infection might give more subtle radiographic findings which can include lower-lobe infiltrates or miliary pattern. However, Perlman *et al.* (1997) reported that 8% of HIV patients with pulmonary TB had normal chest radiographs.

1.3.5.2 Latent infection

In 1890, Koch reported that he had isolated a substance from the tubercle bacilli that could “render harmless the pathogenic bacteria that are found in a living body and do this without disadvantage to the body”, and he called this substance tuberculin. After learning of Koch's discovery, Clemens Freiherr von Pirquet developed the tuberculin skin test in 1907 and used it to demonstrate latent TB infection in asymptomatic children. Charles Mantoux introduced the use of a cannulated needle and syringe for intracutaneous injection of tuberculin, while Florence Seibert developed purified protein derivative (PPD), the more consistent form of tuberculin. The Heaf test, named after Frederick Heaf, was the tuberculin skin test used in the UK until 2005. The test involves

the use of a gun to inject PPD (100,000 units/ml) to the skin over the flexor surface of the left forearm in a circular pattern of six. The Mantoux test is now used in the UK instead; it involves intradermal injection of 2 tuberculin units from the Statens Serum Institute tuberculin RT23 in 0.1-ml solution.

The tuberculin skin test is the widely used method for screening individuals for latent TB infection. However, it has low sensitivity in immunocompromised patients and low specificity due to cross-reactivity with the Bacille Calmette-Guérin (BCG) vaccine and environmental mycobacteria. There is also the need to return 2-3 days after the test for interpretation of results. The interferon- γ release assay, which assesses cell-mediated immunity to the T-cell antigens ESAT-6 and CFP-10, is an alternative method for screening individuals for latent infection. This type of T cell-based assay is not affected by previous BCG vaccination (Mazurek *et al.*, 2001). However, it may show low sensitivity in HIV-infected individuals due to diminished immunological responses (Converse *et al.*, 1997). The modified version of this assay, the enzyme-linked immunospot (ELISPOT) test, is relatively sensitive and specific. This test is marketed as T-SPOT.TB by Oxford Immunotec; it counts the number of activated T cells, which is translated from the number of spots observed in a well of a microtitre plate post-enzyme-linked immunosorbent assay. The ELISPOT assay was used to screen contacts for latent infection during the 2001 school-associated TB outbreak in Leicester, UK (Ewer *et al.*, 2003).

1.3.6 Treatment of tuberculosis

In the late 19th and early 20th centuries, sanatoria were developed for the treatment of patients with TB. Besides providing comfort and relief, surgical collapse procedures such as pneumothorax and thoracoplasty were performed at the sanatoria to rest infected parts of the lungs and to close lung cavities. The discovery of para-aminosalicylic acid and thiosemicarbazone in the 1940s yielded the first therapeutic agents with efficacy in the treatment of TB, although they were only bacteriostatic. The first antibiotic effective against Mtb, streptomycin, was discovered by Albert Schatz, Elizabeth Bugie, and Selman Waksman in 1944. Isoniazid, the first oral mycobactericidal drug, was discovered in 1952, followed by rifampicin in 1957.

The objectives of TB treatment are cure without relapse, to prevent death, to stop transmission, and to prevent the emergence of drug resistance. The latter is achieved by combined chemoprophylaxis with two or more anti-tuberculous drugs. The TB treatment regimens recommended by WHO depend on the severity of disease and history of previous treatment. Standard short-course regimens can cure more than 95% of cases of new drug-susceptible TB. Almost all recommended regimens have two phases (Ormerod *et al.*, 1998). The initial intensive phase aims to kill actively growing and semi-dormant Mtb. At least two bactericidal drugs are required in this phase, common ones being isoniazid and rifampicin. The incorporation of pyrazinamide in most regimens allows the duration of treatment to be reduced from 9 to 6 months, while the inclusion of ethambutol is beneficial if there is a suspicion for initial drug resistance and if TB burden is high. The continuation phase aims to eliminate most remaining bacilli and reduce the numbers of treatment failures and disease relapses.

In the UK, two-month treatment with a combination of four drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) is recommended during the initial phase of pulmonary TB, followed by four-month treatment with isoniazid and rifampicin during the continuation phase (Ormerod *et al.*, 1998). These are all first-line anti-TB drugs which are administered orally. Chemotherapy may be given to contacts with strongly positive tuberculin reactions but without clinical or radiological evidence of TB (Ormerod *et al.*, 2000). In this context, daily administration of isoniazid for 6-12 months has been shown to be 60-90% effective in reducing the risk of progression from latent infection to active disease (Comstock, 1999). Treatment regimens for pulmonary TB are also effective against extrapulmonary TB; there are usually fewer bacilli in the latter case (Reichman and Hershfield, 2000).

Directly observed treatment (DOT), one of the components of the DOTS strategy recommended by WHO, is a standard practice in most countries. As non-adherence to treatment regimens is very common among TB patients, DOT involves a trained observer personally observes each dose of medication being swallowed by the patient. The objectives are to ensure high rates of treatment completion, to reduce the risk of development of drug resistance, and to prevent relapse (WHO, 2010a). The DOTS programmes were successful in curing 36 million TB cases between 1995 and 2008, with as many as 8 million lives saved (WHO, 2010b).

1.3.7 Vaccination against tuberculosis

The first vaccine against TB was developed by Albert Calmette and his associate Camille Guérin, in which *M. bovis* was attenuated after 230 continuous subcultures. Referred to as the BCG vaccination, it has since been extensively used as a live TB vaccine. Randomised and case-control trials have shown consistently high protective efficacy of BCG vaccination against serious forms of TB in children (e.g. meningitis and disseminated disease), but variable efficacy against pulmonary TB in adults (Reichman and Hershfield, 2000). BCG vaccination has also been reported to offer protection against NTM disease among children (Katila *et al.*, 1987). WHO recommends BCG vaccination for children at birth or at first contact with the healthcare services in areas with high prevalence of TB. The national policy in the UK recommends all infants aged 0-12 months living in areas with incidence of TB of 40 per 100,000 or greater to be BCG vaccinated (Leese, 2005). BCG vaccination should only be offered to older contacts if there are special occupational, ethnic, or travel risks.

BCG vaccine can be administered by the intradermal injection or by the multiple puncture percutaneous method. Adverse effects of BCG vaccination include local subcutaneous abscess and ulcers, suppurative lymphadenitis, and, more rarely, disseminated disease (Lotte *et al.*, 1988). This vaccination is not recommended for HIV-infected individuals (Ormerod *et al.*, 2000). Furthermore, BCG vaccination shows poor efficacy in recipients who have prior exposure to environmental mycobacteria (Smith *et al.*, 2000). Exposure of mice to *M. avium*, *M. scrofulaceum*, and *M. vaccae*, has been shown to block replication of *M. bovis* BCG, thereby preventing vaccine protection against Mtb (Brandt *et al.*, 2002). Despite continuous efforts to develop more effective TB vaccines, none has been identified to date. Gagneux and Small (2007) suggested that the significant geographical variation in the protective efficacy of BCG immunisation is partially attributed to regional differences in interaction between the vaccine strain and its human hosts. Therefore, the global Mtb population structure and host-specific pathogen adaptation may need to be taken into account when engineering and evaluating new vaccine candidates.

1.3.8 Emergence of drug-resistant *M. tuberculosis* strains

Drug-resistant TB arises due to inconsistent or partial drug treatment, non-adherence to treatment regimens, inappropriate prescription of treatment regimens, and unreliable drug supply. Multidrug-resistant TB (MDR-TB) is caused by *Mtb* strains that are resistant to at least isoniazid and rifampicin. WHO estimated that 5% of all TB cases have MDR-TB (WHO, 2010b). The rates of MDR-TB incidence are high in the former Soviet Union, the Baltic states, South Asia, and China, attributed to poor or failing TB control programmes in these regions. An alternative combination of first-line drugs (e.g. pyrazinamide, ethambutol, para-aminosalicylic acid, and rifabutin) should be prescribed, if there is good laboratory evidence and clinical history supporting its effectiveness (WHO, 2010a). Otherwise, treatment with second-line drugs, though more expensive and have more side effects, can be considered. These include injectable agents (e.g. kanamycin, amikacin, and capreomycin), fluoroquinolones (e.g. levofloxacin, moxifloxacin, and ofloxacin), and oral bacteriostatic agents (e.g. ethionamide, prothionamide, and cycloserine). The mismanagement of MDR-TB treatment has given rise to extensively drug-resistant (XDR) strains that are resistant to at least isoniazid and rifampicin, and additionally to any of the second-line drugs. At present, XDR-TB has been diagnosed in 57 countries worldwide (WHO, 2010b).

1.3.9 Co-infection with *M. tuberculosis* and HIV

In many parts of the world where AIDS is a pandemic, TB is the leading cause of death in persons infected with HIV. HIV infection and TB form a lethal partnership; each promoting the other's progress in a synergistic fashion. HIV weakens cell-mediated immunity by destroying CD4-positive T lymphocytes and therefore promotes the intracellular replication of *Mtb* and speeds up progression to active TB. Individuals who have TB-HIV co-infection have 20-40 times risk of developing active TB than those not infected with HIV (WHO, 2010b). HIV infection is also the most important risk factor known for the reactivation of latent TB infection (Selwyn *et al.*, 1989). Treatment regimens for TB-HIV patients are similar to pulmonary TB cases but the duration is often extended, although death is much more common during the process (Murray *et al.*, 1999).

1.4 Transmission of Tuberculosis

1.4.1 Early recognition of the infectious nature of tuberculosis

The idea that TB is contagious was possibly first suggested in 1790 by Benjamin Marten, who attributed the disease to “some certain species of animalcula” (Doetsch, 1978). In 1838, Georges Sand expressed the dichotomous views that TB was generally thought to be heritable in Northern Europe but was considered to be infectious in Southern Europe (Daniel, 2006). The infectious nature of TB was first demonstrated by the French military surgeon Jean-Antoine Villemin in his 1865 experiment (Daniel, 2006). Villemin inoculated a rabbit with a small amount of purulent exudate from a tuberculous cavity, originated from an individual who had died of TB. The rabbit was found to have extensive TB when sacrificed and autopsied three months following inoculation. At about the same time, Budd (1867) suggested that TB is spread by specific germs from the individuals suffering from the disease based on the epidemiology of TB in the community at that time. Several early efforts to control the spread of TB in the community then followed. In 1889, Herman Biggs suggested the notification of TB cases in New York City by care providers, which was applauded by Koch but opposed by both the public and the healthcare profession (Daniel, 2006). The development of sanatoria in the late 19th and early 20th centuries for the treatment of TB might reflect an intention to isolate patients with active disease from the general public (Davis, 1996).

1.4.2 Airborne transmission of *M. tuberculosis* in droplet nuclei

TB is a contagious disease that spreads through the air. When a person with pulmonary or laryngeal TB coughs, sneezes, sings or talks, aerosol droplets containing *Mtb* are generated and propelled into the air. Loudon and Roberts (1967) proposed that aerosol droplets were generated by stretching of strings or sheets of secretion between two moistened surfaces that are separated during coughing, and the shattering of these strings or sheets by the subsequent forceful blast of air. The moisture in the aerosol droplets evaporates to form droplet nuclei in less than a fraction of second

(Wells, 1934). Inhalation of droplet nuclei by another person may result in infection or disease.

The existence of droplet nuclei was first postulated by William Wells (1934). He referred to droplet nuclei as airborne infection, the evaporated version of respiratory droplets less than a tenth millimetre in diameter, depending primarily upon air for the buoyancy which kept them suspended for a long time and carried them over long distance. The production of minute droplet nuclei during coughing was described by Loudon and Roberts (1967). From Chapin's hypothesis that most bacterial diseases were spread by direct contact, Wells coined the term "airborne contagion", which means close enough contact for exchange of organisms by air between sick and healthy persons. He indirectly demonstrated that droplet nuclei were responsible for the epidemic spread of disease when he showed that ultraviolet (UV) irradiation of air in a school protected the students and staff during a measles outbreak (Wells, 1934).

When a person with open pulmonary TB coughs violently and frequently, thousands of *Mtb* droplet nuclei are introduced into a room space. The small size of droplet nuclei, 1-5 μm in diameter, enables them to remain suspended in the air for minutes to hours (Wells, 1934), and this facilitates transmission by brief, close contact to an infectious TB case (Raffalli *et al.*, 1996; Golub *et al.*, 2001). It is therefore not surprising that transmission of TB often occurs in spaces that are poorly ventilated and overcrowded such as slum housing, prisons, nursing homes, correctional facilities, and refugee camps.

1.4.3 Epidemiological models for tuberculosis transmission

Epidemiological models can be used to predict the number of new TB cases over time for a given number of susceptible individuals and index under defined ventilatory conditions. Most models for TB transmission assume the following: (1) all susceptible individuals have identical pulmonary ventilation rate, (2) the air in the room space is completely mixed and the tubercle bacilli are evenly dispersed throughout the room space, and (3) all individuals are equally susceptible to the infecting strain (Beggs *et al.*, 2003). One of the earliest epidemiological models used to predict airborne

contagion is the Mass Action model, which is based on the principle that the number of infections due to transmission per index case is a function of the number of susceptible individuals in the population (Riley, 1974). The Gammaitoni and Nucci model revolves around the change of the level of infectious particles in a room space with time (Gammaitoni and Nucci, 1997).

William Wells presented a method for quantifying the relative infectiousness of airborne agents in his book “Airborne Contagion and Air Hygiene”. He postulated the concept of the “quantum of infection”, and defined a quantum as being the number of infectious droplet nuclei required to infect $(1 - e^{-1})$ of susceptible persons (Beggs *et al.*, 2003). Wells stated that the number of persons “who become infected bears a Poisson relation to the number of infective particles which they breathe”, so that 63.2% of a population become infected when, on average, each susceptible person breathes in one quantum of contagion (Beggs *et al.*, 2003). Table 1.2 presents data on quanta production rates for individual TB cases and several TB outbreaks that have been analysed and reported. Based on the concept of the quantum of infection by Wells, Riley *et al.* (1978) devised a model which reflects the exponential behaviour of airborne infections in confined spaces. This model describes the probability of a susceptible individual becoming infected by inhaling a quantum of infection. In relation to TB, one quantum refers to one airborne infectious droplet nuclei which is capable of forming one pulmonary tubercle in the infected host.

Table 1.2: Quanta production rate data for TB cases and outbreaks

Description	Number of quanta produced per hour
Pulmonary TB patient	1.25
TB-HIV patient	8.2
Outbreak in office building	12.7
Laryngeal TB patient	60
Bronchoscopy-related outbreak	360
Outbreak related to irrigation of abscess	2,280
Autopsy-related outbreak	5,400
Intubation-related outbreak	30,840

Adapted from the article by Beggs *et al.* (2003) with permission from the International Union Against Tuberculosis and Lung Diseases. © 2003 IUATLD.

1.4.4 Use of animal models to sample *M. tuberculosis* aerosols

One of the earliest studies investigating the infectiousness of TB patients and transmission of the disease was performed by Richard Riley and colleagues at the Veterans Administration Hospital in Baltimore. This series of studies was the first to provide scientific proof that TB is spread by the airborne route. Their first report dealt with the preparation of a six-room ward attached to an animal exposure chamber, and its validation for both sampling performance and air hygiene (Riley *et al.*, 1957). The amount of fresh air entering the closed circuit air-conditioning system of the ward was carefully regulated and estimated to show the volume of air into which airborne particles would be diluted. Each room was equipped with fixtures for irradiating the upper air with UV light. The effectiveness of UV light in killing artificially atomised *M. bovis* was demonstrated. The animal exposure chamber was constructed in such a way so that the exhaust air from the ward passed through it before being vented. An important feature for this experimental setup was the equivalent infectivity between the air in the TB ward and the air in the exposure chamber. To assess this, Riley and co-workers aerosolised a broth culture of a standard strain of tubercle bacillus called Ravenel VA into the air-conditioning system of the ward to which rabbits and guinea pigs were exposed, in separate experiments. Consistent with what they desired for, similar numbers of tubercles were recovered from animals in the ward and those in the exposure chamber.

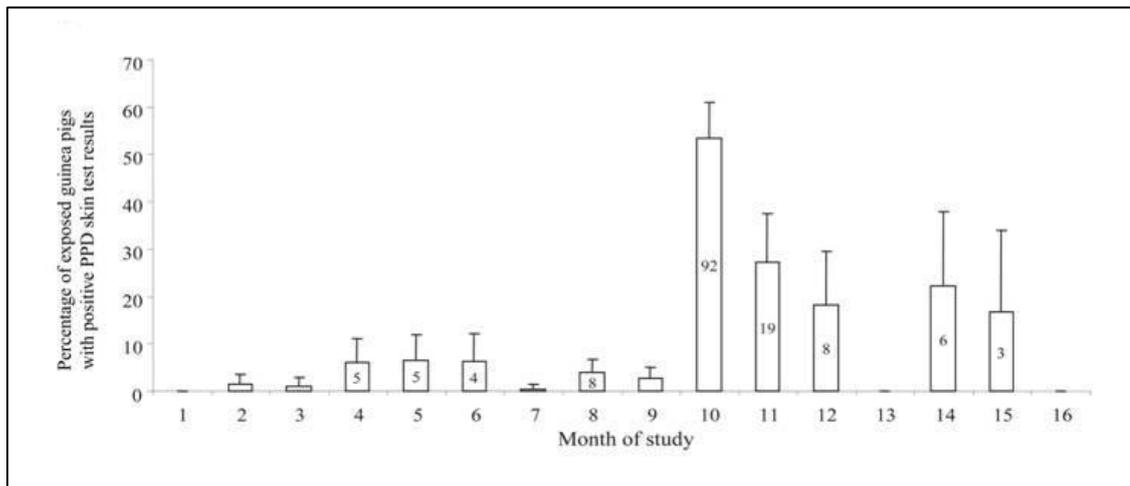
In the studies described below, the TB ward setup was used to investigate the infectiousness of TB patients. Under the conditions of these studies, the characteristic lesion resulting for airborne infection was a single pulmonary tubercle. Riley and colleagues enumerated the number of pulmonary tubercles in infected guinea pigs exposed to the ward air, which was theoretically correlated to the quantity of infectious *Mtb* droplet nuclei inhaled. Infection in guinea pigs was defined by monthly tuberculin skin tests. Patient and guinea pig tubercular infection was matched based on culture-based drug susceptibility profiles, biochemical tests, and temporal exposure patterns. Riley and associates (1959) presented evidence that the ward air was the source of the guinea pig infections. This was further supported by outcomes from UV irradiation of air travelling from the ward to the animal exposure chamber (Riley *et al.*, 1962). The possibilities of guinea pig infections originating from external sources and cross infection among animals were ruled out.

Riley and co-workers found that a relatively small number of TB patients were responsible for the majority of guinea pig infections. In one study, 2 out of 62 patients produced 19 out of 22 infections, and this was attributed to the high levels of smear positivity of their sputa (Riley *et al.*, 1959). Infectivity for guinea pigs exposed to their artificially atomised sputa was also high (Riley *et al.*, 1959). In the next study, 35 of 48 infections were attributable to only 3 out of a total of 77 patients (Sultan *et al.*, 1960). Although these 3 patients had high numbers of Mtb in their sputa, so did other patients who were not associated with transmission. The patient sources of 6 infections could not be identified due to delay in obtaining drug susceptibility results. Sultan and colleagues (1960) also proposed the existence of 'dangerous disseminators' and 'super disseminators'; highly infectious patients gave 1.6 times more days of smear positivity and 2.4 times more days of culture positivity. In another study, only 8 out of 61 patients produced all the infections (Riley *et al.*, 1962). These infectious patients were untreated and had drug-susceptible TB. One of these patients occupied the ward for only 3 days but produced 15 animal infections. This untreated patient had acute laryngeal TB with high sputum smear positivity and severe coughing. It was estimated that he produced 60 quanta per hour; his infectiousness was compared to an average child in the infectious stage of measles who produced only 18 infectious units of airborne measles per hour (Riley *et al.*, 1962). Other patients with high level of smear positivity and extended residence in the ward did not produce any infections. The reasons for the differences in the infectiousness among the patients were not known, apart from suspecting difference in hygienic manners among patients during coughing and variation in resistance to physical stresses of aerosolisation among Mtb strains.

Riley *et al.* (1959) estimated that each patient produced 2-3 quanta per day on average, which was consistent with Chapin's statement that very few tubercle bacilli present in the vicinity of TB patients. They also estimated that a nurse breathing the ward air would take a year to develop infection, which was again consistent with Chapin's statement that the air was not very dangerous to breathe for a short time. Taking into account these estimations, they concluded that the low level of airborne Mtb in spaces occupied by pulmonary TB patients was adequate to account for spread of the disease, at least in healthcare workers.

Escombe and co-workers (2007) recreated the original guinea pig air-sampling model of Riley *et al.* (1959) to investigate the airborne transmission of Mtb from HIV-positive patients in Lima, Peru. Their experimental set-up is shown in Figure 1.6A. A total of 97 patients were recruited and admitted to a mechanically ventilated HIV-TB ward. Ward air was exhausted via ducts to the animal facility on the roof where the guinea pigs were exposed. They demonstrated that the infectiousness of the ward air varied greatly throughout the study period, which reflects the varied infectivity among the patients (Figure 1.5). The ward air was of relatively low infectiousness over the first 9 months, with less than 7% of exposed guinea pigs developing positive tuberculin reactions each month. Furthermore, no tuberculin-positive animals were observed in months 13 and 16. In contrast, ward air was highly infectious prior to month 10, when 92 animals (53%) were tuberculin-positive. The average patient infectiousness each month was highly variable, from production of 0 to 44 quanta per hour. These infectiousness data were well correlated to the theoretical risks of acquiring TB among healthcare staff breathing the ward air.

Figure 1.5: Variation in the infectiousness of the ambient air of the HIV-TB ward

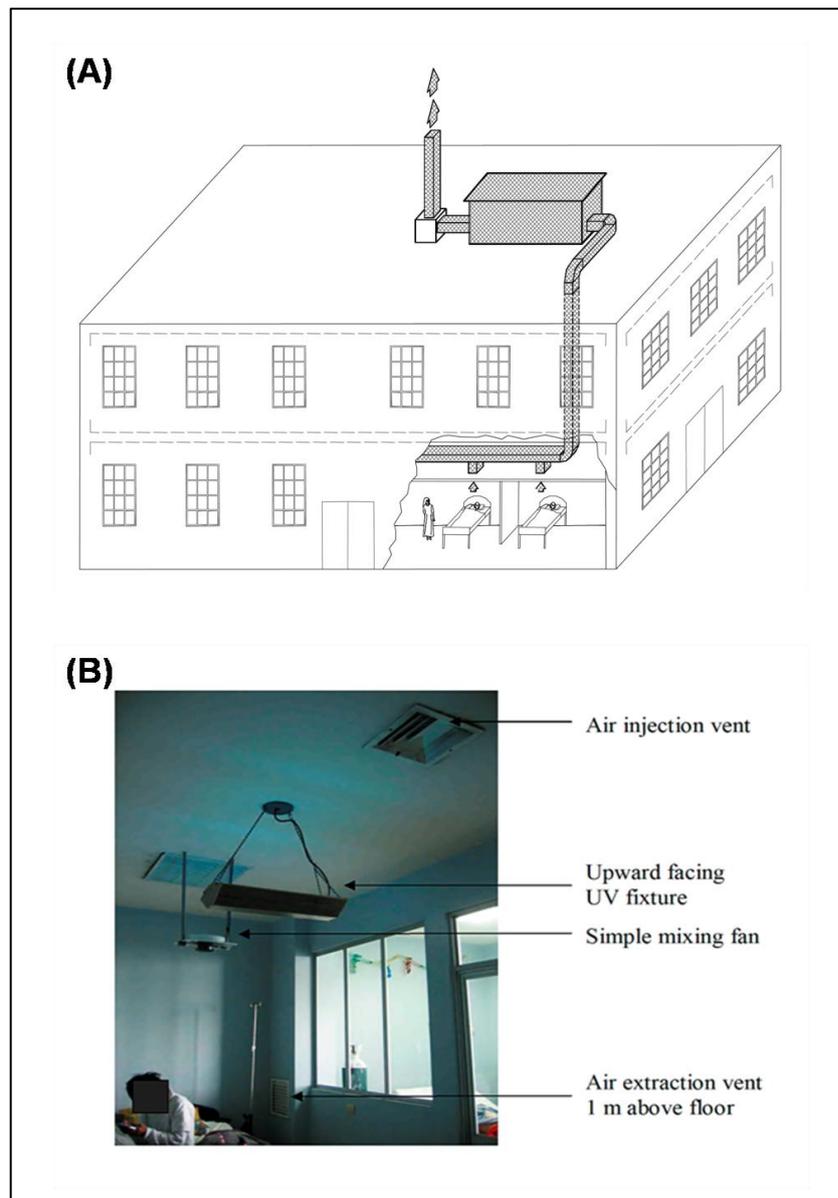


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The guinea pig air-sampling model was used to evaluate upper-room UV light irradiation (Figure 1.6B) for prevention of nosocomial TB transmission, and indeed it was shown to reduce the infectiousness of air from TB wards. Riley *et al.* (1962) showed that there was not one infection among the guinea pigs exposed to the ward

air treated by UV light. In the study by Escombe *et al.* (2009), 35% of guinea pigs in the control group were infected, and this was reduced to 10% by UV irradiation. Active TB was observed in 9% of control animals, and this was reduced to 4% by UV treatment.

Figure 1.6: Guinea pig air-sampling model to study the infectiousness of HIV-TB patients



(A) Schematic illustration of the animal facility on the roof of the HIV-TB ward. (B) Upper-room UV light fixture in the HIV-TB ward. Adjacent to the fixture is a simple mixing fan used to assist mixing of the lower- and upper-room air. A: Reproduced from the article by Escombe *et al.* (2007) with permission from the University of Chicago Press. © 2007 by the Infectious Diseases Society of America. All rights reserved. B: Reproduced from the article by Escombe *et al.* (2009).

1.4.5 Alternatives to animal studies in characterising *M. tuberculosis* aerosols

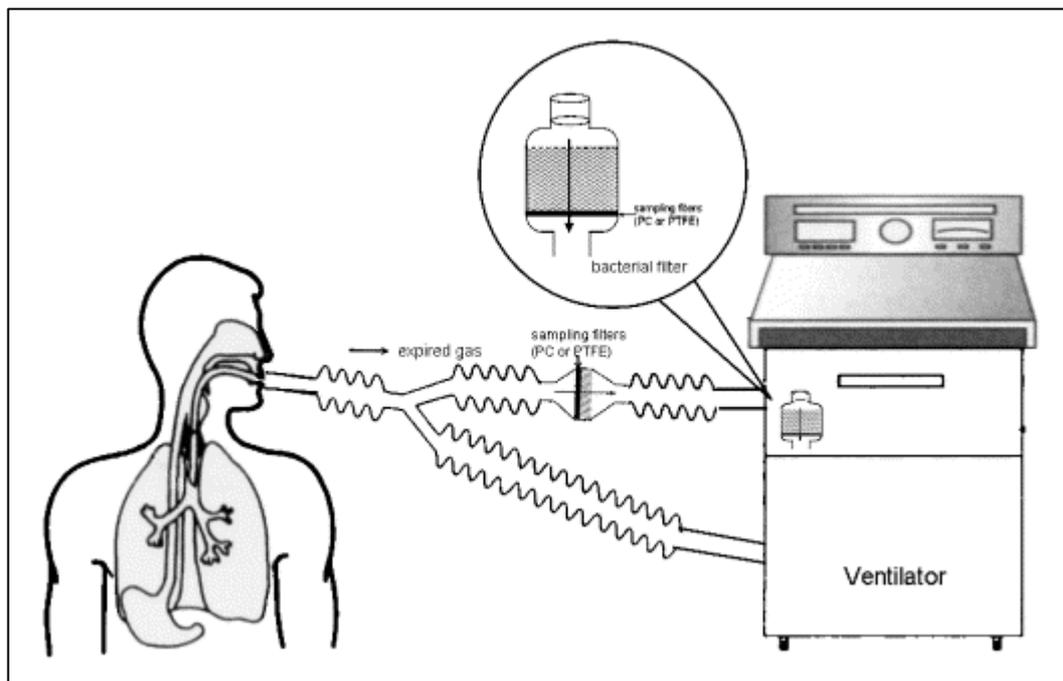
1.4.5.1 Use of conventional PCR for the detection of *M. tuberculosis* aerosols

Mastorides *et al.* (1999) pioneered the use of conventional PCR to detect the presence of Mtb droplet nuclei in the vicinity of TB patients in respiratory isolation rooms. They used a small laboratory pump placed near the patient's bed to draw ambient air through a 0.2- μm polycarbonate membrane filter over a 6-hour period. The filter was then subjected to a one-tube nested PCR and the Mtb-specific sequence was detected by a nucleic acid probe hybridisation assay. Mtb was successfully detected by PCR in 6 of 7 culture-positive patients, but none in 3 patients who were culture-negative. One of these PCR-positive patients was smear-negative at the time of air sampling. The detection of Mtb in the air of isolation rooms raised doubts on the effectiveness of the air handling mechanisms and filtration units of the facility in minimising the level of circulating microorganisms, which might have important implications on nosocomial transmission. The authors proposed the use of their PCR air sampling method to fuel better understanding on the transmission of TB in environments with closed ventilatory systems where large numbers of people are involved, such as aboard ships and aircrafts.

A modified PCR air sampling method was used to detect the presence of Mtb in hospital air samples by Vadrot and co-workers (2004). They sampled ambient air in five hospital rooms housing one TB patient each onto agarose gels over an 8-day period and downstream detection of Mtb was achieved using the Roche Amplicor test. Three smear-positive patients who were coughing gave positive PCR. The remaining two patients, one smear-positive and the other smear-negative, were not coughing and their PCR was negative.

Direct sampling of *Mtb* aerosols from mechanically ventilated TB patients for PCR detection was performed by Wan and associates (2004). They collected the exhaled air from the patients and exhaust air passing through a bacterial filter in the mechanical ventilator onto two sets of polycarbonate or polytetrafluoroethylene filters over the duration of 8 hours, in order to simulate exposure among healthcare workers during working period (Figure 1.7). Among the polytetrafluoroethylene and polycarbonate filters, 75% and 25% of the exhaled air samples were PCR-positive, respectively. These were 14% and 57% respectively for the exhaust air samples. The latter raised the concern on periodic replacement of bacterial filters in the mechanical ventilator. Additional air sampling in the respiratory isolation rooms and outpatient areas gave positive PCR in 40-60% of the filters sampled.

Figure 1.7: Sampling of respiratory-borne *Mtb* aerosols from mechanically ventilated TB patients on membrane filters for PCR detection



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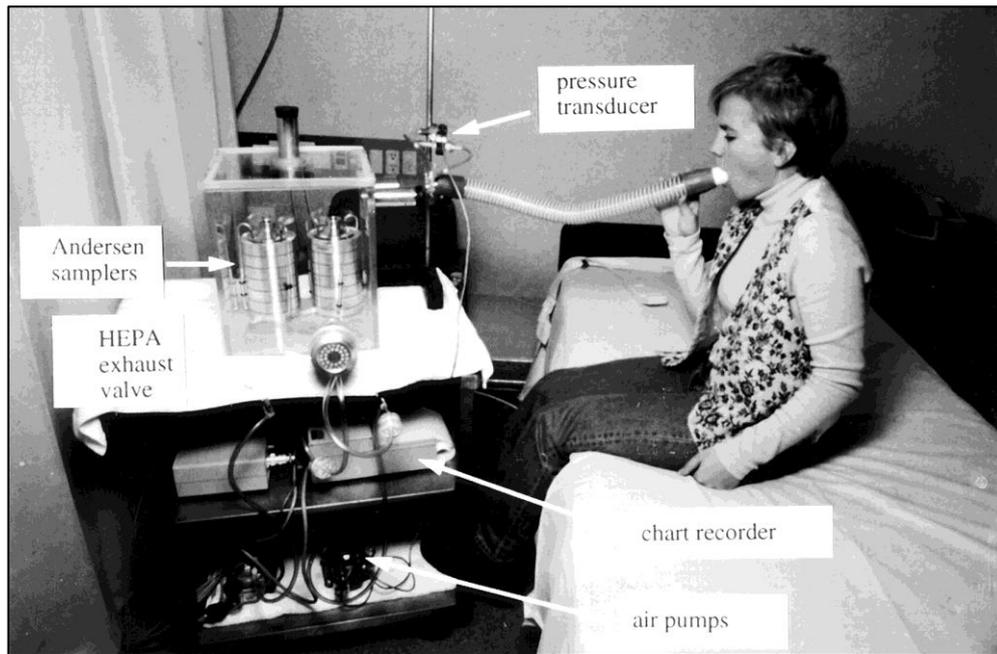
Due to its non-quantitative nature, the use of conventional PCR for detection of airborne *Mtb* in the studies described above does not provide comprehensive information regarding transmission risks posed by TB patients. One study reported the

use of real-time PCR coupled to an air-sampling filter method for quantification of airborne Mtb in respiratory isolation rooms (Chen and Li, 2005). The measured Mtb concentrations in a total of 22 air samples collected from the rooms of six patients varied widely, from 14 to 2×10^5 target gene copies/m³. The discordance between airborne Mtb levels and sputum smear and culture results was evident in some patients during their study, thus raising the question on which of these is a better predictor for TB infectiousness. The findings from these molecular-based studies indicate that the risks posed by the presence of circulating Mtb aerosols in the ambient air of general wards and employee areas should be assessed, taking into account the immunocompetency of individuals exposed in addition to the duration of exposure.

1.4.5.2 Culturable cough-generated aerosols of *M. tuberculosis*

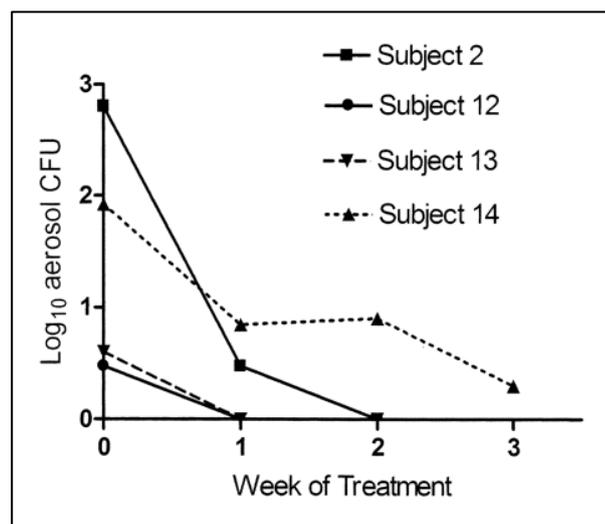
Fennelly *et al.* (2004) pioneered the direct isolation, quantification, and particle size determination of Mtb aerosols expectorated by TB patients. Patients were instructed to cough for 5 minutes into a closed chamber housing the Andersen air samplers in which culturable Mtb aerosols were collected (Figure 1.8). Aerosol colony-forming units (CFU) were confirmed by nucleic acid probes and restriction fragment length polymorphism (RFLP) analyses, which were also performed on subcultures from sputa in parallel. Cough-generated tuberculous aerosols were detected in only 4 out of 16 smear-positive patients, suggesting the variability in the ability to produce potentially infectious aerosols among different patients. The infectiousness of these patients ranged from 18 to 3,798 infectious particles per hour. Fennelly and co-workers also found a trend in the correlation between cough frequency and the quantity of tuberculous aerosols expectorated among these patients. The size distributions of these aerosols were variable but most were 3.3 μm and below, which were immediately respirable. There was a rapid reduction in the culturable aerosols within the first 3 weeks of effective chemotherapy for all the four patients, thus suggesting an association between aerosol production and lack of treatment in the preceding week(s) (Figure 1.9). There were no significant associations between levels of tuberculous aerosols and sputum smear or culture grades, or presence of lung cavitation. Most patients in this study had MDR-TB and were studied during sputum-induction procedures, and therefore their data may not be representative of most patients with drug-susceptible pulmonary TB.

Figure 1.8: Cough aerosol sampling system



Cough-generated aerosols were sampled onto Middlebrook 7H11 agar plates positioned on different stages of two Andersen cascade impactors housed within a closed Plexiglas chamber. Reproduced from the article by Fennelly *et al.* (2004) with permission from the American Thoracic Society. Copyright © American Thoracic Society.

Figure 1.9: Changes in cough-generated Mtb aerosol cultures over the course of anti-tuberculous chemotherapy



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1.4.6 Factors associated with tuberculosis transmission

The experimental studies described in Sections 1.4.4 and 1.4.5.2 reported that the ability to transmit infection varies markedly among different TB patients. This was also shown by contact studies based on the tuberculin skin test and molecular epidemiology approach (Alland *et al.*, 1994; Small *et al.*, 1994a). Apart from knowing that the AFB smear status is a strong predictor for case infectiousness, the determinants of the transmissibility for Mtb are not completely understood.

1.4.6.1 Acid-fast bacilli smear status

When positive for Mtb, it is known that AFB smear-positive patients are more infectious than those who are smear negative. The level of smear positivity might determine the number of bacilli put into airborne suspension. An untreated smear-positive case has been claimed to infect approximately 10 people per annum (Styblo, 1978). Rare outbreaks involving more than 200 secondary infections have also been reported (Raffalli *et al.*, 1996). One of the earliest studies demonstrating the relative infectiousness of smear-positive cases was performed by Shaw and Wynn-Williams (1954). They showed that 65% of children exposed to smear- and culture-positive individuals gave positive tuberculin reactions, while this was 27% and 18% respectively for smear-positive and smear-negative cases that were culture-negative; non-contacts gave a reactor rate of 22%. Similar proportions were reported by Loudon and Spohn (1969). Among culture-positive patients, Shaw and Wynn-Williams (1954) also showed smear-positive cases were more infectious than those who were smear-negative. One study found that progression to active TB is more common among tuberculin-positive contacts of smear-positive cases than those of smear-negative cases (Grzybowski *et al.*, 1975).

AFB smear status is used to gauge the infectiousness of patients with pulmonary TB and in most hospitals; this is the basis on which respiratory isolation should be continued or discontinued. This approach has proven useful in preventing nosocomial and community outbreaks. Transmission of TB from smear-negative index cases has been reported previously (Catanzaro, 1982; Di Perri *et al.*, 1989). One very early study

documented that 53% of children (aged 19-24 months) who were in contact with smear- and culture-negative patients had positive tuberculin reactions (Blahd *et al.*, 1946). The epidemiological study in San Francisco showed that smear-negative patients were responsible for 17% of TB transmission, although the relative transmission rate was higher for those who were smear-positive (Behr *et al.*, 1999). Also, data from several Mtb aerosol studies doubted the reliability of AFB smear grade in predicting infectiousness of TB patients (Sultan *et al.*, 1960; Fennelly *et al.*, 2004; Chen and Li, 2005). Therefore, isolation and discharge decisions should not be made solely on the basis of smear status, but should take other determinants into consideration as well.

1.4.6.2 Anti-tuberculous chemotherapy

Apart from decreasing the number of bacilli in the patient's sputum or saliva (Yeager *et al.*, 1967), chemotherapy introduces drugs into the droplet nuclei. The evaporation of respiratory aerosol droplets to droplet nuclei shrinks them to less than a thousandth of their original size. The concentration of drugs in the saliva and bronchial secretions is similar to that in the bloodstream (Elmendorf *et al.*, 1952). With the evaporation of the moisture, the tubercle bacilli in the droplet nuclei are exposed to a thousand-fold increase in the concentration of the drugs, and in this case survival in the airborne state is governed by their drug resistance (Riley *et al.*, 1959). However, isoniazid-susceptible bacilli in droplet nuclei containing isoniazid were shown to be alive after suspended in the air for 12 hours (Loudon and Spohn, 1969).

Rapid loss of infectiousness has been noted among TB patients undergoing effective anti-tuberculous chemotherapy (Sultan *et al.*, 1960; Yeager *et al.*, 1967; Rouillon *et al.*, 1976; Fennelly *et al.*, 2004). In the study by Riley *et al.* (1962), the relative infectivity of one patient prior to treatment was 3.6 quanta per month; this was reduced to 0.7 quantum following treatment. In medical practice, it is traditionally believed that two weeks of chemotherapy is adequate to render a TB patient sufficiently non-infectious. Therefore, extended isolation of patients beyond this period is uncommon in most hospitals. Laboratory diagnosis of drug resistance cannot be established within two weeks by culture-based methods, and response to standard chemotherapy among

drug-resistant TB patients may be slower or non-existing. The release of drug-resistant patients onto general wards after two weeks of standard therapy has been reported to fuel nosocomial transmission in the USA (Beck-Sague *et al.*, 1992). Farer (1973) wrote, "There is no way to determine an absolute moment at which a patient on therapy becomes non-infectious. Decisions regarding the infectiousness of an individual patient must be individualised for that patient."

1.4.6.3 Expiratory manoeuvres

Loudon and Roberts (1967) were one of the first to carry out investigations to determine the numbers and sizes of particles produced by different individuals during different expiratory manoeuvres. They compared between coughing (series of 15 coughs) and talking (counting loudly from 1 to 100). The subjects, having previously swabbed inside their mouths with a dye, made the appropriate expiratory manoeuvres into an air-tight box through its entry port. A single cough was shown to produce as many droplet nuclei as about 5 minutes of loud continuous talking; the former yielded a higher proportion of droplets that remained airborne after 30 minutes. The numbers of droplets generated by coughing varied between experiments, possibly depending on the amount of secretion present in the mouth and its location, and the placement and movement of lips, tongue, and teeth during coughing. This also suggested that the ability to disperse large cloud of droplet nuclei might depend on the method of coughing habitual to a person, and that incidents of occasional massive dispersion might occur as a result of one cough that is accidentally abnormally productive.

Aerosolisation efficiency governs the amount of Mtb excreted, which might be related to infectiousness; it depends on cough frequency, violence of the cough, and the care with which the mouth is covered while coughing. This last factor was discussed in the study by Riley and associates with reference to the social behaviour of some of the highly infectious patients who might have been careless in covering their mouths during coughing (Riley *et al.*, 1959). In contrast, one quiet and well-mannered patient who had highly infectious sputum probably only infected two guinea pigs. The role of patients' hygienic practice in the spread of TB was also suggested by Chen and Li (2005);

highest levels of airborne Mtb were recorded in the isolation room of one patient who frequently spit sputum and did not cover his mouth when coughing and talking.

There is a close association between cough frequency and the amount of Mtb aerosols excreted; the latter can be related to the infectiousness of TB cases. Loudon and Spohn (1969) showed that 44% of contacts of patients who coughed more than 48 times per night were tuberculin converted as opposed to 27% for those who coughed less than 12 times per night. They reported more than 50% drop in cough count after two weeks of chemotherapy. This decline might represent one of the mechanisms by which chemotherapy decreased the infectiousness of patients and cough count might therefore be used to support decision on patient release from respiratory isolation. However, they demonstrated that cough frequency at the time of hospital admission was less predictive of patient infectiousness than radiological extent and bacteriological status of disease. Furthermore, cough count on admission was not necessarily representative of that during the contact exposure. Vadrot and colleagues (2004) reported the correlation between presence of coughing and PCR positivity of air samples from hospital rooms housing TB patients.

Loudon and Roberts (1967) reported that singing was similar to coughing in terms of the quantity and size of droplet nuclei that were still present after 30 minutes. In a boarding school outbreak, rates of infection were higher among students who were in the choir with the index case than among those who shared a dormitory room or meals with the case but were not in the choir (Bates *et al.*, 1965).

1.4.6.4 Co-infection with tuberculosis and HIV

HIV infection has a poorly defined effect on TB infectiousness, with conflicting results from household contact studies for HIV-positive and HIV-negative patients (Cruciani *et al.*, 2001). One study suggested that HIV-positive TB cases may be more infectious; seven TB cases occurred among employees caring for 85 HIV-infected patients, while only two cases occurred among those who took care of 1,079 HIV-negative patients over the same period (Di Perri *et al.*, 1993). Furthermore, Escombe and colleagues

(2007) reported a mean TB infectiousness of 8.2 quanta per hour among HIV-positive patients in their study, and compared this to an average of 1.25 produced by HIV-negative patients studied by Riley and co-workers (Riley *et al.*, 1959; Sultan *et al.*, 1960; Riley *et al.*, 1962). However, a study in Zaire documented no difference in tuberculin conversion rates among contacts of HIV-infected and HIV-uninfected TB patients (Klausner *et al.*, 1993). Another study in Zambia found that HIV-infected TB cases are less infectious; 52% of contacts of HIV-positive patients were tuberculin-positive, versus 71% of contacts of those uninfected (Elliott *et al.*, 1993). Among HIV-infected individuals, delay in diagnosis due to atypical clinical presentation may result in increased transmission of TB. However, decreased lung cavitation and sputum bacillary load may reduce infectiousness in HIV-positive TB cases (Elliott *et al.*, 1993).

1.4.6.5 Extrapulmonary tuberculosis

There have been reports on transmission of TB from extrapulmonary patients where most cases appeared to be due to aerosolisation of abscess material (Hutton *et al.*, 1990; Frampton, 1992). However, there is the possibility that these patients might have subclinical pulmonary disease. The high transmission potential of individuals with laryngeal TB has been reported. A laryngeal TB patient in the study by Riley and colleagues (1962) produced 15 animal infections within three days of ward occupancy. As many as 23 students exposed to the index case with laryngeal TB in the study by Braden and associates (1995) were tuberculin converted; contact duration was less than 5 hours in all cases. Although uncommon, it has been reported that TB transmitted via the blood through needlestick injuries (Kramer *et al.*, 1993). The current understanding on the transmission potential of extrapulmonary TB cases is far from satisfactory.

1.4.6.6 Characteristics of tubercle bacilli and sputum

Infectiousness of TB patients might be influenced by properties of aerosolised materials, which in turn depend on the sputum bacillary load, the amount of sputum, and the physical characteristics of the sputum. The latter includes the presence of

antibiotics as discussed in Section 1.4.6.2. In a study by Sultan *et al.* (1960), one TB patient had cough and sputum production that were comparable or exceeded by the other patients but was highly infectious. However, his sputum was noted to be exceptionally liquid, a property which might contribute to better aerosolisation.

The phenotypes of infecting Mtb strains might also have important implications for the infectiousness of TB patients. Different strains of Mtb might vary in their ability to resist the physical stress of aerosolisation and survive dehydration in the airborne state, and therefore have different levels of infectiousness. In this regard, Ratcliffe (1952) estimated that more than 99% of bacilli were lost almost immediately following aerosolisation. Perhaps strains differ in this initial survival and this might be reflected in differing levels of dissemination. Molecular fingerprinting analysis demonstrated that one Mtb strain, designated strain C, caused 10% of cases in New York City from 1991 to 1994 (Sepkowitz, 1996). Similarly, Valway *et al.* (1998) reported a community TB outbreak where extremely high tuberculin conversion rates were recorded among contacts and also demonstrated rapid growth of the infecting Mtb strain in a mouse model. This strain was later on referred to as Mtb CDC 1551 (Fleischmann *et al.*, 2002). Recently, the non-replicating phenotype of bacilli in the sputum has been associated with intracellular accumulation of lipids (Garton *et al.*, 2008) and Rpf-dependent dormancy (Mukamolova *et al.*, 2010), which has been proposed as a means of adaptation for onward transmission to new hosts.

Riley and colleagues (1957) demonstrated that an isoniazid-resistant strain of tubercle bacillus isolated from a patient yielded larger number of culturable droplet nuclei following aerosolisation and produced about four times more pulmonary tubercles in guinea pigs, compared to their standard test strain (Ravenel VA). This could possibly reflect the higher resistance of the former to the rigor of aerosolisation. Sultan *et al.* (1960) documented that TB patients harbouring drug-resistant bacilli gave 1.4 and 1.2 times more days of smear and culture positivity, respectively. They also reported that infection of guinea pigs with resistant bacilli was 23 times more frequent than with drug-susceptible ones, which could be rather due to exposure to high concentration of drugs in the droplet nuclei. However, Riley *et al.* (1962) demonstrated that untreated patients with drug-resistant TB were less infectious than those with drug-susceptible bacilli.

1.4.6.7 Duration and proximity of exposure

There is evidence of association between duration and proximity of exposure and transmission of TB. A smear-positive index case has increased risk of transmitting Mtb to other individuals in close contact if the exposure time exceeds 8 hours (Ormerod *et al.*, 2000). This may reflect extended exposure to a low concentration of Mtb in aerosols or the cumulative probability that the index case will produce highly infectious aerosols at some point during the exposure period; the former is consistent with a proposed epidemiological model of TB transmission (Beggs *et al.*, 2003) and the latter is indicative of the possible intermittent nature of Mtb aerosol excretion.

Various reports on transmission in hospitals, on school buses, in classrooms, and during long-haul flights and voyages were published. TB transmission has also been documented in overcrowded and confined environments such as in the prisons, rehabilitation centres, homeless shelters, and care homes. The importance of proximity of exposure was also demonstrated by higher rates of Mtb infection among spouses of index cases as opposed to other family members (Spector, 1939; Hahn, 1943).

In a school bus outbreak where the index case was the driver, 83 of 258 children who rode the bus were tuberculin-converted and 51 acquired active TB (Rogers, 1962). As many as 57% of children riding more than 40 minutes per day had tuberculin-positive reactions, compared to 22% riding less than 10 minutes per day. In one MDR-TB outbreak aboard a trans-oceanic commercial airplane flight, tuberculin conversion occurred in passengers who sat very close to the index case, while those in the forward part of the aircraft were tuberculin-negative (Kenyon *et al.*, 1996).

In a hospital outbreak in England, a mother who spent at least 9 hours per day with her child who had tuberculous abscess, developed cavitary TB (George *et al.*, 1986). Of 129 exposed children, 12 of 82 who had spent 1-2 days as their roommates were tuberculin-positive, compared to 9 of 27 with 3-7 days of exposure, and 8 of 14 with more than 8 days of exposure. Rates of disease were proportional to the duration of

exposure. The close association between the duration of exposure and transmission of TB was also predicted by an epidemiological model (Beggs *et al.*, 2003).

1.4.6.8 Exposure due to closed ventilation

TB transmission associated with closed ventilation has been reported aboard ships (Houk *et al.*, 1968), on school buses (Rogers, 1962), in classrooms (Sacks *et al.*, 1985), and in bars (Kline *et al.*, 1995). During an outbreak aboard the USS *Byrd*, 139 of 308 crew members were tuberculin-converted and TB developed in 7 of them (Houk *et al.*, 1968). Infection was spread by recirculation of contaminated air along closed ventilatory circuits. As many as 79% of crewmen sleeping in the compartments housing six index cases were tuberculin-positive, compared to 57% in the next compartment which shared ventilation with the first. In Miami, a patient with undiagnosed pulmonary TB spread infection to 21 of 60 employees who were exposed for only 57 hours (Ehrenkranz and Kicklighter, 1972). This might be due to circulation of air from the patients' rooms into the central-corridor employee area.

1.4.6.9 Clinical procedures

Clinical procedures that generate large amounts of aerosols are significant risks for TB transmission; these include bronchoscopy or intubation, sputum induction, nebuliser treatment, or abscess irrigation (for extrapulmonary TB). The amounts of quanta produced with these procedures may be so large that dilution by ventilation alone is insufficient to decrease the risk of infection (Table 1.2). An intubated patient with advanced pulmonary TB spread infection to 16 of 112 employees after being present in the emergency department for only 4 hours; the enhanced transmission could be attributed to frequent endotracheal suctioning procedures (Haley *et al.*, 1989). An outbreak related to irrigation of a hip abscess was reported in a hospital in Arkansas in 1985 (Hutton *et al.*, 1990). Therefore, aerosol-generating procedures should be performed in appropriate engineered and ventilated areas, and healthcare workers must wear respirators when conducting them.

1.4.6.10 Susceptibility of tuberculosis contacts

The success of TB transmission from source cases depends on the susceptibility of the contacts to Mtb infection. Previous infection with Mtb is believed by some to provide immunity against second infection, although exogenous reinfection is possible. A meta-analysis demonstrated that BCG vaccination status of contacts may also influence TB transmission; it halved the rates of disease in this case (Colditz *et al.*, 1994). HIV infection is the single greatest risk factor for development of active TB, both in cases of reactivation of latent infection (Selwyn *et al.*, 1989) and in the development of acute disease (Daley *et al.*, 1992). Exogenous reinfection among HIV-infected persons has also been reported (Small *et al.*, 1993). Other medical conditions that compromise the immune system are also important; these include old age, poorly controlled diabetes mellitus, renal failure, underlying malignant disease, chemotherapy, extensive corticosteroid therapy, malnutrition, and deficiency of vitamin D or A (Frieden *et al.*, 2003). Occupations can also be a risk for acquiring Mtb infection, for instance among TB healthcare workers (Meredith *et al.*, 1996).

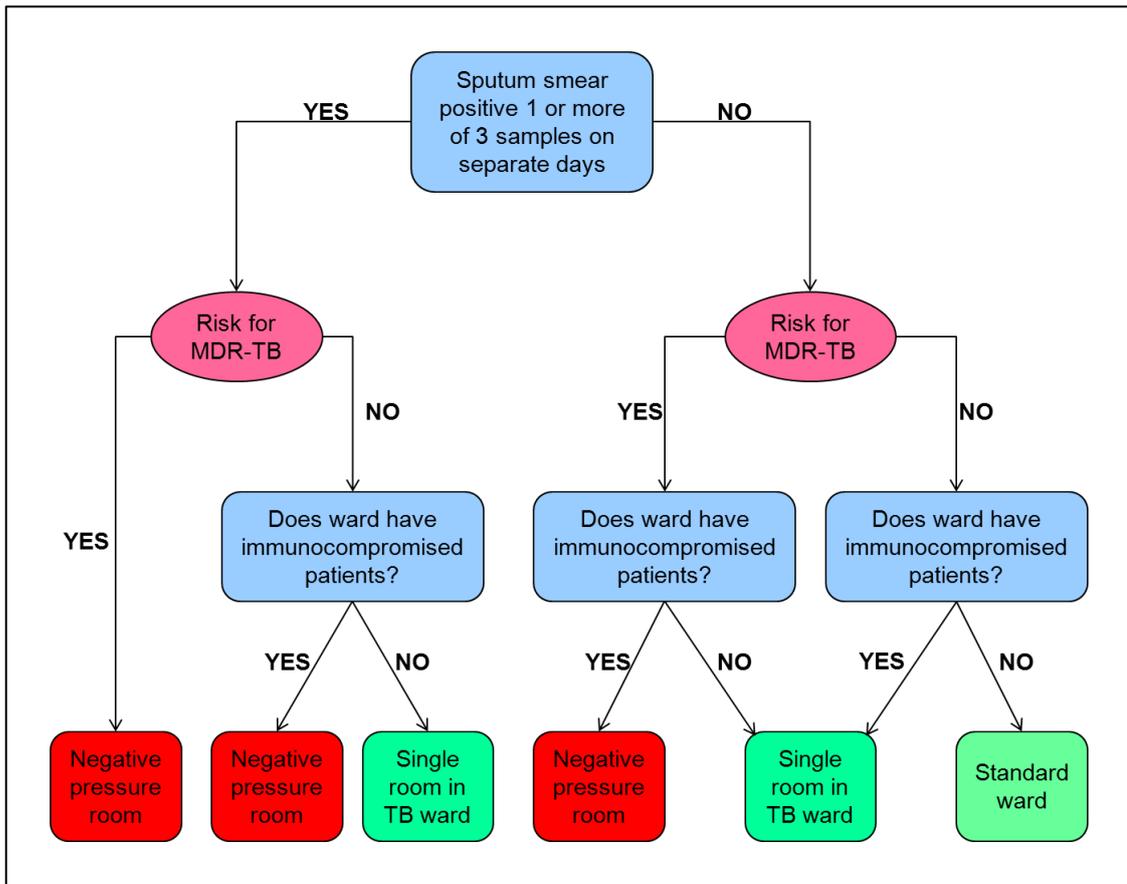
A genetic predisposition to TB may also exist among certain individuals. Population-based studies have found an association between TB and some human leukocyte antigen alleles (Singh *et al.*, 1983), as well as polymorphisms in the genes for natural resistance-associated macrophage protein (Bellamy *et al.*, 1998) and the vitamin D receptor (Bellamy *et al.*, 1999). Defects in the production of cytokines and cytokine receptors might also be important (Jouanguy *et al.*, 1997; Wilkinson *et al.*, 1999; Altare *et al.*, 2001). Also, the difference in the associations between genetic polymorphisms and TB susceptibility among different ethnic groups has also been documented (Delgado *et al.*, 2002).

1.4.7 Control and prevention of tuberculosis transmission in the UK

The guidelines on the control and prevention of TB in the UK are recommended by the Joint Tuberculosis Committee of the British Thoracic Society (Ormerod *et al.*, 2000). All forms of TB are compulsorily notifiable by the clinicians making or suspecting the diagnosis under the Public Health (Control of Disease) Act 1984; this also includes those who are diagnosed following death. The main objective of notification is to trigger contact tracing procedures. Also, notification provides surveillance data to detect TB outbreaks and monitor epidemiological trends, so that preventive measures and treatment services can be planned and targeted appropriately. The responsibility for the local control and prevention of TB rests with the Consultant in Communicable Disease Control in England and Wales. The consultant physician manages the clinical aspects of patients' management, while the specialist nurse or the health visitor provides continuous care through individualised packages.

Hospital admission for TB patients depends on the severity of the disease, the magnitude risk of transmission, the need for investigations to establish the diagnosis, adverse effects of chemotherapy, or social reasons. Patients with suspected pulmonary TB should initially be placed under respiratory isolation until their AFB smear status is known and risk assessments are made for the likelihood of infectiousness and MDR-TB (Figure 1.10). In hospitals and other institutional settings, respiratory isolation for the reason of infectiousness is generally only required for two weeks (Rouillon *et al.*, 1976; Jindani *et al.*, 1980). Every effort should be made to obtain specimens for microscopic examination and culture at the microbiological services; the former permits preliminary prediction of case infectiousness and the latter allows mycobacterial identification and drug susceptibility testing.

Figure 1.10: Risk assessment of infectiousness of pulmonary TB patients



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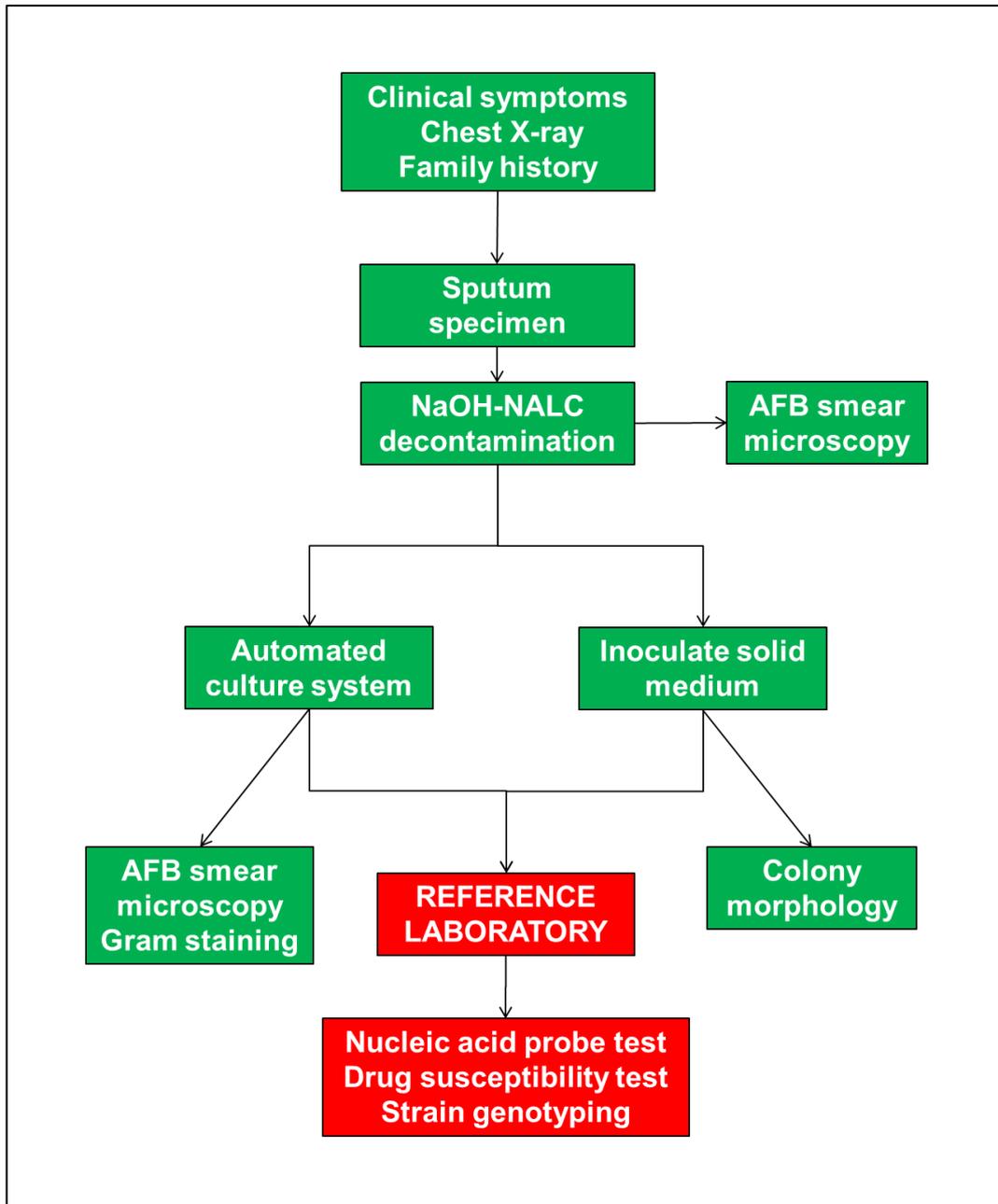
Contact tracing is carried out to detect linked TB cases, to detect latently infected individuals, to identify candidates for BCG vaccination, to screen a local population with a high incidence of TB, and to identify geographical linkage of cases prompting cluster investigation. Up to 10% of TB cases could be diagnosed via contact tracing (Esmonde and Petheram, 1991; Hussain *et al.*, 1992). Screening of new immigrants to the UK is recommended as part of a coordinated local TB control policy. This is recommended for those from countries with a TB incidence of more than 40 per 100,000 per year. Initial screening, normally in the form of chest X-ray, should take place at the port of entry. Suspected TB cases should be reported to the district of intended residence for comprehensive local screening and patient management. In the UK, the incidence of TB remains high in most ethnic minority groups. In this context, subsequent return visits to home countries with a high TB prevalence may be a risk factor.

1.5 Laboratory Diagnosis of Tuberculosis

1.5.1 Laboratory diagnosis of tuberculosis in the UK

The first question that arises when AFB are detected by smear microscopy is whether the infection is due to Mtb or an NTM. Differentiation of Mtb from NTM, in addition to knowledge on the drug susceptibility profile of the infecting strain, is of fundamental importance to both the clinical and public health teams for the management of TB. In this context, prompt identification of Mtb will enable immediate institution of treatment and prevention of further disease transmission to exposed individuals. In most developed countries, primary isolation of Mtb is the mainstay for the definitive diagnosis of TB. Thereafter, in the UK, positive cultures are transferred to a regional reference laboratory for further identification (usually by a molecular assay), drug susceptibility testing, and strain genotyping. These results may take several weeks to become available to the healthcare team, and therefore preliminary response to a particular case in which mycobacteria have been isolated may lead to inappropriate isolation and treatment of patients for TB and unnecessary public health investigations. Numerous rapid methods for the direct detection and identification of Mtb in clinical specimens have been evaluated. However, none approaches the sensitivity of culture and each poses undesirable limitations. Figure 1.11 is a diagnostic algorithm that reflects the practice of processing sputum specimens in many clinical mycobacteriology laboratories in the UK; individual processes are discussed in the following sections. Based on this algorithm, the identification of Mtb in smear-positive cases may take 2-3 weeks, and an additional week is required to obtain drug susceptibility results. This process takes 1-2 weeks longer with smear-negative cases.

Figure 1.11: A diagnostic algorithm reflecting the detection and identification of mycobacteria from sputum specimens



Patients suspected with TB are initially examined and diagnosed by clinicians based on presenting symptoms, radiological results, and family history. Sputa are normally collected for confirmatory diagnosis at the clinical mycobacteriology laboratory. AFB smear microscopy enables preliminary detection of Mtb and prompt prediction of case infectiousness. Smear results, along with initial clinical suspicion of pulmonary TB, will influence decisions on respiratory isolation and patient management. Positive cultures are usually transferred to a regional reference laboratory for confirmatory mycobacterial identification, drug susceptibility testing, and strain genotyping; the latter enables assessments of potential epidemiological links among isolates.

1.5.2 Specimen collection and processing

Early-morning sputa of 5-10 ml are usually collected for the diagnosis of pulmonary TB, when the tubercle bacilli are at their highest concentration. Sputum is produced by deep expectorative cough or, if necessary after inhalation of nebulised 10% NaCl solution. Due to the inconsistent and intermittent release of mycobacteria into the bronchial lumen, a minimum of three sputum specimens are normally collected on successive 24-hour periods to maximise the chance of recovering mycobacteria. If the collection of sputum is not possible, alternative upper respiratory specimens can be considered; these include laryngeal swabs, bronchial washings, bronchoalveolar lavage, and gastric lavage fluids. At the laboratory, specimens are usually homogenised and decontaminated with a combination of N-acetyl-L-cysteine (NALC) and NaOH; the former is a mucolytic agent and the latter kills contaminating organisms that may be present (Kubica *et al.*, 1963).

1.5.3 Conventional diagnostic methods

1.5.3.1 Acid-fast bacilli smear microscopy

Smear examination for AFB, in combination with clinical symptoms and radiological findings, is used for preliminary diagnosis of active TB. The AFB smear status is frequently used in most healthcare settings to gauge the infectiousness of TB patients, to predict the severity of the disease, and to monitor the outcomes of treatment. The American Thoracic Society estimated that about 50-80% of pulmonary TB cases are smear-positive on average (Dunlap *et al.*, 2000). In countries with high TB incidence, more than 95% of suspected TB patients give positive smears (Crampin *et al.*, 2001). For these countries, routine culture for mycobacteria is generally neither practicable nor necessary for disease control.

Smears can be prepared directly from specimens or from concentrated specimens following NaOH-NALC decontamination. Available staining methods for AFB are carbolfuchsin (Ziehl-Neelsen (ZN) or Kinyoun; hot and cold staining, respectively) and auramine O fluorescence staining methods. Mycobacterial cells stain bright red against a blue background in the former and bright green against a dark background in the latter. Acid-fast staining does not differentiate live from dead bacilli, and therefore smear-positive, culture-negative cases are possible. Reports of AFB smear results should include a measure of quantity, such as the actual number of bacilli observed per field or a 1+ to 4+ grading (Table 1.3). AFB smear assessment is however hampered by its inability to distinguish between MTBC and NTM species, low sensitivity of between 5×10^3 to 10^4 bacilli per ml of sputum (Dunlap *et al.*, 2000), and non-specific detection of *Mycobacterium* genus-related actinobacteria such as *Norcadia* and *Rhodococcus spp.*

Table 1.3: Quantitation scale for AFB smears stained by fluorochrome and viewed under the microscope at x250 magnification

Grade	Number of AFB
Negative	0 per 30 fields
Scanty	1-2 per 30 fields
1+	1-9 per 10 fields
2+	1-9 per field
3+	10-90 per field
4+	> 90 per field

Adapted from the article by Dunlap *et al.* (2000) with permission from the American Thoracic Society. Copyright © American Thoracic Society.

1.5.3.2 Primary isolation of mycobacteria

Decontaminated specimens are inoculated to liquid and/or solid culture media. Isolation of mycobacteria by culture is the gold standard required for definite diagnosis of TB and is essential for drug-susceptibility testing. Culture is approximately 1,000 times more sensitive than smear microscopy; it can detect as few as 10 bacilli per ml of decontaminated specimen (Dunlap *et al.*, 2000). Furthermore, cultivation on solid

media enables microscopic examination of colony morphology for preliminary identification of mycobacterial species and detection of mixed infections.

Solid media used for mycobacterial growth can be divided into two categories: the egg-based media (Löwenstein-Jensen (LJ), Petraghani, and American Thoracic Society) and agar-based media (Middlebrook 7H10, Middlebrook 7H11, and Dubois oleic albumin); all contain malachite green to control bacterial contaminants during prolonged incubation. Mtb colonies are buff in colour, non-pigmented, have a rough, dry, and wrinkled surface, with irregular edges. Biochemical tests are most accurate when performed on subcultures from LJ slopes. A well-known biochemical test uses *p*-nitro-acetylamino-hydroxypropiophenone (NAP) to distinguish between MTBC from NTM. NAP selectively inhibits Mtb growth in liquid culture media.

The rate of recovery and time to positivity of mycobacteria from clinical specimens have been improved by the use of liquid culture media. Growth can be observed after 1-3 weeks, as opposed to approximately 8 weeks with solid media. The first selective liquid medium used for the primary isolation of mycobacteria was Middlebrook 7H9 broth containing [¹⁴C] palmitic acid, used in the BACTEC 960 system (Becton Dickinson). Bacterial growth is indicated by radiometric detection of ¹⁴CO₂ released by growing organisms. Due to the problem of radioactive waste disposal, BACTEC 960 system was introduced to detect bacterial growth based on oxygen-quenched fluorescence. The culture vessels in this system are referred to as mycobacterial growth indicator tubes (MGITs). Other non-radiometric automated systems are also available, namely the BacT/Alert system (BioMérieux) and the ESP Culture System II (Trek Diagnostic Systems).

Limitations of the automated culture systems include the cost of instrumentation, the inability to detect mixed cultures, and overgrowth by contaminants. As many as 15-20% adults with TB have negative sputum cultures, and this rate is even higher among children (Dunlap *et al.*, 2000). False-positive cultures due to laboratory cross-contamination, contamination of clinical devices, and clerical errors, are common in liquid media (Burman and Reves, 2000).

1.5.4 Nuclei acid-based technologies

1.5.4.1 Nucleic acid probes

Nuclei acid probes were the first nucleic-acid based technology to be used in the clinical microbiology service for routine identification of mycobacteria in positive cultures. These probes were pioneered by Gen-Probe, currently marketed as the AccuProbe DNA hybridisation test. This test employs chemiluminescent acridinium ester-labeled single-stranded DNA probe complementary to the rDNA for the target organism. Chemiluminescence occurs upon the addition of hydrogen peroxide and is detected by a luminometer. The shortcoming of the AccuProbe system is the ability to detect only limited number of NTM species.

The GenoType DNA hybridisation test was introduced by Hain Lifescience. Three different kits are available for this molecular test. GenoType CM and AS kits are designed for the identification of common mycobacteria and additional NTM species respectively; they enables the identification of a broad range of NTM species. Another kit, GenoType MTBC, enables genetic differentiation of members of the complex based on the gyrase B gene polymorphisms. The GenoType test is based upon reverse hybridisation of biotin-labeled target amplicons to complementary single-stranded DNA probes bound to a nitrocellulose membrane. Upon staining with streptavidin/alkaline phosphatase, the mycobacterial isolate is identified based on the banding pattern obtained. Another reverse hybridisation system is the line probe assay, marketed commercially as the INNO-LiPA Mycobacteria V2 assay by Innogenetics.

1.5.4.2 Nucleic acid amplification assays

Nucleic acid amplification method is most reliable in smear-positive respiratory specimens from patients previously untreated for TB; the sensitivity and specificity can be as high as 95% and 98% respectively relative to culture results (Dunlap *et al.*, 2000). In areas with low TB prevalence but high HIV prevalence, PCR method is useful in differentiating *Mtb* from NTM in positive smears. Since the 1990s, there has been increased interest in the use of PCR methods, both commercial and in-house, for the direct detection of *Mtb* in clinical specimens. However, nucleic acid amplification methods do not replace smear microscopy or culture for the diagnosis of TB.

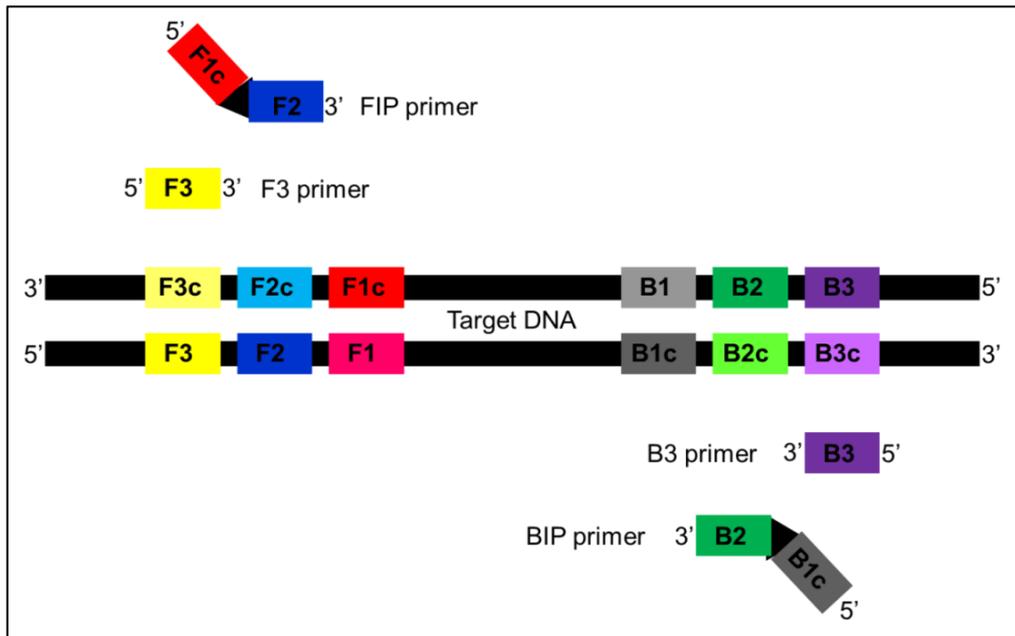
Two commercial PCR assays received the approval of the Food and Drug Administration (FDA) to be used for the detection of *Mtb* in respiratory specimens: the Amplicor *M. tuberculosis* PCR assay (Roche Diagnostics) and the Amplified *M. tuberculosis* Direct test (Gen-Probe). These PCR assays employ transcription-mediated amplification, and only the latter has been approved for the use on smear-negative respiratory specimens. The other two commercial amplification tests, the BD ProbeTec *M. tuberculosis* assay (Becton Dickinson) and the LCx *M. tuberculosis* test (Abbott Laboratories), have not been FDA-approved.

Numerous in-house PCR assays, both conventional and real-time, have been developed and tested by various research groups. Eisenach and co-workers (1991) developed one of the earliest PCR assays for the rapid detection of *Mtb* in sputum specimens. Real-time PCR has simplified and expedited the direct PCR detection of *Mtb* in clinical specimens with the realistic potential for improved sensitivity and specificity (Kraus *et al.*, 2001; Miller *et al.*, 2002). It removes the requirement for post-PCR manipulation and minimises the opportunity for amplicon contamination in the laboratory. Shrestha *et al.* (2003) designed and developed a real-time PCR assay for rapid differentiation of MTBC from NTM isolates based on post-amplification melt analyses of resulting amplicons. The results of real-time PCR assays have also been used for quantitative assessment of bacillary loads in clinical specimens (Rondini *et al.*, 2003) and monitoring of treatment progress (Desjardin *et al.*, 1999).

The recent introduction to the nucleic acid amplification family is the loop-mediated isothermal amplification (LAMP) (Notomi *et al.*, 2000). This amplification method is based on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment. The use of four primers targeting six distinct regions on the target DNA results in the high specificity of this method (Figure 1.12). Amplification is performed under isothermal condition (60-65°C) in just one hour due to the lack of thermal cycling. The amplicons, stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops, are produced in large amount which contributes to the high sensitivity of the LAMP method. Two simple detection formats are available for the amplified products, either based on turbidity or fluorescence. The former relies on the detection of the by-product of the amplification reaction, pyrophosphate, in commercially available turbidimeters (Pandey *et al.*, 2008). The visual fluorescence detection is achieved by incorporating fluorescent dyes into the LAMP reaction. Two fluorescent dyes, SYBR Green (Iwamoto *et al.*, 2003) and calcein (Boehme *et al.*, 2007), have been used in direct LAMP assays on respiratory specimens. SYBR Green dyes bind to the double-stranded amplicons and emit fluorescence; negative reactions remain orange in colour. The fluorescence of calcein is quenched by manganese ions in the reaction, but the depletion of these ions by pyrophosphate during amplification unmasks the fluorescence. Similar to real-time PCR, the closed-tube detection format of LAMP assay minimises the risk of cross contamination. Iwamoto *et al.* (2003) designed a LAMP assay for the detection of MTBC, *M. avium*, and *M. intracellulare* using three different primer sets, where their respective amplicons were further confirmed by restriction digest and gel electrophoresis. A more convenient kit for the LAMP method is currently marketed by Eiken Chemical.

Nucleic acid amplification methods have been shown to be able to detect and identify mycobacteria within hours of receipt of specimens. However, its widespread implementation is limited by the high cost and the potential for poor performance under field conditions. PCR inhibition and subsequent erroneous reporting of false-negative results also poses a problem, although this can be addressed by the use of an internal amplification control. Laboratory cross contamination and occurrence of false-positive results, especially with conventional PCR methods, have been reported previously (Noordhoek *et al.*, 1994).

Figure 1.12: Layout of the LAMP primers



F3 and B3, forward and backward outer primers, respectively; FIP and BIP, forward and backward inner primers, respectively. The 3' and 5' ends of the inner primers are designed to be complementary to two different regions on the target DNA. When the primer-annealed DNA strand is elongated, the 3' end anneals to the complementary region within the self-structure, thus forming a loop structure at the end. This stem-loop structure will allow the 3' end to initiate self-elongation and another inner primer to anneal to its loop region to synthesise new DNA strand in a strand displacement manner. Adapted from the Eiken Chemical website (2005) with permission from Eiken Chemical Co. Ltd. Copyright © 2005 Eiken Chemical Co., Ltd. All Rights Reserved.

1.5.4.3 Nucleic acid sequencing

Sequencing of PCR amplicons is a common method of post-amplification analysis. Analysis of variable regions interspersed between conserved regions of genes that serve as broad-range primer hybridisation sites has become a powerful tool for microbial identification. In mycobacteriology, DNA sequencing has been used for the accurate identification of NTM species, mostly based on the 16S rDNA sequence (Han *et al.*, 2002). A commercial DNA sequencing system, MicroSeq (Applied Biosystems), is available for the mycobacterial identification based on this gene. However, DNA sequencing technology is limited by the requirement for experience with sequence alignment, editing software, and genetic databases.

1.5.5 Mycobacteriophage-based tests

1.5.5.1 Mycobacteriophage and infection of mycobacteria

Mycobacteriophages are viruses that infect mycobacteria. The host range of these phages is unpredictable, but many of them can infect both slow- and rapid-growing mycobacteria. The first mycobacteriophages were described in the 1950s, and more than 250 isolates have been described since then (Hatfull, 2005). Early studies described their use as diagnostic and epidemiological tools for detection and typing of Mtb isolates (Snider *et al.*, 1984). Most mycobacteriophages belong to the morphological class *Siphoviridae*, with a long, flexible tail (130-265 nm in length) attached to an isometric, icosahedral head (~60 nm in diameter) that contains the DNA. Examples are L5, D29, DS6A, TM4, Bxb1, Bxz2, Che8, Che9d, Cjw1, Omega, Rosebush, and Barnyard. Among these, the lysogenic phage L5 is the best characterised. It only infects NTM species but is found to infect *M. bovis* BCG under specific conditions (Fullner and Hatfull, 1997). The widespread distribution of mycobacteriophages reflects the occurrence of their hosts in a plethora of environmental localities. They can be isolated from most soil and compost samples. Mycobacteriophages play central roles in the understanding of mycobacterial genetics and physiology, especially among the slow-growing species. They are used as delivery systems for reporter genes and transposons, mediators of efficient gene replacement, sources of integration-proficient vectors, and systems for studying mycobacterial gene expression (Hatfull, 2005).

Mycobacteriophage D29 was first isolated from soil and demonstrated to be active against Mtb, other members of the MTBC, and a wide variety of NTM species (Froman *et al.*, 1954). The plaques produced on a lawn of mycobacterial cells were clear, indicating that D29 is a lytic phage. Adsorption of phage D29 to *M. leprae* has been documented but it ended up as abortive infection (David *et al.*, 1984). *M. avium* has been reported to resist infection by phage D29 (Froman *et al.*, 1954; Froman and Scammon, 1964). The complete genome sequence of D29 reveals high similarity of this phage to L5 at the DNA level (Ford *et al.*, 1998). A striking difference is the 3.6-kb deletion of the repressor gene *71*, which is responsible for the inability of phage D29 to

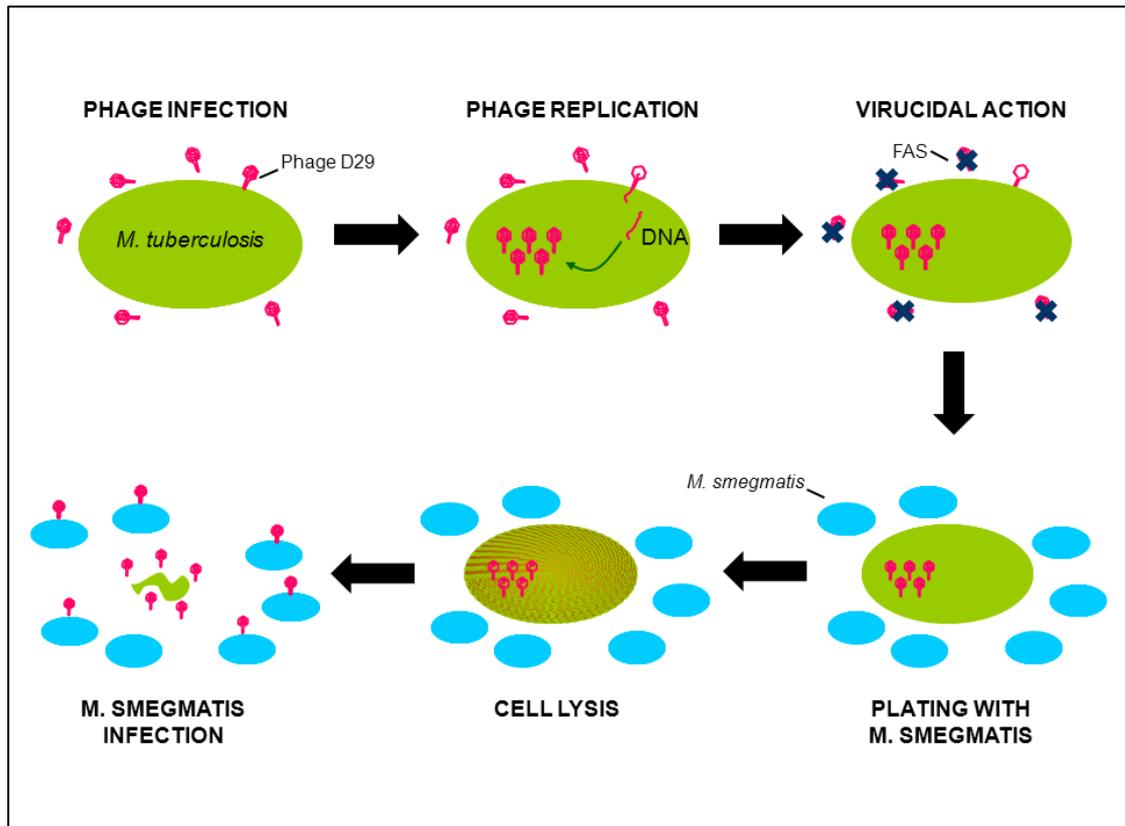
maintain lysogeny in host cells. Multiple insertions, deletions, and substitutions of genes are distributed throughout the genome of D29, consistent with the genetic mosaicism of lambdoid phages.

The identity of the phage D29 receptor remains a mystery, although pyruvylated, glycosylated acyltrehaloses have been implicated in the infection of *M. smegmatis* (Besra *et al.*, 1994). Donnelly-Wu *et al.* (1993) reported that both D29 and L5 use similar mechanisms for infection of *M. smegmatis*. Following infection and proliferation, progeny mycobacteriophages produce two lysis enzymes, Lysin A and Lysin B, to lyse and exit the host cell. Lysin A, which is also produced by phages of Gram-positive bacteria, is a putative endolysin that hydrolyses the peptidoglycan layer (Garcia *et al.*, 2002). Lysin B is a novel mycolylarabinogalactan esterase that cleaves the ester bond linking the mycolate layer to arabinogalactan, and completes host lysis (Payne *et al.*, 2009). The overexpression of the multicopy phage resistance gene (*mpr*) in *M. smegmatis* has been reported to confer resistance to infection by both D29 and L5, possibly via physical alteration to the mycobacterial cell envelope (Donnelly-Wu *et al.*, 1993).

1.5.5.2 Phage amplification assay

The phage amplification technology is based on the production and release of progeny phage particles to reflect the presence of live bacterial cells that can sustain phage infection. This method has been used for the rapid detection of mycobacteria in sputum specimens (McNerney *et al.*, 1998; Albert *et al.*, 2002a; Alcaide *et al.*, 2003) and food samples (Stanley *et al.*, 2007). The principle of the mycobacteriophage assay is illustrated in Figure 1.13. Inactivation of free phages that have not infected the host cells is a crucial step during the phage assay. Inadequate addition or mixing of virucide can lead to breakthrough plaques, i.e. false-positive results. The virucide used, ferrous ammonium sulphate (FAS), does not affect the target organisms or the phages protected within (McNerney *et al.*, 1998). The oxidised form of FAS showed comparable performance for phage inactivation (McNerney *et al.*, 1998). The mode of inactivation is not known, although the role of the oxidative intermediate generated by decomposition of the ferrate ion has been proposed (Kazama, 1995).

Figure 1.13: Principle of the mycobacteriophage amplification assay



Target mycobacteria are rapidly infected by phage D29 during incubation at 37°C. A selective virucide, ferrous ammonium sulphate (FAS), is added to destroy all exogenous phages that have not infected mycobacterial cells. Proliferation of the phages in the target cells is detected by a plating procedure in which the phage-infected mycobacterial suspension is mixed with rapidly growing *M. smegmatis* cells, and the progeny phages released from the lysed target cells infect the *M. smegmatis* cells. Successive rounds of *M. smegmatis* infection result in formation of plaques on the lawn of *M. smegmatis* colonies after 1 day. The number of plaques formed, expressed as plaque-forming units (PFU), corresponds to the number of live mycobacterial cells originally present within the sample. Adapted from the Biotec Laboratories website (2006) with permission from Biotec Laboratories Ltd. © www.biotec.com 2006. All Rights Reserved.

The phage amplification test is available commercially as the *FASTPlaqueTB* kit from Biotec Laboratories. A similar kit is marketed as PhageTek MB by Organon Teknika in the USA. With culture as the gold standard, this commercial test has higher sensitivity than AFB smear microscopy when tested on tuberculous sputum specimens (Albert *et al.*, 2002a; Albay *et al.*, 2003). It has potential to be used to confirm false-positive smear results and diagnose for TB in smear-negative cases (Albert *et al.*, 2002a).

Albay *et al.* (2003) reported that the sensitivity of the *FASTPlaqueTB* test was superior to that of PCR method. In-house developed phage assay also showed higher sensitivity compared to smear microscopy, with good positive predictive value (McNerney *et al.*, 2004). A systematic review and meta-analysis summarised that phage-based detection methods have higher overall accuracy but variable sensitivity relative to sputum microscopy (Kalantri *et al.*, 2005). Sensitivity in smear-positive sputa appeared to be higher than in smear-negative ones, but phage-based assays gave higher specificity with the latter.

The phage detection assay is quick; results are available 48 hours upon receipt of clinical specimens by the laboratory. The sensitivity of this assay with sputum specimens has been reported to approach 100 bacilli per ml (Albay *et al.*, 2003; McNerney *et al.*, 2004). It is cost-efficient and simple to perform, and is therefore well suited for use in developing countries with high TB incidence. However, the broad host range of phage D29 implies that the phage assay does not distinguish between MTBC and NTM species. Stanley and co-workers (2007) pioneered the PCR detection of plaque DNA based on the multicopy DNA insertion sequences for the specific identification of the phage-infected mycobacterial species. An electrochemical sensing assay that exploits the detection of specific cellular compounds released following lysis of phage-infected mycobacterial cells has also been reported (Yemini *et al.*, 2007).

1.5.5.3 Luciferase reporter phage assay

The luciferase phage assay is similar in biological principle to the phage amplification assay, except that mycobacteriophages which have the firefly *fflux* reporter gene inserted within its genome are used. They are called luciferase reporter phages (LRPs). The first generation LRP, phAE40, developed from the lytic phage TM4 was able to detect only 10^4 bacilli per ml, due to the loss of detectable light output following cell lysis (Jacobs *et al.*, 1993). Carriere and associates (1997) reported the incorporation of a temperature-sensitive mutation in the TM4 genome to prevent host lysis at the non-permissive temperature to address this problem. The LRP phGS18 constructed from mycobacteriophage L5 showed more sustained light output and hence better sensitivity, but has a restricted host range (Sarkis *et al.*, 1995). Kumar *et*

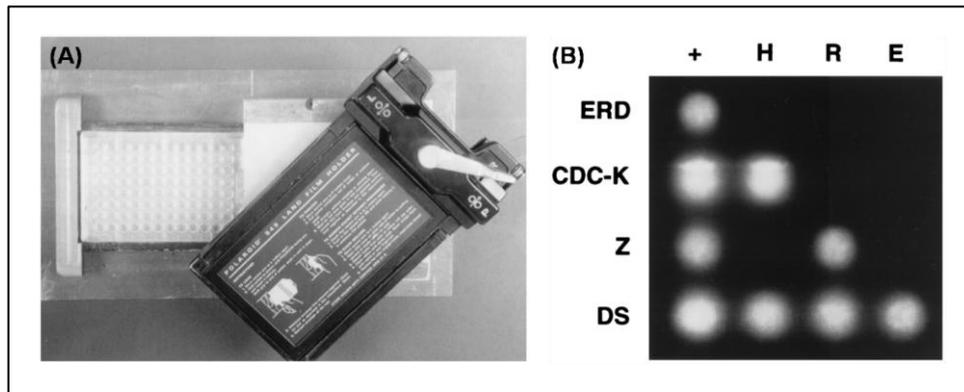
al. (2008) constructed the LRP phAETRC16 from the temperate phage Che12 that can infect *Mtb*. Promoters for genes involved in dormancy of *Mtb* have been exploited for the construction of LRPs, which have potential for the detection of non-replicating bacilli (Dusthacker *et al.*, 2008).

Following infection of mycobacteria, the *fflux* gene is expressed and the luciferase produced catalyses a reaction that releases light in the presence of two substrates, luciferin (added exogenously) and ATP (present in live cells). The *fflux* gene is positioned immediately downstream of a strong promoter to ensure adequate expression and therefore a strong signal. There are two methods to detect light production in the LRP assay. A luminometer can be used to detect and quantify the amount of light emitted from the assay suspension, expressed as relative light units (Carriere *et al.*, 1997). Alternatively, light production can be detected on a photographic film which is placed immediately adjacent to the assay microtitre plate, and the developed film is visualised in a device called the Bronx Box (Figure 1.14) (Riska *et al.*, 1999). The sensitivity of the LRP assay is comparable to that of AFB smear microscopy (Carriere *et al.*, 1997). This phage assay has also been used in combination with the NAP test for the detection and identification of MTBC in positive cultures (Riska *et al.*, 1997; Banaiee *et al.*, 2001). A commercial kit for the LRP assay is yet to be available.

1.5.5.4 Drug susceptibility testing

Mycobacteriophage-based methods have also been used for drug susceptibility testing of isolated *Mtb* strains. These methods are based on the ability of resistant *Mtb* to support phage infection following exposure to antibiotics. Based on the LRP assay, an effective chemotherapy will abrogate light production by decreasing cellular ATP stores, impairing production of luciferase, or interfering with productive phage infection (Jacobs *et al.*, 1993; Carriere *et al.*, 1997). In the presence of a drug-resistant strain, the antibiotic-containing sample will yield similar light output as the antibiotic-free sample (Figure 1.14). This method yields results within 2-3 days. Several research groups reported good concordance between the LRP assay and culture methods for the detection of drug resistance in clinical *Mtb* strains (Riska *et al.*, 1999; Banaiee *et al.*, 2001).

Figure 1.14: Drug susceptibility testing by the luciferase reporter phage assay and the Bronx Box



(A) The Bronx Box. (B) A typical Bronx Box film showing results of drug susceptibility testing with clinical Mtb strains. +, no antibiotic; H, 0.2 µg/ml isoniazid; R, 2 µg/ml rifampicin; E, 2.5 µg/ml ethambutol; ERD, Erdman susceptible reference strain; CDC-K, CDC isoniazid-resistant strain; Z, clinical mono-resistant strain; DS, MDR strain. Reproduced from the article by Riska *et al.* (1999) with permission from the American Society for Microbiology. Copyright © 1999, American Society for Microbiology. All Rights Reserved.

The phage amplification assay has also been used for the detection of rifampicin resistance in clinical Mtb strains. A comparison of the number of plaques is made between samples incubated both in the presence and absence of the test anti-tuberculous drug, in order to screen for drug resistance. This phage assay is available commercially as the *FASTPlaqueTB-RIF* kit from Biotec Laboratories. It demonstrated high correlation with the BACTEC 460 system when tested on clinical Mtb isolates (Albert *et al.*, 2002b; Kisa *et al.*, 2003). In-house phage assays for the detection of rifampicin resistance have also been developed (Gali *et al.*, 2003; Traore *et al.*, 2007). McNerney *et al.* (2007) combined the phage amplification assay with downstream colourimetric detection using redox dyes to negate the need for plating out the reactions. The phage amplification technology has also been applied to the susceptibility testing with other anti-tuberculous drugs including isoniazid, ethambutol, streptomycin, pyrazinamide, and ciprofloxacin (Wilson *et al.*, 1997; Eltringham *et al.*, 1999). However, results with bacteriostatic antibiotics require careful interpretation as knowledge on phage infectivity of susceptible Mtb strains exposed to them is far from satisfactory. The mycobacteriophage-based tests may be considered as an alternative method for susceptibility testing in developing countries, where the prevalence of drug resistance is low and susceptibility tests are performed mainly for surveillance rather than for patient management.

1.6 Phylogeny and Molecular Epidemiology of *M. tuberculosis*

1.6.1 *M. tuberculosis* genome

In 1998, the TB research community entered the genomic era with the establishment of the complete genome sequence of the virulent laboratory strain H37Rv of Mtb (Cole *et al.*, 1998). The genome is 4,411,532 bp in size and has a characteristically high G+C content of 65.6%. A total of 4,043 genes encoding 3,993 proteins and 50 stable RNAs were identified. All open reading frames (ORFs) were given an Rv number and systematic name as appropriate. Mtb has complex gene regulatory systems that consist of 13 sigma factors, 11 two-component systems, and 11 serine/threonine protein kinases. The most striking feature of its genome is the abundance of genes involved in lipid metabolism and the genes for the unusual glycine-rich PE and PPE proteins; the latter occupy about 9% of the genome and exhibit extensive sequence polymorphism. There is also the 23-member *esx* family which encodes the T-cell antigens ESAT-6 and CFP-10, and four large operons involved in mammalian cell entry (*mce*). A set of 15 *mmpL* genes encodes large membrane proteins which may be responsible for lipid transport and virulence (Converse *et al.*, 2003). The genome also has 56 insertion elements and 2 prophages.

Another complete Mtb genome sequence, that of the clinical strain CDC 1551, is available (Fleischmann *et al.*, 2002). This strain was reported to be highly infectious in humans, and is capable of triggering greater immune response but has comparable growth rate in mice relative to Mtb H37Rv (Valway *et al.*, 1998). The genome of Mtb CDC 1551 is highly similar to that of strain H37Rv. However, the former has 86 insertions or deletions and 1,075 single-nucleotide polymorphisms (SNPs) relative to the latter (Fleischmann *et al.*, 2002). Complete genome sequences for other Mtb strains are now available; these include the avirulent laboratory strain H37Ra, strain Haarlem, strain F11, and many other clinical strains. The extensive large-sequence polymorphisms (LSPs) among clinical Mtb strains contribute to the diversity among their genome sequences (Kato-Maeda *et al.*, 2001; Fleischmann *et al.*, 2002; Tsolaki *et al.*, 2004).

1.6.2 Origin and evolution of *M. tuberculosis*

For many years, it was believed that human TB evolved from bovine TB by adaptation of the bovine bacillus to the human host (Stead, 1997). However, this thought was proven wrong by the first major evolutionary phylogeny of the MTBC (Figure 1.2) (Brosch *et al.*, 2002). The origin of TB is the speciation event that led to a population of human pathogenic strains, which then descended through time with a series of most recent common ancestors (MRCAs) (Smith *et al.*, 2009). The current MRCA of Mtb could possibly arise by a selective sweep, by a bottleneck, or simply by the coalescence of all currently existing lineages. Limited nucleotide diversity in Mtb implies that the population is “young” in the sense that the MRCA is relatively recent; however this does not mean that the disease is “young”. Several molecular archaeology studies have attempted to identify ancient Mtb lineages that pre-date the MRCA (Donoghue *et al.*, 2004; Hershkovitz *et al.*, 2008). Brosch *et al.* (2002) claimed that ancestral Mtb strains had intact TbD1. These strains predominate in the Indian subcontinent and South East Asia (Gutierrez *et al.*, 2006; Gagneux and Small, 2007). Mtb strains with deleted TbD1 are exactly the same distance in years from the current MRCA (Smith *et al.*, 2009).

1.6.3 Phylogeny of *M. tuberculosis*

A number of studies have been performed to examine the phylogenetic structure of and relationships among Mtb strains. Both Hirsh *et al.* (2004) and Gagneux *et al.* (2006) reported that a host’s region of origin is predictive of the infecting Mtb strain, and this association remains strong during disease transmission in a cosmopolitan city outside of the region of origin. Gagneux and colleagues (2006) assigned their global collection of Mtb strains into six distinct phylogeographical lineages, based on genomic analyses lineage-defining LSPs (Figure 1.15). The Indo-Oceanic lineage consists of those strains with intact TbD1, and hence the most ancestral of the six lineages (Brosch *et al.*, 2002). The East-Asian lineage predominates in many countries in the Far East and includes, but is not limited to, the Beijing strains. As its name suggests, the East African-Indian strains are highly prevalent in the eastern part of the African continent and South Asia. The Euro-American lineage regroups strains that have generally been

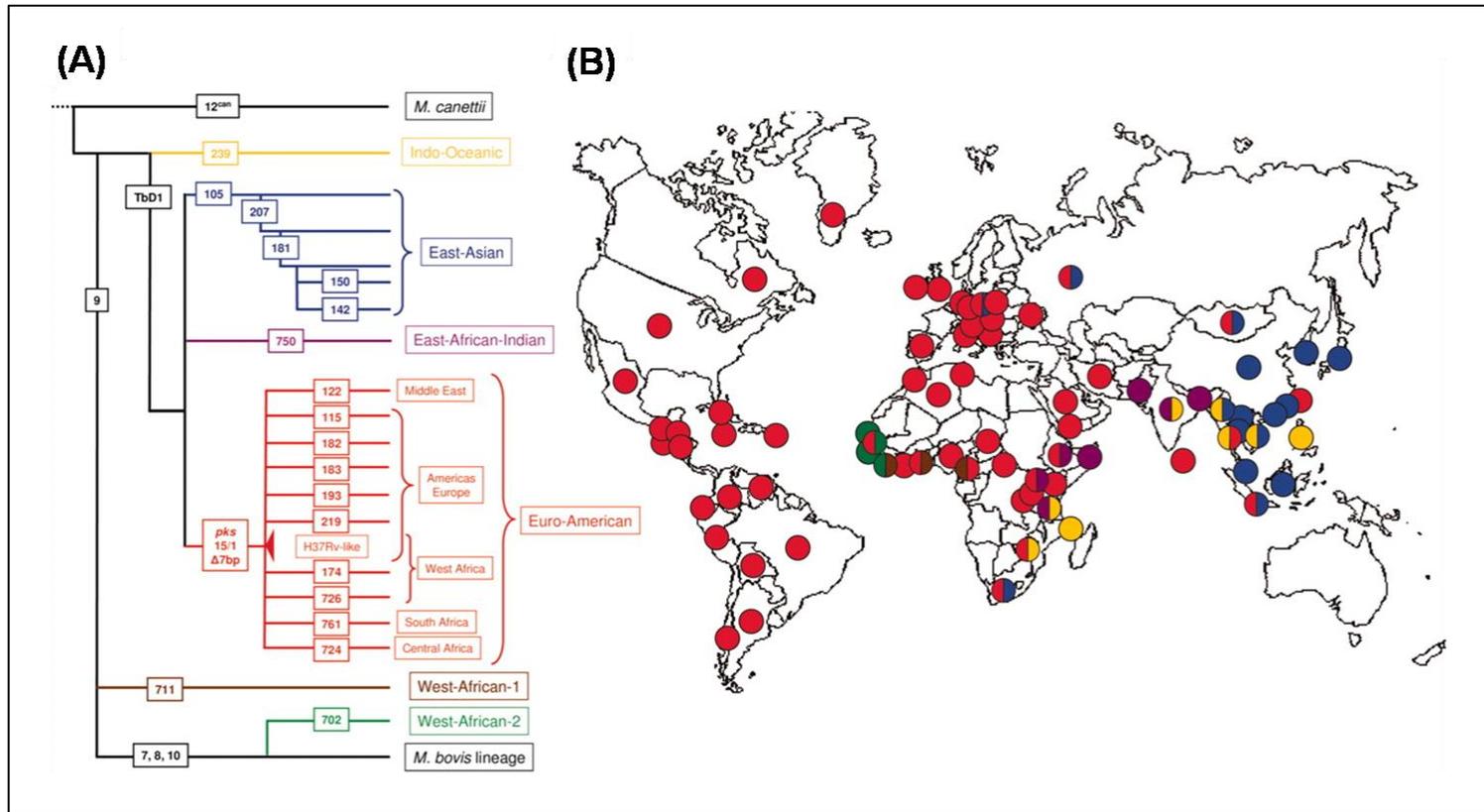
described as principal genetic groups 2 and 3 (Sreevatsan *et al.*, 1997). The West-African lineages 1 and 2 consist of strains of *M. africanum*. All the six phylogeographical lineages proposed by Gagneux *et al.* (2006) are present in the African continent (Figure 1.15). Gutierrez and associates (2005) suggested that ancestral mycobacteria affected the early human populations in East Africa around 3 million years ago, which is consistent with presence of the ancestral Indo-Oceanic lineage in this region (Gagneux *et al.*, 2006). Taken together, these findings suggested that Mtb expanded and diversified during its spread out of East Africa and subsequently adapted to different human populations.

A few research groups constructed the phylogenetic trees for Mtb strains based on SNP polymorphisms (Sreevatsan *et al.*, 1997; Alland *et al.*, 2003; Gutacker *et al.*, 2006). Sreevatsan and co-workers (1997) assigned modern MTBC strains into three principle genetic groups based on neutral non-synonymous SNPs in the catalase-peroxidase-encoding (*katG*) and the A subunit of DNA gyrase (*gyrA*) genes. A more robust analysis by Gutacker *et al.* (2006) divided Mtb isolates from four geographical sites into nine major clusters, based on 36 synonymous SNPs found in four MTBC genomes. SNP-based phylogenetic studies provide confirmation that Mtb is a highly clonal organism and horizontal gene transfer is almost absent.

1.6.4 Molecular epidemiology of tuberculosis

The reliance on conventional epidemiological methods for contact tracing by most TB control programmes often results in underestimation of the level of disease transmission. Large population-based molecular epidemiological studies in San Francisco and Baltimore revealed that only 10-25% of TB cases in designated molecular clusters were linked based on data of conventional contract tracing (Small *et al.*, 1994a; Bishai *et al.*, 1998). Furthermore, casual contact may be responsible for a considerable proportion of molecularly clustered but epidemiologically unlinked cases; this highlights the importance of the molecular epidemiological approach in understanding TB transmission dynamics (Valway *et al.*, 1998).

Figure 1.15: The global population structure and geographical distribution of Mtb



(A) LSPs defining the global population structure of Mtb strains. Numbers in rectangular boxes represent the lineage-defining LSPs or RDEs. (B) The geographical distribution of six main lineages of Mtb. Each coloured circle corresponds to 1 of 80 countries represented in the global strain collection. The colours of the circles correspond to the six lineages defined in (A) and indicate the dominant lineage(s) in respective countries. Reproduced from the article by Gagneux *et al.* (2006) with permission from the National Academy of Sciences. Copyright 2002 National Academy of Sciences, U.S.A.

Molecular clustering is based on identical or similar fingerprints of strains isolated from at least two patients. It is not synonymous with epidemiological clustering, i.e. patient-patient link, especially in areas with low Mtb genetic diversity or in areas of high endemicity. Clustered cases can be considered as part of chains of relatively recent TB transmission, while unique cases are due to endogenous reactivation of latent infection. The degree of polymorphism must be sufficient to distinguish non-epidemiologically linked strains and yet adequately low to reliably link related cases. Degrees of molecular clustering are dependent upon the genotyping method used and the stringency of analysis. The criteria of strain relatedness should be assessed with the appropriate study question and population in mind. Several molecular epidemiological studies in low-incidence populations reported that 35-45% of TB cases were clustered molecularly (Small *et al.*, 1994a; van Soolingen *et al.*, 1999). However, proportions of clustering as high as 70% have been noted in high-incidence populations (Glynn *et al.*, 2005).

Molecular epidemiological studies are also valuable in examining the nature and extent of drug resistance among Mtb strains, and their evolutionary dynamics during transmission (Bifani *et al.*, 1996). The former include investigating the association of specific mutations with phenotypic resistance and the prevalence of specific mutations in a population. Molecular genotyping can also distinguish between recurrent TB due to endogenous reactivation and exogenous reinfection (van Rie *et al.*, 1999), and has proven useful for investigation and confirmation of false-positive cultures due to laboratory error or cross-contamination (Fitzpatrick *et al.*, 2004).

1.6.5 Genotyping methods for *M. tuberculosis*

Most genotyping tools for Mtb make use of repetitive DNA sequences and insertion sequences. The former can exist as interspersed repeats (direct repeats and insertion sequence-like repeats) or tandem repeats (head-to-tail direct uninterrupted repeats). Minisatellites, 10- to 100-bp repeats known as variable-number tandem repeats (VNTRs), are widely distributed in most bacterial genomes. They can be found in intergenic regions, in regulatory regions, or within coding regions. Insertion sequences (IS) are small mobile genetic elements, usually less than 2.5 kb in size, that are

abundant in most prokaryotic genomes. Transposition of IS elements has been identified as one of the sources of genomic variations among *Mtb* strains (Fang *et al.*, 1998).

1.6.5.1 IS6110 restriction fragment length polymorphism analysis

IS6110 is a 1,355-bp member of the IS3 family and is unique to the MTBC (Thierry *et al.*, 1990). IS6110 elements are more or less randomly distributed throughout the genome, although preferred insertion sites exist. These insertion elements present in copy numbers ranging from rare strains lacking any of them to those with 26 copies. IS6110 RFLP analysis has been the gold standard for the genotyping of MTBC strains. The restriction endonuclease PvuII is used to cleave IS6110 at a single asymmetric site, yielding chromosomal fragments that are then subjected to Southern blot hybridisation analysis. The resulting RFLP patterns are analysed by computer software programme. The stability of the IS6110 RFLP patterns has proven to be sufficient for the study of TB transmission (Warren *et al.*, 2002). However, IS6110 fingerprinting has limited resolution when analysing strains with six or fewer IS6110 copies.

1.6.5.2 Polymorphic GC-rich repetitive sequence genotyping

Polymorphic GC-rich repetitive sequence (PGRS) genotyping is another Southern blot technique that involves hybridisation of a specific 3.4-kb probe, which is cloned in plasmid pTBN12, to AluI-digested DNA fragments. IS6110 low-copy-number *Mtb* strains have been discriminated by PGRS genotyping (Chaves *et al.*, 1996). The PGRS-based hybridisation patterns are often too complex for standardisation and computational analysis.

1.6.5.3 Spacer oligonucleotide typing

MTBC complex strains have a distinct chromosomal region consisting of multiple 36-bp direct repeats (DRs) interspersed by 35 to 41-bp unique spacer DNA sequences. Spacer oligonucleotide typing (spoligotyping) is based on the detection of 43 of these spacer sequences, which were originally identified in *Mtb* H37Rv and *M. bovis* BCG strain P3 (Groenen *et al.*, 1993). Membranes spotted with 43 synthetic oligonucleotides are hybridised with labeled PCR-amplified DR locus of the test strain, and the resulting pattern is detected by chemiluminescence. Spoligopatterns are subjected to computational analysis and the resulting data are presented in binary format. The fourth edition of the international spoligotyping database, SpolDB4, contains 1,939 different spoligotypes identified worldwide that are organised into families (Brudey *et al.*, 2006). Spoligotyping has been used to discriminate among *Mtb* strains having few *IS6110* copies (Yang *et al.*, 2000). However, deletions of DRs and spacers can occur multiple times and independently, resulting in the appearance of identical spoligopatterns in phylogenetically unrelated *Mtb* strains (Warren *et al.*, 2002).

1.6.5.4 Variable-number tandem repeat and mycobacterial interspersed repetitive unit genotyping

Frothingham and Meeker-O'Connell (1998) identified 11 VNTR loci in MTBC strains which consist of five major polymorphic tandem repeats (A-E) and six exact tandem repeats (A-F), ranging in size from 53 to 79 bp. Supply *et al.* (2000) identified 41 mycobacterial interspersed repetitive units (MIRUs), which are tandem repeats of 40-100 bp, located in mammalian-like minisatellite regions distributed around the chromosome of *Mtb* H37Rv, *Mtb* CDC 1551, and *M. bovis* AF2122/97. Twelve of the 41 MIRU loci were selected for fingerprinting analysis of clinical *Mtb* strains and were reported in a 12-digit format corresponding to the number of repeats at each locus. However, the combined biological clock of 12-locus MIRU analysis is too slow to be used for the study of endemic strains. The interrogation of 15 MIRU loci increases the resolution power of the genotyping method to that comparable with the *IS6110* RFLP analysis. A high-resolution MIRU-VNTR genotyping system using an automated sequencer and PCR primers tagged fluorescent dyes is now available.

1.6.5.5 Single-nucleotide polymorphism analysis

There appears to be little variation in the genome sequence among different Mtb. Therefore, noted genetic polymorphisms at the nucleotide level, both non-synonymous SNPs and synonymous SNPs, have been useful biomarkers to distinguish among these strains and for phylogenetic analyses. Non-synonymous polymorphisms result in change in amino acids that might arise due to internal or external selection pressure. Synonymous polymorphisms do not alter the amino acid sequence and are not associated with selection pressure. Polymorphisms of this type in structural or housekeeping genes have been exploited to study the phylogenetic structure and relationship of Mtb strains (Sreevatsan *et al.*, 1997; Alland *et al.*, 2003; Gutacker *et al.*, 2006). Despite its high resolution and precision, SNP analysis requires extensive genomic sequencing of multiple chromosomal targets, and is therefore tedious and costly. However, the recent introduction of sequencing technology such as the Solexa system (Illumina) has enabled a more productive and cost-effective whole-genome sequencing and SNP genotyping. This technology is based on large-scale parallel sequencing of millions of DNA fragments using reversible terminator-based fluorescence sequencing chemistry.

1.6.5.6 Genomic deletion analysis

Comparison between the complete genome sequences of Mtb strains H37Rv and CDC 1551 has revealed LSPs in addition to SNPs (Fleischmann *et al.*, 2002). Most LSPs arise as a consequence of genomic deletions and rearrangements rather than via recombination following horizontal DNA transfer (Brosch *et al.*, 2001). Deletions can occur in putative ORFs, intergenic regions, and housekeeping genes (Kato-Maeda *et al.*, 2001; Brosch *et al.*, 2002). Several studies demonstrated that deletions are not always randomly distributed throughout the genome but then to be aggregated (Kato-Maeda *et al.*, 2001; Tsolaki *et al.*, 2004). Deletions of up to 4.2% of the entire genome of clinical Mtb isolates relative to the genome of strain H37Rv have been reported (Tsolaki *et al.*, 2004).

Ancestral and frequent deletions are useful genetic markers for studying the clonal relationship among *Mtb* strains and the resulting data can be used to construct phylogenies. Unique and irreversible genomic deletions have been exploited for evolutionary and phylogenetic analysis of global MTBC (Brosch *et al.*, 2002; Mostowy *et al.*, 2002) and *Mtb* strains (Hirsh *et al.*, 2004; Gagneux *et al.*, 2006). Genomic deletion analysis has also been used to monitor the transmission of TB within urban populations (Rajakumar *et al.*, 2004; Freeman *et al.*, 2005). However, not all LSPs are unique-event polymorphisms and these recurrent LSPs can be a form of convergent evolution. Alland *et al.* (2007) described the occurrence of non-unique LSPs in *Mtb* strains due to positive selection pressure. Furthermore, some genomic deletions can be attributed to IS transposition and can occur independently in different strains. For instance, the RvD5 and DR loci are hot spots for IS6110 insertions (Brosch *et al.*, 2002).

Genomic deletion analysis, also referred to as deligotyping, can be performed using a simple PCR-based method (Brosch *et al.*, 2002; Rajakumar *et al.*, 2004; Gagneux *et al.*, 2006), the GeneChip microarray technique, or by direct DNA sequencing (Tsolaki *et al.*, 2004, Hirsh *et al.*, 2004). The discriminatory power of this genotyping approach can be greatly enhanced if the regions flanking the deletions are sequenced. Recently, a high-throughput deligotyping approach was developed for the rapid screening of clinical isolates (Goguet de la Salmoniere *et al.*, 2004). This method is based on hybridisation of multiplex-PCR products to membrane-bound, highly specific oligonucleotide probes. The use of deligotyping for epidemiology- and phylogenetic-based studies is still at its infancy stage and is yet to be standardised. Evaluation of this approach with different collections of clinical strains and in different epidemiological and geographical setting is required.

1.7 Aims and Objectives

The overall aim of this study was to develop novel analytical methods with potential to increase our understanding of TB transmission and subsequently contribute to public health control and case management.

1.7.1 Molecular assay

The specific aims were:

- ❖ To develop and evaluate a rapid molecular assay that differentiates between MTBC and NTM in positive mycobacterial cultures and ultimately to apply this directly to AFB smear-positive respiratory specimens.
- ❖ To develop and evaluate a rapid binary genotyping assay applicable to cultures positive for Mtb and smear-positive respiratory specimens based on a LSP that is highly prevalent in Leicester in order to facilitate early assessment of potential recent transmission events.

1.7.2 Mask sampling

The specific aims were:

- ❖ To develop methods to assess and evaluate the feasibility a novel mask approach for sampling and quantification of respiratory-borne Mtb from patients with active pulmonary TB.
- ❖ To determine whether there is a correlation between Mtb aerosol output and the infectiousness of TB patients in order to establish the transmission risk presented by particular patients.
- ❖ To investigate the pattern of Mtb excretion among TB patients.

CHAPTER 2

Materials and Methods

2.1 Bacterial Strains, DNA, and Viral Type

Table 2.1: Bacterial strains and DNA used in this study

Bacterial strain	Description	Source/Reference
<i>Corynebacterium glutamicum</i>	Widely used in the industry for the production of amino acids	ATCC 13032
<i>M. bovis</i> BCG Glaxo	TB vaccine strain	GlaxoSmithKline
<i>M. smegmatis</i> MC ² 155	Strain with high transformation efficiency	ATCC 700084
Mtb H37Rv	Virulent laboratory strain	NCTC 10942
<i>Rhodococcus</i> sp.	Unknown species	Departmental stocks
<i>Streptomyces fradiae</i> T59235	Wild-type tylosin producer	E. Cundliffe (Wilson <i>et al.</i> , 1998)

DNA	Description	Source/Reference
<i>Acinetobacter baumannii</i> AYE	Strain with the largest antibiotic resistance island	F. Shaikh (Shaikh <i>et al.</i> , 2009)
<i>Escherichia coli</i> K-12	A well-studied enteric bacterium	Laboratory stocks*
<i>Haemophilus influenzae</i>	Clinical isolate	Laboratory stocks*
<i>Moraxella catarrhalis</i>	Clinical isolate	Laboratory stocks*
<i>M. smegmatis</i> MC ² 155	Strain with high transformation efficiency	Laboratory stocks†
Mtb CDC 1551	Virulent and highly infectious strain	Colorado State University, USA‡
Mtb CH	Index isolate from TB outbreak	Laboratory stocks†
<i>Pseudomonas aeruginosa</i> PA14	Opportunistic pathogen isolated from a burn patient	E. M. Harrison (Harrison <i>et al.</i> , 2010)
<i>Shigella flexneri</i> 2a	Serotype that causes bacillary dysentery in humans	J. D. Lonnen (Lonnen, 2007)
<i>Staphylococcus aureus</i> Newman	Genome-sequenced reference strain	Laboratory stocks*
<i>Streptococcus pneumoniae</i> D39	Virulent encapsulated serotype 2 strain	Laboratory stocks*

* DNA was extracted from liquid cultures using the QIAamp DNA Mini Kit (Qiagen, California, USA) according to the manufacturer's protocol for isolation of genomic DNA from Gram-positive bacteria.

† DNA was extracted from liquid cultures according to the protocol by Belisle and Sonnenberg (1998).

‡ Part of National Institutes of Health, National Institute of Allergy and Infectious Diseases contract HHSN266200400091C, entitled "Tuberculosis Vaccine Testing and Research Materials".

Mycobacteriophage D29 was obtained from laboratory stocks.

2.2 Culture Media and Reagents

All culture media were obtained from Becton Dickinson (Maryland, USA) unless otherwise indicated. All chemicals were obtained from Sigma-Aldrich (Poole) or Fisher Scientific (Loughborough), unless otherwise stated. Media and reagents were sterilised by autoclaving at 121°C for 15 minutes unless otherwise indicated.

2.2.1 Culture media

ADC supplement

USE: Growth supplement for Middlebrook 7H9 broth

Albumin-dextrose-catalase (ADC) supplement was prepared by dissolving 7.5 g of bovine serum albumin (BSA) fraction V, 3 g of D-glucose, 1.28 g of NaCl, and 6 mg of catalase, in distilled water to a final volume of 150 ml. The mixture was centrifuged at 6,371 g for 30 minutes in a Beckman Coulter Avanti J-E refrigerated centrifuge (High Wycombe). The supplement was then sterilised by filtration through a 0.2-µm filter unit (Nalgene, New York, USA) and stored at 4°C away from strong light. Albumin binds toxic free fatty acids, dextrose is an energy source, and catalase destroys toxic peroxides that may be present in the medium (Winn *et al.*, 2006).

AS1 agar

USE: Growth of Streptomyces fradiae

AS1 agar was prepared by dissolving 0.4 g of yeast extract, 0.08 g of L-alanine, 0.08 g of L-arginine, 0.2 g of L-asparagine, 1 g of NaCl, 4 g of Na₂SO₄, 2 g of soluble starch, and 8 g of agar agar (Fisher Scientific), in distilled water to a final volume of 400 ml. The pH of the mixture was adjusted to 8 with 1 M KOH before the addition of soluble starch and agar agar.

LB broth and agar

USE: Non-selective media for growth of bacteria

Luria-Bertani (LB) broth was prepared by dissolving 4 g of tryptone, 2 g of yeast extract, and 2 g of NaCl, in distilled water to a final volume of 400 ml. The pH was adjusted to 7.4. LB agar was prepared as described for LB broth, with the addition of 1.5% w/v agar powder.

Middlebrook 7H9-ADC-Tween broth

USE: Growth of mycobacteria

Middlebrook 7H9 broth was prepared by dissolving 1.88 g of broth powder and 1 g of glycerol in distilled water to a final volume of 360 ml. The broth was supplemented with 10% v/v ADC and 0.05% w/v Tween 80 before use.

Middlebrook 7H9-OADC broth

*USE: Growth of *M. smegmatis* for phage assay lawn*

Middlebrook 7H9 broth was prepared as before, without the addition of Tween 80. The broth was supplemented with 10% v/v oleic acid-albumin-dextrose-catalase (OADC) before use. Tween 80 was excluded as it interferes with adsorption of mycobacteriophages to mycobacterial cells.

Middlebrook 7H9-OGC broth and agar

USE: Phage assay broth and indicator agar, respectively

Middlebrook 7H9 broth was prepared as before, without the addition of Tween 80. The broth was supplemented with 10% v/v OADC and 1 mM CaCl₂ before use. Middlebrook 7H9-OGC agar was prepared as described for the broth, with the addition of 1.5% w/v agar powder. Calcium ions promote adsorption and productive infection of mycobacterial cells by mycobacteriophages (Sellers *et al.*, 1962).

Middlebrook 7H10 agar

USE: Growth of mycobacteria

Middlebrook 7H10 agar was prepared by dissolving 7.6 g of agar powder and 2.5 g of glycerol in distilled water to a final volume of 360 ml. The agar was boiled for 5 minutes to dissolve the powder and then maintained at 55°C until autoclaving. The agar was supplemented with 10% v/v OADC before use.

OADC supplement

USE: Growth supplement for Middlebrook 7H10 agar and phage assay broth and indicator agar

OADC was prepared as described for ADC, with the addition of 8.63 ml of oleic acid (1% w/v in 0.2 M NaOH). Before centrifugation, the mixture was sonicated (Decon FS 100, Ultrasonics, Hove Sussex) for 30 minutes to emulsify the oleic acid. Oleic acid is an important metabolic stimulant for mycobacteria (Winn *et al.*, 2006).

2.2.2 Reagents

Ferrous ammonium sulphate

USE: Virucide for phage D29

FAS was prepared fresh before use. Two concentrations were prepared, 50 mM and 200 mM, depending on the processing volume. FAS was dissolved in distilled water and sterilised by filtration through a 0.2-µm syringe filter (Pall Corporation, Michigan, USA).

Gel loading buffer, 6x

USE: To load and monitor migration of PCR products during gel electrophoresis

Gel loading buffer contained 0.25% w/v Orange G and 30% v/v glycerol. The buffer was diluted 6 times with PCR products prior to loading on the gel.

Glycerol solution, 65% v/v

USE: Long-term storage of bacterial strains at -80°C

Glycerol solution was prepared by mixing 162.5 g of glycerol, 20 ml of 1 M MgSO₄, and 5 ml of 1 M Tris-HCl (pH 8), in a final volume of 200 ml.

Lysozyme buffer

USE: Preparation of lysozyme solution for DNA isolation

Lysozyme buffer contained 20 mM Tris-HCl (pH 8), 2 mM disodium ethylenediaminetetraacetate dihydrate (EDTA), and 1.2% w/v Triton X-100. Lysozyme stock solution of 100 mg/ml was prepared for enzymatic digestion of sputum specimens.

NaOH-NALC solution

USE: Decontamination of mycobacterial samples

NaOH-NALC solution was prepared fresh before use. 4% w/v NaOH and 2.9% w/v sodium citrate dihydrate were mixed in equal proportions. NALC was then added to 0.5% w/v and the mixture was sterilised by filtration through a 0.2-µm syringe filter.

NOA antimicrobial supplement

USE: To inhibit growth of non-target organisms during analysis of clinical mask samples by the phage assay

NOA was prepared fresh before use by mixing 11.25 mg/ml nystatin (dissolved in methanol), 153.5 mg/ml oxacillin (dissolved in sterile distilled water), and 9 mg/ml aztreonam (dissolved in formamide-methanol 1:1 mixture) in equal proportions. The final concentrations were 3, 200, and 4.5 times, higher than published concentrations, respectively (Biotec Laboratories, 2005; Albert *et al.*, 2007; Mole *et al.*, 2007). This NOA cocktail was incorporated into the phage indicator agar.

Phosphate buffer, 67 mM, pH 6.8

USE: To neutralise NaOH during decontamination of mycobacterial samples

Phosphate buffer was prepared by mixing 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 to final concentrations of 34 mM and 33 mM, respectively. In addition to neutralising NaOH, this buffer also washes the sample, dilutes toxic substances, and decreases the specific gravity of the sample (Winn *et al.*, 2006).

Triton-Tris solution

USE: To elute mycobacterial cells from masks

Triton X-100 solution (10% w/v) was prepared by dissolving 10 g of the stock solution in distilled water to a final volume of 100 ml and then warmed at 40°C in a waterbath for 30 minutes. The solution was sterilised by filtration through a 0.2- μm filter unit and stored at 4°C away from strong light. Triton-Tris solution was prepared by mixing 10% w/v Triton X-100 and 1 M Tris-HCl (pH 6.4) to final concentrations of 1% w/v and 10 mM, respectively.

TAE buffer

USE: Buffer for dissolving agarose powder and gel electrophoresis

Tris-acetate-EDTA (TAE) buffer was prepared by dissolving 242 g of Tris base, 37.2 g of EDTA, and 57.1 ml of glacial acetic acid, in distilled water to a final volume of 1 litre. The buffer was diluted 50 times before use.

Tween 80, 10% w/v

USE: To minimise clumping of mycobacterial cells during growth in liquid medium

Tween 80 (10% w/v) was prepared as described for Triton X-100.

2.3 Oligonucleotide Primers and TaqMan Probes

Table 2.2: Oligonucleotide primers and TaqMan probes used in this study

PCR assay	Primer/Probe designation	Nucleotide sequence
16S	MYCO16SF	5'-GAAACTGGGTCTAATACCG-3'
	MYCO16SR	5'-ATCTCAGTCCCAGTGTGG-3'
	MYCO16SPr	5'-ROX-TCCACCACAAGACATGCATCCCGTG-BHQ2-3'
RD	RD750F	5'-CTTAAGCGTCCGCGTATCC-3'
	RD750R1	5'-GCCACAGCTGTACAGGTCAA-3'
	RD750R2	5'-AACTTTCGGCGGTCAAGTGA-3'
	RD750Pr	5'-Cy5-CCGTTGGCGCAGAGCACTCC-BBQ-3'
EB-16S	16S-338F	5'-ACTCCTACGGGNGGCNNGCA-3'
	16S-515R	5'-GTATTACCGCANNCTGCTGGCAC-3'
<i>Rv1519</i>	<i>Rv1519F</i>	5'-GGTGGAGAGCGACGACATCAAG-3'
	<i>Rv1519R</i>	5'-TGTAGCGACAGCATCGTCATCC-3'
LAMP	MTB-FIP	5'-CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT-3'
	MTB-BIP	5'-TCGGGATAAGCCTGGACCACAAGACATGCATCCCGT-3'
	MTB-F3	5'-CTGGCTCAGGACGAACG-3'
	MTB-B3	5'-GTCATCCCACACCGC-3'
	MTB-FLP	5'-GTTCCGCACTCGAGTATCTCCG-3'
	MTB-BLP	5'-GAAACTGGGTCTAATACCGG-3'
IS6110	IS6110F	5'-CCTGCGAGCGTAGGCGTCGG-3'
	IS6110R	5'-CTCGTCCAGCGCCGCTTCGG-3'

TaqMan probes: ROX, 6-carboxy-X-rhodamine (excitation, 585 nm/detection, 610 nm); BHQ2, Black Hole Quencher 2 (absorbance 550-650 nm); Cy5, cyanine 5 (excitation, 625 nm/detection, 660 nm); BBQ, BlackBerry Quencher (absorbance 550-750 nm).

References: 16S primers, R. C. Free, unpublished; MYCO16SPr, Wilkinson *et al.*, 2001; RD primers, Malkin, 2005; EB-16S (eubacterial 16S) primers, Free, 2005; *Rv1519* primers, Rajakumar *et al.*, 2004; LAMP primers, Pandey *et al.*, 2008; IS6110 primers, Negi *et al.*, 2007.

Sources: All primers and MYCO16SPr were synthesised by MWG Biotech (Ebersberg, Germany). RD750Pr was designed and synthesised by TIB MOLBIOL (Berlin, Germany).

2.4 General Methods

2.4.1 Measuring optical density of cultures

The optical density of cultures was measured at a wavelength of 580 nm (OD_{580nm}) using the Sanyo SP75 UV/Vis spectrophotometer (Watford) in the Category 1 Laboratory, and the Jenway 6300 spectrophotometer (Stone) in the Category 3 Laboratory. For the latter, 1 ml of culture was transferred into a 1.5-ml cuvette (Fisher Scientific), which was then sealed with autoclave tape and Nescofilm (Bando Chemical, Kobe, Japan). Dense cultures ($OD_{580nm} > 1$) were diluted ten-fold with appropriate liquid media prior to measurement.

2.4.2 Preparation of stock cultures for long-term storage

Stock cultures of *M. smegmatis*, *M. bovis* BCG, and Mtb were prepared by mixing exponential cultures and 65% v/v glycerol in equal proportions, which were then distributed into 1-ml aliquots in 1.5-ml cryovials (Nalge Nunc, Roskilde, Denmark) for long-term storage at -80°C .

2.4.3 Cultivation of *M. smegmatis*

M. smegmatis was grown by streaking a loopful of glycerol stock or a single isolated colony from a previous plate culture onto Middlebrook 7H10 agar. Plates were incubated at 37°C until isolated colonies became visible; this normally took 2-3 days. A loopful of these colonies was emulsified in Middlebrook 7H9-Tween broth. The OD_{580nm} of the cell suspension was measured, and the appropriate volume of suspension was used to inoculate 25 or 50 ml of Middlebrook 7H9-ADC-Tween broth in a 125- or 250-ml conical flask respectively (SciLabware, Stone) to OD_{580nm} of 0.05 or 0.1. Liquid cultures were incubated at 37°C with shaking at 200 rpm for up to 24 hours. *M. smegmatis* doubles approximately every 3 hours in the exponential phase.

2.4.4 Cultivation of *M. bovis* BCG

Two aliquots of *M. bovis* BCG glycerol stocks were thawed and then used to inoculate 5 ml of Middlebrook 7H9-ADC-Tween broth in a 30-ml Universal tube (Sterilin, Bargoed) each. Cultures were incubated static at 37°C; it took about 10 days to reach OD_{580nm} of 1. *M. bovis* BCG was subcultured from one of these starter cultures into 25 ml of fresh 7H9-ADC-Tween broth in a 125-ml conical flask to OD_{580nm} of 0.05, and grown at 37°C for 3-4 days. *M. bovis* BCG doubles approximately every 24 hours in the exponential phase (based on personal experience).

2.4.5 Cultivation of *M. tuberculosis*

Mtb is classified as a Category 3 hazardous organism by the Advisory Committee on Dangerous Pathogens, and therefore all work with this mycobacterium was carried out in a microbiological safety cabinet within the Category 3 Laboratory, in accordance with the Code of Practice in place. Mtb starter cultures were prepared as described for *M. bovis* BCG (Section 2.4.4). Mtb was then subcultured from one of these into a 125-ml polycarbonate conical flask (Corning Life Sciences, Massachusetts, USA) and incubated with shaking at 100 rpm. Mtb doubles approximately every 14-20 hours in the exponential phase (based on personal experience).

2.4.6 Enumeration of colony-forming units

Colony counting was carried out by the drop plate method (Hoben and Somasegaran, 1982). Ten-fold serial dilutions of a liquid culture or cell suspension were performed in 450- μ l aliquots of Middlebrook 7H9-Tween broth in 1.5-ml microtubes (Eppendorf, Cambridge). Three 20- μ l drops were plated out for each dilution on Middlebrook 7H10 agar in duplicate, on separate plates. Agar plates were incubated at 37°C until isolated colonies became visible. This took about 2-3 days for *M. smegmatis* and at least 3-4 weeks for *M. bovis* BCG and Mtb. The dilution that produced 10-100 colonies was used to calculate the number of CFU in the test sample.

CHAPTER 3

**Development and Evaluation of a
Real-time PCR Assay for
Identification of Mycobacteria and
Binary Genotyping of *M.*
*tuberculosis***

3.1 Introduction

Early detection and identification of Mtb is important for successful treatment and prevention of further person-to-person transmission. In the UK, clinicians rely on preliminary clinical findings and AFB smear results to suspect or dismiss the possibility of pulmonary TB at an early stage. Confirmatory identification of Mtb by culture and by further tests may require 2-3 weeks, particularly for smear-positive cases. Differentiation of Mtb from NTM is of fundamental importance for both clinical management and public health control; infection by the latter is predominantly sporadic and often reflects pre-existing medical conditions in the patients (Campbell *et al.*, 2000; Griffith *et al.*, 2007). Since the findings of identification tests may take several weeks to become available to the healthcare team, a preliminary response to a particular case in which mycobacteria have been isolated may lead to inappropriate isolation and treatment of patients for TB and unnecessary public health investigations.

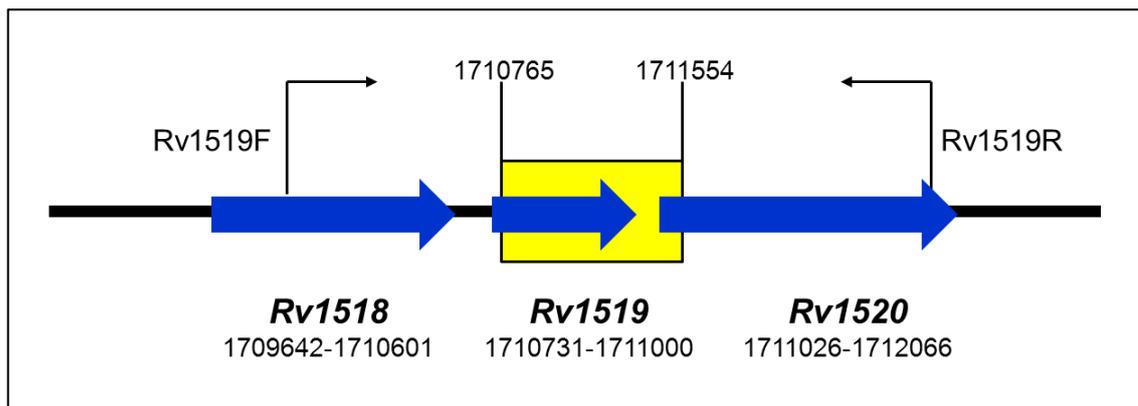
This chapter reports the development and evaluation of a rapid molecular assay based on the mycobacterial 16S rDNA, that is capable of distinguishing between Mtb and NTM to facilitate identification of TB patients for the mask sampling study. We also explored the clinical utility of this assay with clinical mycobacterial isolates and smear-positive respiratory specimens, in relation to patient and public health management. In developed countries, most well-resourced mycobacteriology laboratories rely on automated culture systems as the gold standard for the detection and isolation of mycobacteria. Cultures positive for AFB are normally transferred to a regional reference laboratory for mycobacterial identification, usually by a molecular assay. Therefore, definitive diagnosis of patients with mycobacterial infections may be delayed by as long as 2-3 weeks. In this context, the molecular assay, in conjunction with subsequent DNA sequencing, will yield the identity of the mycobacterial species for both positive cultures and respiratory specimens at an earliest possible time.

We also developed and evaluated a rapid binary genotyping assay applicable to Mtb-positive cultures and smear-positive respiratory specimens, based on a LSP which is highly prevalent among the Mtb strains in the local service. Mtb strain CH was the index isolate responsible for a major school-associated TB outbreak in Leicester in

2001 (Rajakumar *et al.*, 2004). With Mtb H37Rv as the reference strain, microarray analysis was used to interrogate the genome sequence of CH for strain-specific genetic markers (Shafi *et al.*, 2002). Eleven ORFs that were potentially deleted from the CH genome were identified; these consisted of three single and four adjacent pairs of ORFs distributed over seven distinct loci on its chromosome. These seven potential genomic deletions were verified by PCR analysis across the predicted deleted regions. Five of these loci were confirmed to have deletions ranging in size from 0.8 to 2.0 kb; they are *Rv1519*, *Rv0180c*, *echA19/Rv3517*, *PPE66/PPE67*, and *Rv1995/Rv1996*. DNA sequences of the junctions across these five deleted loci were sequenced for further verification of deletions in these genomic regions. The panel of five individual PCRs required for interrogation of these five loci was collectively referred to as the genome level-informed PCR (GLIP) assay. All outbreak-associated Mtb isolates exhibited the five-deletion profile and shared common VNTR-MIRU profiles. None of the non-outbreak-linked isolates showed the presence of all five deletions. During ongoing surveillance months after the outbreak, the GLIP assay identified further outbreak-associated cases and dismissed several epidemiologically suspected cases.

The schematic representation of the *Rv1519* deletion in CH is shown in Figure 3.1. This LSP was subsequently designated RD750 and recognised as one of the six phylogeographical lineage-defining genomic deletions in a global collection of Mtb strains (Gagneux *et al.*, 2006). RD750 defines the East African-Indian (EA-I) lineage (Gagneux *et al.*, 2006), and is also designated as the Central Asian lineage based on spoligotyping (Gagneux and Small, 2007). Intriguingly, the RD750 deletion has been associated with an immunosubversive phenotype in which the CH strain is able to induce anti-inflammatory interleukin 10 production by macrophages (Newton *et al.*, 2006). The RD750 deletion is present in approximately 50% of Leicester Mtb isolates (J. Malkin and H. Patel, personal communication), which likely reflects the city's diverse ethnic population. In the 2001 census, 31% of population in the city of Leicester was of an Asian ethnic background with the majority being Indian from either East Africa or Gujarat (Roberts-Thomson *et al.*, 2008). Through the RD binary genotyping assay, early recognition of whether Mtb strains belong to the EA-I group has potential to assess for the likelihood of recent transmission events.

Figure 3.1: Schematic illustration of the RD750 polymorphism



The coordinates of *Rv1519* are shown below its designation. The yellow box and flanking coordinates indicate the region deleted (RD750) in Mtb CH. The bent arrows represent the approximate positions of the Rv1519 primer annealing sites. Adapted from the article by Rajakumar *et al.* (2004) with permission from the American Society for Microbiology. Copyright © 2004, American Society for Microbiology. All Rights Reserved.

3.2 Materials and Methods

3.2.1 Bacterial strains, DNA, and clinical specimens

The bacterial strains and DNA used are listed in Table 2.1. All clinical isolates (MGIT and LJ cultures) and TB sputum samples were obtained from the Department of Clinical Microbiology, University Hospitals of Leicester (UHL) NHS Trust.

3.2.2 DNA isolation

3.2.2.1 DNA release by boiling

MGIT cultures were centrifuged at 755 *g* for 22 minutes and the resulting pellets were resuspended in 0.2 ml of PCR-grade water (Invitrogen, Paisley). Colonies were scrapped off LJ slopes and resuspended in 0.2 ml of PCR-grade water. Culture suspensions from MGIT and LJ were boiled at 100°C for 30 minutes, centrifuged at 16,100 *g* for 2 minutes, and 5 µl of each resulting supernatant used for PCR analysis.

C. glutamicum and *Rhodococcus sp.* were cultured on LB agar for 1-2 days, and in 5 ml of LB broth overnight with shaking at 200 rpm, both at 37°C. *S. fradiae* was cultured on AS1 agar and in 25 ml of LB broth in a spring-containing conical flask (SciLabware) under the same conditions; the spring facilitates breaking of clumps in the culture. DNA lysates were prepared as described for MGIT and LJ cultures for liquid and solid cultures, respectively.

3.2.2.2 DNA release with *microLYSIS* reagent

DNA was extracted from samples with the *microLYSIS* reagent (Microzone, West Sussex), a specially formulated reagent for lysing cells, according to the manufacturer's protocol. 2 µl of sample was treated with 18 µl of 20x *microLYSIS*. Thermal cycling was performed in the Dyad DNA Engine (MJ Research, Massachusetts, USA) as follows: 65°C for 5 minutes, 96°C for 2 minutes, 65°C for 4 minutes, 96°C for 1 minute, 65°C for 1 minute, 96°C for 30 seconds, and a hold at 20°C.

3.2.2.3 DNA isolation from sputum samples

NaOH decontamination

Each sputum sample was treated with an equal volume of 4% w/v NaOH in a 30-ml Universal tube. Tube contents were mixed on a tube rotator (Bibby Scientific, Stone) at room temperature for 25 minutes. An equal volume of 14% w/v potassium dihydrogen orthophosphate was then added to neutralise the NaOH. The tube was centrifuged at 2,500 *g* for 25 minutes, and the resulting supernatant discarded. The pellet was resuspended in 1 ml of PCR-grade water, and the resulting suspension boiled at 100°C for 30 minutes.

QIAamp DNA Mini Kit

Sputum DNA extraction was performed using the QIAamp DNA Mini Kit according to the manufacturer's protocol for isolation of genomic DNA from Gram-positive bacteria, with minor modifications according to Aldous *et al.* (2005). 100 µl of NaOH-decontaminated sputum sample was centrifuged at 5,000 *g* for 10 minutes in a 1.5-ml Eppendorf tube and the supernatant removed to a new Eppendorf tube and set aside. The pellet was incubated in 200 µl of Buffer ATL (tissue lysis buffer) containing 30 mg/ml lysozyme at 37°C for 30 minutes. Tube contents were then boiled at 100°C for 15 minutes then treated with 20 µl of proteinase K at 56°C for 1 hour, followed by brief centrifugation and treatment with 200 µL of Buffer AL (lysis buffer) at 70°C for 10 minutes. The supernatant which was set aside earlier was made up to a final volume of

200 µl with Buffer AL prior to this incubation. The supernatant and pellet tubes were briefly centrifuged then 200 µl of absolute ethanol added. Following brief centrifugation, tube contents were transferred to a QIAamp Mini spin column held in a 2-ml collection tube, centrifuged at 6,000 *g* for 1 minute, and the column transferred to a new collection tube. The column was washed with 500 µl of Buffer AW1 then the tube centrifuged at 6,000 *g* for 1 minute. The column was transferred to a new collection tube, washed with 500 µl of Buffer AW2, and centrifuged at 16,100 *g* for 3 minutes. The resulting filtrate was discarded then the tube centrifuged at 16,100 *g* for 1 minute to eliminate the chance of possible Buffer AW2 carryover. The column was placed in a new 1.5-ml Eppendorf tube, DNA eluted with 100 µl of Buffer after standing at room temperature for 5 minutes, and centrifuged at 6,000 *g* for 1 minute. The resulting DNA samples were analysed by the 16S and RD assays in volumes of 9.5 and 8.5 µl, respectively (see Section 3.2.4.1).

EliGene MTB Isolation Kit

Sputum DNA extraction was performed using the EliGene MTB Isolation Kit (Elisabeth Pharmacon, Brno, Czech Republic) according to the manufacturer's protocol with minor modifications. The lysis buffer was prepared by reconstituting 15 mg of lysozyme with 1 ml of MI2 solution. Proteinase K was reconstituted with 1.2 ml of PCR-grade water. In a 1.5-ml Eppendorf tube, 100 µl of sputum sample was centrifuged at 14,000 *g* for 10 minutes. The resulting supernatant was set aside. The pellet was incubated in 100 µl of lysis buffer at 37°C for 30 minutes. Tube contents were then treated with 100 µl of MI3 solution and 20 µl of proteinase K at 65°C for 30 minutes, followed by 95°C for 10 minutes. Tube contents were vortexed for 1 minute, briefly centrifuged, left to stand for 5 minutes to equilibrate to room temperature, and then 200 µl of MI4 solution added. The supernatant which was set aside earlier was made up to a final volume of 200 µl with MI4 solution. Contents of both the supernatant and pellet tubes were vortexed for 15 seconds and briefly centrifuged then transferred to the spin filter held in a 2-ml collection tube each, and centrifuged at 13,000 *g* for 1 minute. Following transfer to a new collection tube, the pellet was washed with 500 µl of MI5 solution, centrifuged at 13,000 *g* for 30 seconds, and the resulting filtrate discarded. The filter was washed again with 500 µl of MI6 solution, centrifuged at 13,000 *g* for 30 seconds, transferred to a new collection tube, and 50 µl of MI7 solution (pre-heated at 65°C) added to elute DNA. Following standing at room temperature for 5 minutes, the tube was centrifuged at 13,000 *g* for 1 minute. The elution process was repeated. The resulting DNA

samples were analysed by the 16S and RD assays in volumes of 9.5 µl and 8.5 µl, respectively (see Section 3.2.4.1).

3.2.3 Oligonucleotide primer and TaqMan probe design

The oligonucleotide primers and TaqMan probes used are listed in Table 2.2. Full-length 16S ribosomal DNA sequences representing mycobacteria isolated from humans were retrieved from The Institute for Genomic Research or the Ribosomal Database Project II (Cole *et al.*, 2007). The sequences were aligned using the ClustalX programme with default settings and alignments were processed with a custom built algorithm. MYCO16SF and MYCO16SR primers were designed targeting the 16S rDNA region conserved in all the retrieved mycobacterial sequences (R. C. Free, unpublished). The MTBC-specific 16S TaqMan probe (MYCO16SPr) was that previously reported but with a different fluorophore-quencher pair to allow combination with SYBR Green (Wilkinson *et al.*, 2001). The three RD primers were designed using Primer3 to give products of 126 bp and 193 bp with Mtb strains H37Rv and CH, respectively (Malkin, 2005). RD TaqMan probe (RD750Pr) was designed and synthesised by TIB MOLBIOL. A nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) search demonstrated that none of the primers and TaqMan probes for the 16S and RD assays shared significant similarity with other known nucleotide sequences or each other.

3.2.4 PCR amplification assays

3.2.4.1 Combined 16S-RD assay

For the 16S assay, PCRs were carried out in 25-µl volumes containing 250 nM each primer MYCO16SF and MYCO16SR, 150 nM MYCO16SPr, and 1x ABsolute QPCR SYBR Green Mix (ABgene, Epsom). For the RD assay, PCRs were carried out in 25-µl volumes containing 80 nM of each primer RD750F, RD750R1 and RD750R2, 150 nM RD750Pr, and 1x ABsolute QPCR SYBR Green Mix. Volumes of template DNA tested

varied among different experiments and were specified under individual sections in Results (Section 3.3). PCRs were performed in a Rotor-Gene machine (Qiagen) as follows: 95°C for 15 minutes to activate the *Taq* polymerase, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, 72°C for 20 seconds, and 84°C for 20 seconds. ROX and Cy5 signals were acquired at 60°C while SYBR Green signals were acquired at 84°C. Melt analyses for the 16S and RD assays were performed after the PCR amplification with 1°C increments from 72-95°C, holding 90 seconds for pre-melt conditioning on the first step and 5 seconds on the following steps. SYBR Green signals were monitored during these analyses. Melt curves were examined for all assays. All samples were assayed in duplicates unless specified otherwise.

Real-time PCR signals were analysed by the Rotor-Gene 6000 Series Software 1.7. Dynamic tube normalisation was used for every run, and threshold was set based on agreement between raw and analysed data. The functions “slope correct” and “ignore cycles” were applied to analyses, as necessary. Only runs with correlation coefficients (R^2) and reaction efficiencies above 0.98 and 0.7 respectively were considered legitimate (Dorak, 2006). Replicate reactions were considered reproducible if the difference in cycle threshold (Ct) was less than 1.

3.2.4.2 Rv1519 PCR

This conventional PCR assay differentiates between MTBC strains with intact (RD750⁺) and deleted RD750 (RD750⁻) (Rajakumar *et al.*, 2004). PCRs were performed in 10- μ l volumes containing 1 μ l of DNA lysate, 1 μ M each primer Rv1519F and Rv1519R, 200 μ M dNTPs (Promega, Southampton), 1x PCR buffer (Qiagen), 1x Q solution (Qiagen), and 1 U of Hot start *Taq* polymerase (Qiagen). Amplification was performed in the Dyad DNA Engine as follows: 95°C for 15 minutes to activate the *Taq* polymerase, followed by 40 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 3 minutes. PCR was repeated for all samples yielding no bands or multiple bands in 25- μ l volumes with 5 μ l of neat and diluted DNA lysates (1:10 and 1:100).

3.2.4.3 Loop-mediated isothermal amplification assay

The LAMP assay was performed using the Loopamp DNA Amplification Kit and Fluorescent Detection Reagent (Eiken Chemical, Tochigi, Japan) according to the manufacturer's protocol with minor modifications according to Pandey *et al.* (2008). Sample reactions were performed in a total volume of 25 μ l containing 2 μ l of DNA, 30 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol each of loop primers FLP and BLP, 1x reaction mix, 1 μ l of *Bst* DNA polymerase, and 1 μ l of Fluorescence Detection Reagent. Positive control for the sample reactions with 16S primers was performed with Mtb CDC 1551 DNA, while the negative control was performed with distilled water. Kit controls were performed in a total volume of 25 μ l containing 2.5 μ l of PrimerMix DNA, 1x reaction mix, 1 μ l of *Bst* DNA polymerase, and 1 μ l of Fluorescence Detection Reagent. Positive kit control was performed with the control DNA, which is a plasmid DNA inserted with a 6,557-bp HindIII fragment of lambda phage DNA, while negative kit control was performed with distilled water. Reactions were incubated at 63°C for 1 hour for amplification. The polymerase was inactivated and the amplification terminated following incubation at 80°C for 5 minutes. Visual fluorescence detection was achieved by exposing reaction tubes to the UV light from a transilluminator (Kodak, London).

3.2.5 Post-PCR analyses

3.2.5.1 Gel analysis of PCR amplicons

PCR amplicons from the 16S-RD and LAMP assays were separated by electrophoresis on 2% w/v agarose gels. PCR amplicons from the *Rv1519* assay were separated on 1% w/v agarose gels. All agarose gels contained 0.5 μ g/ml ethidium bromide. All PCR amplicons contained 1x gel loading dye; 10 μ l each was analysed on the gel along with 2.5 μ g of GeneRuler 100-bp DNA ladder (Fermentas Life Sciences, York). Gel electrophoresis was performed at 80-100 V for 1-2 hours, depending on the size of the amplicons to be separated. PCR amplicons were visualised under the G:BOX UV transilluminator (Syngene, Cambridge) following electrophoresis.

3.2.5.2 Gel purification of PCR amplicons

Electrophoresed PCR amplicons were purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. All centrifugation steps were performed at 16,100 *g* for 1 minute. PCR amplicons were excised from the agarose gel with a scalpel then weighed out. Three volumes of Buffer QG were added to one volume of gel in a 1.5-ml Eppendorf tube, i.e. 300 μ l of buffer for a 100-mg gel. The sample was incubated at 50°C for 10 minutes, and mixed by vortexing every 2-3 minutes during the incubation. One gel volume of isopropanol was then added to the dissolved gel suspension, and this mixture applied to a QIAquick spin column, and centrifuged to bind the DNA. 0.5 ml of Buffer QG was added to the column. Following centrifugation, the flow-through was discarded and 0.75 ml of Buffer PE applied for 5 minutes, followed by centrifugation to wash the column. The flow-through was discarded, centrifugation repeated, and the column transferred to a 1.5-ml Eppendorf tube. Finally, 30 μ l of PCR-grade water was applied to the QIAquick membrane for 1 minute then centrifuged to elute DNA. One volume of Loading Dye was added to 5 volumes of purified DNA, and the mixture analysed on agarose gel as described in Section 3.2.5.1. Purified PCR amplicons were used for downstream molecular applications such as cloning and DNA sequencing.

3.2.5.3 Cloning of PCR amplicons

Purified 16S PCR amplicons were ligated into the multiple cloning region of pGEM-T Easy Vectors (Promega) and cloned according to the manufacturer's protocol. The multiple cloning region is located within the α -peptide coding region of the enzyme β -galactosidase; insertional inactivation of the α -peptide allows recombinant clones to be directly identified by blue-white screening on indicator plates. Ligation was performed in 10- μ l volumes containing 2 μ l of PCR amplicons, 1 μ l of pGEM-T Easy Vectors, 1x ligation buffer, and 1 μ l of T4 DNA ligase. Positive control was performed with pUC19 DNA (Biolone, London) to determine the transformation efficiency of the α -Select Gold Efficiency Competent Cells (Biolone). Reactions were incubated at 4°C overnight. 2 μ l of each ligation reaction was added to a 1.5-ml Eppendorf tube on ice. 50 μ l of the competent cells was then added to each reaction, mixed by gentle flicking, and placed

on ice for 20 minutes. Cells were heat-shocked in a water bath at exactly 42°C for 45-50 seconds for transformation, returned immediately to ice for 2 minutes, 950 µl of LB broth added, and the tubes incubated at 37°C with shaking at 200 rpm for 1.5 hours. 100 µl each of neat and 1:10 diluted transformation culture was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. Plates were incubated at 37°C for 16-24 hours. White colonies containing inserts were randomly selected for analysis by the 16S assay and subsequent sequencing of the 16S amplicons.

3.2.5.4 DNA sequencing and analysis

All gel-purified PCR amplicons were sent to MWG Biotech for DNA sequencing, as necessary. Resulting DNA sequences were analysed by BLASTn and/or the Ribosomal Differentiation of Medical Microorganisms (RIDOM) database. DNA alignment was performed online using ALIGN Query at the GENESTREAM network server IGH Montpellier, France (<http://xylian.igh.cnrs.fr/bin/align-guess.cgi>).

3.2.6 Statistical analysis

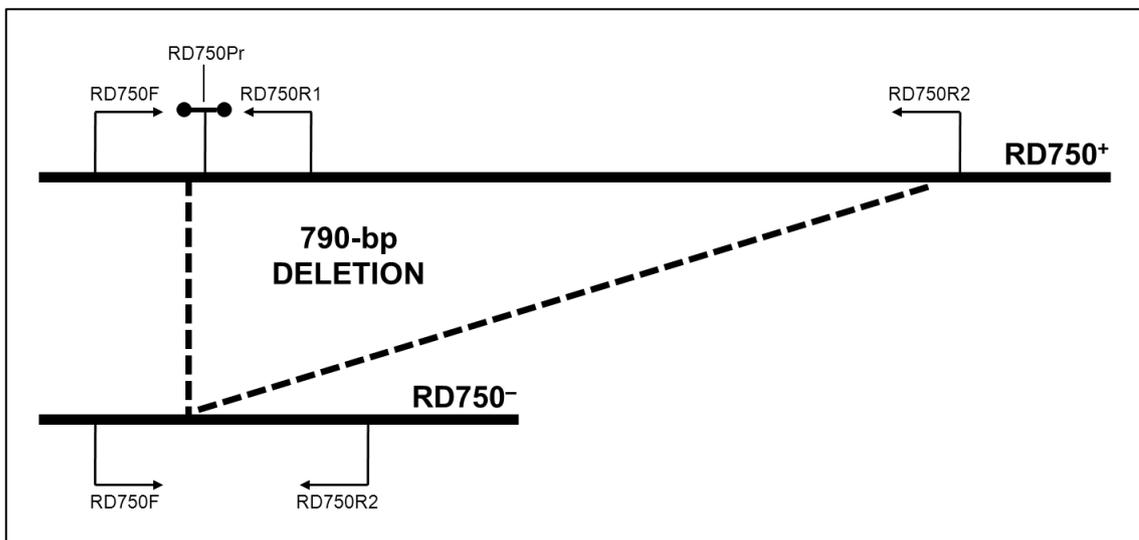
All data in this chapter were analysed by the two-tailed paired *t* test, as required, unless otherwise indicated.

3.3 Results

3.3.1 Principle of the 16S-RD assay

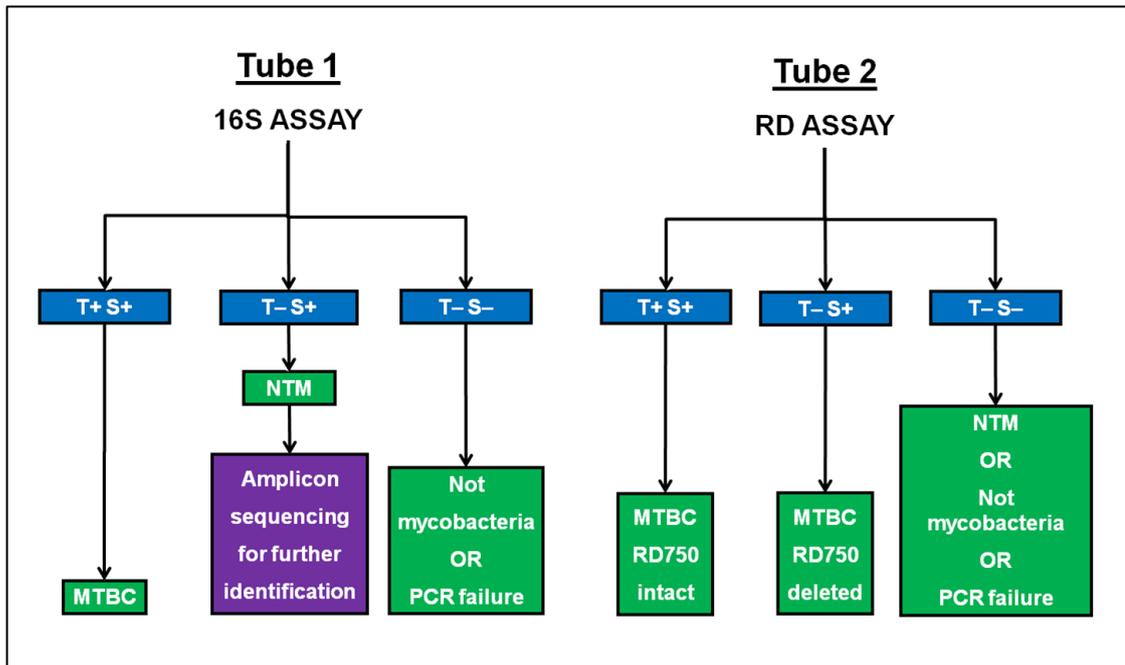
The real-time PCR assay targets the mycobacterial 16S rDNA and the EA-I lineage-defining RD750 polymorphism in separate reaction tubes within a single thermocycler run. It utilises a combination of TaqMan and SYBR Green chemistries to simultaneously differentiate two products in each PCR tube. The 16S assay differentiates between MTBC and NTM by combining *Mycobacterium* genus-specific 16S primers and a MTBC-specific 16S-targeted TaqMan probe. The RD assay distinguishes between MTBC strains with intact (RD750⁺) and deleted RD750 (RD750⁻) regions by use of hemi-nested primers and an RD750⁺ specific TaqMan probe. The principles of the 16S-RD assay are shown in Figures 3.2-3.4.

Figure 3.2: Diagrammatic illustration of the RD assay targets and oligonucleotide binding sites



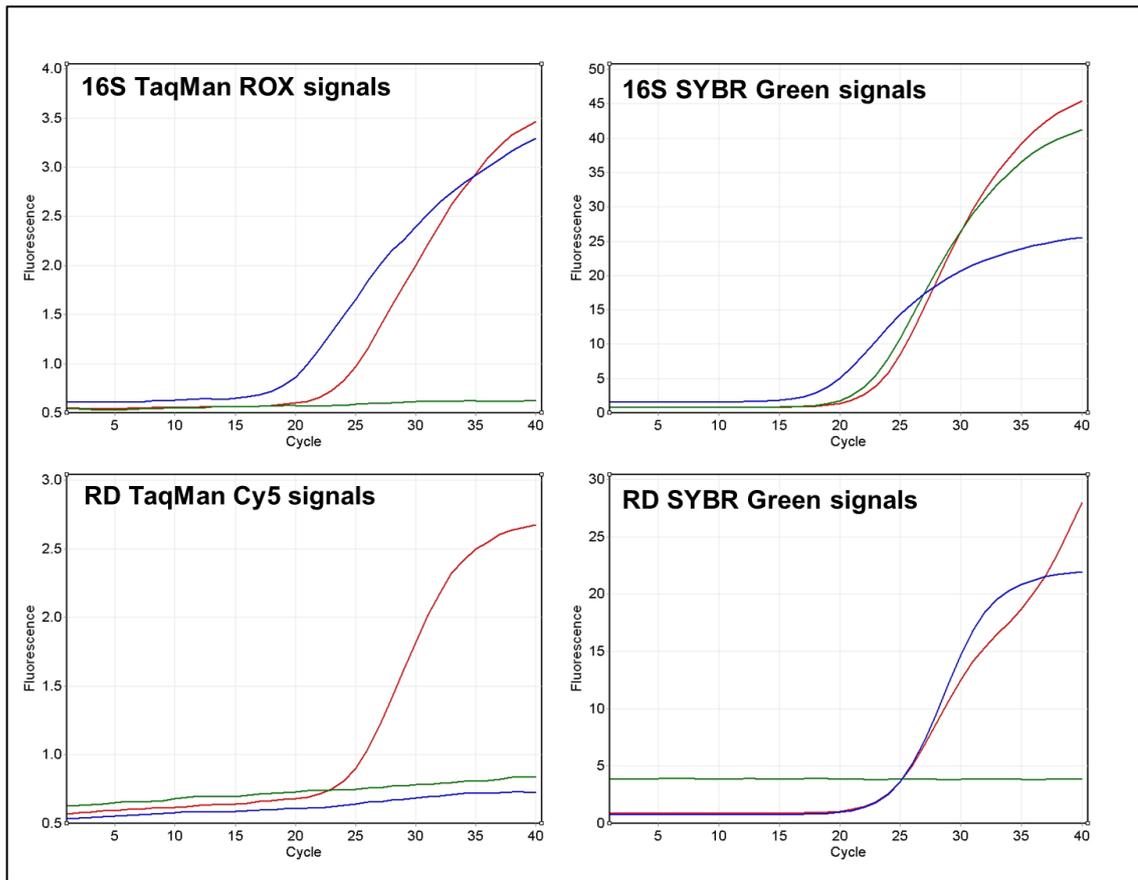
The bent arrows represent the RD primer binding sites. Both the TaqMan probe (RD750Pr) and RD750R1 were designed to anneal within the deleted region and are therefore specific to RD750⁺ strains. Amplification of RD750⁺ sequence by RD750F and RD750R1 gives a product of 126 bp in size, while amplification of RD750⁻ sequence by RD750F and RD750R2 yields a 193-bp amplicon. Although RD750R2 is specific to both RD750⁺ and RD750⁻ strains, there will only be a product detected with the latter as the 1,027-bp amplicon with the former is too large in size for efficient detection by real-time PCR.

Figure 3.3: Schematic illustration of the combined 16S-RD assay



For the 16S assay (tube 1), TaqMan (T) signals show the presence or absence of MTBC-specific 16S rDNA sequence while the SYBR Green (S) signals indicate the presence or absence of *Mycobacterium* genus-specific 16S rDNA sequence. For the RD assay (tube 2), T signals show the presence or absence of RD750 while the S signals act as further confirmation of MTBC-specific sequence and a positive assay control when the T signal is negative. T+ S- signals would be anomalous in both assays, indicating amplicons that do not interact with SYBR Green; no results were obtained in this category during the study.

Figure 3.4: Illustrative signal tracings of the combined 16S-RD assay



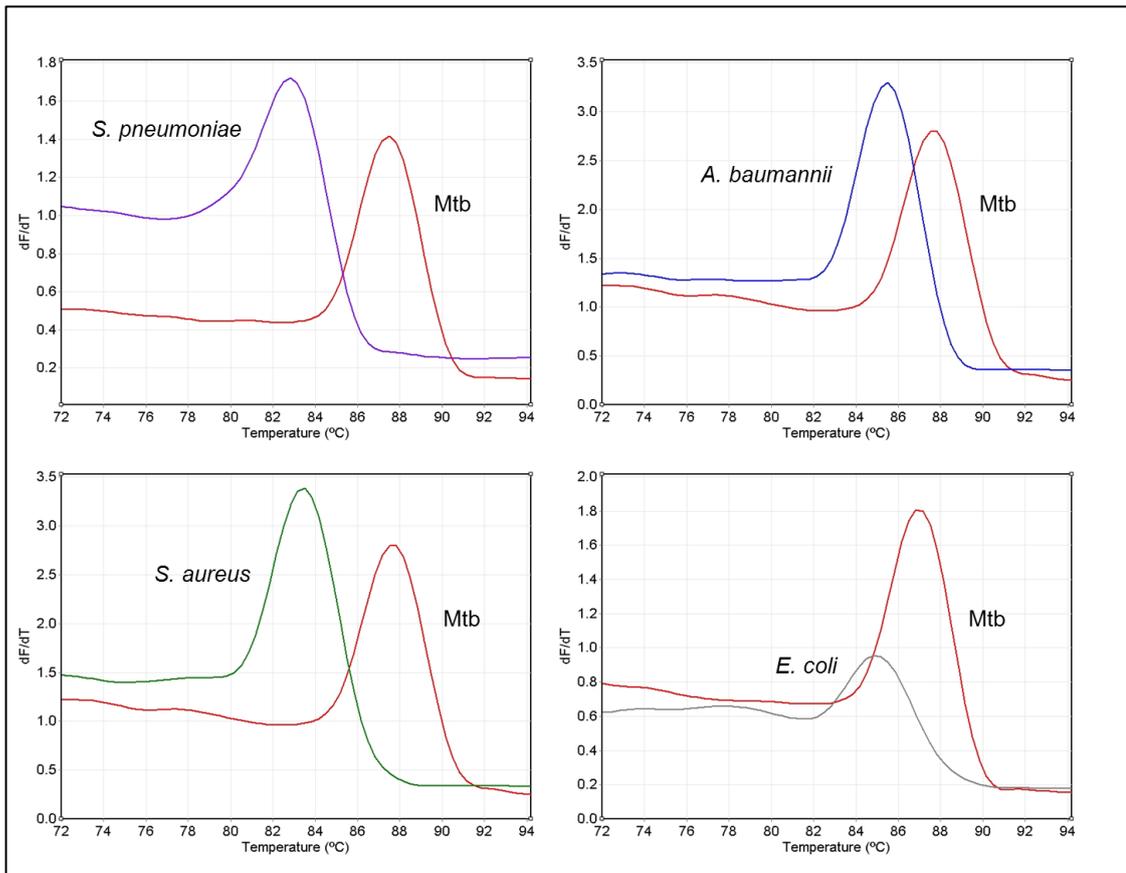
Red lines represent Mtb CDC 1551 (RD750⁺) reactions. Green and blue lines represent *M. smegmatis* and Mtb CH (RD750⁻) reactions, respectively.

3.3.2 Development of the 16S-RD assay

3.3.2.1 Optimising the annealing temperature

Preliminary run of the 16S assay with annealing temperature of 56°C revealed that several non-mycobacterial species gave positive SYBR Green signals (T⁻ S⁺) (Appendix 1, Table I). However, the T_ms of their amplicons were distinctive from those of mycobacteria (87.0 ± 0.4°C), which made interpretation unambiguous (Figure 3.5). Sequencing of 16S amplicons yielded concordant identities for all these non-mycobacterial species except for *A. baumannii* which was identified as uncultured *Bacillus* sp.

Figure 3.5: Melt profiles of non-mycobacterial species versus Mtb



Only one replicate is shown for each sample.

Conventional 16S PCR was performed on the gradient mode with annealing temperatures of 56-70°C to estimate the temperature where non-specific amplification would be abolished without affecting specific amplification. This was determined based on gel electrophoresis results (data not shown). Approximately 10 ng of Mtb CDC 1551 and *S. pneumoniae* DNA each were tested. The lowest annealing temperature to see no amplification of *S. pneumoniae* DNA was 60°C and the highest temperature tolerable for successful amplification of Mtb CDC 1551 DNA was 62°C. The 16S PCR profile for this test is described in Appendix 1.

Mixtures of Mtb and non-mycobacterial DNA were tested by real-time 16S PCR with different annealing temperatures, starting from 60°C. PCR with 56°C was also performed for comparison. Reaction specificity was assessed by melt analyses of 16S amplicons. The results of this experiment are displayed in Table II (Appendix 1). 60°C

was chosen for the 16S-RD assay as this annealing temperature is optimal temperature for cleavage of TaqMan probes by the 5' to 3' exonuclease activity of *Taq* polymerase (Dorak, 2006), and no amplification of non-mycobacterial DNA took place in the mixtures at this temperature. While pure samples of non-mycobacterial DNA gave amplicons at 60°C, the melt curves clearly reveal that they were not mycobacterial.

3.3.2.2 Optimising the magnesium ion concentration

The 16S-RD assay was optimised for the magnesium ion concentration from 3 to 5 mM in 0.5-mM increments (Appendix 1, Table III). Theoretically, the concentration yielding the lowest Ct is considered optimal and difference in Ct of more than 1 is significant (Dorak, 2006). Since there was very little difference in Ct values among the concentrations tested, 3 mM magnesium ion was chosen to maintain the specificity of the reaction.

3.3.2.3 Optimising the primer and TaqMan probe concentrations

The 16S-RD assay was also optimised for the primer and TaqMan probe concentrations in various combinations as shown in Table IV (Appendix 1). For the 16S assay, there was very little difference in Ct values (< 1 Ct) among the different combinations and therefore the combination, 250 nM primers and 150 nM probe, used during the initial pilot study was retained (Malkin, 2005). Although the use of higher concentrations of primers (250 and 500 nM) increased the sensitivity of the RD assay, it compromised the specificity of the reactions; no-template controls (NTCs) yielded late TaqMan and SYBR Green signals (T+ S+). NTCs with 80 nM primers gave no signals (T- S-). Among the probe combinations with this primer concentration, 150 nM was chosen for both sensitivity and cost effectiveness.

3.3.2.4 Background signals in 16S no-template controls

NTCs for the 16S assay always yielded background SYBR Green signals (T– S+). The amplicon size (173 bp) was similar to those of mycobacteria following gel electrophoresis (Figure 3.6). NTC amplicons consistently yielded T_m s of $87.4 \pm 0.3^\circ\text{C}$, which are about 1°C lower than those of mycobacterial 16S amplicons. DNA sequencing of NTC amplicons from five different PCR runs indicated that they were those of 16S rDNA from non-mycobacterial actinobacteria (see Section 3.3.3).

Contamination of PCR reagent(s) was initially suspected. 16S PCR with SYBR Green alone (i.e. no TaqMan probe) yielded T– S+ signal. The use of alternative SYBR Green mix, SensiMix (Quantace, London), resulted in similar outcome. The 173-bp gel band was observed following the conventional 16S PCR (i.e. no Absolute SYBR Green Mix) with or without the TaqMan probe. The use of new stocks of SYBR Green mix, primers, and PCR-grade water did not solve the problem. Treatment of PCR master mixes with different dosage of UV did not have any effect on background SYBR Green signals in NTCs; reproducibility of replicates for the three high UV dosages was rather poor (Table 3.6).

Investigation of the background SYBR Green signals in 16S NTCs was not conclusive. Sample reactions were always compared against NTC in every PCR run; those with Ct values equal to or higher than that of the latter were considered insignificant.

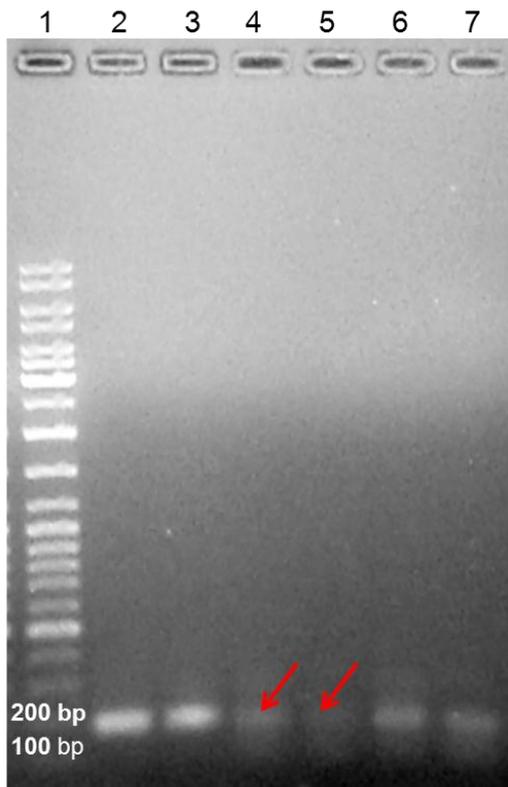


Figure 3.6: Gel analysis of NTC amplicons from the 16S assay

Lane 1, GeneRuler 100-bp DNA ladder; lane 2, Mtb CDC 1551 DNA; lane 3, *M. smegmatis* DNA; NTC reactions: lane 4, conventional PCR with TaqMan probe; lane 5, real-time PCR with Absolute SYBR Green Mix; lane 6, conventional PCR; lane 7, real-time PCR with SensiMix.

Faint bands are shown by red arrows for clarity.

Table 3.1: Effect of different UV dosages on SYBR Green signals of no-template and template reactions

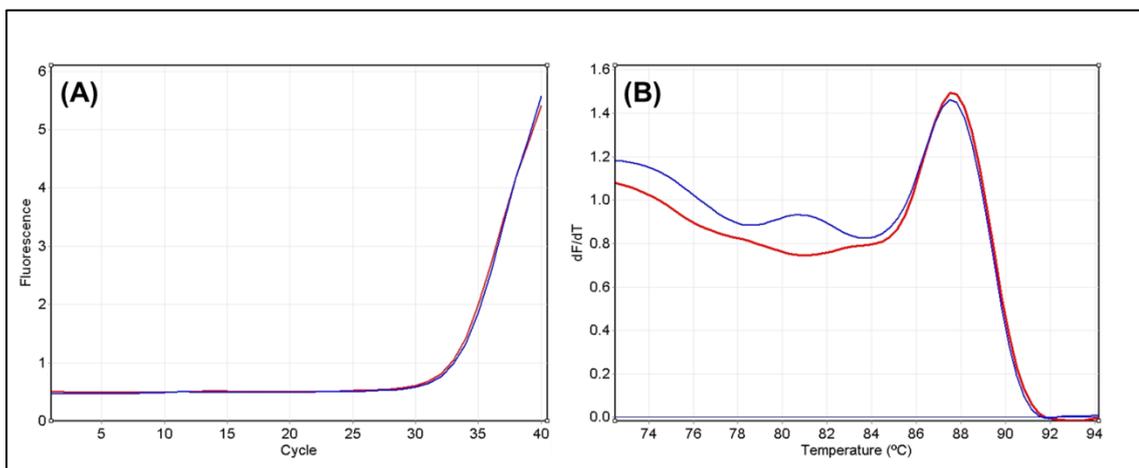
UV dosage (μJ)	16S rDNA copies detected (Log_{10})	
	NTC	Mtb CDC 1551
25,000	1.95	5.21
	1.80	5.18
50,000	1.71	5.26
	1.72	5.20
75,000	1.60	5.22
	2.04	5.22
100,000	2.17	5.23
	1.63	5.18
120,000	2.16	5.23
	1.51	5.20

Master mixes were treated with different dosages of UV in the Stratalinker UV Crosslinker (Stratagene, California, USA) prior to the 16S PCR. Reactions containing approximately 10^5 Mtb CDC 1551 genome equivalents each were performed in parallel to assess the effect of UV treatment on the sensitivity of detection.

3.3.3 Detection limit and specificity of the 16S-RD assay

Based on results of Mtb CDC 1551 genomic DNA standards from multiple runs of the 16S-RD assay, the sensitivity fell between 10^2 and 10^3 genome equivalents. The magnitude of SYBR Green signals of 10^2 standards always approached those of NTCs in the 16S assay. Furthermore, the T_m s for their 16S amplicons ($87.6 \pm 0.4^\circ\text{C}$) also approximated those of NTCs (Figure 3.7). Sequencing of these amplicons failed but the residual DNA sequence aligned well with that for Mtb 16S rDNA. Therefore, the sensitivity of the 16S assay was assessed at 10^3 genome equivalents. The RD assay was more sensitive, approaching 100 mycobacterial genomes.

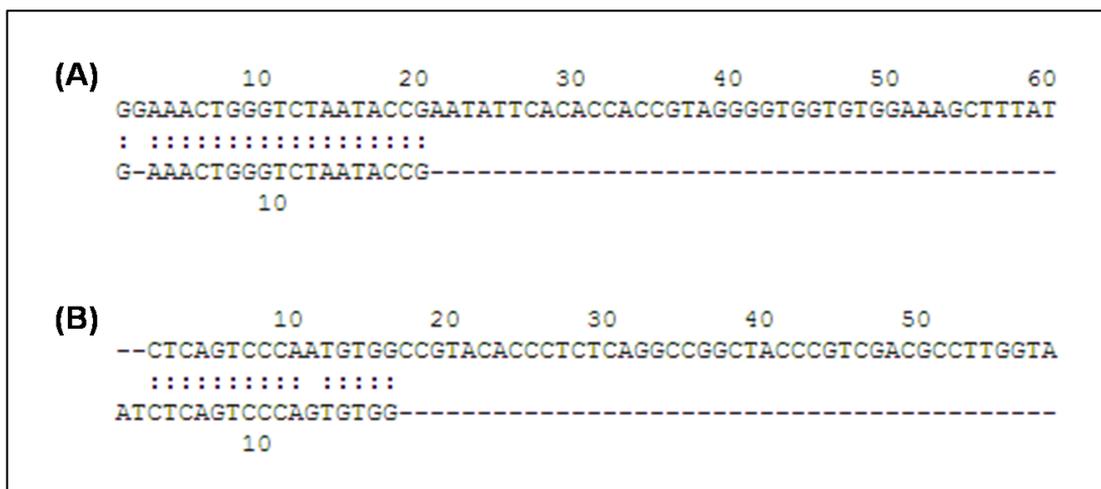
Figure 3.7: 16S assay with 10^2 Mtb genome equivalents and NTC



Red line represents Mtb CDC 1551. Blue line represents NTC. (A) Late SYBR Green signals during PCR amplification. (B) Melt analyses.

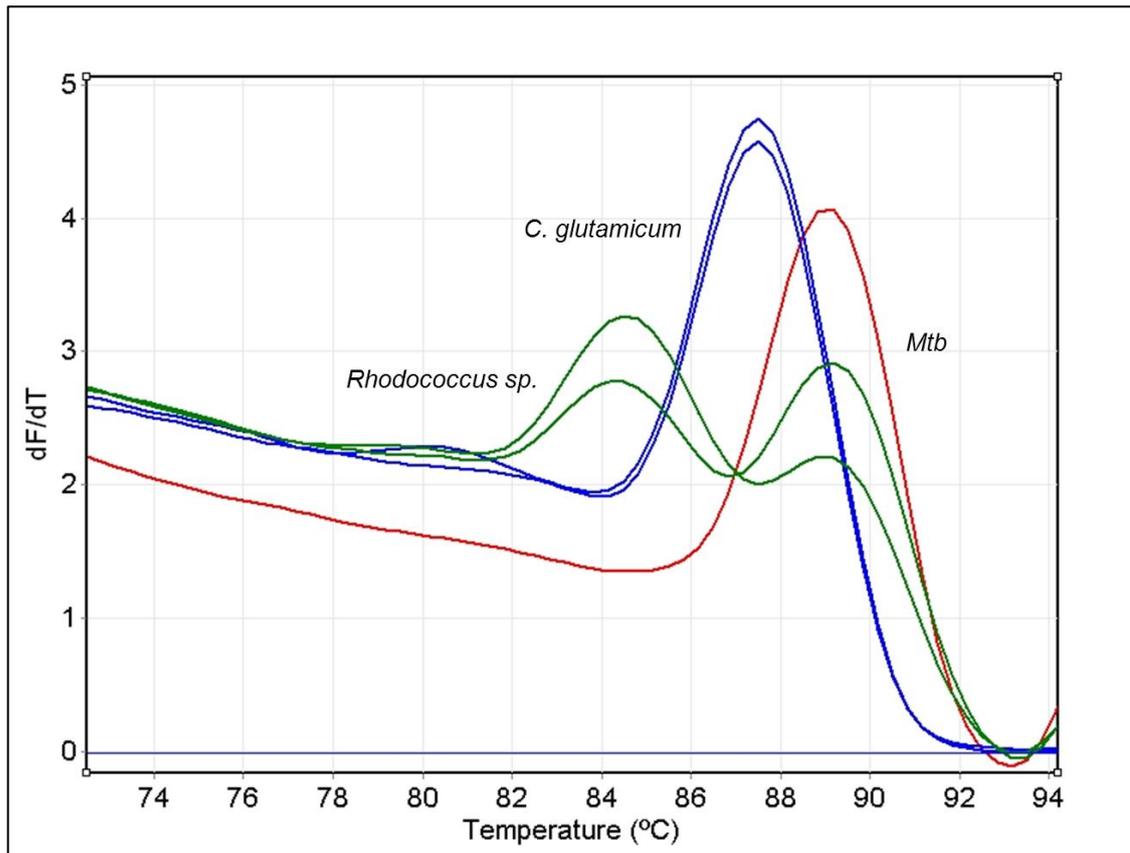
Specificity of the 16S-RD assay was evaluated with DNA lysates from several non-mycobacterial actinobacteria and pure DNAs from a selection of respiratory and Gram-negative organisms. Approximately 10^5 16S rDNA copies were assayed per PCR for the latter. BLASTn analyses revealed that several non-mycobacterial actinobacteria may produce amplicons with the mycobacterial 16S primers (Figure 3.8). Although *C. glutamicum* and *Rhodococcus sp.* yielded positive 16S SYBR Green signals, TaqMan signals were absent and the RD assay was negative for both signals (16S T– S+; RD T– S–). *S. fradiae* was not detected during the 16S-RD assay (16S T– S–; RD T– S–); subsequent eubacterial 16S assay (see Appendix I for PCR profile) ruled out failure of DNA extraction. The 16S amplicon T_m s for *C. glutamicum* and *Rhodococcus sp.*, a single peak of $87.5 \pm 0.1^\circ\text{C}$ and two peaks at $84.4 \pm 0.1^\circ\text{C}$ and $89.1 \pm 0.1^\circ\text{C}$ respectively against the mycobacterial T_m of $88.2 \pm 0.2^\circ\text{C}$, made interpretation unambiguous (Figure 3.9). The latter could possibly be explained by the presence of more than one DNA molecules in some rhodococcal species (McLeod *et al.*, 2006). The DNA sequence of *C. glutamicum* amplicon aligned poorly with its 16S rDNA sequence (data not shown). Amplicons of *Rhodococcus sp.* failed DNA sequencing. There were no significant 16S signals detected for *S. pneumoniae*, *S. aureus*, *A. baumannii*, *P. aeruginosa*, *E. coli*, *H. influenzae*, and *M. catarrhalis*.

Figure 3.8: Alignment of the partial 16S rDNA sequence of *C. glutamicum* ATCC 13032 with the mycobacterial 16S primers



(A) Forward strand and MYCO16SF. (B) Reverse strand and MYCO16SR.

Figure 3.9: Melt profiles of non-mycobacterial actinobacteria versus Mtb



3.3.4 Effect of combining TaqMan and SYBR Green chemistries in single reactions

The effect of combining TaqMan and SYBR Green chemistries in the same reaction tube was tested with *Mtb* CDC 1551 and *M. smegmatis* DNA for the 16S assay and *Mtb* CDC 1551 and CH DNA for the RD assay with target amounts in the range of 10^3 - 10^9 copies. Numeric data and illustrative signal tracings from this experiment are displayed in Table 3.2 and Figure 3.10, respectively. Ct values generated by TaqMan and SYBR Green for single and combined assays (six pairs of values for each chemistry) were compared by the paired *t* tests. While the presence of TaqMan probe had no measurable effect on SYBR Green Ct values irrespective of the probe binding status of the reaction ($P > 0.1$), adding SYBR Green significantly increased the Ct for TaqMan signals ($P < 0.05$). The effect was more pronounced for the RD assay (two-sample *t* test, $P < 0.01$). Magnitudes of Ct difference were generally higher at low

target concentrations; these were approximately 0.7 and 3 for the 16S and RD assays, respectively.

Table 3.2: Effect of combining TaqMan and SYBR Green chemistries in single reactions

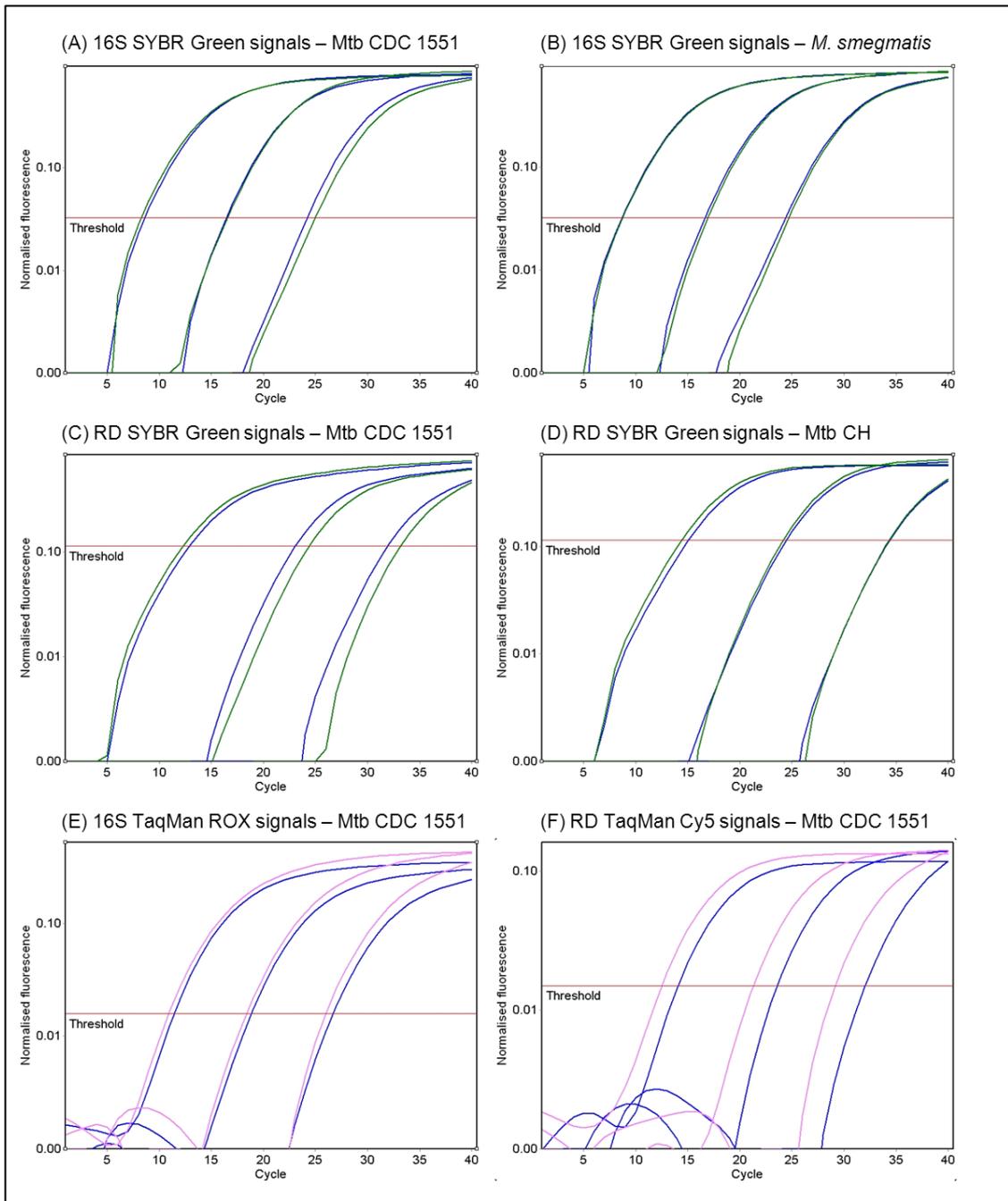
3.2 (A) TaqMan-SYBR Green versus TaqMan alone

Molecular assay	Target organism	Target gene copies	TaqMan Ct		Ct difference
			Combined chemistries	Single chemistry	
16S	Mtb CDC 1551	10 ⁷	11.46 ± 0.11	10.94 ± 0.33	0.52
		10 ⁵	18.86 ± 0.26	18.40 ± 0.04	0.46
		10 ³	26.72 ± 0.42	26.01 ± 0.11	0.71
RD	Mtb CDC 1551	10 ⁷	14.11 ± 0.74	12.55 ± 0.54	1.56
		10 ⁵	23.60 ± 0.10	21.32 ± 0.15	2.28
		10 ³	32.04 ± 0.18	29.14 ± 0.27	2.90

3.2 (B) TaqMan-SYBR Green versus SYBR Green alone

Molecular assay	Target organism	Target gene copies	SYBR Green Ct		Ct difference
			Combined chemistries	Single chemistry	
16S	Mtb CDC 1551	10 ⁷	8.62 ± 0.11	8.30 ± 0.47	0.32
		10 ⁵	16.45 ± 0.12	16.56 ± 0.07	-0.11
		10 ³	24.24 ± 0.21	25.08 ± 0.64	-0.84
16S	<i>M. smegmatis</i>	10 ⁷	8.70 ± 0.21	8.76 ± 0.06	-0.06
		10 ⁵	16.70 ± 0.11	16.96 ± 0.10	-0.26
		10 ³	24.46 ± 0.25	24.76 ± 0.29	-0.30
RD	Mtb CDC 1551	10 ⁷	12.91 ± 0.64	12.32 ± 0.46	0.59
		10 ⁵	23.11 ± 0.46	24.43 ± 0.16	-1.32
		10 ³	31.93 ± 0.26	33.16 ± 0.06	-1.23
RD	Mtb CH	10 ⁷	15.09 ± 0.06	14.45 ± 0.16	0.64
		10 ⁵	24.49 ± 0.14	24.19 ± 0.03	0.30
		10 ³	34.40 ± 0.23	34.34 ± 0.17	0.06

Figure 3.10: Effect of combining TaqMan and SYBR Green chemistries in single reactions



Mycobacterial targets at 10^7 gene copies and two 100-fold serial dilutions were tested. TaqMan only reactions were performed using Absolute QPCR Mix (ABgene), and all determinations were assayed in triplicates. Blue lines represent reactions containing both TaqMan and SYBR Green. Green and pink lines represent reactions containing SYBR Green and TaqMan alone, respectively. Panels A -D and E-F show the effect of combining TaqMan and SYBR Green chemistries on SYBR Green and TaqMan Ct, respectively.

3.3.5 Evaluation of the 16S-RD assay for analysis of clinical mycobacterial isolates

3.3.5.1 Assessment of simple methods for releasing DNA from clinical mycobacterial isolates

Two rapid methods for preparing DNA lysates from clinical mycobacterial cultures were compared. One method involved simple boiling to release DNA from mycobacterial cells while the other method involved additional treatment of the boiled culture lysates with *micro*LYSIS reagent. There was no significant difference in the amount of DNA released by these two methods (Table 3.3; $P > 0.1$). Therefore, boiling was used in subsequent experiments.

Table 3.3: Comparison between two simple methods for preparing DNA lysates from clinical mycobacterial isolates

Culture type	Sample	Average 16S copies detected per reaction (Log ₁₀)	
		Boiling	Boiling + <i>micro</i> LYSIS
MGIT	1	5.13	4.86
	2	7.04	7.12
	3	6.66	6.72
LJ	1	6.23	6.28
	2	7.30	7.30
	3	5.58	5.64

Each PCR was performed with 1 µl of DNA lysate. Results were based on SYBR Green signals.

3.3.5.2 The 16S-RD assay differentiates between *M. tuberculosis* complex and non-tuberculous mycobacteria

A total of 88 cultures from different patients deemed probable mycobacteria-positive following ZN acid-fast staining obtained from the routine microbiology service at the UHL between January and November 2007 were analysed operator-blinded. Of these 88 positive cultures, 42 were MGITs and 46 were LJ cultures. The results for analysis of these cultures by the combined 16S-RD assay are displayed in Table 3.4. All mycobacterial isolates were also identified as part of the routine service by the Regional Centre for Mycobacteriology, Birmingham Heartlands Hospital (reference laboratory) using the GenoType kits from Hain Lifescience (Nehren, Germany). Both the reference laboratory and the 16S-RD assay identified 87 cultures to be positive for mycobacteria. Out of these, 70 were MTBC-positive and 17 were NTM-positive. The only mycobacteria-negative culture in the sample set was derived from MGIT and its DNA lysate yielded no signal in both the 16S and RD assays (T- S-), which was consistent with the reference laboratory report of “no AFB seen”.

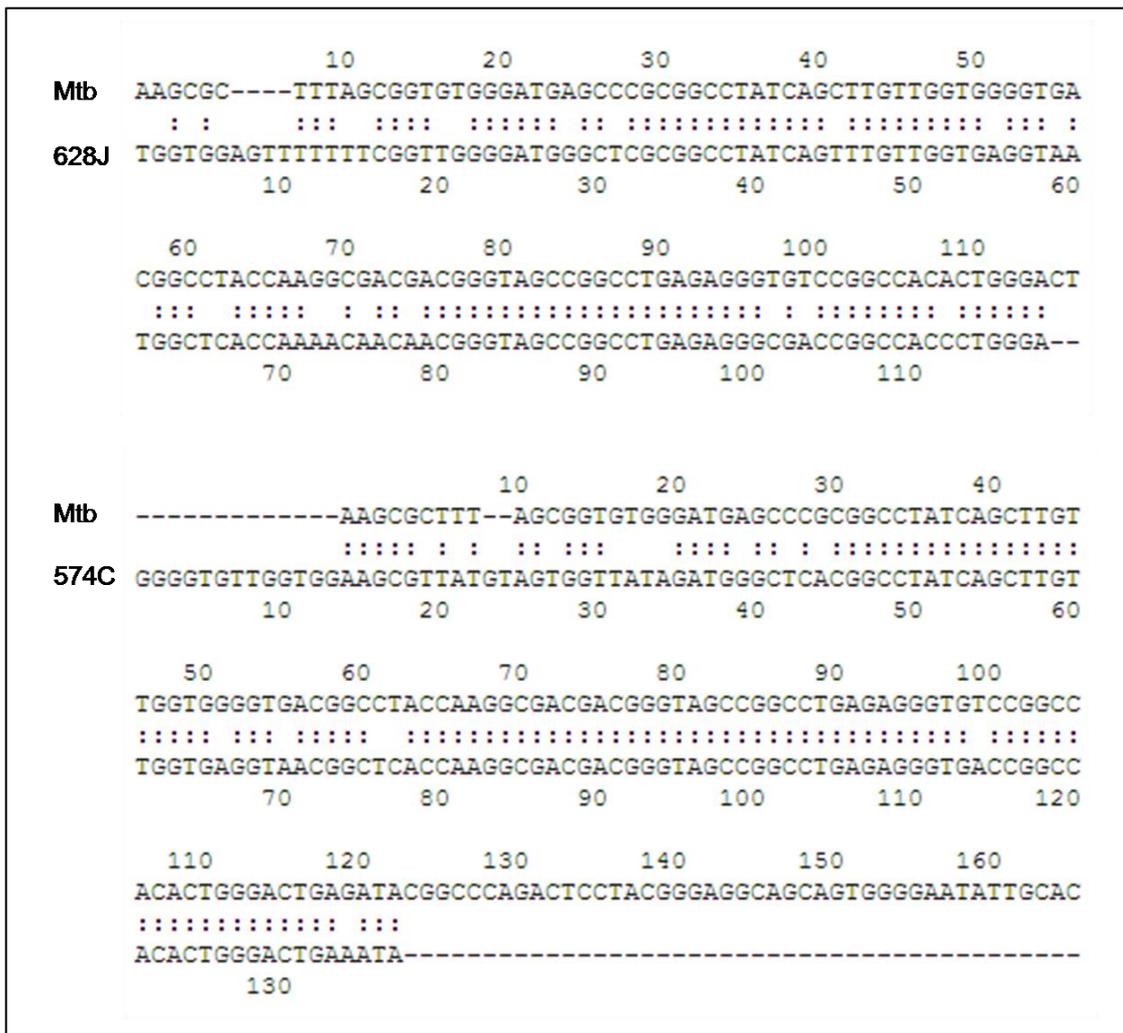
The 16S assay identified 68 (16S T+ S+) out of the 70 MTBC-positive cultures identified by the RD assay (RD T+ S+; T- S+). Two MGIT lysates did not give an MTBC signal with the 16S TaqMan probe but were positive for 16S SYBR Green (T- S+) and for both RD TaqMan and SYBR Green signals (T+ S+). Their 16S amplicons gave T_{mS} of $86.9 \pm 0.1^{\circ}\text{C}$ and $86.4 \pm 0.1^{\circ}\text{C}$ respectively, which were more than 1°C lower than those of mycobacterial amplicons tested previously ($88.2 \pm 0.2^{\circ}\text{C}$). The sequences of these amplicons, both from initial and repeated PCRs, aligned poorly with the partial 16S rDNA sequence of Mtb H37Rv with multiple mismatches (Figure 3.11). Examples of good alignment between query and test mycobacterial DNA sequences are shown in Figure 3.12. Cloning of these ‘anomalous’ amplicons was also performed to investigate possible interference by non-mycobacterial signals in these MGIT lysates. However, subsequent analysis of randomly selected clones by the 16S assay and sequencing of the resulting amplicons yielded similarly ambiguous outcomes.

Table 3.4: Combined 16S-RD assay applied to 88 clinical cultures deemed probable mycobacteria-positive

		RD assay			Total
		T+ S+	T- S+	T- S-	
16S assay	T+ S+	42	26	0	68
	T- S+	2	0	17	19
	T- S-	0	0	1	1
Total		44	26	18	88

PCRs were repeated for all samples yielding no or anomalous signals with neat and diluted DNA lysates (1:10 and 1:100).

Figure 3.11: Alignment of the partial 16S rDNA sequence of Mtb H37Rv with sequences of 16S TaqMan-negative MTBC amplicons



3.3.5.4 The RD assay differentiates between *M. tuberculosis* complex strains with intact and deleted RD750

Data from the RD assay indicated that 44 of the MTBC strains were RD750⁺ (T+ S+) and 26 were RD750⁻ (T- S+) (Table 3.4). These 70 DNA lysates were analysed by the established *Rv1519* PCR assay in parallel (Rajakumar *et al.*, 2004). The results from these two assays were compared against the 5-locus VNTR genotyping results from the reference laboratory, where the 10-locus MIRU analysis was also performed. While the majority of the RD750⁻ isolates in Leicester were genotyped as VNTR 42234/5, a more extensive study has revealed that all can be encompassed within the profile x2234/5 (R. J. Smith, unpublished) and this latter profile was used to recognise concordance or otherwise with the in-house PCR assays. For 62 lysates, the results of all three genotyping assays (RD, *Rv1519* PCR, and VNTR) were concordant. The *Rv1519* PCR yielded no amplicons in eight cases but their RD and VNTR results were concordant.

3.3.6 The 16S-RD assay shows potential in detecting samples containing mixed mycobacterial species or strains

A by-product of the 16S-RD assay design is that it has potential to detect mixed infections involving either a combination of MTBC and NTM or MTBC RD750⁺ and RD750⁻ strains; this was examined and evaluated using mixtures of purified DNA. *Mtb* CDC 1551 was used to represent MTBC and RD750⁺ strain in both combinations, respectively. *M. smegmatis* was used as an example NTM, while *Mtb* CH represented RD750⁻ strain. Target DNA was diluted to 10⁵, 10⁴, and 10³ gene copies per µl, and added to individual PCR reactions in a checker board format as detailed in Table 3.5.

The results show that it is only possible to reliably recognise the target detected solely by SYBR Green chemistry (NTM or RD750⁻ in the 16S and RD assays, respectively) when the copy number detected by the SYBR Green signal significantly exceeds that detected by the TaqMan probe in the same sample. Only in this situation can the higher SYBR Green signal be confidently interpreted to demonstrate the presence of

the second TaqMan-negative target. This implies that the combined assay will not detect mixed infections when the SYBR Green signals are approximately equal to the TaqMan signals, i.e. when the TaqMan-positive species or strain is present in significantly large excess. In this case, gel analysis of mixed RD amplicons has higher resolution (Figure 3.13). Mixed targets, indicated by two bands within a single lane on the gel (193 bp for RD750⁻ and 126 bp for RD750⁺), were evident for mixed proportions as high as 1:10 (Figure 3.13, lanes 5-6 and 9-10). The observation of the 1,027-bp amplicon, product of primers RD750F and RD750R2 (Figure 3.2), was consistent with PCR amplification of large amounts of RD750⁺ DNA. However, detection of mixed amplicons by gel analysis was not possible when a particular MTBC strain was present in amounts 100 times excessive of the other. In this case, only the bands for the former were visible on the gel (Figure 3.13, lanes 7-8 and 11-12). The presence of both MTBC and NTM species in the same samples did not affect the performance of the RD assay (Table 3.6).

Table 3.5: Potential of the combined 16S-RD assay to detect mixed mycobacterial infections

		<i>M. smegmatis</i> (16S SYBR Green signal)			Mtb CH (RD SYBR Green signal)		
		10 ⁵	10 ⁴	10 ³	10 ⁵	10 ⁴	10 ³
Mtb CDC 1551 signals [§]	10 ⁵	BOTH [†]	CDC	CDC	BOTH	CDC	CDC
	10 ⁴	BOTH	BOTH [‡]	CDC	BOTH	BOTH	CDC
	10 ³	MS	BOTH	CDC	BOTH	BOTH	BOTH

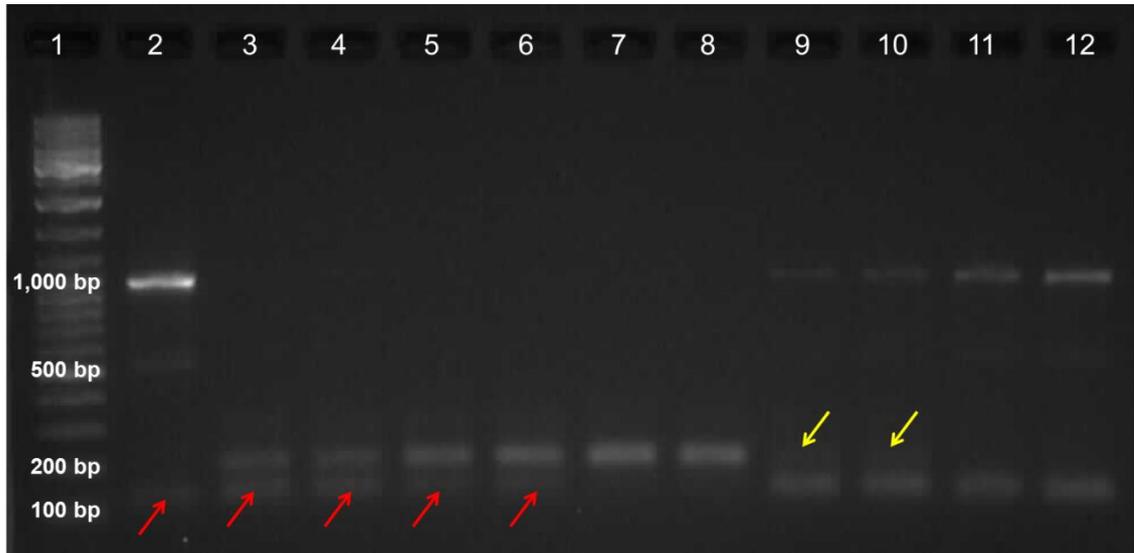
* Figures denote number of target gene copies tested.

† BOTH, both targets detected; CDC, only Mtb CDC 1551 (RD750⁺) detected; MS, only *M. smegmatis* detected. No Mtb CH (RD750⁻) only signals were obtained.

‡ Borderline *M. smegmatis* signal.

§ TaqMan signals from both the 16S and RD assays.

Figure 3.13: Gel analysis of PCR reactions containing mixed RD amplicons from Mtb RD750⁺ and RD750⁻ strains



Lane 1, GeneRuler 100-bp DNA marker; lane 2, Mtb CDC 1551 DNA; lanes 3-4, RD750⁺:RD750⁻ 1:1; lanes 5-6, RD750⁺:RD750⁻ 1:10; lanes 7-8, RD750⁺:RD750⁻ 1:100; lanes 9-10, RD750⁻:RD750⁺ 1:10; lanes 11-12, RD750⁻:RD750⁺ 1:100.

This gel image was from a separate experiment than that described in Section 3.3.6. Approximately 10⁵ mixed genome equivalents were tested per PCR reaction. Faint RD750⁺ 126-bp and RD750⁻ 193-bp bands are shown by red and yellow arrows respectively for clarity.

Table 3.6: Performance of the RD assay with mixed MTBC-NTM samples

		Mtb CDC1551			Mtb CH		
		10 ⁵	10 ⁴	10 ³	10 ⁵	10 ⁴	10 ³
<i>M. smegmatis</i>	10 ⁵	5.48 [†]	3.98	3.91	4.89	3.98	3.25
	10 ⁴	5.50	4.72	4.01	4.90	3.99	3.31
	10 ³	5.50	4.72	3.93	4.91	3.98	3.32

* Figures denote number of target gene copies tested.

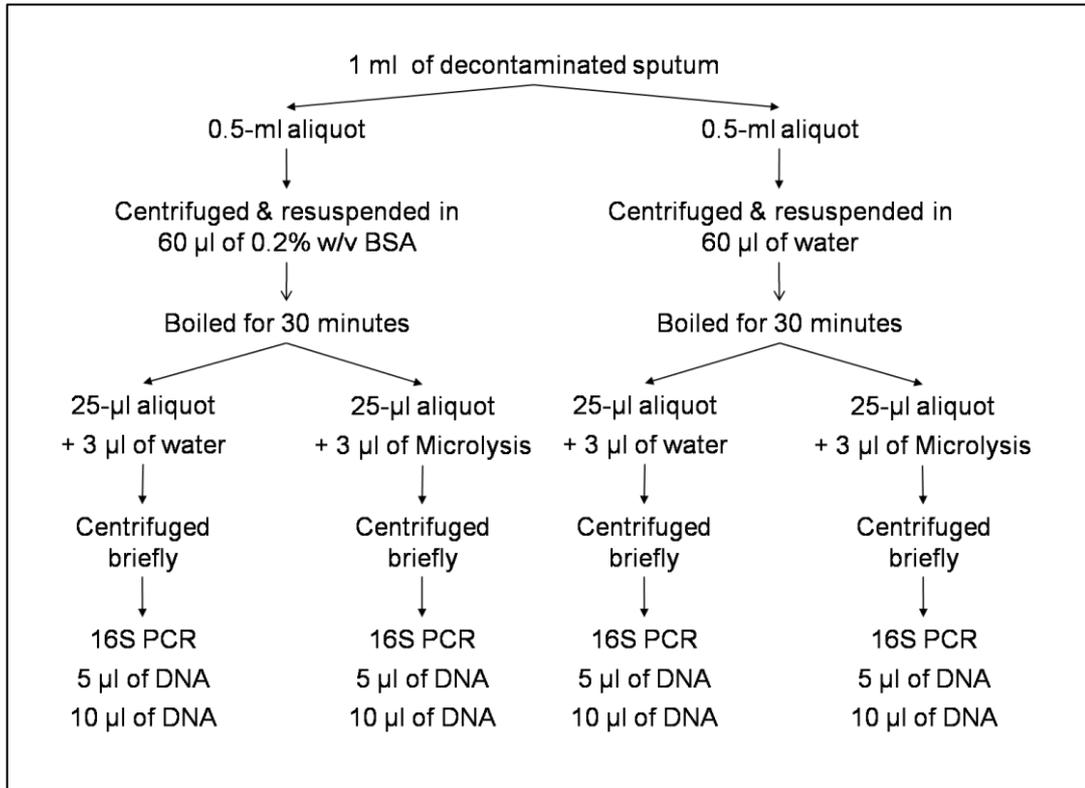
† Average RD gene copies detected based on SYBR Green signals (Log₁₀).

3.3.7 Preliminary evaluation of the 16S-RD assay for direct analysis of respiratory specimens

3.3.7.1 Assessment of simple methods for sputum DNA extraction

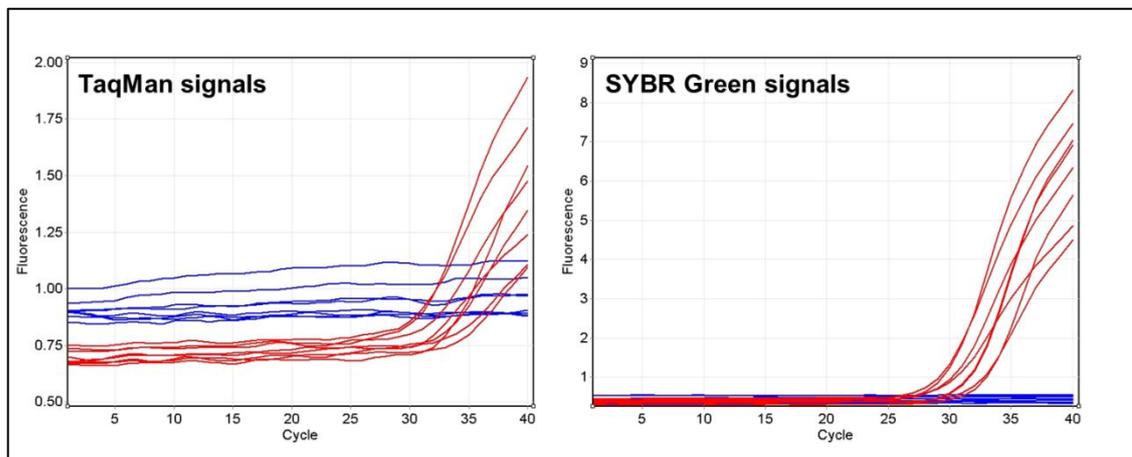
Two simple methods of sputum DNA extraction were compared for two Mtb smear-positive sputum samples; one involved boiling to release DNA from the mycobacterial cells in NaOH-decontaminated sputa while the other involved additional treatment of the boiled sputa with *micro*LYSIS reagent. The effect of BSA on downstream PCR detection and different volumes of template DNA were also tested. The experimental design is summarised in Figure 3.14. All test samples gave no signals in the 16S assay (T– S–) and no bands upon gel electrophoresis. Repetition of the assay with ten-fold diluted template DNA yielded both TaqMan and SYBR Green signals (Figure 3.15), and the results are displayed in Table 3.7. The resulting 16S amplicons yielded T_m s in the range of 86.7-88.0°C; mycobacterial T_m s were $88.2 \pm 0.2^\circ\text{C}$. There was no significant difference between the two DNA extraction methods tested, and inclusion of BSA had no significant effect on downstream PCR ($P > 0.1$).

Figure 3.14: Experimental design for validating simple methods for sputum DNA extraction



Sputum samples were decontaminated as described in Section 3.2.2.3. All determinations were tested only once by the 16S assay.

Figure 3.15: Application of the 16S assay to undiluted and diluted DNA of Mtb smear-positive sputum samples



Blue lines represent reactions with undiluted template DNA. Red lines represent reactions with ten-fold diluted template DNA. All determinations were assayed once.

Table 3.7: Assessment of simple methods for extracting DNA from Mtb smear-positive sputum samples

3.7 (A) Resuspending medium – BSA vs. Water

Sputum sample	Resuspending medium	DNA release method	TaqMan Ct	SYBR Green Ct	TaqMan Copies	SYBR Green Copies	T _m (°C)
414	BSA	Boiling + <i>micro</i> LYSIS	34.58	25.08	373	1,860	87.2
		Boiling only	34.30	25.71	439	1,276	87.7
	Water	Boiling + <i>micro</i> LYSIS	32.24	23.57	1,473	4,548	87.8
		Boiling only	31.80	23.55	1,915	4,609	88.0
202	BSA	Boiling + <i>micro</i> LYSIS	33.88	24.74	563	2,271	87.3
		Boiling only	35.32	25.76	241	1,241	87.2
	Water	Boiling + <i>micro</i> LYSIS	37.56	27.28	64	502	86.7
		Boiling only	37.61	27.12	63	550	87.0

3.7 (B) DNA release method – Boiling + *micro*LYSIS vs. Boiling only

Sputum sample	DNA release method	Resuspending medium	TaqMan Copies
414	Boiling + <i>micro</i> LYSIS	BSA	373
		Water	1,473
	Boiling only	BSA	439
		Water	1,915
202	Boiling + <i>micro</i> LYSIS	BSA	563
		Water	64
	Boiling only	BSA	241
		Water	63

All determinations were tested once by the 16S assay with ten-fold diluted template.

3.3.7.2 Assessment of commercial kits for sputum DNA extraction

Two commercial kits, QIAamp DNA Mini Kit and EliGene MTB Isolation Kit, were compared for extraction of DNA from Mtb culture-positive sputum samples, and the results are shown in Table 3.8. The working principles of these two kits are similar; samples are digested and lysed with lysozyme and proteinase K, and DNAs are subsequently purified and isolated by binding to silica membrane (see Section 3.2.2.3). There was no significant difference between these two kits in extracting DNA from tuberculous sputum samples ($P > 0.1$). However, the detection of more signals (up to 10 fold) for some sputum samples with only one kit might reflect an unequal split of the sample. Signals for the DNA samples eluted in water for the QIAamp DNA Mini Kit dropped by ten fold after storage at -20°C for one month, whereas signals for those samples eluted in the buffer provided by the EliGene MTB Isolation Kit remained unchanged (data not shown).

Table 3.8: Comparison between QIAamp DNA Mini kit and EliGene MTB Isolation kit for extracting DNA from Mtb culture-positive sputum samples

Sputum sample	AFB smear positivity	RD gene copies detected per reaction	
		QIAamp DNA Mini kit	EliGene MTB Isolation kit
579C	Negative	601	353
796K	Positive	1,510	2,452
549Y	Positive	3,419	27,518
891F	Positive	9,447	4,540
U4808350	Unknown	56,910	12,675

Results were based on TaqMan signals. Samples 579C and 796K, and samples 891F and 549Y, were from the same patients. Two sputum samples from Patient U4808350 were smear-positive and one was smear-negative.

3.3.7.3 Tuberculous sputum samples yielding anomalous 16S signals

The full qualitative 16S-RD assay results for the sputum samples processed in Section 3.3.7.2 are shown in Table 3.9. Samples 579C, 796K, and 891F did not give an MTBC signal with the 16S TaqMan probe but were positive for 16S SYBR Green (T– S+) and for both RD TaqMan and SYBR Green signals (T+ S+). The first two samples were from the same TB patient whose MGIT culture had given similarly anomalous PCR signals (see Section 3.3.5.2). The 16S amplicon T_m s for these samples were at least 2°C lower than those expected for mycobacterial amplicons ($88.2 \pm 0.2^\circ\text{C}$). Furthermore, the sequences of these amplicons showed poor alignment with the Mtb 16S rDNA sequence with multiple gaps (Figure 3.16). These two samples were also negative by the LAMP assay (see Section 3.3.8). Although several sputum samples gave ‘false-negative’ 16S TaqMan results (T– S+), the RD signals (T+ S+) for all five samples were concordant with the culture results from the reference laboratory; both indicated the presence of MTBC in these samples.

Table 3.9: Combined 16S-RD assay applied to Mtb culture-positive sputum samples

Sputum sample	16S ASSAY		RD ASSAY	
	PCR signals	T_m (°C)	PCR signals	T_m (°C)
579C	T– S+	86.0	T+ S+	87.7
796K	T– S+	86.2	T+ S+	88.0
549Y	T+ S+	87.8	T+ S+	87.7
891F	T– S+	86.9	T+ S+	87.7
U4808350	T+ S+	87.5	T+ S+	87.7

Results for sputum samples processed by the QIAamp DNA Mini kit are shown. Both samples 549Y and U4808350 were positive for MTBC based on 16S sequencing data. The alignment of the 16S amplicon sequence of 549Y with the Mtb 16S rDNA sequence is shown in Figure 3.16. 16S amplicons for 891F were not sequenced.

3.3.7.4 Use of PCR additives to improve the specificity of the 16S assay

The 'false-negative' 16S TaqMan signal (T– S+) for several sputum samples in Section 3.3.7.3 might have been due to cross-reactive non-mycobacterial DNA present within the samples. It was thought the incorporation of PCR additives might solve this problem by improving the specificity of the reaction (Sarkar *et al.*, 1990). The effect of two PCR additives, formamide and dimethyl sulphoxide (DMSO), on the 16S assay was tested at various concentrations in two-fold increments starting from 1.25% v/v, and the results are displayed in Table 3.10. There was a concentration-dependent decline in the sensitivity of reactions and T_m s of 16S amplicons; the effect was more pronounced with formamide. Although amplicon T_m s were lowered by nearly 1°C, both 1.25% v/v formamide and DMSO did not appear to affect the sensitivity of the reaction. Formamide was chosen to re-test the tuberculous sputum samples yielding anomalous 16S signals. However, there was still no TaqMan signal detected during the 16S assay (T– S+) and amplicon T_m s dropped by more than 1°C.

Table 3.10: Effect of different types and concentrations of PCR additives on the 16S assay

PCR additive	Concentration (% v/v)	TaqMan Ct	SYBR Green Ct	T_m (°C)
Formamide DMSO	1.25	25.81 25.70	20.56 20.74	87.8 87.7
Formamide DMSO	2.5	34.60 26.20	27.13 21.26	87.0 86.9
Formamide DMSO	5	Negative 32.85	Negative 27.58	Negative 85.0
Formamide DMSO	10	Negative Negative	Negative Negative	Negative Negative
Water	-	25.76	20.77	88.5

Stocks of 6.25, 12.5, 25, and 50% v/v PCR additives were prepared in PCR-grade water to give the final concentrations listed in the table when 5 µl was added to each 25-µl reaction. Each PCR was tested with 2×10^5 Mtb CDC 1551 genome equivalents. For the analysis of sputum samples, 1 µl of 31.25% v/v formamide and 8.5 µl of DNA were added to a 25-µl reaction.

3.3.7.5 Bovine serum albumin improves the sensitivity of molecular assay with sputum and pure mycobacterial DNA

The effect of 1 mg/ml non-acetylated BSA on the sensitivity and positivity of molecular assay was studied with sputum and pure mycobacterial DNA, and the results are shown in Table 3.11. Although not statistically significant ($P > 0.1$), all sputum samples which were initially positive by the 16S-RD assay yielded more signals in the presence of BSA than in the controls when the RD assay was repeated. The magnitudes of difference varied among different sputum samples; as much as ten-fold higher signals were yielded in the presence of BSA for 549Y. The inclusion of BSA in PCRs with pure Mtb DNA also improved their sensitivity, more pronounced for CDC 1551 than the CH strain. The presence of BSA did not improve the positive detection rate for the sputum samples that were negative by the initial 16S-RD assay.

Table 3.11: Effect of BSA on the sensitivity of RD assay with sputum and pure mycobacterial DNA

Sample		RD gene copies detected per reaction	
		Control	BSA
Sputum	891F	1,031	1,077
	549Y	127	1,809
	U4808350	6,297	8,762
	804AC	63	154
Pure DNA	CDC 1551	62,339	150,549
	CH	100,831	129,069

A stock of 10 mg/ml non-acetylated BSA was prepared in PCR-grade water and sterilised by filtration through a 0.25- μ m syringe filter. A final concentration of 1 mg/ml was used in the PCR. Each RD PCR was tested with 6.5 μ l of sputum DNA. Approximately 10^5 Mtb CDC 1551 and CH genome equivalents each was assayed per reaction. Results were based on SYBR Green signals.

3.3.8 Loop-mediated isothermal amplification assay has higher positivity rate in the detection of *M. tuberculosis* in respiratory specimens

The 16S-RD assay was developed to perform a specific function within the TB transmission studies in Leicester. Sensitive detection was not a primary concern. However, the negative assays on patients recruited into our studies were still of some concern. Therefore, a brief evaluation of a recently developed assay reported to have high sensitivity was undertaken. The LAMP assay was applied to 19 *Mtb* culture-positive sputum samples which had been tested by the combined 16S-RD assay, and the results are displayed in Table 3.12. Detection of LAMP products was achieved by visual assessment of the fluorescence intensity of sample reactions (Figure 3.17). Reactions were scored as *Mtb*-positive if their fluorescence intensities were similar to and higher than those of the positive and negative controls, respectively. Reactions with fluorescence intensities similar to or lower than those of the negative controls were considered negative. Alternatively, positive LAMP reactions were demonstrated by the appearance of ladder patterns or smeared bands upon gel electrophoresis, which were due to the amplified products consisting of inverted repeats of the target sequence in various sizes (Figure 3.18). The absence of bands for Loopamp Kit positive control might have been due to analysis of wrong reaction tube (lanes 6 in both gel panels).

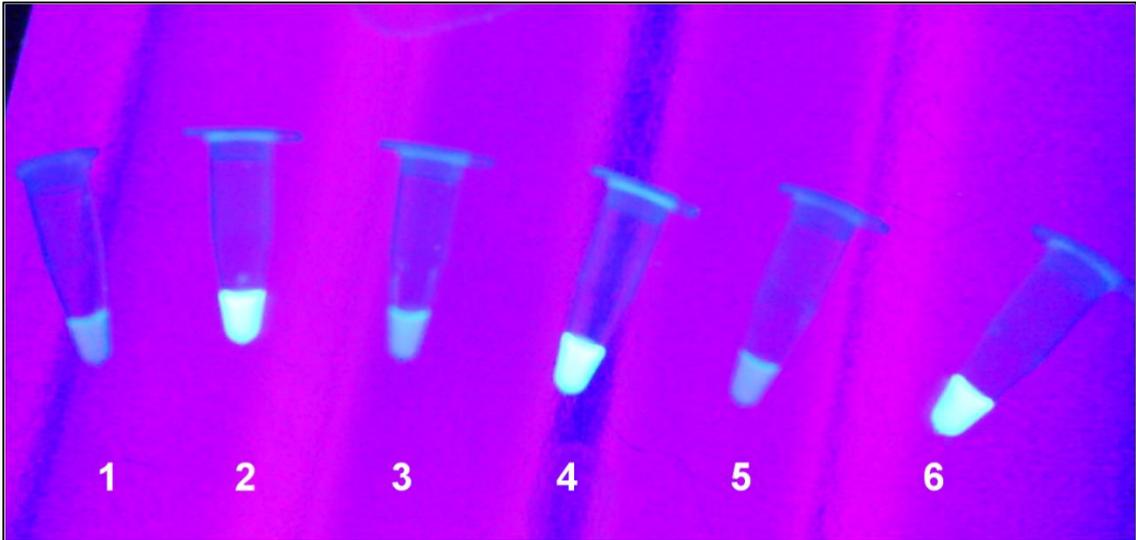
Four out of 14 sputum samples which were negative by the 16S-RD assay were positive by the LAMP assay. Twelve of these samples were AFB smear-negative; LAMP assay detected *Mtb* in three of them. Another LAMP-positive sample which was negative by the 16S-RD assay was smear-positive. The smear-positive sample from Patient 9 (296JJ) which was negative by the LAMP assay had scanty AFB. Two out of three sputum samples (579C and 796K) which did not give positive 16S TaqMan signals but were positive for the 16S SYBR Green (T– S+) and for both the RD TaqMan and SYBR Green signals (T+ S+), were negative by the LAMP assay. Sample 891F was LAMP-positive (see Section 3.3.7.3). The two samples which were graded *Mtb*-positive by both the 16S and RD assays were also positive by the LAMP assay. Among the 13 smear-negative sputa tested, the LAMP assay was able to detect *Mtb* in three, while the 16S-RD assay only detected one. Different samples from the same patients gave different LAMP results in two out of six cases (Patients 4 and 6 in Table 3.12).

Table 3.12: LAMP assay applied to Mtb culture-positive sputum samples

Patient no.	Sputum sample	AFB smear positivity	Combined assay*		LAMP assay
			16S	RD	
1	620PB	Negative	T- S-	T- S-	Positive
2	137DG	Negative	T- S-	T- S-	Negative
	134DG	Negative	T- S-	T- S-	Negative
3	666CN	Negative	T- S-	T- S-	Negative
4	804AC	Negative	T- S-	T- S-	Positive
	826AC	Negative	T- S-	T- S-	Negative
5	638NS	Negative	T- S-	T- S-	Negative
6	314CB	Negative	T- S-	T- S-	Positive
	284CB	Negative	T- S-	T- S-	Negative
7	634MB	Positive	T- S-	T- S-	Positive
8	833RV	Negative	T- S-	T- S-	Negative
	834RV	Negative	T- S-	T- S-	Negative
	801RV	Negative	T- S-	T- S-	Negative
9	296JJ	Positive	T- S-	T- S-	Negative
10	579C	Negative	T- S+	T+ S+	Negative
	796K	Positive	T- S+	T+ S+	Negative
11	891F	Positive	T- S+	T+ S+	Positive
	549Y	Positive	T+ S+	T+ S+	Positive
12	U4808350	Unknown	T+ S+	T+ S+	Positive

* Different shades of blue categorise sputum samples into three groups: those negative by the combined assay (16S T- S-; RD T- S-), those yielding anomalous 16S signals (T- S+) but expected RD signals (T+ S+), and those positive for MTBC by the combined assay (16S T+ S+; RD T+ S+).

Figure 3.17: Visual assessment of LAMP reactions by calcein fluorescence



Tubes 1 and 2, negative and positive controls with the Loopamp Kit primers, respectively; tubes 3 and 4, negative and Mtb CDC 1551 positive controls with the test 16S primers, respectively; tubes 5 and 6, negative and positive sample reactions, respectively.

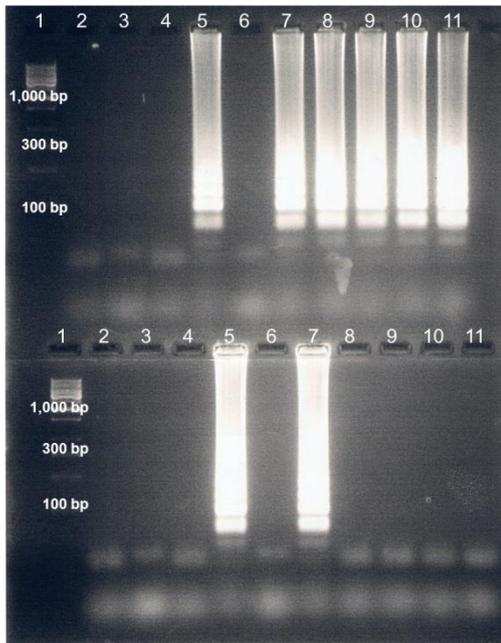


Figure 3.18: Gel analysis of LAMP amplicons

Upper gel panel: lane 1, GeneRuler 100-bp DNA marker; lanes 2 and 3, negative controls with test 16S and Loopamp Kit primers, respectively; lane 4, *M. smegmatis* DNA; lane 5, Mtb CDC 1551 DNA; lane 6, Loopamp Kit positive control; lane 7, 891F; lane 8, 549Y; lane 9, U4808350; lane 10, 804AC; lane 11, 634MB.

Lower gel panel: lane 1-6, same as above; lane 7, 620PB; lane 8, 666CN; lane 9, 833RV; lane 10, 137DG; lane 11, 296JJ.

3.4 Discussion

3.4.1 Principle of the 16S-RD assay

The real-time PCR assay developed in this study facilitates differentiation between MTBC and NTM; further identification of NTM species is achieved by 16S rDNA sequencing. This molecular assay is also capable of binary genotyping of MTBC strains based on the EA-I lineage-defining RD750 polymorphism. The 16S and RD assays are performed in separate reaction tubes within a single thermocycler run; each utilises a novel combination of TaqMan and SYBR Green technologies to simultaneously differentiate two products within a single tube. SYBR Green detection provides a total PCR amplicon readout, and this serves as the positive assay control for TaqMan results.

3.4.2 Development of the 16S-RD assay

Initial testing of the 16S assay showed that the universal mycobacterial 16S primers could non-specifically amplify DNA from other genera at the low annealing temperature (56°C) used. Although these primers were designed to target the 16S rDNA region specific to the genus *Mycobacterium*, random priming of template DNA could occur under conditions of low PCR stringency. However, the non-specific amplicons could be distinguished from those of mycobacteria by subsequent melt analyses and DNA sequencing. Based on the former, their T_m s were distinctively lower than those of mycobacterial 16S amplicons. The specificity of the 16S assay was improved with the use of higher annealing temperatures; none of the non-mycobacterial species tested gave significant PCR signals at temperatures above 63°C. Specific detection of Mtb DNA in samples spiked with non-mycobacterial DNA in various proportions reflects the preferential amplification of the former and interruption of non-specific priming in mixed DNA preparations.

Optimisation of the concentration of PCR reagents was carried out to further improve the specificity and maximise the sensitivity of the 16S-RD assay. These reagents included magnesium chloride, primers, and TaqMan probes. Excess in these reagents during PCR could compromise the specificity of the reaction, but at the same time contribute towards increased sensitivity. In the former case, oligonucleotide primers and probes annealing to non-target sites on the template DNA are stabilised in the presence of excessive magnesium ions, thereby reflecting the synergistic interaction between these reagents in decreasing PCR specificity (Bartlett and Stirling, 2003). Furthermore, excess in magnesium ions also stabilises and prevents complete denaturation of DNA during PCR and therefore reducing amplicon yield (Bartlett and Stirling, 2003).

PCR signals were constantly detected for NTCs in the 16S assay, thus indicating that the 16S primers were binding to contaminating DNA present in those reactions. The resulting amplicons were sequenced to be those of non-mycobacterial actinobacteria, consistent with the potential of these organisms to produce amplicons with the mycobacterial 16S primers. Source of contamination could not be traced. Since non-mycobacterial actinobacteria are frequently found in environmental reservoirs (Winn *et al.*, 2006), it is possible that the water used in the PCR contained the DNAs of these organisms and manufacturer's quality control procedure failed to eliminate them. Exposure of PCR reagents to UV light did not prevent subsequent amplification of these contaminating DNAs. Therefore, PCR signals of sample reactions were always compared against that yielded for NTC in every 16S assay in order to assign the final results.

3.4.3 Detection limit and specificity of the 16S-RD assay

The detection limit of the 16S-RD assay was estimated to be between 100 and 1,000 mycobacterial genomes per reaction. In relation to its specificity, the combined assay gave 16S amplicons with some non-mycobacterial actinobacteria but was universally negative with the other bacterial species tested. Nevertheless, these actinobacterial species are stained Gram-positive and appear only 'partially acid fast' during ZN staining (Winn *et al.*, 2006). These distinctive staining characteristics distinguish them

from mycobacteria. Mycobacterial cells appear either beaded or almost as negative image against the counterstained background during Gram staining due to their mycolate-rich cell envelope (Winn *et al.*, 2006). Thus, when applied to both clinical specimens and isolates which are routinely ZN stained, additional consideration of Gram staining results and the results of conventional cultures should make interpretation unambiguous. Furthermore, results from melt analyses and direct sequencing of amplicons from the 16S assay would rule out any doubt in interpretation. On a long-term basis, re-designing of the 16S primers or targeting alternative genes specific to the genus *Mycobacterium* (i.e. those involve in lipid metabolism) should be considered to address the specificity issue.

3.4.4 Effect of combining TaqMan and SYBR Green chemistries in single reactions

The simultaneous use of TaqMan and SYBR Green chemistries in each reaction tube had very minimal consequence for the performance of the 16S-RD assay on positive mycobacterial cultures. Inclusion of TaqMan probes had no detectable effect on SYBR Green results. In contrast, inclusion of SYBR Green led to a decline in the sensitivity of TaqMan-based detection, which was more pronounced with low target concentrations and the RD assay. This might reflect the impact of SYBR Green molecules binding to the same region as the TaqMan probes, thus resulting in lower rate of probe cleavage. Although this has little consequence for analysis of clinical mycobacterial isolates, it could lead to decreased assay sensitivity for clinical specimens. The inclusion of additional RD reactions utilising TaqMan chemistry alone would be beneficial in this case, although this would increase the cost and decrease the throughput per assay run.

3.4.5 Evaluation of the 16S-RD assay for analysis of clinical mycobacterial isolates

3.4.5.1 Assessment of simple methods for releasing DNA from clinical mycobacterial isolates

Additional treatment of boiled lysates from LJ and MGIT cultures with *microLYSIS* reagent did not give more signals in the 16S assay. If the preceding boiling process released DNA from most mycobacterial cells in the cultures, the additional treatment with *microLYSIS* reagent would not make a significant difference. Therefore, all clinical cultures in this study were only subjected to boiling for simplicity and to minimise the cost of processing. Perhaps the boiling step was not even necessary if the real-time PCR thermocycler was housed within the microbiological safety cabinet in the Category 3 Laboratory. In this case, DNAs would be released directly from mycobacterial cells in the cultures during the initial PCR hold at 95°C.

3.4.5.2 The 16S-RD assay differentiates between *M. tuberculosis* complex and non-tuberculous mycobacteria

16S-RD assay results for 87 cultures were concordant with those from the reference laboratory in which 70 were graded MTBC-positive and 17 NTM-positive. Two MTBC-positive cultures failed to yield TaqMan signals in the 16S assay. These results derived from MGIT culture lysates in which the possible interference by non-mycobacterial signals may have been a confounding factor. Interestingly, the T_m s of their 16S amplicons were more than 1°C lower than those obtained for confirmed mycobacterial amplicons. Furthermore, the sequences of their amplicons showed poor alignment with the partial 16S rDNA sequence of Mtb H37Rv. Therefore, anomalous T_m s and sequencing results from the 16S assay would raise an initial suspicion on the identity of the samples concerned. Although the 16S assay failed to confirm the mycobacterial identity of the two MGIT cultures, the parallel RD assay detected MTBC DNA in them. Thus, there seems to be limitations to the negative predictive value of the 16S assay alone for MTBC. At present, when applied to cultures, the combined 16S-RD assay

reliably excludes the presence of MTBC when the RD results are negative. Further confirmation of 'false-negative' 16S TaqMan signals from mycobacterial isolates can be achieved via the NAP test.

One positive MGIT culture failed to yield any signals in the 16S-RD assay and was tested to be negative for mycobacteria by the reference laboratory. This occurs in up to 5% of the MGIT cultures graded positive for AFB by the routine microbiology service at the UHL (J. Malkin, personal communication). Therefore, analysis of AFB-positive cultures by the 16S-RD assay has the potential to recognise such true negatives. This would avoid unnecessary transfer of these samples to the reference laboratory for further molecular tests. Since a preliminary report indicating the possible isolation of mycobacteria is routinely issued to clinicians when cultures are forwarded to the reference laboratory, use of the combined assay to recognise these false-positive samples warrants further analysis. This would have important implications with regard to appropriate case management.

3.4.5.3 Direct sequencing of 16S amplicons identifies non-tuberculous mycobacterial species

The 16S-RD assay correctly identified the presence of NTM in all 17 reference laboratory-confirmed cultures. Subsequent sequencing of their 16S amplicons produced NTM identities concordant with those reported by reference laboratory in 15 cases. There were two primary sequencing failures, both from MGIT cultures yielding *M. goodnae*. One culture was from a HIV-positive patient. Three sputum samples were collected from this patient at the same time period, of which two were identified by the reference laboratory as containing *M. goodnae* and another one with *M. celatum*. Mixed mycobacterial infections have been reported in individuals with HIV infection (Libanore *et al.*, 1992; Campbell *et al.*, 2000). The likelihood of mixed mycobacterial amplicons causing sequencing failure in the two NTM-positive cultures could be demonstrated by sequencing of 16S clones. Also, the possible interference by non-mycobacterial signals in MGIT cultures should be taken into account.

3.4.5.4 The RD assay differentiates between *M. tuberculosis* complex strains with intact and deleted RD750

Out of 70 MTBC isolates, the RD assay assigned 44 to RD750 intact and 26 to RD750 deleted. RD750 identities for 62 of these isolates were concordant with those assigned by the conventional *Rv1519* PCR assay. Occasional failures of the *Rv1519* PCR are expected given the relatively large 1- to 3-kb amplicon sought and the crude DNA preparation used for clinical isolates. The substitution of water for an appropriate suspension buffer might be beneficial in this case to minimise acid hydrolysis and fragmentation of DNA molecules (Bartlett and Stirling, 2003). As expected, all the RD750⁻ strains fell into the VNTR x2234/5 group; both have been shown to be molecular signatures unique to Indian ethnic groups from East Africa and South Asia (Gagneux *et al.*, 2006; Menendez *et al.*, 2007). Since MTBC strains of the EA-I lineage are highly prevalent in Leicester, the results of the RD assay provide an opportunity for preliminary epidemiological discrimination. For example where two potentially linked cases yield RD750⁺ and RD750⁻ isolates, transmission can be excluded.

3.4.6 The 16S-RD assay shows potential in detecting samples containing mixed mycobacterial species or strains

Assay of mixed DNA samples demonstrated that the combined 16S-RD assay has potential to detect mixed infections due to MTBC strains belonging to different lineages and mixed MTBC/NTM infections. Indeed, both these versions of mixed infections have been documented previously, both in immunocompromised and immunocompetent TB patients (Libanore *et al.*, 1992; Das *et al.*, 2004; Warren *et al.*, 2004). One of them reported the detection of different Mtb strains in the same sputum specimen via a PCR approach (Warren *et al.*, 2004). The potential of the 16S-RD assay to detect mixed infections is limited to samples in which the SYBR Green signals generated by the assay indicate a significant excess of input DNAs over those attributable to the TaqMan probe in the same reaction. In the event of detecting PCR signals suggestive of a mixed infection, further analyses such as cloning and sequencing in the case of the 16S assay and gel electrophoresis in the case of the RD assay could be performed to obtain further confirmation. This attractive potential of the combined assay requires

further evaluation with mixed clinical specimens or at least, artificially seeded specimens.

3.4.7 Preliminary evaluation of the 16S-RD assay for direct analysis of respiratory specimens

Direct application of the 16S assay to boiled Mtb-positive sputa with or without *microLYSIS* treatment did not yield any PCR signals. Repetition of the assay with diluted DNA samples yielded both TaqMan and SYBR Green signals. One experiment showed the reduction in PCR signals for pure mycobacterial DNA samples spiked with non-tuberculous sputum (data not shown). All these observations suggest the presence of substances inhibitory to PCR in sputum samples which are not eliminated by simple methods of DNA extraction, although the manufacturer claimed that purification of DNA is not necessary following treatment with *microLYSIS*. Indeed, inhibition of PCR for the direct detection of Mtb in sputum specimens has been reported previously; higher rates of inhibition with simple extraction procedures (Forbes and Hickes, 1993; 1996; Kox *et al.*, 1994). Proteinaceous inhibitors can inhibit the *Taq* DNA polymerase directly or by reducing the availability of magnesium ions, which are co-factors for the normal functioning of the polymerase during PCR (Bartlett and Stirling, 2003).

Although PCR inhibition with these simple extraction methods can be resolved by dilution, the assay sensitivity for clinical specimens can be compromised. The use of commercial DNA extraction kits involving enzymatic digestion and column-based purification, although more labourious and costly, would be beneficial in this case. Considering the cost of the kits, the QIAamp DNA Mini Kit was preferred over the EliGene DNA Mini Kit. Therefore, the former was routinely used for processing of sputum samples at the UHL routine microbiology service by another researcher in the same group. Elution of DNA in the buffer provided in the commercial kit will be beneficial for long-term storage and repeated analyses of sputum DNA samples (Bartlett and Stirling, 2003). The inclusion of BSA in PCRs with sputum DNA improved the PCR detection for some samples. Indeed, this beneficial property of BSA in PCR has been reported previously (Forbes and Hickes, 1996). It is possible that BSA binds and isolates inhibitors in the clinical specimens from the ongoing PCRS; varied effects

among different samples might reflect the different loads of inhibitors that were present. The extended heating of decontaminated sputum samples during the initial evaluation might have destroyed this property of BSA (Figure 3.14). Thomson and associates (2005) reported successful PCRs in clinical specimens processed by similar procedures but with shorter heating duration. The inclusion of an internal amplification control in the 16S-RD assay to rule out the possibility of PCR inhibition should definitely be considered.

Three Mtb-positive sputum samples tested in this study were anomalously negative for the 16S TaqMan signal. Two of these samples were from the patient whose MGIT isolate gave similar PCR signals during the combined 16S-RD assay. Both clinical isolate and samples gave 16S amplicons with lowered T_m s relative to that predicted for mycobacterial amplicons. Their DNA sequence also aligned poorly with the partial 16S rDNA sequence of Mtb H37Rv. It was thought that this could be due to interference by non-mycobacterial signals in the samples. However, these sputum samples were also negative by the LAMP assay that targets the MTBC 16S rDNA, which might suggest the possible role of novel 16S sequence variation among clinical strains.

3.4.8 Loop-mediated isothermal amplification assay has higher positivity rate in the detection of *M. tuberculosis* in respiratory specimens

LAMP reactions were positive for 4 out of 14 Mtb-positive sputum samples which were negative by the 16S-RD assay. Three of these LAMP-positive samples were graded smear-negative. The LAMP method is highly specific and sensitive due to the use of multiple primers and the generation of large amount of amplified products, respectively. This method is quick and simple to perform; the duration of amplification for the current assay evaluated can be shortened to 40 minutes due to the additional use of loop primers. By having a LAMP assay which targets MTBC and clinically important NTM with subsequent restriction analysis for species identification (Iwamoto *et al.*, 2003), this method can be used to confirm the negative results of the 16S-RD assay and detect mycobacteria in smear-negative samples (Boehme *et al.*, 2007). With simple DNA isolation procedure, the LAMP method has potential in facilitating bedside diagnosis.

3.5 Conclusion

The 16S-RD assay, based on the novel combination of TaqMan and SYBR Green chemistries, is capable of distinguishing between MTBC and NTM, and assigning MTBC isolates to within or outside the EA-I lineage. The former is important with regard to the varied clinical and public health implications and interventions between MTBC and NTM infections, while the latter enables recognition or exclusion of links among MTBC strains which is of great value for assessments of potential recent transmission events. This combined molecular assay is reliable when applied to clinical mycobacterial isolates, which has potentially beneficial clinical utility. Also, the results from this study show that it can be used to recognise false AFB-positive cultures prior to forwarding to the reference laboratory for further analyses, and this warrants further investigation.

Most encouraging of all, the 16S-RD assay is showing promising results for direct application to the analysis of smear-positive respiratory specimens, which, if successfully set up and validated, can be employed to influence decisions on clinical and public health management prior to obtaining positive cultures. Indeed, a researcher at the UHL routine microbiology service reported the successful use of the assay to exclude TB in one case and to recognise possible infection due to strain CH in another. In this latter case, CH infection was subsequently confirmed by GLIP analysis and an appropriate public health response instituted prior to obtaining positive cultures. Furthermore, direct specimen analysis by the 16S-RD assay will facilitate identification of TB patients for future mask sampling studies, which was one of the main aims of its development and evaluation in the current study.

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CHAPTER 4

Methods for Detecting Face Mask Contamination

4.1 Introduction

Medical practice relies on AFB smear status to assess the infectiousness of TB patients; however, this remains controversial as transmission of Mtb from smear-negative cases has often been reported (Blaht *et al.*, 1946; Catanzaro, 1982; Di Perri *et al.*, 1989; Behr *et al.*, 1999). In this context, measuring the concentration of Mtb in respiratory aerosols might be a more reliable means for determining case infectivity, and perhaps monitoring the progress of chemotherapy. Various methods for collecting and quantifying tuberculous droplet nuclei have been developed and evaluated, both with TB patients and pure Mtb cultures, but no reliable method has been identified to date. Only a few methods are amenable for direct sampling of respiratory-borne Mtb and many suffer from various limitations.

Various mechanical air samplers for the collection of airborne microbes are available and some have been used to sample Mtb aerosols. The Andersen cascade impactor (Graseby Andersen Division) is used for the collection and particle size determination of culturable bioaerosols (Andersen, 1958). It consists of 6 size-determining stages in which agar plates are loaded, one on each stage. The micro-orifice uniform deposit impactor (MOUDI) (MSP Corporation) is similar to the Andersen sampler, except that it is available in various numbers of size-determining stages and normally collects aerosols onto membrane filters for downstream PCR analysis (Figure 4.1). MOUDI has been validated with aerosolised suspensions of MTBC, and its performance was compared to that of a plastic, disposable filter cassette (Schafer *et al.*, 1998; 1999). Both the Andersen sampler and MOUDI have also been used to sample and detect airborne NTM associated with natural waters (Falkinham *et al.*, 1990) and public swimming pools (Schafer *et al.*, 2003), respectively.



Figure 4.1: Ten-stage rotating MOUDI for the collection and particle size determination of bioaerosols

The cut-size diameters are from 0.056 to 18 μm . The stage rotation allows particle deposit on the impaction plate to spread out over a circular area to form a nearly uniform layer. Reproduced from the MSP Corporation website (2008) with permission from MSP Corporation. Copyright © MSP Corporation 2008. All rights reserved.

The use of animal models to study TB infectiousness provides useful data and enabled extended sampling but has several limitations (Riley *et al.*, 1959; Escombe *et al.*, 2007). This approach is costly, requires ethical approval, and necessitates specially constructed facilities. Furthermore, interpretation of results is limited by the need to identify patient source of infection based on bacillary phenotype or genotype and temporal exposure patterns, and also possible variation in susceptibility of animals to Mtb infection.

Direct sampling of respiratory Mtb aerosols for downstream detection by culture has several disadvantages including the delay in obtaining results, the presence of bacilli that may be infective but are non-culturable by conventional methods, problems associated with extended sampling, fungal contamination of culture, and desiccation of media and bacilli (Fennelly *et al.*, 2004). Furthermore, correlation between the culturability of Mtb cells from close proximity of sampling and prolonged aerial suspension is not established.

The use of air sampling-PCR method, although rapid, does not differentiate between live and dead organisms and is prone to assay inhibition. Most reported studies employed this method to detect Mtb in TB wards and were more relevant to hospital air hygiene; discrete infectiousness of individual TB patients was not directly assessed (Mastorides *et al.*, 1999; Vadrot *et al.*, 2004; Chen and Li, 2005).

Bioaerosol mass spectrometry has also been evaluated for the detection of artificial Mtb H37Ra aerosols generated from liquid culture (Tobias *et al.*, 2005). Individual aerosolised Mtb-containing particles were sampled and concentrated by an impactor. The molecules in these particles were then desorbed and ionised by the laser light. The resulting positive and negative ions were simultaneously extracted in opposite directions and chemically analysed by the time-of-flight mass spectrometer, in which Mtb aerosols could be identified from their distinctive mass spectral signatures. This method is rapid and sensitive, and it enables both automation and real-time operation. However, the equipment is bulky, expensive, and requires high-end skills for operation and result interpretation.

In the current study, a novel approach based on use of surgical masks was developed and evaluated for sampling and quantification of Mtb expectorated by pulmonary TB patients. The masks that were chosen for this study are the TECNOL Standard Surgical Masks from Kimberly-Clark, which are easily available in most hospitals and other healthcare settings (Figure 4.2). Mask sampling is economical, non-invasive, and simple to perform; it enables direct collection of Mtb aerosols from TB patients. Also, frequent wearing of masks by TB patients confers additional protection to healthcare staff, other patients, and visitors (Chen *et al.*, 1994; Chen and Li, 2005). Detection of Mtb organisms sampled on masks was attempted by real-time PCR and mycobacteriophage amplification methods due to their quantitative potential and rapid nature. Both these methods yield results within 1 and 2 days of sample receipt respectively, as opposed to culture-based approaches that require 2-3 weeks. Furthermore, sensitive detection of Mtb in sputum has been demonstrated for the real-time PCR and phage methods (Miller *et al.*, 2002; Albay *et al.*, 2003; McNerney *et al.*, 2004). This chapter reports the development of methods to assess and evaluate the feasibility of the mask approach for sampling and enumeration of respiratory-borne Mtb aerosols.

Figure 4.2: TECNOL Standard Surgical Masks from Kimberly-Clark



The mask is made of polypropylene fibres and consists of three layers: the outer shell, the middle filter layer, and the inner cover web. It is pleated and can come with ear loops (A) or ties (B) as the method of fastening. Reproduced from the Kimberly-Clark website (2001) with permission from Kimberly-Clark Worldwide, Inc. Images are properties of Kimberly-Clark Worldwide, Inc. © Copyright 2001 Kimberly-Clark Worldwide, Inc. All Rights Reserved.

4.2 Materials and Methods

All experiments in this chapter were performed with mycobacterial cultures grown to exponential phase in Middlebrook 7H9-ADC-Tween broth unless otherwise indicated (see Sections 2.4.3-2.4.5). All CFU counts for test mycobacterial cultures were performed on Middlebrook 7H10 agar as described in Section 2.4.6.

4.2.1 Detection of face mask contamination by quantitative real-time PCR

4.2.1.1 DNA release by lysing matrices with downstream phenol-chloroform purification

Ten-fold serial dilutions of *M. smegmatis* culture were made in phosphate-buffered saline (PBS). Mask segments (1 cm²) were spiked with 20 µl of *M. smegmatis* suspension each. One mask segment was spiked with PBS as a negative control. Spiked mask segments were left to dry under laminar air flow then transferred to a tube containing Lysing Matrix D (1.4-mm ceramic spheres; MP Biomedicals, California, USA) each. The mask segment was homogenised in the presence of 0.6 ml of 1% w/v Triton X-100 containing 0.1 mg/ml proteinase K in a cell disrupter (Hybaid, Teddington) at full speed for 45 seconds. The homogenised mixture was centrifuged at 15,700 g for 15 minutes and 0.4 ml of the resulting supernatant transferred to a tube containing Lysing Matrix B (0.1-mm silica spheres; MP Biomedicals). The remaining mixture was centrifuged and 0.2 ml of supernatant transferred to the same Lysing Matrix B tube, after which homogenisation and centrifugation were repeated. Proteinase K was added to a final concentration of 0.3 mg/ml and the mixture incubated at 55°C overnight. Homogenisation and centrifugation were repeated again. Equal volume of 25:24:1 v/v/v phenol-chloroform-isoamyl alcohol was added, the mixture centrifuged, the resulting upper aqueous phase transferred to a 1.5-ml Eppendorf tube, and an equal volume of 24:1 v/v chloroform-isoamyl alcohol added. The mixture was centrifuged and the resulting aqueous phase transferred to a new 1.5-ml Eppendorf tube. DNA precipitation was performed at -20°C for 1 hour in the presence of 5 µg of glycogen, 0.05 volume of

3 M sodium acetate (pH 5.2), and 0.7 volume of isopropanol. Centrifugation was performed, the resulting DNA pellet washed sequentially in 70% and 95% v/v ethanol, and the final DNA pellet resuspended in 50 µl of PCR-grade water. 16S SYBR Green PCR was performed with 2 µl of the resulting DNA suspension as described in Section 3.2.4.1, except that MYCO16SPr was not included in the reaction.

4.2.1.2 DNA release by *micro*LYSIS reagent

Protocols were as described in Section 4.2.1.1 except that homogenised samples were not treated with Lysing Matrix B. Following overnight treatment with proteinase K and homogenisation, the suspension was transferred to a 0.2-ml microtube (ABgene) and centrifuged at 15,700 *g* for 2 minutes. The resulting pellet was resuspended in 20 µl of *micro*LYSIS and thermal cycling performed as in Section 3.2.2.2. 16S SYBR Green PCR was performed as in Section 4.2.1.1.

4.2.1.3 DNA release by mechanical action and boiling with downstream purification using QIAamp DNA Mini Kit

Ten-fold serial dilutions of Mtb culture were made in 1.8-ml aliquots of Middlebrook 7H9-Tween broth in 7-ml Bijoux tubes (Sterilin). Half mask segments (9.0 x 9.5 or 85.5 cm²) were spiked with twenty 50-µl drops of Mtb suspension each. One mask segment was spiked with 7H9-Tween broth as a negative control. Spiked mask segments were left to dry then transferred to a 50-ml Falcon tube (Corning Life Sciences) each, to which 20 ml of Triton-Tris solution was added. Tube contents were vortexed for 2 minutes then mixed on the tube rotator for 30 minutes. The mask segment was removed and the suspension centrifuged at 3,023 *g* for 15 minutes. Supernatant was removed leaving behind approximately 1 ml to resuspend the pellet. The suspension was then transferred to a 1.5-ml Eppendorf tube and centrifuged at 14,100 *g* for 5 minutes. The resulting pellet was resuspended in 50 µl of PCR-grade water, boiled for 30 minutes, and then treated with 1 mg/ml proteinase K at 56°C for 2 hours. RD assay was performed with 8.5 µl of the resulting DNA suspension as in Section 3.2.4.1. RD

assay was repeated on samples purified through the QIAamp DNA Mini Kit (see Section 3.2.2.3).

4.2.2 Detection of face mask contamination by the mycobacteriophage assay

4.2.2.1 Harvesting and preparation of mycobacterial cultures

Preparation of test mycobacterial cultures

Aliquots of test mycobacterial cultures (usually 10 ml) were harvested and centrifuged at 3,000 g for 15 minutes. The resulting cell pellets were resuspended in Middlebrook 7H9-OGC broth. These steps were important as Tween 80 in the initial cultures would inhibit the phage assay. Dilutions of culture suspensions were prepared as required in 7H9-OGC broth.

Preparation of phage indicator plates from *M. smegmatis* culture

M. smegmatis was grown in Middlebrook 7H9-OADC broth at 37°C with shaking at 200 rpm for 24 hours from starting OD₅₈₀ of 0.05. Clumps in the culture were allowed to sediment for at least 30 minutes in a 50-ml Falcon tube. Phage indicator plates were prepared by mixing 1 ml of the upper homogenous suspension with 9 ml of molten 7H9-OGC agar, which was maintained in 30-ml Universal tubes at 50°C. Plates were left to dry under laminar air flow prior to use.

Preparation of mycobacterial supernatants

The effect of mycobacterial Rpf on phage assay with stationary-phase mycobacteria was also assessed in this study. Mycobacterial supernatants for this purpose were prepared as follows. *M. smegmatis* was grown in Middlebrook 7H9-OADC broth at 37°C with shaking at 200 rpm for 20 hours. Culture was centrifuged at 3,000 g for 15 minutes and the supernatant sterilised by filtration through a 0.2-µm syringe filter. An aliquot of the filtered supernatant (containing Rpf) was incubated at 37°C to check for

carry-over mycobacterial cells. *M. bovis* BCG supernatant was prepared as described for *M. smegmatis* from culture that was grown static to OD_{580nm} of between 0.5 and 1.

4.2.2.2 Propagation and enumeration of phage D29

A phage indicator plate was inoculated with 100 µl of phage D29 at approximately 4×10^3 PFU/ml in Middlebrook 7H9-OGC broth, and incubated at 37°C overnight. There should be a large number of plaques but not complete lysis of *M. smegmatis* lawn following incubation. After adding 10 ml of 7H9-OGC broth, the plate was returned to the 37°C incubator for a further overnight incubation. The broth was then pipetted into a 30-ml Universal tube and filtered through two 0.45-µm syringe filters (Pall Corporation). The resulting phage D29 suspension was stored in 1-ml aliquots in 1.5-ml Eppendorf tubes at 4°C. To quantify the phage suspension, ten-fold serial dilutions were made in 7H9-OGC broth and triplicate 10-µl drops of each dilution were spotted onto a phage indicator plate (two dilutions per plate). Plates were allowed to dry and then incubated at 37°C overnight. The dilution that yielded 10-100 plaques was chosen to calculate the number of PFU. The phage stock generally contained 10^9 - 10^{10} PFU/ml.

4.2.2.3 Contamination of face masks with mycobacteria

All mask segments were treated with 600,000 µJ of UV in the Stratalinker UV Crosslinker prior to spiking with mycobacterial cells unless otherwise indicated. For 4-cm² mask segments, each of these was spiked with six 20-µl drops of mycobacterial culture suspension in Middlebrook 7H9-OGC broth (see Section 4.2.2.1). Spiked mask segments were left to dry under laminar air flow, transferred to a 1.5-ml microtube each, and 1 ml of 7H9-OGC broth added. Tube contents were vortexed for 30 seconds and all of the resulting suspension transferred to an appropriate reaction vessel for analysis by the phage assay. For half mask segments (9.0 x 9.5 or 85.5 cm²), each of these was spiked with twenty 50-µl drops of culture suspension and left to dry. Dry mask segments were transferred to a 50-ml centrifuge tube each to which 20 ml of an appropriate eluting medium added. In both cases, negative controls were performed with mask segments spiked with 7H9-OGC broth.

4.2.2.4 NaOH-NALC decontamination

Biotec method

NaOH-NALC decontamination for the Biotec *FASTPlaqueTB* (FPTB) assay was performed according to the protocol recommended by the manufacturer (Biotec Laboratories, 2004). In a 50-ml Falcon tube, 1 ml of sample was treated with 1 ml of 2% w/v NaOH-NALC at room temperature for 15 minutes and vortexed for no more than 30 seconds. NaOH was then neutralised by the addition of 67 mM phosphate buffer up to the 45-ml mark on the tube. Tube contents were centrifuged at 3,000 *g* for 15 minutes and the resulting pellet resuspended in 15 ml of FPTB Medium Plus. Tube contents were centrifuged again and the resulting pellet resuspended in 1 ml of FPTB Medium Plus. All the suspension was transferred to the FPTB reaction vessel and incubated at 37°C for 18-24 hours to resuscitate chemically stressed mycobacterial cells.

In-house method

Protocol for NaOH-NALC decontamination for the in-house phage assay was similar to the Biotec method with a few modifications. Following decontamination, NaOH was neutralised with 20 ml of 67 mM phosphate buffer. Upon centrifugation, the resulting pellet was resuspended in 2 ml of Middlebrook 7H9-OGC broth and then subjected to similar resuscitation condition.

4.2.2.5 Mycobacteriophage amplification assay

Biotec FASTPlaqueTB assay

Preparation of Biotec media and reagents is described in Appendix 2. Biotec FPTB assay was performed according to the manufacturer's protocol (Biotec Laboratories, 2004). Briefly, mycobacterial cells in 1 ml of sample were infected with 100 µl of Actiphage at 37°C for 1 hour in the reaction vessel provided. Exogenous phages that did not infect the target cells were inactivated with 100 µl of Virusol solution at room temperature for 5 minutes. Virucide action was terminated by dilution with 5 ml of FPTB Medium Plus, which also served as a source of nutrients for the growth of Sensor cells and propagation of progeny phage particles from the infected hosts. Finally, 1 ml of Sensor cells was added and the entire reaction mixture was plated out with 5 ml of FPTB Agar. Plates were left to set and then incubated at 37°C for up to 24 hours. The number of plaques formed was enumerated, aided by a light box (Jessop, London). Negative control was performed with 1 ml of FPTB Medium Plus and should not yield more than 10 plaques. Positive control was performed with 10⁻⁶ dilution of Sensor cells and should result in 20 plaques or more.

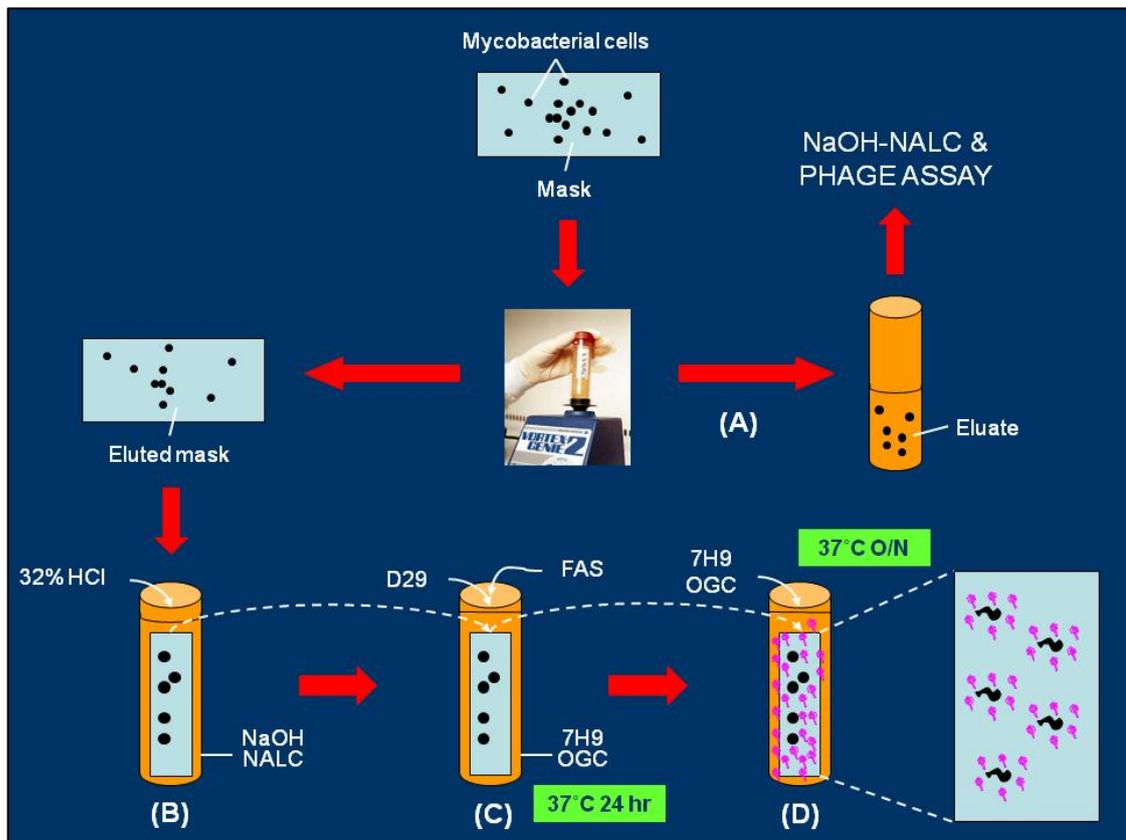
In-house phage assay

Negative control was performed with Middlebrook 7H9-OGC broth. Positive control was performed with 10⁻⁵ or 10⁻⁶ dilution of *M. smegmatis* culture for lawn formation. To 1 ml of sample or control in a 7-ml Bijoux tube, 0.5 volume of phage D29 at 10⁸ PFU/ml was added. The reaction vessel was shaken gently to mix its contents and then incubated at 37°C for 1 hour. Following incubation, 0.2 volume of 50 mM FAS was added and tube contents were vortexed. Virucide action was terminated by diluting 1 ml of tube contents in 9 ml of molten 7H9-OGC agar. Upon addition of 1 ml of *M. smegmatis* lawn culture (see Section 4.2.2.1), tube contents were mixed by inversion and then poured into a Petri dish. Plates were left to set and then incubated at 37°C for up to 24 hours. The number of plaques formed was enumerated over the light box.

4.2.2.6 On-mask phage infection and cell lysis

Figure 4.3 summarises the initial protocol tested for on-mask phage infection and cell lysis. Half mask segments were spiked with approximately 10^7 *M. bovis* BCG CFU each and left to dry.

Figure 4.3: On-mask phage infection and cell lysis



Processing of mask eluates (A)

Each dry mask segment was transferred to a 50-ml Falcon tube, 20 ml of Middlebrook 7H9-OGC broth added, and tube contents vortexed for 2 minutes. The eluted mask segment was transferred to a new 50-ml Falcon tube (for Process B). The eluate was centrifuged at 3000 *g* for 15 minutes and the resulting supernatant removed leaving behind about 1 ml to resuspend the pellet. NaOH-NALC decontamination and phage assay were performed as in Sections 4.2.2.4 and 4.2.2.5, respectively.

Processing of eluted masks (B)

The eluted mask segment was treated with 35 ml of 1% w/v NaOH-NALC at room temperature for 15 minutes. NaOH was then neutralised with 1.5 ml of 32% v/v HCl and the decontaminated mask segment transferred to a new 50-ml Falcon tube (for Process C). The eluate was centrifuged at 3,000 *g* for 15 minutes, the resulting supernatant discarded, and the pellet resuspended in 2 ml of 7H9-OGC broth. Resuscitation was performed at 37°C for 24 hours. Phage assay was performed as in Section 4.2.2.5.

On-mask phage infection and pre-lysis plating (C)

To the decontaminated mask segment, 40 ml of 7H9-OGC broth was added and resuscitation was performed at 37°C for 24 hours. Phage D29 was then added to a final concentration of 3×10^7 PFU/ml and the reaction incubated at 37°C for 1 hour. Following this, 200 mM FAS was added to a final concentration of 8.3 mM and the reaction incubated at room temperature for 5 minutes. The mask segment was then transferred to a new 50-ml Falcon tube (for Process D). Finally, 1 ml of tube contents was plated out with 9 ml of molten 7H9-OGC agar and 1 ml of *M. smegmatis* lawn culture.

On-mask cell lysis and release of progeny phage particles (D)

The mask segment was incubated in 40 ml of 7H9-OGC broth at 37°C for 24 hours to allow for lysis of host cells and release of large numbers of progeny phage particles. Following this, tube contents were plated out as for Process C.

4.2.3 Statistical analysis

All data in this chapter were analysed by the two-tailed paired *t* test, as required.

4.3 Results

4.3.1 Detection of face mask contamination by quantitative real-time PCR is insensitive and subject to inhibitory activity

The utility of quantitative real-time PCR was examined to detect mycobacteria from face masks. This method produced results on the day of processing. However, several experiments in which different procedures for releasing mycobacterial DNA were tested, demonstrated poor sensitivity. The aim was to detect less than 100 mycobacterial cells from face masks. The method involving DNA release by treatment with lysing matrices followed by phenol-chloroform purification yielded detection limit of only 10^4 - 10^5 *M. smegmatis* cells spiked per mask segment (Method 4.2.1.1; Table 4.1A). DNA release with *micro*LYSIS in the absence of downstream purification improved sensitivity by ten fold (Method 4.4.1.2; Table 4.1B). Analysis of larger mask segments spiked with Mtb cells involving DNA release by combined mechanical action and boiling followed by purification using the QIAamp DNA Mini Kit yielded a lower detection limit of 10^5 - 10^6 cells spiked per mask segment (Method 4.2.1.3; Table 4.1C). For each of the three methods, one mask segment was analysed for each amount of mycobacterial CFU spiked. Results were based on 16S SYBR Green signals for both Methods 4.2.1.1 and 4.2.1.2. For Method 4.2.1.3, results were based on SYBR Green signals of the RD assay.

Table 4.1: Different methods for releasing DNA from mycobacterial cells applied to face masks for detection by quantitative real-time PCR

4.1 (A) DNA release by lysing matrices with downstream phenol-chloroform purification

<i>M. smegmatis</i> CFU spiked per 1-cm ² mask segment (Log ₁₀)	<i>M. smegmatis</i> genome equivalents recovered per 1-cm ² mask segment (Log ₁₀)
6.36	7.02
5.36	5.47
4.36	3.79
3.36	Not detected
2.37	Not detected
1.36	Not detected
0.30	Not detected

4.1 (B) DNA release by *micro*LYSIS without downstream purification

<i>M. smegmatis</i> CFU spiked per 1-cm ² mask segment (Log ₁₀)	<i>M. smegmatis</i> genome equivalents recovered per 1-cm ² mask segment (Log ₁₀)
5.68	6.62
4.68	6.19
3.68	4.94
2.68	Not detected
1.68	Not detected
0.70	Not detected

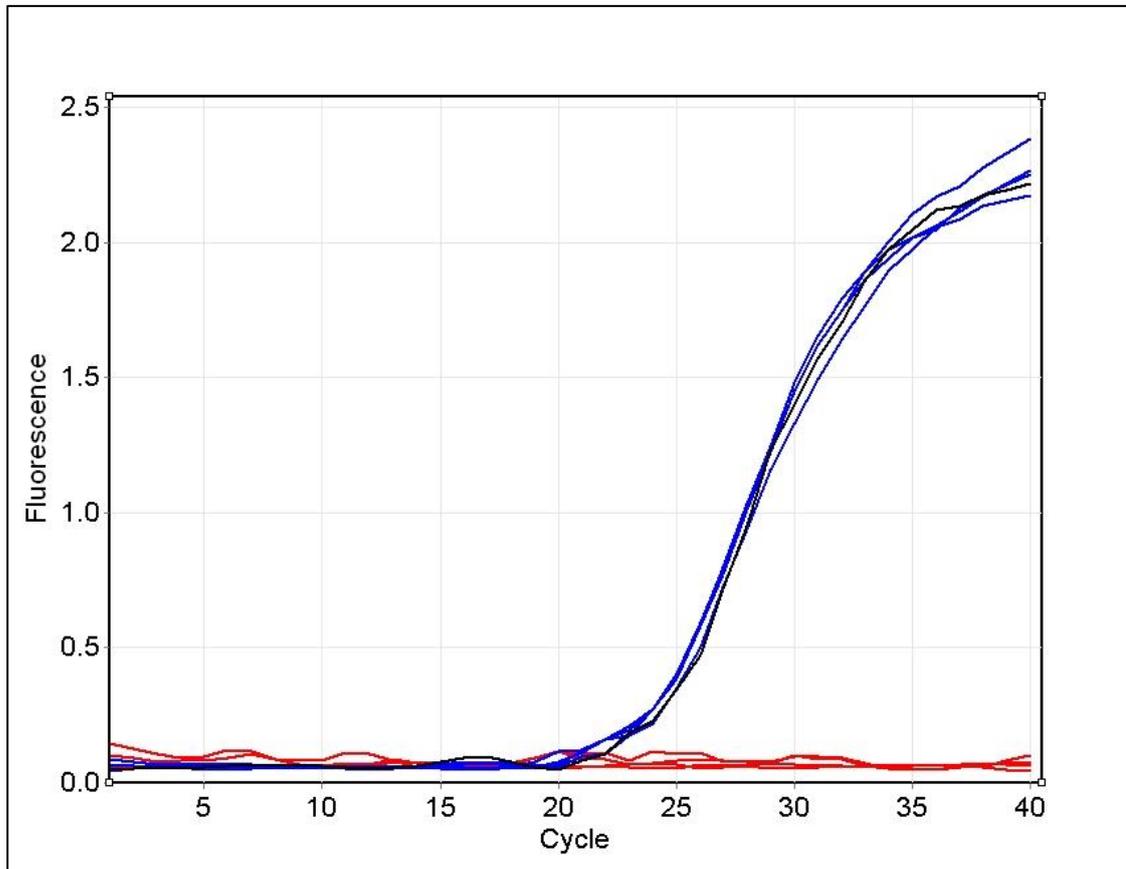
4.1 (C) DNA release by mechanical action and boiling with downstream purification using QIAamp DNA Mini Kit

Mtb CFU spiked per 85.5-cm ² mask segment (Log ₁₀)	Mtb genome equivalents recovered per 85.5-cm ² mask segment (Log ₁₀)
7.83	7.07
6.83	5.02
5.83	3.75
4.83	Not detected
3.83	Not detected
2.83	Not detected
1.83	Not detected
0.85	Not detected

Standard deviation for the test CFU is no more than 0.11 for each method.

It seemed likely that the poor sensitivity with this molecular method might be due to inhibition of downstream PCR by mask materials and/or processing reagents. With regard to Method 4.2.1.3 described above, no PCR signals were detected for all mask samples spiked with Mtb cells in the absence of purification with the QIAamp DNA Mini Kit. One test showed that the addition of extracts from non-contaminated masks (16% and 34% v/v) resulted in complete inhibition of PCRs with Mtb CDC 1551 DNA. PCRs spiked with mask extracts that had been purified by the QIAamp DNA Mini Kit yielded PCR signals equivalent to those of positive control reactions in which no mask extracts were added (Figure 4.4). Therefore, this test demonstrated that the kit-based purification step following DNA release in Method 4.2.1.3 removed PCR inhibitors, which could derive from the face mask itself and/or the mask analysis procedure. A similar outcome could be achieved by the phenol-chloroform step in Method 4.2.1.1. Since DNA extracts were purified in both these methods, PCR inhibition should not be the limiting factor to their low sensitivities. Interestingly, Method 4.2.1.2 gave the best sensitivity among the three methods tested although the DNA extracts were not purified prior to PCR analysis. Therefore, the low sensitivity of the real-time PCR method renders it non-amenable for the analysis of face masks.

Figure 4.4: Spiking of PCRs with non-contaminated mask extracts to investigate the possibility of PCR inhibition by mask materials and/or the processing procedure



Four non-contaminated mask segments (85.5 cm²) were processed as described in Section 4.2.1.3. Two of the final mask extracts were purified using the QIAamp DNA Mini Kit while the other two were not purified. 16S PCRs with approximately 2×10^4 Mtb CDC 1551 genome equivalents per reaction were spiked with two different amounts of purified and non-purified mask extracts, 16% and 34% v/v. SYBR Green signals were shown. Red lines represent PCRs with non-purified mask extracts added. Blue lines represent reactions with purified mask extracts. The black line represents positive control reaction without mask extracts.

4.3.2 Detection of face mask contamination by the mycobacteriophage assay is sensitive and cost effective

4.3.2.1 The phage assay can detect less than 100 mycobacterial CFU in pure cultures

An initial trial of the Biotec FPTB assay with test *M. smegmatis* culture yielded a detection limit of less than 100 CFU/ml. Long-term dependence on FPTB kits would be costly. Therefore, an in-house phage assay was replicated following the methods of McNerney *et al.* (2004) with further optimisation and modification. All optimisation procedures were carried out using *M. smegmatis* as the target. Phage adsorption and uptake was compared at 60 minutes (Albert *et al.*, 2002a) and 90 minutes (McNerney *et al.*, 2004); there was no significant difference in the PFU yield between these two durations ($P > 0.05$). With regard to the effect of *M. smegmatis* lawn density on plaque formation, there was no obvious difference in the PFU yield among the different densities tested (cultures with starting OD_{580nm} of 0.02, 0.05, 0.07, and 0.1). Plaque size and lawn confluence appeared similar for all culture densities by observation.

The detergent Tween 80 is inhibitory to mycobacteriophage infection (White and Knight, 1958; Riska and Jacobs, 1998). Growing *M. smegmatis* in Middlebrook 7H9-OADC broth (no Tween 80) resulted in a clumpy culture and therefore standing was necessary to sediment large clumps before using it for lawn formation in the phage assay. Tyloxapol was tested as an alternative to Tween 80 in 7H9 broth for growing *M. smegmatis* to see if it could be used to produce a confluent lawn without interfering with phage adsorption to mycobacterial cells. Similar to Tween 80, the phage assay was completely inhibited in the presence of 0.05% w/v tyloxapol. It has been proposed that phage receptors may be disrupted or washed from the surface of mycobacterial cells in the presence of detergent and/or detergent may directly affect their integrity (Park *et al.*, 2003).

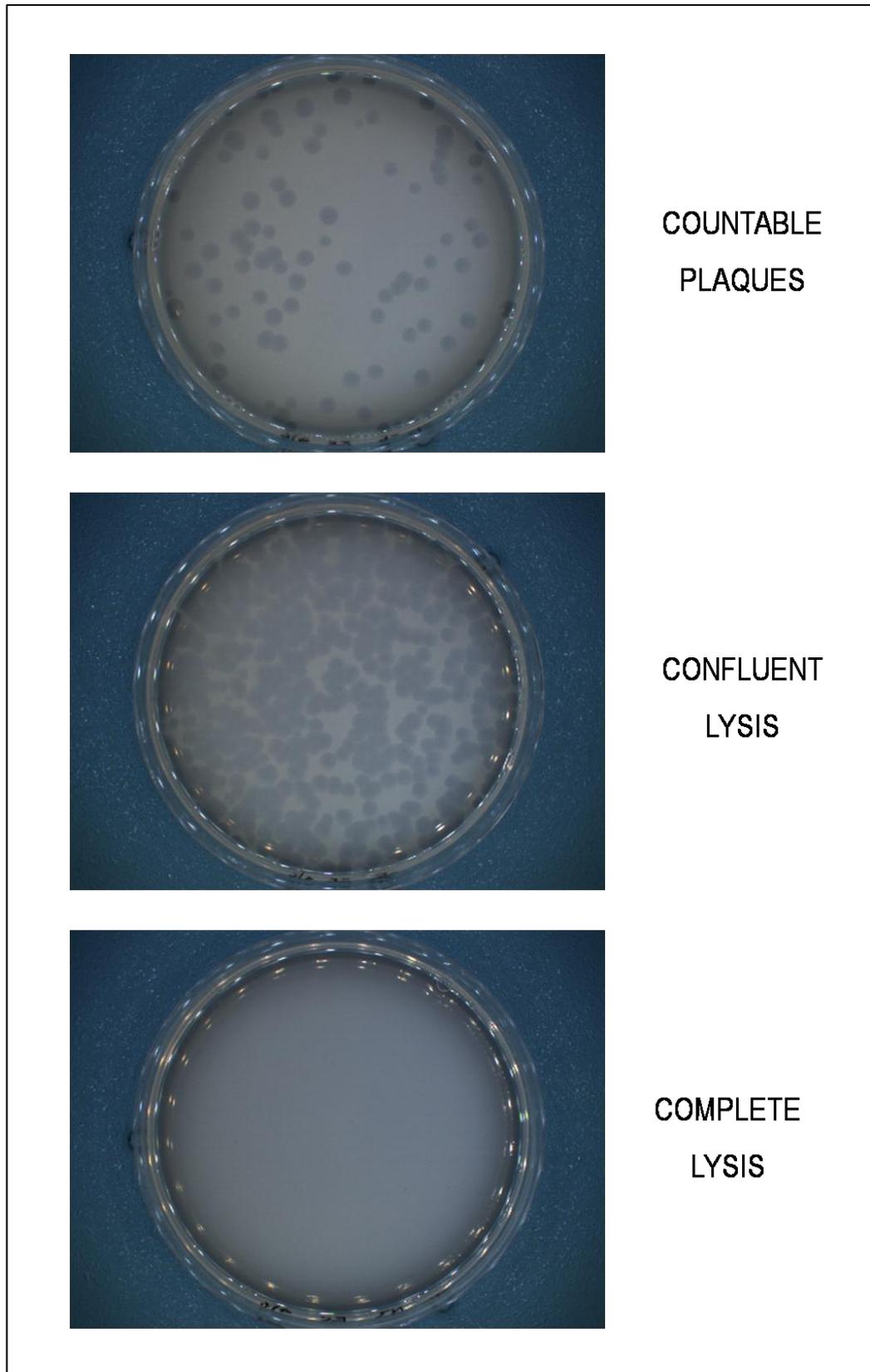
The final optimised in-house phage assay used 60 minutes as the duration of phage infection and *M. smegmatis* for lawn formation was grown in 7H9-OADC broth from a starting OD_{580nm} of 0.05. The optimised phage assay was tested with pure cultures of *M. smegmatis*, *M. bovis* BCG, and Mtb; the detection sensitivity was less than 100 CFU in each case (Table 4.2). Figure 4.5 shows the typical appearance of mycobacteria-derived plaques on phage indicator plates.

Table 4.2: Summary of detection sensitivities of the in-house phage assay with pure mycobacterial cultures

Mycobacterial CFU/ml tested (Log ₁₀)	Phage D29 PFU/ml recovered (Log ₁₀)		
	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	Mtb
7 to 8	Complete lysis	Complete lysis	Complete lysis
6 to 7	Complete lysis	Complete lysis	Complete lysis
5 to 6	Complete lysis	Complete lysis	Complete lysis
4 to 5	Confluent lysis	Confluent lysis	Confluent lysis
3 to 4	3.25	2.86	2.84
2 to 3	2.38	1.94	1.90
1 to 2	1.36	1.04	0.85
0 to 1	0.60	Not detected	0.30

One replicate was tested for each target dilution. Standard deviation for the test CFU is no more than 0.23 for each organism. Complete lysis refers to complete lysis of *M. smegmatis* lawn by progeny phage particles which lysed and exited target mycobacterial cells. Confluent lysis refers to lysis of the *M. smegmatis* lawn with phage D29 such that plaques were merging with each other. See Figure 4.5 for illustration.

Figure 4.5: Phage indicator plates showing plaques from infection of *M. smegmatis* cells in culture by phage D29



4.3.2.2 The phage assay can detect less than 100 mycobacterial CFU applied to face masks

The detection sensitivity of the phage assay on masks contaminated with pure mycobacterial cultures was less than 100 CFU per mask segment for most cases, except for in-house assay on mask segments spiked with *M. bovis* BCG (Table 4.3). Therefore, the phage assay performed better than the real-time PCR-based methods tested previously which yielded sensitivity in the range of 10^3 - 10^6 CFU per mask segment. The sensitivity of Biotec FPTB assay was 10 and 100 fold better than the in-house assay for mask segments spiked with *M. smegmatis* and *M. bovis* BCG, respectively. Mask segments contaminated with Mtb were not tested by the Biotec FPTB assay and therefore comparison could not be made. The PFU/CFU ratios (CFU of inoculated mycobacterial cultures) derived from contaminated masks were lower than those with pure cultures; this was more pronounced with *M. bovis* BCG and Mtb (Table 4.4).

Table 4.3: Summary of results comparing the detection sensitivity between Biotec FPTB and in-house phage assays with face masks contaminated with pure mycobacterial cultures

Mycobacterial CFU applied per 4-cm ² mask segment (Log ₁₀)	BIOTEC FPTB ASSAY		IN-HOUSE PHAGE ASSAY		
	Phage D29 PFU recovered per 4-cm ² mask segment (Log ₁₀)		Phage D29 PFU recovered per 4-cm ² mask segment (Log ₁₀)		
	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	Mtb
6 to 7	Complete lysis	Complete lysis	Complete lysis	Confluent lysis	Confluent lysis
5 to 6	Complete lysis	Complete lysis	Confluent lysis	Confluent lysis	Confluent lysis
4 to 5	Confluent lysis	Confluent lysis	3.41	2.94	2.96
3 to 4	2.78	2.30	2.48	1.93	1.97
2 to 3	1.83	1.32	1.34	0.85	0.70
1 to 2	0.95	0.48	0.60	Not detected	0.60
0 to 1	0.48	0	Not detected	Not detected	Not detected

One mask segment was analysed for each level of mycobacterial CFU applied. Standard deviation for the test CFU is no more than 0.1 in each case.

Table 4.4: Comparison of PFU/CFU between pure mycobacterial cultures and face masks contaminated with mycobacterial cultures analysed by the in-house phage assay

Mycobacterial CFU tested (Log ₁₀)	PFU/CFU with pure cultures (%)			PFU/CFU with masks spiked with pure cultures (%)		
	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	Mtb	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	Mtb
3 to 4	51	25	31	17	2	2
2 to 3	69	30	36	12	2	1
1 to 2	66	38	32	22	Not detected	10

CFU for the ratios were of inoculated mycobacterial cultures.

4.3.3 Mycobacterial cell suspensions are retained in the first mask layer

An experiment was performed to determine if it was necessary to process contaminated masks in separate mask layers or the three layers intact. The results are displayed in Table 4.5. The majority of *M. smegmatis* cells applied to mask segments were retained in the first layer contaminated. A small amount of penetration was detected in the second layer but none in the third. Therefore, all mask segments were processed intact in further spiking experiments reported in this chapter.

Table 4.5: Penetration of *M. smegmatis* cells applied to face masks

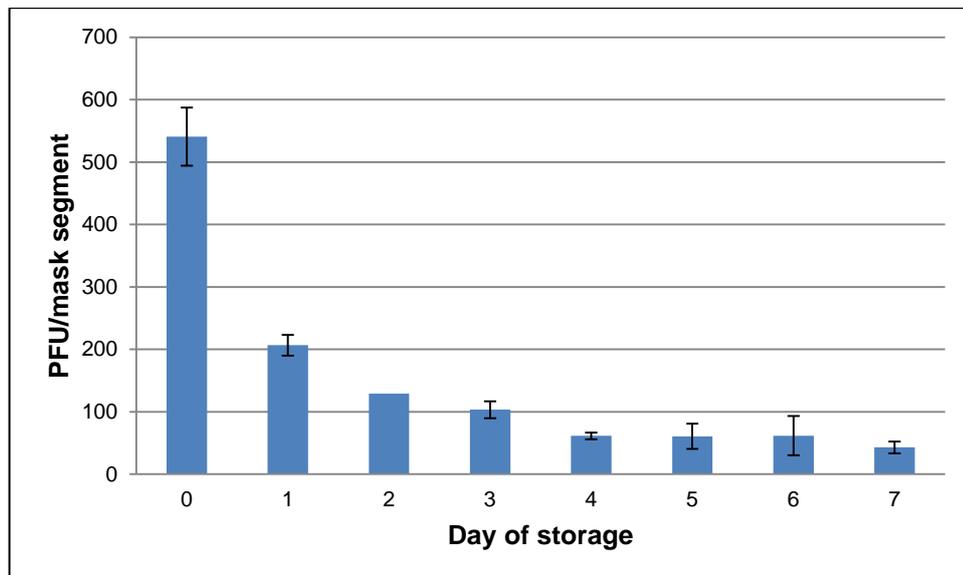
Mask layer	Phage D29 PFU recovered per 4-cm ² mask layer		
	Replicate 1	Replicate 2	Replicate 3
FIRST	Complete lysis	Complete lysis	Complete lysis
SECOND	2	26	0
THIRD	0	0	0

Each 4-cm² mask segment was spiked with approximately 10⁵ CFU of *M. smegmatis*. Mask layers were separated and processed individually by the Biotec FPTB assay.

4.3.4 Storage of contaminated face masks at 4°C leads to a ten-fold decline in PFU count over 4 days

An experiment was performed to determine the maximum duration for storage of contaminated masks at 4°C before PFU counts were affected. The number of PFU recovered from *M. smegmatis* cells applied to masks decreased by more than 50% after 1 day and ten fold after 4 days (Figure 4.6). The PFU counts appeared to stabilise at day 4 onwards.

Figure 4.6: Phage D29 PFU recovered from face masks contaminated with *M. smegmatis* cells after storage at 4°C



Approximately 3,000 *M. smegmatis* CFU were applied to each 4-cm² mask segment in triplicate for each day of storage. Masks were processed by the Biotec FPTB assay. The average for duplicate data was shown for day 2 due to contamination of another replicate.

4.3.5 Assessment of methods for releasing mycobacteria applied to face masks for detection by the phage assay

Alternate elution methods, duration of elution, and eluting medium were tested with half mask segments contaminated with 10^7 - 10^8 *M. smegmatis* CFU each in triplicates. All CFU counts were performed on LB agar. Elution efficiencies were determined by comparing the numbers of CFU recovered from with that applied to mask segments. Comparison was made between vortexing and stomaching for the elution method, with 1% w/v peptone (Becton Dickinson) + 0.05% w/v Tween 80 broth as the suspending medium. Stomaching is a process of homogenising biological samples in a machine that uses a beating mechanism. In this experiment, each half mask segment was cut into four segments and stomached in 20 ml of the eluting medium above in a stomacher bag (Seward, West Sussex). There was no significant difference in the number of CFU recovered between these two methods ($P > 0.1$). In a separate experiment with the same eluting medium, there was also no significant difference in CFU yield among most of the different durations of vortexing tested ($P > 0.1$). Although 5-minute vortexing recovered more *M. smegmatis* CFU than 2 minutes on average, the difference was marginal (4.22×10^7 vs. 3.41×10^7 CFU per mask segment; $P < 0.01$).

A mixture of Tween 80 and peptone has been used in quality control laboratories of industries manufacturing medical supplies to elute contaminating microbes on surgical swabs; the former minimise the hydrophobic interaction between organisms and mask materials while the latter resuscitates physically stressed cells via provision of nutrients (E. Thornton, personal communication). The elution efficiency of peptone broths containing different concentration of Tween 80 (0.05%, 0.5%, and 1% w/v) and Middlebrook 7H9-OGC broth were compared. All mask segments were vortexed for 2 minutes in this experiment. Peptone broth with 0.5% Tween 80 (30-55% CFU recovered) performed significantly better than 7H9-OGC broth (5-10% CFU recovered) in eluting *M. smegmatis* cells from mask segments ($P < 0.05$). All other comparisons showed no significant difference ($P > 0.05$). If using detergent-based medium, complete resuspension of cell pellet in fresh medium without detergent following centrifugation is obligatory but this will result in cell loss, which might counterbalance the lower elution efficiency with 7H9-OGC broth. The method of vortexing in 7H9-OGC broth for 2 minutes was chosen to release mycobacteria from clinical mask samples.

4.3.6 NaOH-NALC decontamination

4.3.6.1 Optimising NaOH-NALC decontamination for the phage assay

The effect of different durations of NaOH-NALC treatment (5, 10, 15, and 20 minutes) and NALC concentration (0%, 0.03%, 0.1%, and 0.5% w/v) on downstream phage assay were tested with *M. bovis* BCG cultures. Each determination was tested with approximately 10^7 CFU in triplicate. There was no significant difference in PFU yield among most of the NaOH-NALC contact times tested ($P > 0.05$). The PFU count at 5 minutes was significantly higher than 15 minutes on average (2,965 vs. 2,386 PFU/ml; $P < 0.05$). There was no significant difference in the number of PFU recovered among the different concentrations of NALC tested ($P > 0.1$). The published method of 15-minute contact time and 0.5% NALC (Master, 1992) was followed during analyses of mask samples from patients. Another experiment demonstrated that the resuscitation of *M. bovis* BCG cells in Middlebrook 7H9-OGC broth at 37°C for 24 hours was necessary following NaOH-NALC treatment. Although not statistically significant, there was more than 15-fold lower PFU count in the absence of resuscitation ($P > 0.1$).

4.3.6.2 NaOH-NALC decontamination affects PFU and CFU counts of mycobacterial cultures

NaOH-NALC treatment showed different magnitudes of effect on PFU and CFU counts, depending on the species of mycobacteria exposed (Table 4.6). This chemical treatment decreased the number of Mtb PFU by 3 fold during in-house phage assay ($P < 0.05$). Following overnight resuscitation in Middlebrook 7H9-OGC broth, there was a similar magnitude of reduction in its CFU count ($P < 0.01$). With *M. bovis* BCG, PFU and CFU yields were decreased by as much as 10^4 ($P < 0.05$) and 10^3 fold following decontamination, respectively. With resuscitation in FPTB Medium Plus and detection by the Biotec FPTB assay, both these counts showed a lower decline of about 100 fold. The difference in the numbers of PFU was statistically significant ($P < 0.05$). The substitution of 7H9-OGC broth for FPTB Medium Plus during the in-house assay resulted in 10^3 fold reduction in PFU and CFU counts ($P < 0.05$).

Table 4.6: Effect of NaOH-NALC decontamination on PFU and CFU counts of mycobacterial cultures

4.6 (A) *M. bovis* BCG

Treatment	In-house phage assay		Biotec FPTB assay		In-house phage assay with Biotec FPTB Medium Plus	
	Phage D29 PFU/ml (Log ₁₀)	<i>M. bovis</i> BCG CFU/ml (Log ₁₀)	Phage D29 PFU/ml (Log ₁₀)	<i>M. bovis</i> BCG CFU/ml (Log ₁₀)	Phage D29 PFU/ml (Log ₁₀)	<i>M. bovis</i> BCG CFU/ml (Log ₁₀)
Without decontamination	5.61 ± 0.12	5.77 ± 0.08	5.83 ± 0.15	5.90 ± 0.17	6.68 ± 0.14	6.40 ± 0.07
With decontamination	1.81 ± 0.33	2.28*	3.16 ± 0.02	3.55 ± 0.29	3.37 ± 0.05	3.85 ± 0.07

* The average of duplicate data was shown.

4.6 (B) *Mtb*

Treatment	Phage D29 PFU/ml (Log ₁₀)	<i>Mtb</i> CFU/ml (Log ₁₀)
Without decontamination	6.94 ± 0.04	7.40 ± 0.02
With decontamination	6.42 ± 0.05	6.91 ± 0.09

Each determination was tested with approximately 10⁷-10⁸ CFU in triplicate.

4.3.7 Attempts to improve sensitivity of method via on-mask phage infection and cell lysis

4.3.7.1 Direct NaOH-NALC decontamination of face masks yields high background PFU counts

Vortexing in Middlebrook 7H9-OGC broth was only 5-10% efficient in releasing *M. smegmatis* cells applied to masks (see Section 4.3.5). Therefore, a protocol was designed to process eluted masks in addition to their initial eluates (see Section 4.2.2.6). This protocol involved direct application of NaOH-NALC decontamination on eluted masks, followed by infection of mycobacterial cells remaining on masks by phage D29. The ultimate aim was to increase the detection sensitivity by the release of multiple phage particles from the infected cells, a strategy that has been reported previously (McNerney *et al.*, 1998; Park *et al.*, 2003). However, processing of half mask segments contaminated with *M. bovis* BCG culture by this protocol resulted in high background PFU counts (Table 4.7).

Table 4.7: On-mask phage infection and lysis of *M. bovis* BCG cells to improve method sensitivity

Process	Sample	PFU/plate
Processing of mask eluates (A)	Negative control	0
	Contaminated mask	0
Processing of eluted masks (B)	Negative control	157
	Contaminated mask	134
On-mask phage infection and pre-lysis plating (C)	Negative control	130
	Contaminated mask	72
On-mask cell lysis and release of progeny phage particles (D)	Negative control	202
	Contaminated mask	34
	Negative control	Confluent lysis
	Contaminated mask	Confluent lysis
	Negative control	Confluent lysis
	Contaminated mask	Complete lysis
	Negative control	Complete lysis
	Contaminated mask	Complete lysis

4.3.7.2 Use of an antibiotic cocktail obviates the need for NaOH-NALC decontamination of face masks prior to the phage assay

Direct treatment of masks with NaOH-NALC solution might have contributed to the high background PFU counts in the method of on-mask phage infection and cell lysis. Furthermore, NaOH-NALC decontamination is time consuming and affects the sensitivity of downstream phage assay for the detection of mycobacterial cells (see Section 4.3.6.2). The mask processing method described in Section 4.2.2.6 was modified to include use of an antibiotic cocktail (nystatin, oxacillin, and aztreonam; collectively known as NOA) in the indicator plates to control contamination. Two different concentrations of NOA were used during processing of clinical mask samples: the published level (Biotec Laboratories, 2005; Albert *et al.*, 2007; Mole *et al.*, 2007) during resuscitation of masks and increased concentration in final phage indicator plates.

The published concentration of NOA antimicrobial supplement does not affect PFU and CFU counts of mycobacteria

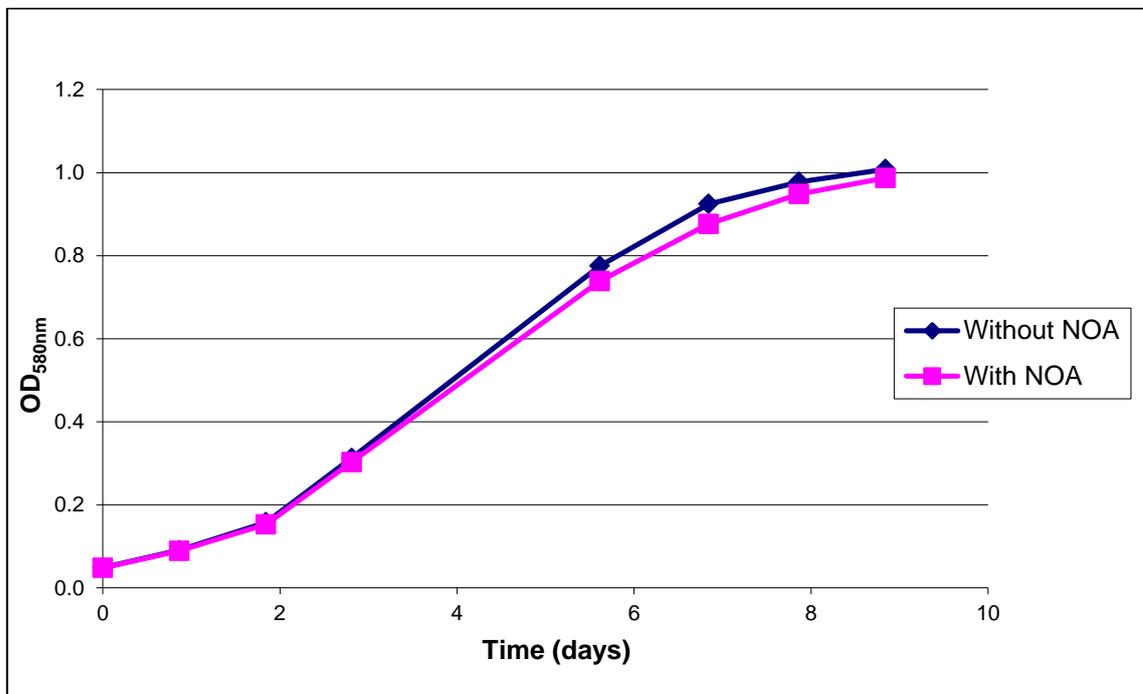
The effect of the published level of NOA on pure *M. bovis* BCG culture and NaOH-NALC-treated cell suspension was evaluated. Both the culture and suspension were exposed to NOA in 2-ml volumes for 24 hours prior to PFU analysis by the in-house phage assay. Each determination was performed with approximately 10^7 CFU in triplicate, and results are displayed in Table 4.8. Overnight exposure to NOA did not affect the PFU and CFU yields of both pure culture and decontaminated suspension. This antibiotic cocktail also did not affect the growth of Mtb in liquid medium (Figure 4.7). Therefore, the published concentration of NOA was used to control overgrowth by non-target organisms during resuscitation of clinical mask samples prior to the phage assay.

Table 4.8: Effect of the published concentration of NOA antimicrobial supplement on PFU and CFU counts of *M. bovis* BCG

Treatment	<i>M. bovis</i> BCG culture		NaOH-NALC-treated <i>M. bovis</i> BCG	
	Phage D29 PFU/ml (Log ₁₀)	<i>M. bovis</i> BCG CFU/ml (Log ₁₀)	Phage D29 PFU/ml (Log ₁₀)	<i>M. bovis</i> BCG CFU/ml (Log ₁₀)
Without NOA	2.77 ± 0.02	3.21 ± 0.12	2.85 ± 0.28	2.87*
With NOA	2.83 ± 0.01	3.27 ± 0.12	3.11 ± 0.19	2.91*

* The average of duplicate data was shown.

Figure 4.7: Effect of the published concentration of NOA antimicrobial supplement on the growth of *Mtb* in Middlebrook 7H9-ADC-Tween broth



Triplicate cultures were tested for each treatment.

An increased concentration of NOA antimicrobial supplement does not affect phage D29 and *M. smegmatis* lawn confluence

Different concentrations of antibiotics were incorporated into phage indicator plates and spotted with ten-fold serial dilutions of phage D29 suspension (approximately 10^9 PFU/ml). No-antibiotic controls were performed in parallel. Oxacillin was tested from the initial concentration of 0.8 $\mu\text{g/ml}$ in two-fold increments. The highest level tested, 410 $\mu\text{g/ml}$ (200 times higher than the published concentration), did not affect the PFU yield and there was no observable difference in confluence of *M. smegmatis* lawn. Aztreonam was evaluated in concentration range of 10 to 400 $\mu\text{g/ml}$, also in two-fold increments. The maximal level tested (10 times higher than the published concentration) showed similar outcomes with oxacillin. Combination of antibiotics (oxacillin 410 $\mu\text{g/ml}$, aztreonam 135 $\mu\text{g/ml}$, and nystatin 30 $\mu\text{g/ml}$) did not affect both the PFU yield and *M. smegmatis* lawn confluence. The aztreonam and nystatin levels in the mixture are 4.5 and 3 times higher than the published concentration, respectively. This NOA cocktail was incorporated into phage indicator plates to control contamination by rapid-growing organisms present in mask samples from patients.

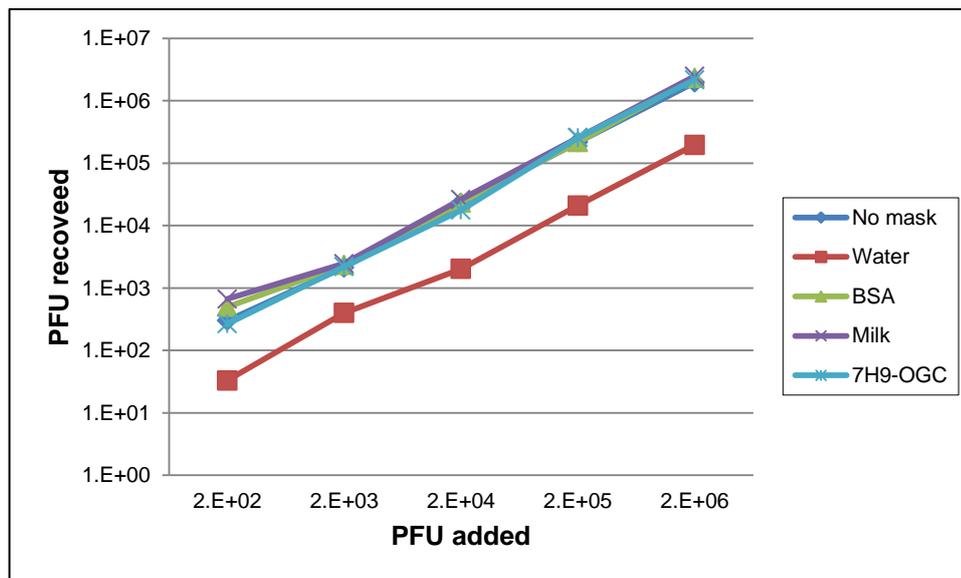
4.3.7.3 Phage D29 adheres to face masks

Although residual counts were occasionally seen in the negative controls for clinical mask analysis, in the absence of direct NaOH-NALC decontamination of face masks, high background PFU counts were not observed in reactions where unlysed cells were plated out from suspensions of culture-spiked masks following on-mask infection (Process C, Section 4.2.2.6). However, such high backgrounds were a problem for on-mask cell lysis and direct release of progeny phage particles (Process D, Section 4.2.2.6). Extended exposure to FAS following on-mask infection for up to 45 minutes did not resolve this problem. It was postulated that the background PFUs were due to exogenous phage particles adhering to masks, and possibly protecting them from virucidal inactivation. The possible presence of these 'phage binding sites' was investigated by incubating mask segments pre-blocked by different reagents with phage D29 suspension, and assessing the change in PFU. There was ten-fold decline in PFUs recovered when mask segments were 'not blocked' in controls with water (Figure 4.8). The PFU recovered were similar to no-mask controls when mask segments were blocked with BSA, milk, or Middlebrook 7H9-OGC broth. These results

suggest that the overnight resuscitation of masks in 7H9-OGC prior to on-mask infection should have blocked the ‘binding sites’ adequately, and therefore rule out its contribution to the background PFU counts.

Based on the outcome of the experiment above, the role of BSA in the interaction between phage D29 and its receptor on the host cell was investigated. Since 2% BSA minimised adherence of phage D29 to masks (Figure 4.8), it was hypothesised that BSA in 7H9-OGC broth (though lower concentration) might affect adsorption of phage to mycobacterial cells during phage assay. However, when the experiment was performed with 7H9-OGC broth in which BSA and oleic acid were omitted from the OADC supplement, no plaques were observed. This demonstrated the importance of BSA for the phage assay (White and Knight, 1959).

Figure 4.8: Effect of treating face masks with different blocking reagents on PFU counts



Mask segments (4 cm²) were treated with 1 ml of different blocking reagents each (2% w/v BSA, 2% w/v dried skimmed milk, and Middlebrook 7H9-OGC broth) for 1 hour with continuous mixing on the tube rotator. Both BSA and milk powder were dissolved in distilled water and sterilised by filtration through 0.2-µm syringe filters. Controls were performed with mask segments incubated in 1 ml of sterile distilled water ('unblocked'). Following blocking, mask segments were washed once in water. Mask segments from each treatment and control were then incubated with different amount of phage D29 (2 x 10⁶ PFU and its five 10-fold serial dilutions) at 37°C for 24 hours. Controls without masks were also performed. PFU enumeration of suspensions was then performed as described in Section 4.2.2.2.

4.3.7.4 Mask layers trap phage D29 particles

An alternate hypothesis to the background PFU counts during on-mask infection and cell lysis was protection of exogenous phage particles trapped among mask layers from virucidal inactivation. To investigate this phenomenon, a phage D29 suspension was incubated with mask segments in different layers. The change in the numbers of PFU recovered was then assessed, and the results are shown in Table 4.9. The amount of phage D29 recovered decreased from 1 to 3 layers of masks, consistent with higher degree of trapping when more mask layers were present during analyses. When mask segments were processed intact, i.e. 3 layers, there was a five-fold reduction in the number of PFU recovered. Therefore, it was highly probable that the trapping of phage particles among mask layers contributed to the background PFU yields.

Table 4.9: Effect of different mask layers on PFU counts

Mask layers	Phage D29 added PFU/ml (Log ₁₀)	Phage D29 recovered PFU/ml (Log ₁₀)
1 layer	6.85 ± 0.09	6.12 ± 0.07
2 layers	6.85 ± 0.09	6.02 ± 0.13
3 layers	6.85 ± 0.09	5.82 ± 0.06

Each 4-cm² mask segment (in 1, 2, or 3 layers) was incubated with 7.15 x 10⁶ phage D29 PFU at 37°C for 24 hours. All determinations were tested in triplicates. PFU enumeration of suspensions was performed as described in Section 4.2.2.2.

4.3.8 Phage infection of mycobacterial cells in stationary phase of growth

4.3.8.1 Stationary-phase mycobacteria yield lower PFU counts than exponential-phase mycobacteria assayed in suspensions

The cell wall composition and thickness for mycobacterial cells vary in different phases of growth; thickened peptidoglycan layers among cells in stationary cultures have been suggested. This alteration in cell wall morphology might have important implication on their susceptibility to phage infection (Piuri and Hatfull, 2006; Dusthacker *et al.*, 2008a). A comparison was made between infection of mycobacterial cells in exponential and stationary cultures by phage D29. Each determination was tested once and the results are summarised in Table 4.10. For all mycobacterial species and sizes of inocula tested, the PFU/CFU ratios were always lower for stationary-phase mycobacterial cultures. Irrespective of growth phases and sizes of inocula tested, these ratios were lower for the two MTBC species relative to *M. smegmatis*. The detection sensitivity for stationary-phase Mtb appeared rather poor.

Table 4.10: Comparison between phage D29 infection of mycobacterial cells in exponential and stationary phases of growth

Target mycobacteria	Growth stage	PFU/CFU (%)		
		10 ³ -10 ⁴ CFU/ml	10 ² -10 ³ CFU/ml	10 ¹ -10 ² CFU/ml
<i>M. smegmatis</i>	Exponential	51	69	66
	Stationary	44	38	33
<i>M. bovis</i> BCG	Exponential	25	30	38
	Stationary	7	8	15
Mtb	Exponential	31	36	32
	Stationary	0.1	Not detected	Not detected

Standard deviation for the test CFU is no more than 0.23 in each case.

4.3.8.2 Mycobacterial resuscitation-promoting factors have no effect on phage assay with stationary-phase *M. bovis* BCG

Rpf proteins have muralytic activity and function to stimulate the growth of dormant bacteria, including those in stationary cultures (Mukamolova *et al.*, 1998a; 1998b; 2006; Cohen-Gonsaud *et al.*, 2005). Indeed, stationary-phase *M. bovis* BCG cells exhibit positive growth in response to these proteins (Mukamolova *et al.*, 2002). The following experiments were performed to investigate if similar response could be achieved for phage infection in Rpf-treated stationary mycobacterial cultures. If this was the case, this treatment might enhance the PFU/CFU ratios to the levels yielded by exponential cultures. Treatment with *M. smegmatis* supernatant (containing Rpfs) for up to 3 hours did not have any effect on the PFU yield of stationary *M. bovis* BCG culture (Table 4.11). The increase in PFU count from 1 to 3 hours was possibly due to combined nutrient- and temperature-assisted resuscitation of metabolically inactive mycobacterial cells. Exposure to *M. smegmatis* supernatant and purified Mtb Rpfs for 24 hours also did not show any effect ($P > 0.05$; Table 4.12). Interestingly, treatment with *M. bovis* BCG supernatant resulted in a slight reduction in the PFU count ($P < 0.01$).

Table 4.11: Effect of *M. smegmatis* supernatant on PFU counts of stationary-phase *M. bovis* BCG

Duration of treatment	Treatment	
	Middlebrook 7H9-OADC broth PFU/ml (Log ₁₀)	<i>M. smegmatis</i> supernatant PFU/ml (Log ₁₀)
1 hour	2.29	2.23
2 hours	2.46	2.38
3 hours	2.69	2.65

Stationary *M. bovis* BCG culture (about 1-month old) was tested. Cell suspensions were treated with either 50% v/v 7H9-OADC broth (control) or *M. smegmatis* supernatant at 37°C for 1, 2, and 3 hours. In-house phage assay was then performed to assess PFU yields. Each exposure time was tested in duplicate for both treatments.

Table 4.12: Effect of *M. bovis* BCG supernatant and purified Mtb Rpf s on PFU counts of stationary-phase *M. bovis* BCG

Treatment	Phage D29 PFU/ml (Log ₁₀)
Middlebrook 7H9-OADC broth	3.49 ± 0.01
<i>M. smegmatis</i> supernatant	3.56 ± 0.04
<i>M. bovis</i> BCG supernatant	3.35 ± 0.01
Mtb Rpf B*	3.47 ± 0.01
Mtb Rpf E*	3.48 ± 0.03

Stationary *M. bovis* BCG culture (more than 1-month old) was tested. Cell suspensions were treated with either 1 volume of 7H9-OADC broth (control), mycobacterial supernatant, or purified Rpf proteins at 37°C for 24 hours. In-house phage assay was then performed to assess PFU yields. Each treatment was tested in triplicate.

* Kind gifts from G. V. Mukamolova.

4.4 Discussion

4.4.1 Detection of face mask contamination by quantitative real-time PCR is insensitive and subject to inhibitory activity

Experiments with several DNA release procedures demonstrated poor sensitivity (10^3 - 10^6 mycobacterial cells per mask segment) with the real-time PCR method. The aim was to detect less than 100 cells from face masks. The two DNA release methods involving downstream purification were 10-100 fold less sensitive compared to the *microLYSIS* method, which was very likely due to significant loss of materials through multiple centrifugation and other manipulation steps during the clean-up stages.

Purification of DNA extracts removed inhibitory mask materials or processing reagents which would otherwise interfere with downstream PCRs. Both proteinase K and phenol-chloroform treatments in the method with lysing matrices inactivated and removed proteinaceous inhibitors from the DNA preparations, respectively (Hilz *et al.*, 1975; Bartlett and Stirling, 2003). For the column-based DNA clean-up, the chaotropic guanidine hydrochloride in the buffer denatured and subsequently washed these inhibitors out of the DNA extracts. Inactivation by proteinase K alone in the absence of this column-based purification was inadequate in removing PCR inhibitors during mask processing. The absence of purification in the *microLYSIS* method did not result in complete PCR inhibition, in agreement with the beneficial property claimed by the manufacturer. Therefore, PCR inhibition was not the major limiting factor to the sensitivities of the three DNA extraction methods evaluated.

The low sensitivity with the DNA quantification method could be due to a combination of factors. The poor detection limit of the PCR assays (100-1,000 genome equivalents per reaction) was immediately an issue; the lowest amounts of DNA detected in all three methods fell in the range of 10^3 - 10^5 genome equivalents. Furthermore, DNA purification might not have removed PCR inhibitors completely, especially for samples with lower amount of targets in which the inhibitors were present in great excess. The efficiency in releasing mycobacterial cells from masks could also be a limiting factor to

the method sensitivity. The method involving cell release by mechanical action of vortexing and mixing was the least sensitive among the three methods tested. It is very likely that this mechanical action was not as efficient as the vigorous homogenisation of masks with lysing matrices. The use of these matrices on larger mask segments processed in this method would not be cost effective. Also, the processing of more mask materials would introduce larger amount of PCR inhibitors into the DNA preparations.

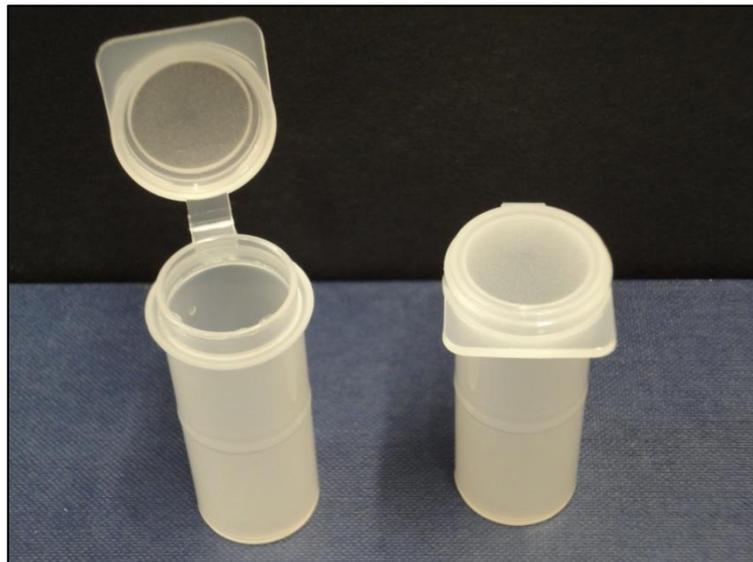
The sensitivity of the molecular-based mask analysis method could be improved by the use of a more sensitive DNA amplification assay, for instance a nested derivative of the current assay or a PCR assay targeting gene that is present in multiple copies within the genome (Chen and Li, 2005; Negi *et al.*; 2007; Stanley *et al.*, 2007). The sensitive LAMP assay could also be an alternative; target quantification might be possible via real-time turbidity detection (Pandey *et al.*, 2008). Further optimisation and modification of existing methods in relation to cell release from masks would also improve the method sensitivity. Alternatively, direct DNA release from mycobacterial cells on masks without the additional effort of elution might also improve its sensitivity.

4.4.2 Detection of face mask contamination by the mycobacteriophage assay is sensitive and cost effective

4.4.2.1 The phage assay can detect less than 100 mycobacterial CFU in pure cultures

Both the Biotec FPTB and in-house phage assays were able to detect less than 100 CFU/ml with exponential mycobacterial cultures. Use of a phage assay developed in-house minimised the cost of the study. The in-house phage assay produces reliable results and is safer to be carried out in the Category 3 Laboratory, though more labour intensive. The pouring action during FPTB procedure runs the risk of transferring phage particles on the wall of reaction vessels that escape virucidal inactivation, resulting in false-positive plaques (R. McNerney, personal communication). The pouring action also runs the risk of generating aerosols, which is unacceptable in the containment facility. These risks were minimised during the in-house procedure by pipetting of reaction mixture into molten agar before pouring the entire tube content into a Petri dish. There was also difficulty in handling FPTB reaction vessels which operate via the 'flip' mechanism (Figure 4.9).

Figure 4.9: Reaction vessels provided in the Biotec FPTB kit



4.4.2.2 The phage assay can detect less than 100 mycobacterial CFU applied to face masks

Processing of face masks by the phage assay gave better sensitivity than by the real-time PCR method. The sensitivity with phage assay was less than 100 mycobacterial CFU per mask segment, while the latter gave lower sensitivities in the range of 10^3 - 10^6 CFU per mask segment. This might be due to larger volumes of samples (50-100%) processed through phage assays as opposed to only 4-20% for DNA quantitation methods. In addition, sensitivity of the latter could be limited by the poor detection level of PCR assays and the presence of PCR inhibitors, as discussed in Section 4.4.1. PFU/CFU ratios were lower with contaminated masks than with pure cultures, possibly limited by the efficiency of cell release from masks.

The Biotec FPTB assay was 10-100 fold more sensitive than the in-house phage assay. Since most media and reagents were prepared fresh prior to the in-house assay, the most obvious limiting factor to its sensitivity was the stability of phage D29 suspensions over extended storage at 4°C. PFU counts of phage suspensions dropped by 5-10 fold from an initial count of approximately 10^{10} PFU/ml after about one-year storage (data not shown). This implies that as much as 50-90% of viruses used during the phage assay were not able to infect target mycobacterial cells. Although the phage concentration was adjusted accordingly prior to the assay, the presence of large number of these 'non-infective' phage particles could have interfered with adsorption of their 'infective' counterparts to host cells. This could be either by blocking access of 'infective' phage particles or 'non-infective' binding to the receptors on target cells; the latter action mimics abortive infection. With regard to this problem, phage breeding to produce an enhanced extracellular stability could increase the sensitivity of the in-house assay via improvement of infection efficiency (Chatterjee *et al.*, 2000). Phage D29 is supplied in lyophilised form in the Biotec FPTB kit with a shelf life of one year, and the manufacturer recommends the storage of the reconstituted suspension at 2-8°C for up to 7 days. The higher sensitivity of the Biotec FPTB assay might also reflect the difference in media used and the highly skilled and therefore better level of assay optimisation.

4.4.3 Mycobacterial cell suspensions are retained in the first mask layer

Most mycobacterial cells were retained in the contaminated mask layer with very little penetration to the second layer and none to the third. Penetration of aerosol particles through respiratory filters depends on their flow rate (Brosseau *et al.*, 1997). Application of mycobacterial suspension to masks by pipetting involved lower flow rate compared to expulsion of Mtb aerosols onto face masks by TB patients during vigorous respiratory activities. Indeed, Nicas (1995) reported that 42% of Mtb droplet nuclei penetrated into surgical masks. Therefore, the use of an aerosoliser to spike masks with pure mycobacterial culture would have better simulated the process of sampling tuberculous aerosols from patients.

4.4.4 Storage of contaminated face masks at 4°C leads to a ten-fold decline in PFU count after 4 days

Overnight storage of mask segments contaminated with *M. smegmatis* resulted in more than 50% decline in PFU counts. After 4 days of storage, PFU counts decreased by approximately 90%. Brosseau and colleagues (1997) reported that bacteria on respiratory filters were not able to grow and were only able to survive for no longer than 3 days. In their experiment, they demonstrated that the culturability of *M. abscessus* dropped from 35-100% to 1-60% after 5 days of storage. All these findings suggested that face masks from TB patients should be processed at the earliest possible time after sampling.

4.4.5 Assessment of methods for releasing mycobacteria applied to face masks for detection by the phage assay

Between vortexing and stomaching, the former was chosen as the elution method to avoid the risks and complications of operating the stomacher in the Category 3 Laboratory. The choice of two-minute vortexing was consistent with a report that this duration managed to recover maximal number of bacteria from respiratory filters (Wang *et al.*, 1999). Middlebrook 7H9-OGC broth was the eluting medium of choice as the lower elution efficiency would be counterbalanced by the loss of cells via the centrifugation step with detergent-based medium.

4.4.6 NaOH-NALC decontamination affects PFU and CFU counts of mycobacterial cultures

The choice of 1% w/v final concentration of NaOH was aimed to balance the requirement for Mtb survival against the need to control contamination by non-target organisms and remove potential inhibitors in respiratory secretions (Park *et al.*, 2003). Following an optimisation experiment, 15 minutes was chosen for treatment of mycobacterial suspensions with the NaOH-NALC mixture. Although 5-minute exposure gave higher PFU counts than 15 minutes, this short duration was thought to be inadequate for efficient killing of rapid growing non-target organisms in clinical mask samples. On the other hand, prolonged contact with NaOH-NALC for 20 minutes could have affected the phage infectivity of mycobacterial cells or the integrity of phage receptors on their surface; the latter was proposed by Park and associates (2003). Overnight resuscitation in nutritionally rich medium is obligatory following the decontamination process for the recovery of chemically stressed cells, larger fold drop in the PFU count than that recorded in this study has been reported for NaOH-treated Mtb culture (Park *et al.*, 2003).

NaOH-NALC treatment affected the sensitivity of phage assay with and culture of mycobacteria; the effect was more pronounced with *M. bovis* BCG than Mtb. This might reflect the difference in the composition and organisation of cell envelope between these two MTBC species and hence their survival and resistance in harsh chemicals. The difference in the speed of recovery following decontamination could also play a role. However, Yesilkaya and co-workers (2004) showed that higher NaOH concentrations and longer durations of exposure affected CFU counts of sputum-seeded *M. bovis* BCG by similar log folds with the pure cultures tested in this study. Another study reported that similar NaOH concentration and exposure time did not significantly affect the CFU count of sputum-spiked Mtb H37Ra (Burdz *et al.*, 2003). Both these findings might reflect the protective effect of sputum components on bacilli during the alkaline decontamination; protection by buffering activity and/or physical shielding has been suggested previously (Park *et al.*, 2003). The better recovery of NaOH-NALC-treated *M. bovis* BCG cells in FPTB Medium Plus and higher sensitivity with the Biotec FPTB assay were as discussed in Section 4.4.2.2.

4.4.7 Attempt to improve sensitivity of method via on-mask phage infection and cell lysis

Processing of mask eluates alone would miss out on the 90-95% of mycobacterial cells that were not released from masks. A method was designed and evaluated for processing eluted masks based on phage infection of mycobacterial cells that remained on masks after the initial elution process. It was hypothesised that signal amplification through phage D29-associated lytic burst following extended incubation would improve the method sensitivity; a 10- to 20-fold increase in signal has been reported for infected Mtb culture plated out 24 hours post-infection (Park *et al.*, 2003). However, the high background PFU counts with this proposed mask processing method was immediately a problem.

Neutralisation of NaOH with concentrated HCl during direct decontamination of eluted masks (spiked with culture) resulted in the formation of heavy precipitates which sequestered ferrous ions during virucide treatment, thereby affecting inactivation of exogenous phage particles (Park *et al.*, 2003). Carry-over of precipitates and surviving exogenous phages resulted in high background PFU counts following on-mask phage infection and cell lysis, respectively. The use of published NOA concentration during overnight resuscitation of masks and increased concentration in the final phage indicator plates to control contamination by non-target organisms obviates the need for NaOH-NALC decontamination, which is time consuming and affects the sensitivity of phage assay. High background PFU counts were not observed for mask suspensions following infection, but remained a problem for on-mask cell lysis.

It was initially thought that exogenous phage particles adhered to face masks and in this way were protected from virucide action. However, the 'mask binding sites' for phage D29 should have been adequately blocked during overnight resuscitation in Middlebrook 7H9-OGC broth prior to the phage assay. The background PFU counts were more likely due to trapping of phage particles among mask layers and therefore shielded from virucidal inactivation. In this case, processing of individual mask layers would have overcome this problem. Also, the possible protection of exogenous phages

from virucide action by mask materials released after prolonged incubation warrants further investigation.

4.4.8 Phage infection of mycobacterial cells in stationary phase of growth

The bacteriophage-encoded tape measure protein facilitates efficient infection and DNA injection into mycobacterial cells in stationary cultures (Piuri and Hatfull, 2006; Dusthacker *et al.*, 2008a). Motif 1 of this protein shares similar sequence with Rpf proteins that stimulate the growth of stationary-phase mycobacterial cells (Mukamolova *et al.*, 2002), while motif 3 has peptidoglycan-hydrolysing activity (Piuri and Hatfull, 2006). The absence of motif 3 in the tape measure protein of phage D29 probably renders it incapable of penetrating the thickened peptidoglycan outer layer of cells in stationary growth, and therefore resulted in lower PFU/CFU ratios relative to exponential-phase cells. Interestingly, the lower ratios for MTBC species compared to the rapid-growing *M. smegmatis* might reflect the association between cell wall composition and phage infection.

Most tuberculous sputum samples are dominated by a large number of Mtb cells which can only be grown in the presence of Rpfs (Mukamolova *et al.*, 2010). If these Mtb cells are also present in aerosol particles sampled on face masks, resuscitation in Rpfs prior to the phage assay might improve phage infectivity and therefore the sensitivity of detection. The current study demonstrated that both mycobacterial supernatants and purified Rpfs had no effect on phage assay with stationary cultures. However, all supernatants used in this study were prepared in media without Tween 80; the effect of growth media on the quality of supernatants, if any, remains to be investigated. Most-probable-number analysis of stationary *M. bovis* BCG and Mtb cultures showed that exposure to Rpfs for more than 24 hours might be necessary to yield significant effect (method and data not reported); therefore, the importance of duration of exposure to Rpfs warrants further assessment.

4.5 Conclusion

When choosing and assessing methods for the detection of Mtb in face masks from TB patients, two important aspects were considered: the sensitivity and quantitative potential. With regard to the former, the mycobacteriophage amplification test is superior to real-time PCR-based approaches. Sensitivity with the latter method is compromised by the poor detection level of PCR assays and PCR inhibitory activity associated with mask materials and/or analytical procedure. The phage assay is able to detect less than 100 mycobacterial cells applied to masks, and was therefore the method of choice for processing of clinical mask samples.

Initially, analysis by the phage assay was proposed for decontaminated mask materials, which were released by the mechanical action of vortexing. However, this elution method is inefficient and therefore, the procedure was modified to process the eluted masks based upon on-mask phage infection and pre-lysis plating. In order to circumvent the detrimental effect of alkaline decontamination on phage infection of target bacilli, a combination of three antibiotics (NOA) would be used in the modified method to control contamination by non-target organisms. This strategy is novel and would simplify the method. An attempt to enhance the method sensitivity by plating post-lytic burst was hampered by detection of high background PFU counts.

Preliminary findings indicate that delay in processing masks could lead to underestimated levels of mask positivity, and should therefore be avoided, if possible, for clinical mask analyses. The potential release of mycobacterial cells from masks during overnight resuscitation and following phage infection warrants further investigation.

CHAPTER 5

Use of Mask Sampling to Study

Expectoration of *M. tuberculosis*

5.1 Introduction

TB is contagious. WHO estimated that TB is spreading at the rate of one person per second and an untreated active TB case can go on to infect 10-15 people in the course of a year (WHO, 2010b). Therefore, prompt identification of infectious index cases is important in preventing and interrupting person-to-person transmission; this can be achieved via successful treatment and respiratory isolation. Although many studies have been performed, knowledge pertaining to the infectiousness of individual TB cases and mechanisms of transmission is far from satisfactory. The marked variation in transmission of infection from different TB cases is just one area in which our knowledge and understanding is weak (Riley *et al.*, 1959; 1962; Fennelly *et al.*, 2004; Escombe *et al.*, 2007); the existence of “dangerous disseminators” and “super disseminators” has been proposed (Sultan *et al.*, 1960).

In medical practice, clinicians generally rely on the AFB smear status to gauge the infectiousness of TB patients. It is also standard practice that patients are released from respiratory isolation after two weeks of chemotherapy. Reliance on smear positivity has been shown to be unsound in several studies (Sultan *et al.*, 1960; Fennelly *et al.*, 2004; Chen and Li, 2005); furthermore, disease transmission from smear-negative patients has been documented (Blaht *et al.*, 1946; Catanzaro, 1982; Di Perri *et al.*, 1989; Behr *et al.*, 1999). Release of patients after two weeks has also been criticised as a misjudgement that runs the risk of community and nosocomial transmission; there have been documented incidents of nosocomial transmission following movement of TB patients to general wards (Beck-Sague *et al.*, 1992).

A correlation between cough frequency and TB infectiousness has been reported (Loudon and Spohn, 1969). Furthermore, hygienic manners among TB patients during coughing have been implicated in determining the airborne levels of Mtb (Chen and Li, 2005) and the number of secondary infections (Riley *et al.*, 1962). A drop in cough frequency following two weeks of chemotherapy has also been documented and has been proposed as a means of enabling decisions on patient release from respiratory isolation (Loudon and Spohn, 1969). All these findings reflect the potential for

assessing the infectivity of TB cases by sampling and measuring the concentration of bacilli in respiratory aerosols, for which a reliable method has yet to be available.

In the current study, the feasibility of a novel mask approach for sampling and quantification of Mtb in TB patients' expectorates was assessed and evaluated. Quantitative data from mask sampling has potential to reveal the correlation between Mtb aerosol output and infectiousness of TB cases. A positive correlation would support subsequent aim of establishing the transmission risk presented by particular cases. A preliminary study was also performed to investigate whether TB patients are infectious all the time or if there is a pattern to their infectivity. This chapter reports application of the mask sampling method for collection and quantification of respiratory-borne Mtb from TB patients.

5.2 Materials and Methods

5.2.1 Recruitment of patients

AFB smear-positive patients were recruited by a clinical fellow in the UHL Respiratory and Infectious Diseases wards as well as in their own homes, between June 2007 and March 2009. AFB smear microscopy was performed on respiratory specimens (sputum, induced sputum, and/or bronchoalveolar lavage) received by the UHL routine microbiology service. Patients were also identified by a TB specialist as being highly likely to be AFB smear-positive, prior to microscopy, based on clinical and radiological findings. Informed consent was obtained from each patient. This study received ethics approval (no. 10148) from the Leicester, Northampton, and Rutland Ethics Committee.

5.2.2 Mask sampling

Face masks with ties were used initially but subsequently ear-loop masks were preferred for ease of use (Figure 4.2). Apart from the fastening style, there was no difference in the composition and architecture of both these masks and this was confirmed by the manufacturer. All mask sampling studies were performed on patients who were isolated according to the UHL NHS trust isolation policy for patients with confirmed or suspected AFB smear-positive pulmonary TB. All healthcare staff wore N-95 respirators or standard surgical masks, gowns, and non-sterile examination gloves when attending to patients. When visiting patients in their own homes, no personal protective equipment was worn as per UHL policy. The clinical fellow was sometimes accompanied by a translator when required.

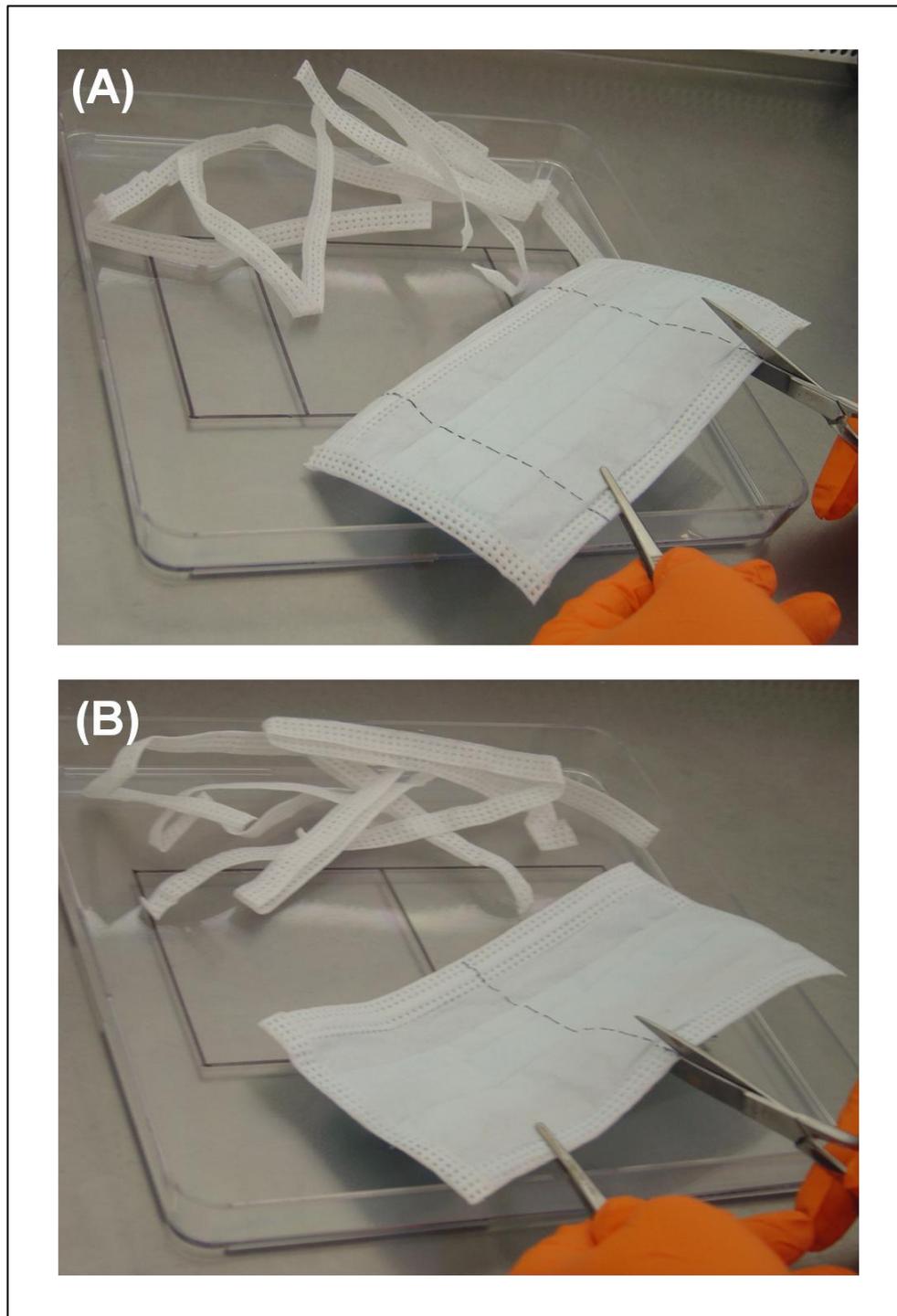
Where possible, two mask sampling studies were performed for each patient after meals, one in the morning and one in the evening. Patients were instructed to wear and fasten masks with the “Kimberly-Clark” inscribed surface facing outward. The mask was placed in such a way that it covered from the middle of the nasal bridge to the bottom of the chin, ensuring that it fitted comfortably and symmetrically to the face. If

using face masks with ties, the upper and lower ties were fastened to behind the head and neck, respectively. Figure 4.2 illustrates how face masks were worn by the patients. Despite no standardisation on the types of respiratory activities, patients were encouraged to read aloud if they were not talking naturally or coughing during mask sampling. There was no specified sampling duration but patients were encouraged to wear masks for at least one hour. Nevertheless, patients were pre-advised to remove masks in the event of discomfort or obstruction to breathing. If sputum was produced during sampling, this would be transferred to a sputum pot and then a new mask put on. Following sampling, patients folded masks into quarters with the sampling surface facing inwards and put them into a 250-ml plastic jar (Sarstedt, Leicester) each. Mask samples were then transported to the Category 3 Laboratory for processing. Masks were stored at 4°C if not processed on the day of sampling.

5.2.3 Splitting of face masks into smaller segments

A large plastic bioassay dish (24.5 x 24.5 cm²; Nalge Nunc) was sanitised with 70% v/v ethanol. Face masks were removed from the jars and placed on this dish one at a time, and unfolded so that the sampling surface was not in contact with the dish. Ties or ear loops were trimmed off and not processed for all mask samples. For routine processing, each mask was cut into 3 segments: 10.0 x 9.5 cm² (middle segment, M) and 4.0 x 9.5 cm² (left and right segments, L&R) to assess the distribution of mycobacterial aerosols upon impaction. Left and right segments for each mask were processed in a single 50-ml Falcon tube, as was the middle segment. For patients 016-020, each mask was cut into two equal-size segments (L and R) of 9.0 x 9.5 cm² each for comparison between two different processing methods as described in the Results section. Each of these mask segments was processed in a 50-ml Falcon tube. The splitting of masks into smaller segments was guided by the grids drawn on the bioassay dishes. All mask segments were rolled up with their sampling surfaces facing inwards before being transferred into the processing tubes. Forceps were used to handle masks throughout the processing. Both scissors and forceps were sanitised in between processing steps and samples by soaking in 70% v/v ethanol in a 50-ml Falcon tube. The splitting of masks into segments of different size is illustrated in Figure 5.1.

Figure 5.1: Cutting face masks into smaller segments

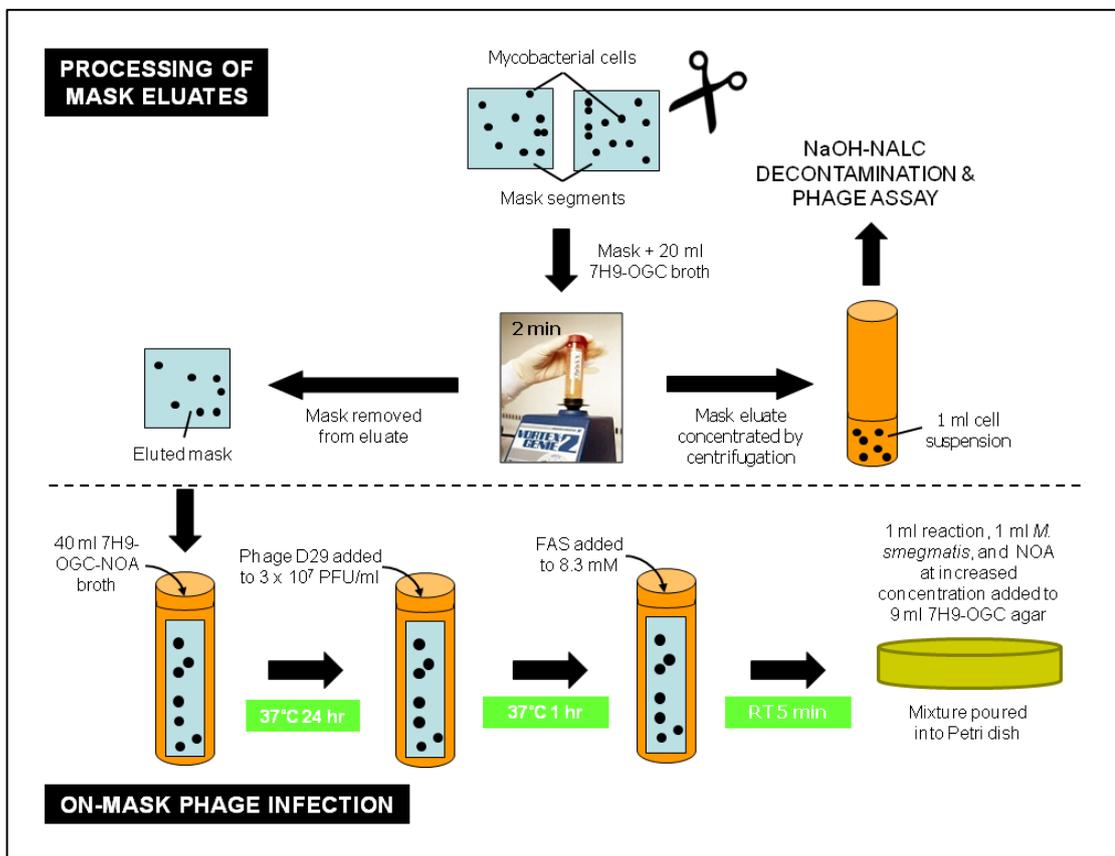


(A) Cutting mask into three segments: $10.0 \times 9.5 \text{ cm}^2$ (middle segment, M) and $4.0 \times 9.5 \text{ cm}^2$ (left and right segments, L&R). (B) Cutting mask into two equal-size segments (L and R) of $9.0 \times 9.5 \text{ cm}^2$ each.

5.2.4 Processing of face masks through the phage assay

Figure 5.2 summarises the protocol for the processing of a clinical mask sample through the phage assay. Only mask eluates were processed for patients 001-012. Both mask eluates and eluted masks were processed for patients 014-020. For patient 021, face masks were processed directly by the on-mask phage infection and pre-lysis plating method without the initial elution step. All tubes to be vortexed horizontally were sealed with two layers of Nescofilm.

Figure 5.2: Processing of a clinical mask sample through the phage assay



RT, room temperature.

5.2.4.1 Processing of mask eluates

This was performed as described for Process A in Section 4.2.2.6. For patients 001 and 003, mask segments were eluted in FPTB Medium Plus. The resulting mask eluates were subjected to the Biotec NaOH-NALC decontamination and phage assay as described in Sections 4.2.2.4 and 4.2.2.5, respectively.

5.2.4.2 On-mask phage infection and pre-lysis plating

For patients 014-020, eluted mask segments were transferred to new 50-ml Falcon tubes. On-mask phage infection and pre-lysis plating was performed as described for Process C in Section 4.2.2.6. Vortexing was performed for 2 minutes following the overnight resuscitation, with the tube held horizontally. Biotec NOA was added to Middlebrook 7H9-OGC broth at the published concentration (nystatin 10 µg/ml, oxacillin 2 µg/ml, and aztreonam 30 µg/ml) (Biotec Laboratories, 2005; Albert *et al.*, 2007; Mole *et al.*, 2007). In-house prepared NOA was added to 7H9-OGC agar at an increased concentration (nystatin 37.5 µg/ml, oxacillin 512 µg/ml, and aztreonam 30 µg/ml) (see Section 2.2.2).

5.2.5 Mask sampling with healthy volunteers

This was performed to assess the distribution of respiratory aerosols on face masks by healthy volunteers during voluntary coughing. Nine volunteers were recruited and were asked to wear two masks each, one with voluntary coughing and another without coughing, both for 1 minute each. The negative control was performed with an unexposed mask. Each mask was cut into middle, left, and right segments as in Section 5.2.3. The middle segment was transferred to a 50-ml Falcon tube, to which 20 ml of 1% w/v peptone + 0.05% w/v Tween 80 broth was added. The left and right segments were transferred to a 50-ml Falcon tube each and 10 ml of the same eluting medium added. Vortexing was performed for 2 minutes with the tube held horizontally. Following this, 100 µl of the resulting suspension were plated on LB agar in duplicate.

The agar plates were incubated at 37°C for up to 4 days. The number of CFU on each plate was enumerated.

5.2.6 Preparation of plaque DNA and IS6110 PCR

Plaque DNA was prepared according to the method described by Stanley *et al.* (2007). A minimum of three plaques from each plate were processed. Plaques were excised from phage indicator agar using 200- μ l pipette tips and transferred to a 1.5-ml screw-cap microtube (STARLAB, Milton Keynes) each, to which 10 μ l of PCR-grade water was added. Tube contents were heated at 100°C for 30 minutes, centrifuged briefly, and then left at -20°C for 15 minutes. The tubes were then centrifuged at 16,100 *g* for 2 minutes and the resulting supernatant used for the IS6110 PCR.

IS6110 PCR was performed according to the method described by Negi and associates (2007). PCRs were carried out in 50- μ l volumes containing 10 μ l of DNA suspension, 200 nM each primer IS6110F and IS6110R, 1.25 U of Thermoprime *Taq* DNA Polymerase (ABgene), 1x buffer (ABgene), 1.5 mM MgCl₂ (ABgene), and 200 μ M dNTPs. PCRs were performed in the Dyad DNA Engine as follows: 94°C for 5 minutes to activate the *Taq* polymerase, followed by 40 cycles of 94°C, 63°C, and 72°C for 1 minute each step. Final elongation was performed at 72°C for 7 minutes. The resulting PCR amplicons were separated by electrophoresis on 2% w/v agarose gels as described in Section 3.2.5.1.

5.2.7 Statistical analysis

All data in this chapter were analysed by the two-tailed paired *t* test, as required, unless otherwise indicated.

5.3 Results

5.3.1 Patients

A total of 17 patients were recruited for the mask sampling study. There were 10 men and 7 women. Two patients were below 20 years of age, eleven were between 20 and 60 years old, and four were over 60 years of age. There were seven Asians, three Africans, two African-Indians, four Whites, and one White and Black-Caribbean mix. Twelve patients were born outside the UK. Among the 17 patients, two had NTM infection, one *M. kansasii* and one *M. avium-intracellulare*. One TB patient had concomitant HIV infection. Eleven patients had 1+ to 2+ AFB smears, and six patients had scanty smears. All TB patients had isolates susceptible to all drugs. Nine patients had cavitory lung disease. A total of 29 mask sampling studies were conducted. Twelve patients had two studies, and five patients were studied only once. Twelve TB patients received treatment prior to studies, one had no treatment, and two were treated before one of two studies.

5.3.2 The phage assay can be used to detect mycobacteria in face masks from patients

Face masks from patients were processed by the phage assay in which mycobacteria were detected as plaques in the phage indicator plates. The number of plaques from each mask sample was enumerated and expressed as PFU. Negative mask controls were performed in parallel for every processing. The results for the processing of clinical mask samples using two different methods are displayed in Table 5.1.

Initially, the method was developed to process materials released from face masks. These mask eluates were first decontaminated with NaOH-NALC mixture and then subjected to the phage assay. Overall, processing of mask eluates yielded less than 10 PFU per plate in all positive cases. These low PFU counts were considered questionable positives.

It was hypothesised that NaOH-NALC decontamination affected the sensitivity of method for processing mask eluates. This chemical treatment has been shown to affect the sensitivity of the phage assay with pure mycobacterial cultures, 3 fold with Mtb and as much as 10^4 fold with *M. bovis* BCG Glaxo (see Section 4.3.6.2). Another limiting factor to the processing of mask eluates was the need to elute mycobacteria from face masks and the subsequent concentration of cells by centrifugation. The elution method used was only 5-10% efficient in releasing mycobacteria applied to masks (see Section 4.3.5), and centrifugation also results in cell loss. To circumvent these two limiting factors, a simplified method was developed that involved direct infection of mycobacteria on masks by phage D29 followed by plating before the D29 lytic burst. The NOA antibiotic cocktail was used to control contamination by non-target organisms during processing, using the published concentration (Biotec Laboratories, 2005; Albert *et al.*, 2007; Mole *et al.*, 2007) during overnight resuscitation of masks and an increased concentration (nystatin and oxacillin only) in phage indicator plates. The interference of contamination was minimal with these precautions.

The masks from Patients 016 and 017 were split into two equal-size segments and one segment was processed by the real-time PCR method (see Section 4.2.1.3). Masks from Patients 018-020 were also split into two equal-size segments each to assess the effect of mycobacterial Rpfs on PFU yields (see Section 5.3.9). Equal distribution of mycobacteria between equally split mask segments was assumed.

Table 5.1: PFU yields of face masks from patients

5.1 (A) Processing of mask eluates (NaOH-NALC)

Patient no.	Sampling duration	Mask no.	Mask segment(s) [§]	Sample PFU/plate	Control PFU/plate
001	5 hr	1	M	0	0
			L&R	0	0
	2 hr	2	M	0	0
			L&R	0	0
003	2 hr	1	M	0	0
			L&R	0	0
006	10 min	1	M	5	0
			L&R	0	0
	Unknown	2	M	1	0
			L&R	0	0
007	< 1 hr	1	M	0	0
			L&R	0	0
	< 1 hr	2	M	1	0
			L&R	0	0
008*	30 min	1	M	1	0
			L&R	0	0
	30 min	2	M	0	0
			L&R	0	0
009	2 hr	1	M	7	0
			L&R	2	0
	2 hr	2†	M	No result	0
			L&R	No result	0
010	50 min	1‡	M	6	0
			L&R	0	0
	25 min	2	M	3	0
			L&R	0	0
011	40 min	1	M	0	0
			L&R	0	0
012	1 hr	1‡	M	2	0
			L&R	0	0

* *M. kansasii* infection based on reference laboratory result.

† No results due to error during processing.

‡ Pre-chemotherapy sample.

§ Each mask was split into three segments: 10.0 x 9.5 cm² (middle segment, M) and 4.0 x 9.5 cm² (left and right segments, L&R).

5.1 (B) Processing of both mask eluates (NaOH-NALC) and eluted masks (NOA)

Patient no.	Sampling duration	Mask no.	Mask segment(s) ^c	MASK ELUATES ^f		ELUTED MASKS ^f	
				Sample PFU/plate	Control PFU/plate	Sample PFU/plate	Control PFU/plate
014	30 min	1 [§]	M	4	4	0	0
			L&R	0	0	1	0
	50 min	2	M	0	4	4	0
			L&R	0	0	0	0
015	Unknown	1	M	1	2	0	0
			L&R	0	0	0	0
016	40 min	1	L	0	0	133	0
017	1 hr	1	L	9	3	71	0
	1 hr	2	L	1		93	
018*	1 hr	1	L -Rpf	0	1	221	3
			R +Rpf	0	0	12	31
	1 hr	2	L -Rpf	0	1	399	3
			R +Rpf	0	0	350	31
019 [†]	1 hr	1	L -Rpf	1	1	172	3
			R +Rpf	0	0	0	31
	1 hr	2	L -Rpf	0	1	144	3
			R +Rpf	0	0	Confluent lysis	31
020*	45 min	1	L -Rpf	0	1	166	3
			R +Rpf	0	0	324	31
	45 min	2	L -Rpf	0	1	213	3
			R +Rpf	0	0	219	31
021 [‡]	30 min	1	M	NOT DONE		2	0
			L&R			35	2
	30 min	2	M	NOT DONE		10	3
			L&R			0	10

Table 5.1 (B) (Continue.....)

* These samples relate to one control mask.

† *M. avium-intracellulare* infection based on reference laboratory result.

‡ Masks were directly subjected to on-mask phage infection and pre-lysis plating without the initial elution step.

§ Pre-chemotherapy sample.

© M and L&R, each mask was split into three segments: 10.0 x 9.5 cm² (middle segment, M) and 4.0 x 9.5 cm² (left and right segments, L&R); L and R, each mask was split into two equal-size segments (L and R) of 9.0 x 9.5 cm² each. R segments for Patients 016-017 were processed by the real-time PCR method.

¶ Mask eluates refer to materials released from masks, NaOH-NALC decontaminated, and then phage assay performed. Eluted masks were subjected to on-mask phage infection followed by plating before D29 lytic burst; published and increased concentrations of NOA were used to control contamination by non-target organisms during overnight resuscitation and in phage indicator plates, respectively.

The on-mask phage infection and pre-lysis plating method had a better level of sensitivity; PFU were detected in 19 out of 25 (76%) mask reactions and 11 out of these (58%) yielded more than 100 PFU in 2% v/v of mask suspension plated out each. For processing of mask eluates, PFU were detected in only 14 out of 49 (29%) mask reactions and 10 out of these (71%) gave less than 5 PFU in 56% v/v of mask suspension plated out each. The detection of large numbers of PFU from eluted masks as opposed to very low PFU yields from mask eluates suggested both the effects of NaOH-NALC treatment and poor elution efficiency on the sensitivity of processing of the latter (Table 5.1B). Since mask eluates gave only very low PFU counts, it was decided that all further mask samples would be processed directly by the on-mask phage infection and pre-lysis plating method.

5.3.3 Background PFU counts are detected occasionally during mask processing

Background PFU were detected in negative controls during processing of mask eluates and on-mask analyses, more pronounced for the latter in which more than 30 plaques per plate had been recorded (Tables 5.1 and 5.2A). Background PFU were also recovered from face masks of healthy volunteers processed via the on-mask phage infection and pre-lysis plating method (Table 5.2B). Overall, these PFU counts varied widely from one processing to another; they ranged from 1 to 32 PFU per plate and gave an average of 9 PFU. The cut-off for verification of mask positivity by on-mask analysis was set at 45 PFU; i.e. five times the average.

For processing of mask eluates, the clustered incidence of background PFU in negative controls where eluted masks were processed simultaneously came as a surprise. No PFU were detected in negative mask controls for the first nine patients and all negative controls for the phage assay in this study. Overall, positive mask eluates yielded 1-9 PFU per plate while negative controls gave 1-4 PFU. As a result of these low PFU yields, it was not possible to estimate the cut-off for background counts. This was another pitfall with the method for processing mask eluates. The cut-off for interpretation of Biotec FPTB results with sputum samples is set at 20 PFU (Biotec Laboratories, 2004).

Table 5.2: Background PFU counts during on-mask analyses

5.2 (A) Negative mask controls

Negative control	Mask segment(s)	PFU/plate	PFU/mask segment (Log ₁₀)		
014	M	0	Not detected	PATIENT STUDY	
	L&R	0	Not detected		
015	M	0	Not detected		
	L&R	0	Not detected		
016*	L	0	Not detected		
017*	L	0	Not detected		
018-020 -Rpf	L	3	2.10		
018-020 +Rpf	R	31	3.11		
021 R1†	M	0	Not detected		
	L&R	2	1.92		
021 R2†	M	3	2.10		
	L&R	10	2.62		
R1†	M	8	2.52		HEALTHY VOLUNTEER STUDY
	L&R	0	Not detected		
R2†	M	7	2.47		
	L&R	0	Not detected		

* Half mask segment (9.0 x 9.5 cm²) was processed as the negative control.

† R1, replicate 1; R2, replicate 2.

5.2 (B) Face masks from healthy volunteers

Subject no.	Mask segment(s)	PFU/plate	PFU/mask segment (Log ₁₀)
1	M	1	1.62
	L&R	32	3.13
2	M	2	1.92
	L&R	0	Not detected
3	M	8	2.52
	L&R	0	Not detected
4	M	Contamination	No result
	L&R	6	2.40

5.3.4 Plaques from face masks may be positive by the confirmatory IS6110 PCR for *M. tuberculosis* DNA

Plaque DNA extracts from mask samples and negative mask controls were subjected to IS6110 PCR to check for the presence of Mtb-specific sequence. If single infected cells are well suspended, there should be one DNA molecule from a lysed Mtb cell within a plaque and by targeting IS6110 which is present in multiple copies throughout the genome, detection by PCR is possible. For phage indicator plates yielding 10 PFU or less, all plaques were tested. For those plates with more than 10 PFU, only 3-5 plaques were randomly selected for testing. The results are shown in Table 5.3.

Plaque DNAs from clinical mask samples were rarely positive by the IS6110 PCR during this study. Mtb-specific IS6110 sequence was only detected in plaque DNAs from both face masks of Patient 006 and the first mask sample of Patient 017, which were 13 out of a total of 88 (15%) mask plaques tested. Plaques from face masks of patients with NTM infections (008 and 019) were expected to be negative by the IS6110 PCR. A typical gel analysis of IS6110 PCR amplicons is shown in Figure 5.4. The IS6110-positive plaques for Patient 017 were from the mask eluate; plaques from the corresponding eluted mask were PCR-negative. Furthermore, plaques from the negative mask control for the former were also positive by the IS6110 PCR, which might suggest cross contamination during processing. Both these observations suggest ambiguity in the identity of IS6110-positive plaques from the first mask sample of Patient 017. For Patients 018-020, PCR-positive plaques from mask samples and control treated with mycobacterial Rpf were very likely due to Mtb H37Rv DNA, which was present in abundance in the culture supernatant used (G.V. Mukamolova, personal communication). Apart from the negative mask control for Patient 017 and that processed in the presence of culture supernatant, all other controls that yielded background PFU gave negative PCR results. Plaques from face masks of healthy volunteers were also PCR-negative.

Table 5.3: IS6110 PCR results for plaque DNAs from clinical mask samples and negative mask controls

5.3 (A) Processing of mask eluates – PATIENT STUDY

Mask segment(s)*	Sample PFU/plate	No. of plaque(s) tested by IS6110 PCR	No. of IS6110-positive plaque(s)
006 (1) M	5	5	5
006 (2) M	1	1	1
007 (2) M	1	1	0
008 (1) M	1	1	0
009 (1) M	7	7	0
009 (1) L&R	2	2	0
010 (1) M	6	6	0
010 (2) M	3	3	0
012 (1) M	2	2	0
014 (1) M	4	4	0
NEG M	4	4	0
015 (1) M	1	1	0
NEG M	2	2	0
017 (1) L	9	8	7
017 (2) R	1	1	0
NEG L	3	3	3
019 (1) L	1	1	0
NEG L	1	1	0

* Numbers in brackets refer to mask numbers. NEG denotes negative mask control.

5.3 (B) On-mask phage infection and pre-lysis plating – PATIENT STUDY

Mask segment(s)*	Sample PFU/plate	No. of plaque(s) tested by IS6110 PCR	No. of IS6110-positive plaque(s)
014 (1) L&R	1	1	0
014 (2) M	4	4	0
016 (1) L	133	5	0
017 (1) L	71	5	0
017 (2) L	93	5	0
018 (1) L -Rpf	221	3	0
018 (1) R +Rpf	12	3	3
018 (2) L -Rpf	399	3	0
018 (2) R +Rpf	350	3	3
019 (1) L -Rpf	172	3	0
019 (2) L -Rpf	144	3	0
019 (2) R +Rpf	Confluent lysis	Not done	Not done
020 (1) L -Rpf	166	3	0
020 (1) R +Rpf	324	3	3
020 (2) L -Rpf	213	3	0
020 (2) R +Rpf	219	3	3
NEG L -Rpf	3	3	0
NEG R +Rpf	31	3	3
021 (1) M	2	2	0
021 (1) L&R	35	3	0
021 (2) M	10	10	0
NEG (1) L&R	2	2	0
NEG (2) M	3	3	0
NEG (2) L&R	10	10	0

* Numbers in brackets refer to mask numbers. NEG denotes negative mask control.

5.3 (C) On-mask phage infection and pre-lysis plating – HEALTHY VOLUNTEER STUDY

Mask segment(s)*	Sample PFU/plate	No. of plaque(s) tested by IS6110 PCR	No. of IS6110-positive plaque(s)
S1 M	1	1	0
S1 L&R	32	5	0
S2 M	2	2	0
S3 M	8	8	0
S4 L&R	6	6	0
NEG (R1) M	8	8	0
NEG (R2) M	7	7	0

* S denotes subject. NEG denotes negative mask control. R1, replicate 1; R2, replicate 2.



Figure 5.4: IS6110 PCR of plaque DNAs from face masks of Patient 006

Upper panel: Lane 1, GeneRuler 100-bp DNA marker; lane 2, NTC; lane 3, Mtb CDC 1551 DNA (positive control); lane 4, *M. smegmatis* DNA; lanes 5-6, *M. smegmatis* plaque DNAs; lanes 7-11, plaque DNAs from mask no.1.

Lower panel: Lane 1, GeneRuler 100-bp DNA marker; lane 2, NTC; lane 3, Mtb CDC 1551 DNA (positive control); lane 4, *M. smegmatis* DNA; lanes 5-8, *M. smegmatis* plaque DNAs; lane 9, plaque DNA from mask no.2.

IS6110 amplicons of plaque DNAs from face masks are framed in red for clarity.

5.3.5 Final assignment of face mask positivity for mycobacteria

Table 5.4 collates and summarises the data from Sections 5.3.2-5.3.4 for final assignment of face mask positivity for mycobacteria. Assuming equal distribution of mycobacteria among equally split mask segments, the PFU counts for mask samples from Patients 016 and 017 were multiplied by a factor of two. The other half mask segments did not show positive detection by the real-time PCR method. For Patients 018-020, PFU counts (above the set threshold of 45 PFU) for mask segments treated and untreated with mycobacterial Rpf were summated to obtain the final PFU yield per intact face mask.

Table 5.4: Final assignment of face mask positivity for mycobacteria

5.4 (A) Processing of mask eluates

Patient no.	Mask no.	Mask segment(s) [§]	Sample PFU/plate	Control PFU/plate	IS6110 PCR Sample, Control	PFU/mask (Log ₁₀)																																																																																																																																											
001	1	M	0	0	N/A [©]	Not detected																																																																																																																																											
		L&R	0	0				2	M	0	0	N/A	Not detected	L&R	0	0	003	1	M	0	0	N/A	Not detected	L&R	0	0	006	1	M	5	0	Positive, N/A	0.95	L&R	0	0	N/A	2	M	1	0	Positive, N/A	0.48	L&R	0	0	N/A	007	1	M	0	0	N/A	Not detected	L&R	0	0	2	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0	Negative, N/A	2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85
	2	M	0	0	N/A	Not detected																																																																																																																																											
		L&R	0	0			003	1	M	0	0	N/A	Not detected	L&R	0	0	006	1	M	5	0	Positive, N/A	0.95	L&R	0	0		N/A	2	M	1	0	Positive, N/A	0.48	L&R	0	0	N/A	007	1	M	0	0	N/A	Not detected	L&R	0		0	2	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R		0	0	N/A	2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51		L&R	2	0	Negative, N/A	2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A		1.34	L&R	0	0	N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A	
003	1	M	0	0	N/A	Not detected																																																																																																																																											
		L&R	0	0			006	1	M	5	0	Positive, N/A	0.95	L&R	0	0		N/A	2	M	1	0	Positive, N/A	0.48	L&R	0	0	N/A	007	1	M	0	0	N/A	Not detected	L&R	0	0		2	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0		N/A	2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0		Negative, N/A	2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0		N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A											
006	1	M	5	0	Positive, N/A	0.95																																																																																																																																											
		L&R	0	0	N/A			2	M	1	0	Positive, N/A	0.48	L&R	0	0	N/A	007	1	M	0	0	N/A	Not detected	L&R	0	0	2		M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A		2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0	Negative, N/A		2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A		2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																						
	2	M	1	0	Positive, N/A	0.48																																																																																																																																											
		L&R	0	0	N/A		007	1	M	0	0	N/A	Not detected	L&R	0	0	2		M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A		2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0	Negative, N/A		2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A		2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																	
007	1	M	0	0	N/A	Not detected																																																																																																																																											
		L&R	0	0				2	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A	008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0		N/A	2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0		Negative, N/A	2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0		N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																											
	2	M	1	0	Negative, N/A	0.60																																																																																																																																											
		L&R	0	0	N/A		008*	1	M	1	0	Negative, N/A	0.60	L&R	0	0	N/A		2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0	Negative, N/A		2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A		2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																						
008*	1	M	1	0	Negative, N/A	0.60																																																																																																																																											
		L&R	0	0	N/A			2	M	0	0	N/A	Not detected	L&R	0	0	009	1	M	7	0	Negative, N/A	1.51	L&R	2	0	Negative, N/A		2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A		2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																	
	2	M	0	0	N/A	Not detected																																																																																																																																											
		L&R	0	0			009	1	M	7	0	Negative, N/A	1.51	L&R	2	0		Negative, N/A	2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0		N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																											
009	1	M	7	0	Negative, N/A	1.51																																																																																																																																											
		L&R	2	0	Negative, N/A			2 [†]	M	No result	0	N/A	No result	L&R	No result	0	010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0	N/A		2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																																						
	2 [†]	M	No result	0	N/A	No result																																																																																																																																											
		L&R	No result	0			010	1 [‡]	M	6	0	Negative, N/A	1.34	L&R	0	0		N/A	2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																																																
010	1 [‡]	M	6	0	Negative, N/A	1.34																																																																																																																																											
		L&R	0	0	N/A			2	M	3	0	Negative, N/A	1.04	L&R	0	0	N/A	011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																																																											
	2	M	3	0	Negative, N/A	1.04																																																																																																																																											
		L&R	0	0	N/A		011	1	M	0	0	N/A	Not detected	L&R	0	0	012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																																																																						
011	1	M	0	0	N/A	Not detected																																																																																																																																											
		L&R	0	0			012	1 [‡]	M	2	0	Negative, N/A	0.85	L&R	0	0	N/A																																																																																																																																
012	1 [‡]	M	2	0	Negative, N/A	0.85																																																																																																																																											
		L&R	0	0	N/A																																																																																																																																												

* *M. kansasii* infection based on reference laboratory result.

† No results due to error during processing.

‡ Pre-chemotherapy sample.

§ Each mask was split into three segments: 10.0 x 9.5 cm² (middle segment, M) and 4.0 x 9.5 cm² (left and right segments, L&R).

© N/A denotes not applicable.

5.4 (B) Processing of both mask eluates and eluted masks

Patient no.	Mask no.	Mask segment(s) [§]	MASK ELUATES [¶]				ELUTED MASKS [¶]			
			Sample PFU/plate	Control PFU/plate	IS6110 PCR Sample, Control	PFU/mask (Log ₁₀)	Sample PFU/plate	Control PFU/plate	IS6110 PCR Sample, Control	PFU/mask (Log ₁₀)
014	1 [§]	M	4	4	Negative, Negative	Invalid	0	0	N/A	Invalid
		L&R	0	0	N/A [§]		1	0	Negative, N/A	
	2	M	0	4	N/A	Not detected	4	0	Negative, N/A	Invalid
		L&R	0	0			N/A	0	0	
015	1	M	1	2	Negative, Negative	Invalid	0	0	N/A	Not detected
		L&R	0	0	N/A		0	0		
016	1	L	0	0	N/A	Not detected	133	0	Negative, N/A	4.05
017	1	L	9	3	Positive, Positive	Invalid	71	0	Negative, N/A	3.78
	2	L	1	3	Negative, Positive		93	0	Negative, N/A	3.90
018*	1	L -Rpf	0	1	N/A, Negative	Not detected	221	3	Negative, Negative	3.97
		R +Rpf	0	0	N/A, Negative		12	31	Positive, Positive	
	2	L -Rpf	0	1	N/A, Negative	Not detected	399	3	Negative, Negative	
		R +Rpf	0	0	N/A, Negative		350	31	Positive, Positive	
019 [†]	1	L -Rpf	1	1	Negative, Negative	Invalid	172	3	Negative, Negative	3.86
		R +Rpf	0	0	N/A, Negative		0	31	N/A, Positive	
	2	L -Rpf	0	1	N/A, Negative	Not detected	144	3	Negative, Negative	
		R +Rpf	0	0	N/A, Negative		CL [§]	31	Not done, Positive	
020*	1	L -Rpf	0	1	N/A, Negative	Not detected	166	3	Negative, Negative	4.31
		R +Rpf	0	0	N/A, Negative		324	31	Positive, Positive	
	2	L -Rpf	0	1	N/A, Negative	Not detected	213	3	Negative, Negative	
		R +Rpf	0	0	N/A, Negative		219	31	Positive, Positive	
021 [‡]	1	M	NOT DONE				2	0	Negative, N/A	Invalid
		L&R	NOT DONE				35	2	Negative, Negative	
	2	M	NOT DONE				10	3	Negative, Negative	Invalid
		L&R	NOT DONE				0	10	N/A, Negative	

Table 5.4 (B) (Continue.....)

* These samples relate to one control mask.

† *M. avium-intracellulare* infection based on reference laboratory result.

‡ Masks were directly subjected to on-mask phage infection and pre-lysis plating without the initial elution step.

§ Pre-chemotherapy sample.

© M and L&R, each mask was split into three segments: 10.0 x 9.5 cm² (middle segment, M) and 4.0 x 9.5 cm² (left and right segments, L&R); L and R, each mask was split into two equal-size segments (L and R) of 9.0 x 9.5 cm² each. R segments for Patients 016-017 were processed by the real-time PCR method.

¶ Mask eluates refer to materials released from masks, NaOH-NALC decontaminated, and then phage assay performed. Eluted masks were subjected to on-mask phage infection followed by plating before D29 lytic burst; published and increased concentrations of NOA were used to control contamination by non-target organisms during overnight resuscitation and in phage indicator plates, respectively.

ξ N/A denotes not applicable.

Due to the low PFU yields of positive mask eluates, it was not possible to set a cut-off for background counts. In this case, only mask PFU counts higher than those of controls were considered as true positives. Based on this criterion, Mtb was detected in 7 out of 13 (54%) clinical mask samples (Table 5.4A). Another positive mask was from a patient (008) with *M. kansasii* infection. All mask eluates in which their corresponding eluted masks were processed in parallel were assigned mycobacteria-negative (Table 5.4B). With the on-mask analysis, 7 out of 12 (58%) face masks were positive for Mtb (Tables 5.4B and C). One patient (009) with *M. avium-intracellulare* infection also gave positive masks. Only face masks from Patient 006 were confirmed to be positive for Mtb based on IS6110 PCR results. Overall, mycobacteria were detected in 17 out of 29 (59%) face masks processed and these were from 11 out of 17 (65%) patients recruited in this study.

A total of 22 out of 29 mask sampling studies were performed post-chemotherapy, but Mtb was detected in more than 50% (12 out of 22) of them. The time lapse between mask sampling and processing ranged from 0 to 8 days for processing of mask eluates alone, and 4 to 15 days for processing by the on-mask phage infection and pre-lysis plating method. The overall median was 5 days. The effect of delay in processing on PFU yields of clinical mask samples was not assessed in this study.

5.3.6 CFU analyses of mask eluates

Mask eluates from Patients 006-020 were also subjected to CFU analyses on Middlebrook 7H10 agar, in addition to processing by the phage assay, to compare between two different means of end-point mycobacterial detection. Results are shown in Table 5.5. Mycobacterial CFU were only detected in eluates from 15% (4 out of 27) of clinical mask samples; PFU were detected in 30% of these (Table 5.4A). The lower detection rate with the former might be explained by lesser volume of mask suspension tested, 60 μ l as opposed to 1 ml by the phage assay. CFU plates for the first masks from Patients 008, 010, and 017 were heavily contaminated. All colonies appeared off-white in colour and dry breadcrumb-like in appearance, characteristic of those of *Mtb*. They were also positive by auramine O staining. Colony lysates were subjected to the combined 16S-RD assay for further confirmation.

Table 5.5: CFU yields of mask eluates

Mask sample	Mask segment	CFU per 60 μ l [*]	CFU/mask (Log ₁₀)	PFU/mask (Log ₁₀)	Molecular assay	
					16S signals	RD signals
006 mask no. 2	M	2	1.85	0.48	T- S+ [†]	T- S-
	L&R	1			T- S+	T+ S+
007 mask no. 2	M	0	2.52	0.60	N/A [‡]	N/A
	L&R	1			T+ S+	T+ S+
008 mask no. 2	M	0	2.12	Not detected	NA	N/A
	L&R	4			Not done	Not done
010 mask no. 2	M	3	2.00	1.04	T+ S+	T+ S+
	L&R	0			N/A	N/A

* CFU counts were based on neat mask eluates except for that of 007 mask eluate which was based on 10⁻¹ dilution. No colonies were detected in the neat eluate plated out for the latter, suggesting the possibility of cross-contamination.

† 16S amplicons of one mycobacterial colony that was merged with a contaminant colony gave T_m of 86.8°C. Those of another colony which was well isolated yielded T_m of 87.8°C. 16S amplicons of MGIT and LJ mycobacterial isolates consistently yielded T_ms of 88.2 ± 0.2°C.

‡ N/A denotes not applicable.

5.3.7 Patients expectorate different amounts of *M. tuberculosis* throughout the day

A comparison was made between PFU yields for mask sampling performed at two different times of the day, and the results are displayed in Table 5.6. Although sampling results for only four patients were considered, three consistently gave slightly higher PFU yields in the morning than in the afternoon or evening. The other patient (019) who showed a reverse trend had *M. avium-intracellulare* infection.

Table 5.6: Comparison of PFU counts for face masks between sampling in the morning and sampling in the afternoon or evening

Patient no.	Sampling time [†]	PFU/mask (Log ₁₀)
017	AM	3.90
	PM	3.78
018	AM	4.50
	PM	3.97
019*	AM	3.86
	PM	4.43
020	AM	4.31
	PM	4.26

* *M. avium-intracellulare* infection based on reference laboratory result.

† AM denotes morning. PM denotes afternoon or evening.

5.3.8 Mycobacteria are predominantly sampled in the middle section of face masks

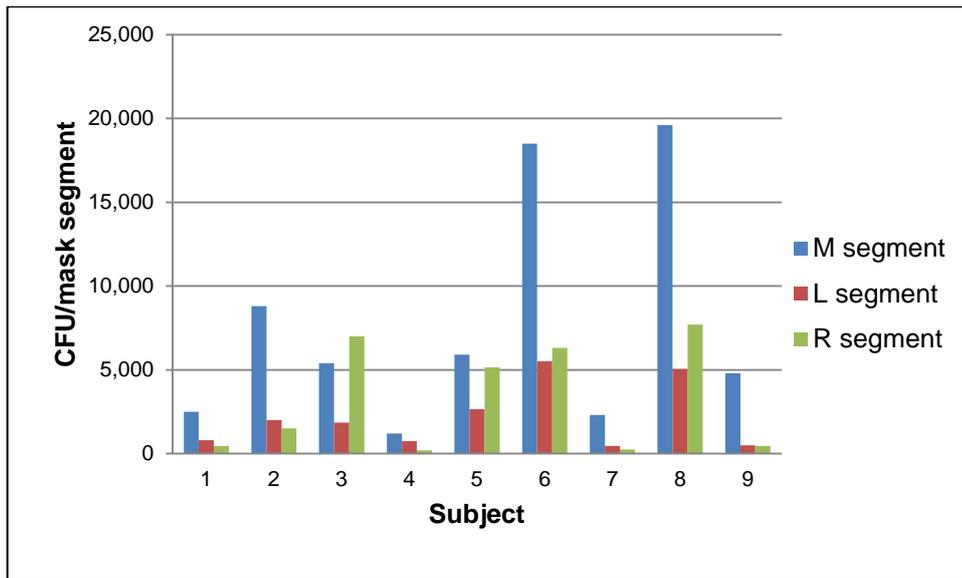
Although it was not possible to assess the specific distribution of mycobacteria, some patients showed a skewed distribution of mycobacteria sampled on the face masks. Based on results of mask eluates, all positive mask samples showed sampling of all or more mycobacteria in the middle segments and no or fewer mycobacteria in the left and right segments (Table 5.7).

Table 5.7: Distribution of PFU on different sections of face masks

Patient no.	Mask no.	PFU/mask segment (Log ₁₀)	
		M	L&R
006	1	0.95	0
	2	0.48	0
007	2	0.60	0
008	1	0.60	0
009	1	1.40	0.85
010	1	1.34	0
	2	1.04	0
012	1	0.85	0

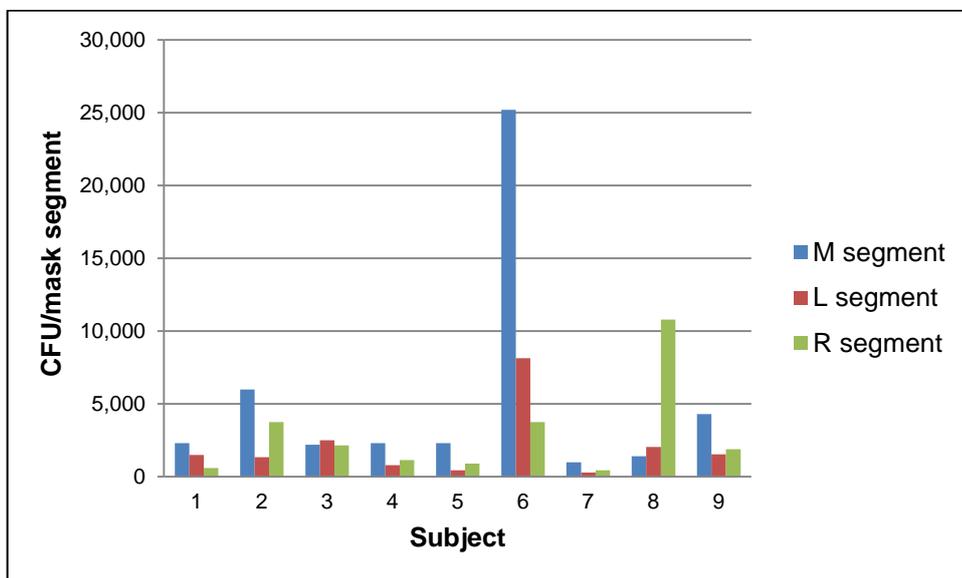
An experiment with healthy volunteers showed that there were more CFU in the middle segments of face masks on average compared to the left and right segments (processed separately) following coughing for 1 minute ($P < 0.05$, two-sample *t* test; Figure 5.4). There was no significant difference in CFU between the left and right mask segments ($P > 0.1$). When masks were worn for 1 minute without coughing, there was no significant difference in CFU among all the mask segments ($P > 0.1$; Figure 5.5).

Figure 5.4: Distribution of CFU on face masks of 9 healthy volunteers during 1-minute coughing



Average of duplicate CFU counts is shown for each mask segment.

Figure 5.5: Distribution of CFU on face masks of 9 healthy volunteers worn for 1 minute without coughing



Average of duplicate CFU counts is shown for each mask segment.

5.3.9 Effect of mycobacterial culture supernatant on phage assay with face masks

Most tuberculous sputum samples are dominated by a population of Mtb cells that can only be grown in the presence of Rpf (Mukamolova *et al.*, 2010). If Mtb bacilli in similar physiological state are also present in respiratory droplets, pre-treatment of face masks with Rpf might improve phage infectivity of cells and ultimately increase the sensitivity of the phage detection method. The effect of Mtb H37Rv culture supernatant (containing Rpf) on PFU yields of face masks was investigated, and the results are displayed in Table 5.8. This experiment assumed equal distribution of mycobacteria between two equal-size segments of individual mask samples. The tested mask samples showed a no clear trend in their PFU counts, some higher and some lower with supernatant; this might actually reflect the difference in distribution of mycobacteria between equal-size segments of corresponding mask samples, rather than genuine effect of culture supernatant.

Table 5.8: Effect of Mtb H37Rv culture supernatant on PFU counts of face masks from patients with mycobacterial infections

Patient no.	Mask no.	Treatment	PFU/mask segment (Log ₁₀)
018	1	- Rpf	3.97
		+ Rpf	Invalid*
	2	- Rpf	4.22
		+ Rpf	4.16
019	1	- Rpf	3.86
		+ Rpf	Not detected
	2	- Rpf	3.78
		+ Rpf	4.32
020	1	- Rpf	3.84
		+ Rpf	4.13
	2	- Rpf	3.95
		+ Rpf	3.96

Mtb was grown in Middlebrook 7H9-OGC broth in a roller culture bottle (Greiner Bio-One, Stonehouse) at 37°C to OD_{580nm} of 0.5-1.0. The culture was centrifuged at 3,023 g for 15 minutes and the resulting supernatant sterilised by filtration through a 0.2-µm filter unit. The filtrate was mixed with 7H9-OGC broth in 1:1 and then Biotec NOA added to the published concentration (Albert *et al.*, 2007; Mole *et al.*, 2007).

* PFU value is below the cut-off for verification of mask positivity by the on-mask phage infection method.

5.4 Discussion

5.4.1 The phage assay can be used to detect mycobacteria in face masks from patients

The phage amplification assay was used in this study to detect patient-excreted mycobacteria sampled on face masks. The approach is economical and produces results within 48 hours of receipt of mask samples. The phage detection method is also sensitive, detecting less than 100 mycobacterial CFU applied to face masks, and has quantitative potential.

Analysis of materials released from masks by the phage assay only gave low levels of detection; less than 50 PFU in all positive cases. Consequently, the results with this processing method could only be interpreted qualitatively, i.e. masks being assigned as positive or negative for mycobacteria. The sensitivity of this method is very likely limited by the detrimental effect of NaOH-NALC decontamination and the low efficiency of mycobacteria elution from masks.

Direct processing of masks using the NOA antibiotic cocktail to control contamination by non-target organisms overcomes both these limitations. This method is simpler to perform; it relies on phage particles infecting mycobacterial cells on masks without the need for prior mechanical elution. The absence of centrifugation steps minimises cell loss during the procedure. With more mask samples, it might be possible to investigate if the resuscitation process and phage infection assisted in releasing mycobacterial cells from masks. Taken together, all these would contribute to sensitive detection of mycobacteria with the direct method; all positive masks yielded between 5,000 and 50,000 PFU. Therefore, direct phage infection on masks enhances the quantitative potential of the mask sampling approach.

The method of processing materials released from masks is less sensitive than the direct mask processing method although the volumetric ratio of mask suspension

analysed with the latter is nearly 30 times lower than the former. If infected cells are well suspended, the maximal sensitivity achievable with the direct method should theoretically be 40 PFU per mask reaction. Concentration by centrifugation would stretch the sensitivity further but it runs the risk of mycobacteria rendered fragile by infection being lysed by collisions during the procedure. Multiple platings of mask suspension following virucide treatment would increase the chance of recovering very low numbers of PFU (less than 40 PFU per mask reaction) and could demonstrate reproducibility of PFU counts, although this would impose an increase in labour and processing time. An alternative is to use membrane filtration to concentrate the infected cells followed by plating with *M. smegmatis* lawn. However, the cut-off value for background PFU counts used in this study would not validate any counts below 45 PFU per ml of mask suspension plated out.

5.4.2 Background PFU counts are detected occasionally during mask processing

Background PFU yields are a problem during direct phage infection on masks. It was possible that background PFU were due to protection of exogenous phage particles trapped among mask layers from virucide inactivation. In this case, removal of masks from the processing tubes following phage infection could solve this problem, but would result in cell loss and make it difficult to standardise the volume of virucide to be added. A better and sensible solution would be to process layers of intact masks in separate tubes; this would simultaneously address the question on penetration of expectorated bacilli through mask layers, which has been reported previously (Nicas, 1995). The detection of background PFU from masks worn by healthy volunteers could be due to the protection of exogenous phage by respiratory secretions, which has been suggested before (Albert *et al.*, 2002a). The spontaneous reassociation of virucide-disrupted D29 particles has also been proposed (Park *et al.*, 2003). During this study, mask positivity with the direct processing method was assigned based on the cut-off set for background PFU counts. Based on this cut-off, the maximal sensitivity achievable with this method would only be about 2,000 PFU per mask reaction. Therefore, a solution to this problem of breakthrough PFU is important for reliable detection and quantification of *Mtb* cells on masks; an approach involving double infection of *Mtb* by dual reporter phages has been proposed (Park *et al.*, 2003)

5.4.3 Plaques from face masks may be positive by the confirmatory IS6110 PCR for *M. tuberculosis* DNA

IS6110 PCR positivity rate for mask plaques was rather low in this study. The single or very few copies of genomic DNA within a plaque are prone to losses which might occur during the extraction process. Despite targeting an IS that is present in multiple copies throughout Mtb genome, the need for single-cell detection might stretch the sensitivity of the IS6110 PCR. In both cases, development of a more efficient DNA extraction method and nested PCR could improve the sensitivity of this test (Stanley *et al.*, 2007). It has also been suggested that the media used for the in-house phage assay might not be favourable to downstream IS6110 PCR on mycobacterial plaques; similar molecular test has only been validated on plaques generated by the Biotec FPTB test (C. E. D. Rees, personal communication). Furthermore, there was significant delay in PCR analysis of plaques from most mask samples during this study, and the effect of this delay on PCR positivity remains to be investigated.

5.4.4 Final assignment of face mask positivity for mycobacteria

A total of 9 out of 15 (60%) AFB smear-positive TB patients gave positive masks in this study. Mtb was detected in 14 out of a total of 25 (56%) masks processed from these patients. In the cough aerosol sampling study by Fennelly and co-workers (2004), Mtb was cultured from respiratory aerosols expectorated by only 4 out of 16 (25%) smear-positive patients. This was more than 2 times lower than the rate of positive mask detection in this study. The highest bacillary yield per hour for a single patient was about 3,500 CFU in Fennelly's study, while this was approximately 10 times higher based on PFU count in the mask sampling study. The lower detection rate in Fennelly's study might be explained by the shorter sampling duration, 5 minutes as opposed to more than 30 minutes for most patients in this study. In addition, the former might be underestimated by the presence of non-culturable bacilli in aerosol droplets, which might arise from physical stress of aerosolisation and airborne dehydration. Furthermore, the possible presence of Rpf-dependent Mtb cells in respiratory droplets might contribute to the lower positivity rate in their study.

The mask sampling study was initially aimed at smear-positive TB patients, but direct sputum analysis by the 16S-RD assay to distinguish between MTBC and NTM was still in development at the time of the study. Two patients in this study had NTM infections, one *M. kansasii* and one *M. avium-intracellulare*. Human infections due to NTM aerosols from natural and domestic waters have been reported (Falkinham, 1996; Kreiss and Cox-Ganser, 1997; Embil *et al.*, 1997), but there is no evidence for human-to-human transmission. The phage D29 used in the detection assay is not specific for Mtb and will infect all species of the genus *Mycobacterium*. However, *M. avium* has been reported as not susceptible to infection by D29 (Froman *et al.*, 1954; Froman and Scammon, 1964). The detection of *M. avium-intracellulare* in this study might reflect strain difference in the susceptibility to phage infection. With more optimised sample preparation and sensitive detection procedures, the IS6110 PCR can be reliably used to differentiate TB from NTM infections. Nevertheless, there is still doubt regarding the specificity of IS6110 as the signature sequence for MTBC; NTM species with this insertion element and MTBC strains lacking IS6110 have been reported (Thierry *et al.*, 1990; Coros *et al.*, 2008). An alternative for the differentiation of MTBC from NTM would be to incorporate NAP into the phage assay; this biochemical test has been used with the luciferase phage assay (Riska *et al.*, 1997; Banaiee *et al.*, 2001). However, the selective inhibition of MTBC by NAP means there will be no quantitative data for Mtb.

5.4.5 Patients expectorate different amounts of *M. tuberculosis* throughout the day

Preliminary data suggested that TB patients might have a specific pattern to their infectivity. Although the difference is marginal, a small number of patients in this study expectorated more Mtb bacilli in the morning than in the afternoon or evening. The former is the time of the day when frequent contact with visitors and other patients is expected, thus justifying the compulsory wearing of masks by patients when leaving the wards and suggesting higher level of precaution. Early-morning sputum specimens are believed to contain higher bacillary load (J. Malkin, personal communication), and therefore aerosolisation of larger quantities of Mtb would be expected (Riley *et al.*, 1959). However, the small number of patients in this study limited the statistical power to justify this conclusion. Furthermore, the marginal difference recorded might not be meaningful.

5.4.6 Mycobacteria are predominantly sampled in the middle section of face masks

Splitting and processing different segments of face masks showed that most mycobacteria are sampled in the middle segments. This section of the mask covers the nose and mouth, and therefore predominant sampling of aerosol droplets in this area during oral and respiratory activities would be expected. If further clinical data show consistent trend, selective processing of the frequently contaminated area on face masks could be considered. It would be a waste in resources to process the perimeter of masks if it is less likely to be contaminated with respiratory aerosols. An experimental design to locate specific sites on face masks for deposition of mycobacteria by the phage assay did not yield any meaningful results (see Appendix 3).

5.4.7 Effect of mycobacterial culture supernatant on phage assay with face masks

There was no evidence for the effect of mycobacterial culture supernatant (contains Rpfs) on PFU yields of face masks in this study. The major limitation of the test performed was the assumption that mycobacteria were equally distributed between two equal-size segments of individual mask samples, which might not be the case. Even if this assumption is true, the magnitude increase in PFU yields for those mask samples treated with culture supernatant might be marginal to be considered significant. Mukamolova and co-workers (2010) reported that as much as 80-100% of *Mtb* cells in sputum samples demonstrated by culture could only be detected with Rpf stimulation. Possible effects of other factors such as the quality of culture supernatant and the duration of exposure on PFU yield remain to be investigated, as discussed in Section 4.4.8. Perhaps it would be more possible to investigate the effect of mycobacterial culture supernatant on phage assay with sputa in relation to the distribution of organisms within samples. Nevertheless, the effect of Rpfs on phage infectivity of mycobacteria is still not known.

5.5 Conclusion

The mask sampling coupled to phage detection method developed in this study can be used to detect *Mtb* expectorated by smear-positive TB cases. This method is cost effective and produces results within 48 hours of receipt of mask samples. As opposed to processing of materials released from face masks, the method developed for direct phage infection on masks is relatively sensitive and yielded data that are potentially quantitative. Background PFU counts remains a problem but can be resolved by interpreting results against the cut-off and modifying the existing processing method; the latter includes analysis of masks in separate layers so that the question pertaining to mask penetration by bacilli could be addressed at the same time. The low positivity rate of the confirmatory PCR for phage-extracted *Mtb* DNA warrants further technical investigation.

With further work, the correlation between quantitative data of the mask sampling approach and TB infectiousness could be assessed. In the event of a positive correlation, results of *Mtb* aerosol output could be used to establish the transmission risk presented by particular TB patients; this might be of greater value than the reliance on AFB smear status to guide clinicians regarding decisions on respiratory isolation. Preliminary data from the current study postulate the presence of a specific pattern of infectivity among TB cases; they are more infectious early in the morning than later on during the day. However, this necessitates further study. Due to the nature of the mycobacteriophage used in the detection assay, the mask sampling method can also be used for patients with NTM infections who might be infectious by the airborne route.

CHAPTER 6

General Discussion

6.1 General Discussion

The main aim of this study was to develop novel analytical methods with potential to improve our understanding of the transmission of TB. Such developments could contribute to public health control and clinical management of the disease. Two methods were developed and evaluated, a rapid molecular assay and a mask aerosol sampling approach. The molecular assay, which is capable of rapid differentiation between MTBC and NTM based on the mycobacterial 16S rDNA, was developed in parallel to facilitate identification of TB patients for the mask sampling study. Another molecular assay, which targets a global Mtb lineage-defining LSP that predominates locally, was developed for preliminary genotyping of Mtb strains in order to facilitate early assessment of potential recent transmission events. The mask approach involved sampling with standard face masks with subsequent detection and measurement of amounts of Mtb in aerosols expectorated by individuals with pulmonary TB.

Application of the 16S assay to direct analysis of sputum is still being evaluated at the time of writing and therefore could not fulfill its intended role during the mask sampling study. Nevertheless, we explored the potential clinical utility of the assay on mycobacterial isolates. Until identity and genotyping information is available from the reference laboratory, a precautionary response to a particular case in which mycobacteria have been detected may lead to inappropriate isolation and treatment of patients for TB and unnecessary public health investigations. Wrong assignment of tuberculous treatment regimen to patients with atypical infections can lead to serious consequences; furthermore irrelevant public health efforts are wasteful in time and resources. In this context, application of the 16S-RD assay to mycobacterial isolates, in addition to clinical and AFB smear assessments, enables rapid recognition of infections as MTBC or NTM and the detection of potential epidemiological links between contemporary Mtb strains, all within a total processing time of 4 hours. These may be delayed by days in the former case and weeks in the latter at the reference laboratory. Within our local service, where TB and NTM infections affect between 200 and 400 patients per annum, these delays may affect the management of a significant number of patients (M. R. Barer, personal communication). Of even greater value, direct specimen analysis by the combined assay has potential to provide this information within the same day of sample receipt; preliminary utility of this has been demonstrated

by a clinical fellow within our research group. In the case of positive Mtb detection, prompt treatment of patients and prevention of further person-to-person transmission will be a priority of the clinical and public health teams. In the case of negative clinical findings and smear results, the outcomes from the 16S-RD assay on clinical specimens will provide additional confirmation. Misdiagnosis and discharge of TB patients pose serious consequence to public health.

With regard to the potential utility of the 16S-RD assay for direct analysis of smear-positive respiratory specimens, there is a need to improve its current sensitivity, which can be achieved by further optimisation or by modifying it into a nested PCR format. An alternate method for optimal and maximal removal of PCR inhibitors should also be considered. When the assay sensitivity is sufficiently high to accommodate a quantitative potential, it is then possible to measure the bacillary load of samples which can be exploited in assessing its correlation to case infectiousness, besides monitoring the progress of chemotherapy. An improved assay with higher sensitivity also has potential for analysis of smear-negative specimens.

With regard to genotyping for assessment of potential epidemiological links, the combined assay can be adapted to target other lineage-defining polymorphisms relevant to different geographic locations, depending on the epidemiological questions to be resolved. It can therefore be adapted to complement the local, routine mycobacterial service in specified populations. Apart from assessing for potential recent transmission events, genotyping of Mtb strains can be useful in distinguishing between recurrent TB due exogenous reinfection and endogenous reactivation (van Rie *et al.*, 1999), and investigating and confirming false-positive cultures due to laboratory cross-contamination (Fitzpatrick *et al.*, 2004). Also, in the longer term, specific identification of Mtb lineages will also facilitate detection of associations between bacterial genotypes and clinical phenotypes (Caws *et al.*, 2008; de Jong *et al.*, 2008; Thwaites *et al.*, 2008).

The mask sampling study was envisaged to facilitate better understanding on the infectiousness of pulmonary TB cases with respect to quantities of Mtb aerosols produced and the pattern of their excretion. There was a significant challenge in the recruitment of patients into the study; very few subjects were recruited in the first year of the study but this picked up in the following year. Two detection assays were developed to assess and evaluate the sampling and quantitative potential of the mask approach for respiratory-borne Mtb, real-time PCR and the mycobacteriophage amplification assay; the latter was chosen as the analytical method for masks due to its higher sensitivity. Direct phage infection on masks using an antibiotic cocktail to control contamination by non-target organisms gave results that are potentially quantitative.

The preliminary evidence that mask sampling produces quantitative results with the method of direct phage infection on masks will facilitate investigation on the possible correlation between respiratory Mtb output and infectiousness of TB cases; the latter can be defined by epidemiological screening of both close and casual contacts for latent infection. Mask sampling data from a few patients in this study suggested the presence of a specific pattern of Mtb excretion; more bacilli are put into aerial suspension early in the morning than later in the day during expiratory manoeuvres. If this pattern of expectoration is proven true, it is plausible to speculate the role of host-pathogen interaction in sensing the difference in human throughout the day. The data from this study are preliminary and further investigation is required to verify this hypothesis.

Mask sampling enables direct collection of Mtb aerosols produced during expiratory activities. It is economical, non-invasive, and simple to perform. Mask sampling can be carried out under the comfort of patients' homes; sampling was performed from home for a few cases in the current study. The masks that were chosen for this study are standard face masks which are easily available in most hospitals and other healthcare institutions. Due to the nature of the processing method used, saturation of cells as a result of extended sampling is not an issue with the mask approach. Also, frequent wearing of face masks by TB patients might be beneficial in minimising the risk of infecting their contacts; similar types of face masks have been suggested to execute this role (Chen *et al.*, 1994; Chen and Li, 2005). The long-term benefit of this hygienic practice among TB patients with regard to community and nosocomial transmission warrants further investigation.

There was poor standardisation in sampling procedures during this study; duration of sampling varied among patients and there was no clear record on any expiratory manoeuvres which might have taken place. The latter can be resolved by the simultaneous use of a vocal monitor during mask sampling. The extremely close proximity to the patients during sampling made it unclear whether some bacilli would remain infectious after prolonged aerial suspension and long-distance spread. Most patients had initiated chemotherapy prior to mask sampling studies; however 50% of these still showed positive detection for Mtb. The effect of delay in processing mask samples on bacillary load was not assessed in this study. With regard to face masks as sampling devices, the consequence of consecutive expiratory activities on organisms already sampled on masks was not known. Besides that, face seal leak and re-aerosolisation of organisms from masks would need to be taken into consideration. Several patients experienced discomfort when wearing masks.

It is generally believed that AFB smear status is a useful predictor of case infectiousness; however, the discordance between smear positivity (Sultan *et al.*, 1960; Fennelly *et al.*, 2004) and case infectivity as well as TB transmission from smear-negative individuals (Blaht *et al.*, 1946; Catanzaro, 1982; Di Perri *et al.*, 1989; Behr *et al.*, 1999) have questioned this association. More reliable predictors for TB infectiousness are needed, and one of these is Mtb aerosol output, consistent with a study that demonstrated the correlation between cough frequency and infectivity (Loudon and Spohn, 1969). The mask sampling approach should therefore be exploited for assessment of case infectiousness in relation to smear status in order to verify the reliability and relevance of the latter in clinical practice. Upon this, the potential of the mask method in detecting Mtb from smear-negative patients warrants investigation. The outcomes of this investigation might show that mask infectivity results might be more useful than smear status in establishing the transmission risks of TB patients, and subsequently aiding decisions on respiratory isolation and ward discharge. In addition, mask sampling data might be potentially useful for monitoring treatment outcome and might contradict the general practice of releasing patients from respiratory isolation after two weeks of chemotherapy. Also, the mask approach could be used to assess the relative rates of Mtb aerosol production during different expiratory manoeuvres and therefore, their risks of infection could be established. The mask method could also be used to study the airborne transmission of Mtb from laryngeal TB patients. The concordance between mask sampling and quantitative 16S-

RD assay data could also be assessed, and together, would be valuable for better understanding of TB infectiousness.

If the sensitivity issue is resolved, the use of PCR-based approach to detect mask contamination would be ideal in the longer term, taking into account the possible shorter duration in yielding results and method versatility. With regard to the latter, the mask sampling approach has potential to be adapted for other diseases that are infectious by the airborne route. Indeed, Huynh and co-workers (2008) have developed and tested a simple mask-like device for sampling of exhaled respiratory virus aerosols for analysis by PCR. Apart from *Mtb*, membrane air sampling coupled to PCR detection method has also been used to detect airborne varicella-zoster virus (Sawyer *et al.*, 1994) and cytomegalovirus (McCluskey *et al.*, 1996) in hospital rooms. By combining phage infection and real-time PCR detection of host DNA, it might be possible to discriminate between live and dead organisms; the former could be a better predictor for case infectiousness and progress of treatment.

6.2 Conclusion

In conclusion, application of the combined 16S-RD assay to both clinical isolates and specimens will yield valuable information for clinical management as well as public health control and investigation. Meanwhile, the mask sampling approach has potential to be a better and promising alternative to AFB smear microscopy in gauging the infectiousness and assessing the transmission risks of TB patients, thereby fuelling subsequent public health efforts to prevent or interrupt disease transmission. With further validation and evaluation, the novel analytical methods developed in this study could be incorporated into routine TB diagnostics to complement or even replace conventional methods in current practice.

6.3 Summary of Aims Fulfilled

- ❖ When applied to clinical mycobacterial isolates, the 16S-RD assay is able to distinguish between MTBC and NTM, and assign MTBC strains to within or outside the EA-I lineage; the latter facilitates assessment of potential recent transmission events. Preliminary results indicate that the combined assay could be applied to direct specimen analysis to yield this information.
- ❖ The mask sampling coupled to phage detection method can be used to sample, detect, and potentially quantify respiratory-borne Mtb from AFB smear-positive pulmonary TB patients.

6.4 Summary of Future Studies

- ❖ To improve the sensitivity, specificity, and predictive values of the 16S-RD assay via modification of assay design and/or technical optimisation in order to improve its reliability and render it amenable for direct specimen analysis.
- ❖ To explore the potential utility of direct specimen analysis by the 16S-RD assay in assessing case infectiousness and monitoring progress of chemotherapy.
- ❖ To improve the sensitivity and quantitative potential of the mask sampling approach via modification of analytical procedure and/or technical optimisation for a more reliable assessment of the correlation between Mtb aerosol output and case infectiousness via standardised sampling parameters.
- ❖ To produce further data to investigate the possible presence of infectivity pattern among TB patients.
- ❖ To investigate the dynamics of Mtb aerosol output in relation to different stages of chemotherapy, expiratory manoeuvres, and clinical TB manifestations.
- ❖ To develop and evaluate a real-time PCR method for detection of mask contamination.

APPENDICES

Appendix 1

Development of the 16S-RD assay

Table I: Specificity test for the 16S assay with non-mycobacterial species

Organism	Amount of DNA tested (ng)	16S assay signals	Average T_m (°C)*
<i>Streptococcus pneumoniae</i> D39	128	T- S+	82.8
<i>Acinetobacter baumannii</i> AYE	2,655	T- S+	85.4
<i>Staphylococcus aureus</i> Newman	165	T- S+	83.5
<i>Escherichia coli</i> K-12	15	T- S+	85.0
<i>Pseudomonas aeruginosa</i> PA14	38	T- S-	N/A
<i>Shigella flexneri</i> 2a	2,125	T- S-	N/A

PCR conditions were as described in Section 3.2.4.1 except that SYBR Green signals were acquired at 82°C. SYBR Green-based melt analysis was performed with 1°C increments from 50-99°C, holding 5 seconds on the first step and 30 seconds on the following steps. Each sample was assayed in triplicate.

16S amplicon sequencing identities: *S. pneumoniae*, *S. pneumoniae*; *A. baumannii*, uncultured *Bacillus* sp.; *S. aureus*, uncultured *Staphylococcus* sp.; *E. coli*, uncultured Enterobacteriaceae species.

* N/A denotes not applicable.

Conventional 16S PCR

A gradient PCR was performed during the initial annealing temperature optimisation for the 16S assay. PCRs were carried out in 25- μ l volumes containing 10 ng of DNA, 250 nM each primer MYCO16SF and MYCO16SR, 1.25 U of Thermoprime *Taq* DNA Polymerase, 1x buffer, 3 mM MgCl₂, and 200 μ M dNTPs. PCRs were performed in the Dyad DNA Engine as follows: 94°C for 5 minutes to activate the *Taq* polymerase, followed by 40 cycles of 94°C for 20 seconds, 56-70°C for 1 minute, and 72°C for 1 minute. The resulting PCR amplicons were separated by electrophoresis on 2% w/v agarose gels as described in Section 3.2.5.1.

Table II: Effect of increasing the annealing temperature on the specificity of the 16S assay in mixed and undiluted non-mycobacterial samples

DNA mixture		Identification by melt analyses at different annealing temperatures				
		56°C	60°C	63°C	64°C	65°C
1+4	SP	Mtb	Mtb	Mtb	ND	ND
	AB	Mtb	Mtb	Mtb	ND	ND
	SA	Mtb	Mtb	Mtb	ND	ND
	EC	Mtb	Mtb	Mtb	ND	ND
1+10	SP	Mtb	Mtb	Mtb	Mtb	ND
	AB	Mtb	Mtb	Mtb	Mtb	ND
	SA	Mtb	Mtb	Mtb	Mtb	ND
	EC	Mtb	Mtb	Mtb	Mtb	ND
1+100	SP	Mtb	Mtb	Mtb	Mtb	Mtb
	AB	AB	Mtb	AB	Mtb	Mtb
	SA	Mtb	Mtb	Mtb	Mtb	Mtb
	EC	Mtb	Mtb	Mtb	Mtb	ND
Undiluted non-mycobacterial DNA	SP	SP	SP	Negative	Negative	Negative
	AB	AB	AB	AB	Negative	Negative
	SA	SA	SA	Negative	Negative	Negative
	EC	EC	Negative	Negative	Negative	ND

SP, *S. pneumoniae*; AB, *A. baumannii*; SA, *S. aureus*; EC, *E. coli*; ND, not done.

DNA samples were mixed Mtb CDC 1551 + non-mycobacterial species. Approximately 2×10^5 total 16S rDNA copies were tested per PCR for both mixed and undiluted DNA samples. Amount of undiluted DNA tested in pg: *S. pneumoniae*, 123; *A. baumannii*, 117; *S. aureus*, 106; and *E. coli*, 179. PCR conditions were as described in Table I.

Mtb was specifically detected by the 16S assay in almost all the DNA mixtures in various proportions except for the 1+100 mixtures of Mtb and *A. baumannii* with annealing temperatures of 56°C and 63°C. The detection of *A. baumannii* in the latter came as a surprise, since Mtb was specifically detected in similar mixture proportion at lower temperature of 60°C. This was possibly due to mispipetting of small-volume Mtb DNA. All the non-mycobacterial species tested undiluted were detected by the 16S assay with annealing temperature of 56°C. This was consistent with previous findings although more than 100-fold lower amounts of DNA were tested (Appendix 1, Table I). Only *E. coli* went undetected at annealing temperature of 60°C. Only *A. baumannii* remained detected when the temperature was increased to 63°C, indicating that this non-mycobacterial species was the most problematic to the 16S assay out of the four tested. At 64°C onwards, non-mycobacterial species in all the undiluted samples were not detected.

Table III: Optimisation of the 16S-RD assay for the magnesium ion concentration

Magnesium concentration (mM)	Average SYBR Green Ct	
	16S assay	RD assay
3.0	17.63	20.98
3.5	17.83	20.66
4.0	18.05	20.01
4.5	18.58	20.21
5.0	18.47	20.08

Each PCR was performed with approximately 10^5 Mtb CDC 1551 genome equivalents.

Table IV: Optimisation of the 16S-RD assay for primer and TaqMan probe concentrations

Primer concentration (nM)	TaqMan probe concentration (nM)	TaqMan Ct	
		16S assay	RD assay
50	50	28.00	ND
	150	25.88	ND
	250	26.52	ND
80	50	ND	26.27
	150	ND	23.54
	250	ND	23.37
250	50	20.25	21.90
	150	19.95	20.72
	250	19.72	20.40
500	50	20.35	21.70
	150	19.68	20.36
	250	19.83	19.71
900	50	20.40	ND
	150	19.77	ND
	250	19.97	ND

Each primer-probe combination was assayed once with approximately 10^5 Mtb CDC 1551 genome equivalents. ND denotes not done.

Eubacterial 16S assay

This was performed according to the protocol by K. Haldar (unpublished). PCRs were carried out in 25- μ l volumes containing 1 μ l of DNA lysate, 250 nM each primer 16S-338F and 16S-515R, and 1x ABsolute QPCR SYBR Green Mix. Amplification was performed in the Rotor-Gene machine as follows: 95°C for 15 minutes to activate the *Taq* polymerase, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 20 seconds, and 78°C for 20 seconds. SYBR Green signals were acquired at 78°C. Melt and PCR signal analyses were performed as in Section 3.2.4.1. All samples were assayed in duplicates.

Appendix 2

Preparation of *FASTPlaqueTB* media and reagents

FPTB Medium Plus

FPTB Medium was reconstituted by mixing the contents of the sachet with 270 ml of distilled water in a 500-ml Duran bottle. The powder was allowed to hydrate for 10 minutes and then mixed gently before autoclaving at 121°C for 10 minutes. One vial of FPTB Growth Supplement was added to sterile cooled FPTB Medium.

Actiphage

Actiphage was reconstituted by adding 1.1 ml of FPTB Medium Plus to the vial and mixed gently to resuspend.

Sensor cells

Sensor cells were reconstituted by adding 11 ml of FPTB Medium Plus to the vial and mixed gently to resuspend.

Virusol solution

5 ml of sterile distilled water was added to Virusol tablet in the vial and allowed to dissolve.

FPTB Agar

The agar was reconstituted by mixing the contents of the sachet with 60 ml of distilled water in a 100-ml Duran Bottle. The powder was allowed to hydrate for 10 minutes and then mixed gently before autoclaving at 121°C for 10 minutes.

Appendix 3

Detection of distribution of mycobacteria on face masks

Method

Mask segments (25 cm²), filter papers (25 cm²; Fisher Scientific), and polyvinyl chloride cling film (Terinex, Bedford) were treated with 600,000 µJ of UV in the Stratalinker UV Crosslinker. Each mask segment was spiked with eight 20-µl drops of *M. smegmatis* culture suspension (4 x 10⁵ CFU and its 7 ten-fold serial dilutions) and 20 µl of Middlebrook 7H9-OGC broth at known sites, and then left to dry. One filter paper was permeated with 0.5 ml of phage D29 (6 x 10⁷ PFU/ml), laid over a mask segment, wrapped in cling film, and incubated at 37°C for 1 hour. The filter paper and the cling film were then removed and discarded. Another filter paper was permeated with 0.5 ml of 10 mM FAS, laid over the mask segment, wrapped in cling film, and left at room temperature for 5 minutes. The filter paper and the cling film were removed and the mask segment placed on the surface of pre-poured 10-ml 7H9-OGC agar. Approximately 20 ml of molten overlay agar (7H9-OGC-0.75% w/v agar containing 50 µg/ml iodinitrophenyltetrazolium chloride and 10% v/v *M. smegmatis* lawn culture) was then poured over the mask segment, agar allowed to solidify, and the plate incubated at 37°C for up to 24 hours.

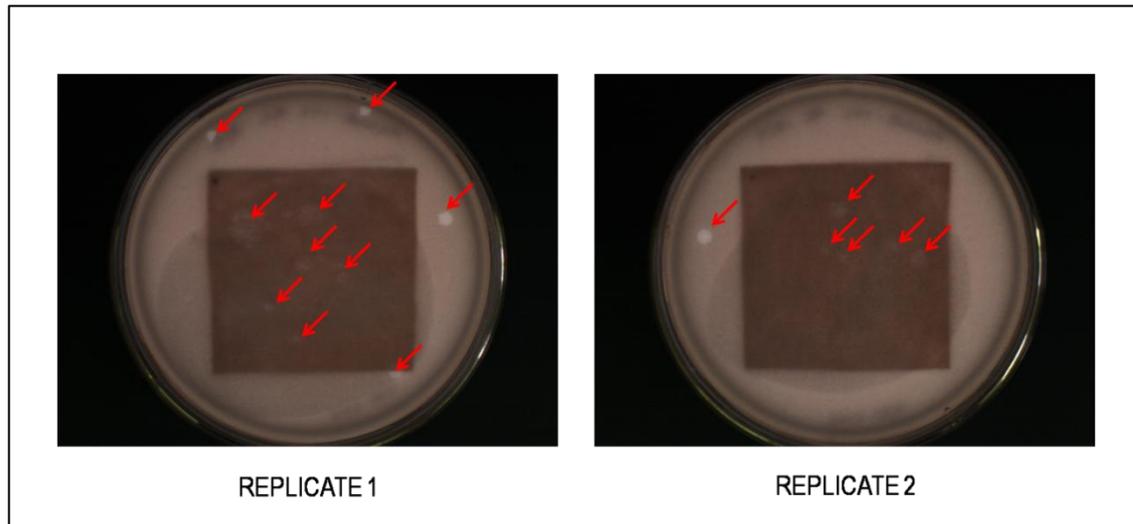
Results and Discussion

Detection of distribution of mycobacteria on face masks would reveal 'hot spots' for deposition of Mtb aerosols produced by TB patients, possibly vary among different respiratory activities. This would aid selective processing of areas on masks frequently contaminated by Mtb cells to save on the time and cost. One method designed and tested involved the use of tetrazolium salts to produce a coloured lawn of sensor cells in order to visualise plaques corresponding to sites of mycobacteria impaction on masks in good contrast. Following an experiment described in the method above, the distribution of plaques did not correspond to the sites on the mask segments contaminated with *M. smegmatis* cells (Figure I). A few plaques appeared on the *M. smegmatis* lawn outside the mask segments, which indicated that mycobacterial cells

Appendices

were easily dislodged when the molten overlay agar was poured over the masks. In a separate experiment, application of phage D29- and FAS-treated mask segments to pre-grown *M. smegmatis* lawn failed to yield any plaques, possibly because virucide was inadequately diluted by agar and it inactivated progeny phage particles liberated from target cells.

Figure I: Detection of distribution of *M. smegmatis* cells applied to face masks



Plaques are shown by red arrows for clarity.

Appendix 4

A two-tube combined TaqMan/SYBR Green to identify mycobacteria and detect single global lineage-defining polymorphisms in *Mycobacterium tuberculosis*

A Two-Tube Combined TaqMan/SYBR Green Assay to Identify Mycobacteria and Detect Single Global Lineage-Defining Polymorphisms in *Mycobacterium tuberculosis*

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We have developed a novel real-time PCR assay to identify and perform preliminary genotyping of mycobacteria in a manner tailored to our local service. Within a single thermocycler run, mycobacterial 16S rDNA and the *Mycobacterium tuberculosis* global lineage-defining RD750 polymorphism are targeted in separate reaction tubes, each of which includes both TaqMan and SYBR Green chemistries. The results of this 16S-RD assay differentiate *M. tuberculosis* complex (MTBC) from nontuberculous mycobacteria (NTM) and recognize whether or not MTBC isolates belong to the East African-Indian lineage, the single most frequently isolated global MTBC lineage in our service. If required, NTM amplicons may be sequenced to provide more specific identities. We report the technical performance of this assay on 88 mycobacteria-positive cultures and discuss its use in the initial management of mycobacterial infections. The 16S-RD assay correctly identified all 70 MTBC-positive cultures and 17 NTM-positive cultures while contemporaneously recognizing 26 MTBC isolates as within and 44 outside the East African-Indian lineage. In artificial samples, the combined assay also showed limited potential to detect mixed mycobacterial infections (MTBC/NTM) and tuberculosis infections involving more than one global MTBC lineage. The approach we have established can be readily tailored to targets of particular value for any mycobacterial diagnostic service, thereby optimizing the value of the results for local clinical and public health management of mycobacte-

rial infections. (J Mol Diagn 2010, 12:250–256; DOI: 10.2353/jmoldx.2010.090030)

When a possible case of mycobacterial infection is detected, both the clinical and public health management depend critically on whether the causative agent is *Mycobacterium tuberculosis* or a nontuberculous mycobacterium (NTM). In the former case, treatment and prevention of further person-to-person transmission are urgent concerns while, in the latter, infections are predominantly sporadic, derive from environmental sources of infection, and often reflect pre-existing conditions in the patient.^{1–3} Although there are several promising direct molecular assays for *M. tuberculosis*,^{4–9} culture is the most sensitive means to detect specific mycobacterial infections and remains the mainstay of diagnostic services in well-resourced laboratories. However, when cultures are detected positive and the presence of mycobacteria confirmed by acid fast staining, it is not known whether the isolated mycobacteria belong to the *Mycobacterium tuberculosis* complex (MTBC) or the NTM group. Furthermore, when the possibility of recent transmission of tuberculosis (TB) arises, no information is available regarding the relatedness of the isolate to other contemporary isolates with potential epidemiological connections to the new case.

Cultures positive for mycobacteria are referred for further testing to address these points and, in the UK, this generally involves transmission to a reference laboratory where the caseload enables economies of scale in deploying established molecular identification and MTBC genotyping procedures. Thus, definitive recognition of

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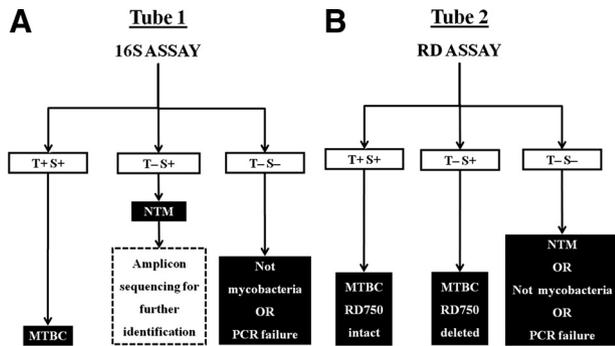


Figure 1. Schematic illustration of the combined 16S-RD assay. **A:** For the 16S assay (tube 1), TaqMan (T) signals show the presence or absence of MTBC-specific 16S rDNA sequence, whereas the SYBR Green (S) signals indicate the presence or absence of *Mycobacterium* genus-specific 16S rDNA sequence. **B:** For the RD assay (tube 2), T signals show the presence or absence of RD750, whereas the S signals act as further confirmation of MTBC-specific sequence and a positive assay control when the T signal is negative. T+ S- signals would be anomalous in both assays, indicating amplicons that do not interact with SYBR Green; no results were obtained in this category.

infections as MTBC or NTM and the detection of potential epidemiological links may be delayed by days in the former case and weeks in the latter. In our service, where TB and NTM infections affect between 200 and 400 patients per annum, these delays may affect the management of a significant number of patients. Until identity and genotyping information is available, a precautionary response to a particular case in which mycobacteria have been isolated may lead to inappropriate isolation and treatment of patients for TB and unnecessary public health investigations.

To address these issues, we have developed a real-time PCR assay for analysis of mycobacteria-positive cultures. The assay targets the mycobacterial 16S rDNA and the global lineage-defining RD750 polymorphism in separate reactions within a single PCR run; it also combines TaqMan and SYBR Green technologies to simultaneously differentiate two products in each reaction tube. The 16S assay distinguishes between MTBC and NTM by combining newly designed universal mycobacterial 16S primers and a MTBC-specific 16S-targeted TaqMan probe. The RD assay enables differentiation of MTBC strains with intact (RD750⁺) and deleted RD750 (RD750⁻) regions by use of hemi-nested primers and an RD750⁺-specific TaqMan probe. SYBR Green chemistry is used in both assays to obtain a total PCR amplicon readout. The principles of the combined 16S-RD assay are illustrated in Figures 1 and 2.

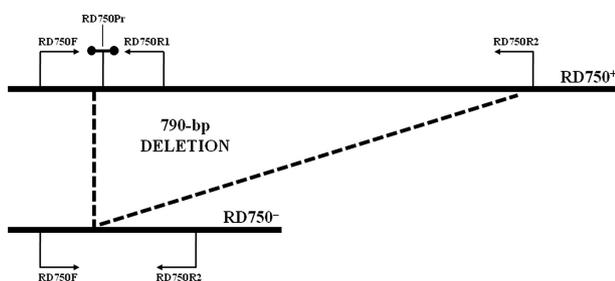


Figure 2. Diagrammatic representation of the RD assay targets.

RD750 was first described as a deletion in *Rv1519* present in *M. tuberculosis* strain CH, a local isolate responsible for a large school-associated TB outbreak in Leicester in 2001.¹⁰ This large sequence polymorphism was subsequently designated RD750 and recognized as one of six phylogeographical lineage-defining deletions in a global collection of *M. tuberculosis* strains.¹¹ It defines the East African-Indian (EA-I) lineage,¹¹ the differentiation of which is also supported by genomic and multilocus sequencing data^{11,12} as well as spoligotyping. By the latter method, it is also designated as the Central Asian lineage.¹³ Intriguingly, the RD750 deletion has been associated with an immunosubversive phenotype¹⁴; it also occurs in approximately half of our local *M. tuberculosis* isolates (J. Malkin and H. Perera, unpublished data). The predominance of this lineage among Leicester *M. tuberculosis* isolates likely reflects our diverse ethnic population. In the 2001 census, 31% of Leicester city was reported to have an Asian ethnic background with the majority being Indian from either East Africa or Gujarat.¹⁵ In accordance with previous studies in the UK, our regional genotyping service reports a preponderance of the five-locus variable-number tandem repeat (VNTR) profile x2234/5 in this ethnic group.¹⁶ In this context, it will be appreciated that, in our service, early recognition of whether new *M. tuberculosis* isolates belong to the EA-I group through a single rapid assay offers the highest probability of obtaining strain information that might be discriminatory in a local context. For example, where two potentially linked cases yield RD750⁺ and RD750⁻ isolates, transmission can be excluded. Also, in the longer term, specific identification of *M. tuberculosis* lineages will facilitate detection of associations between clinical phenotypes and bacterial genotypes.¹⁷⁻¹⁹ This approach can be adapted to target any lineage-defining polymorphisms that are appropriate to a particular local service.

In the present study, we report evaluation of the 16S-RD assay on positive cultures. A by-product of the assay design (Figure 1, A and B) is that it has the potential to detect mixed infections (MTBC + NTM or RD750⁺ + RD750⁻) in clinical specimens, a phenomenon previously recognized by molecular analysis.²⁰ We therefore also report on the performance of our combined assay to detect mixed mycobacterial infections.

Materials and Methods

Bacterial Strains

Strains and DNA used in this study included clinical isolates from the Department of Clinical Microbiology, University Hospitals of Leicester (UHL), departmental stocks (DS), and research materials from Colorado State University (CSU). The following were used: RD750⁺ (CDC 1551²¹ from CSU) and RD750⁻ (CH¹⁰ from DS) strains of *M. tuberculosis*, *M. smegmatis* MC²155 (DS), *Corynebacterium glutamicum* ATCC 13032 (DS), *Streptomyces fradiae* T59235 (DS), *Rhodococcus* spp. (DS), *Streptococcus pneumoniae* D39 (DS), *Staphylococcus aureus* Newman (DS), *Acinetobacter baumannii* AYE (DS),

Table 1. Oligonucleotide Primers and TaqMan Probes for the Combined 16S-RD Assay

PCR assay	Primer/Probe designation	Nucleotide sequence
16S	MYCO16SF	5'-GAAACTGGGTCTAATACCG-3'
	MYCO16SR	5'-ATCTCAGTCCCAGTGTGG-3'
	MYCO16SPr	5'-ROX-TCCACCACAAGACATGCATCCCCTG-BHQ2-3'
RD	RD750F	5'-CTTAAGCGTCCGCGTATCC-3'
	RD750R1	5'-GCCACAGCTGTACAGGTCAA-3'
	RD750R2	5'-AACTTTCGGCGGTCACTGTA-3'
	RD750Pr	5'-Cy5-CCGTTGGCGCAGAGCACTCC-BBQ-3'
<i>Rv1519</i>	Rv1519F	5'-GGTGAGAGCGACGACATCAAG-3'
	Rv1519R	5'-TGTAGCGACAGCATCGTCATCC-3'

ROX, 6-carboxy-X-rhodamine (excitation, 585 nm/detection, 610 nm); BHQ2, Black Hole Quencher 2 (absorbance 550–650 nm); Cy5, cyanine 5 (excitation 625 nm/detection 660 nm); BBQ, BlackBerry Quencher (absorbance 550–750 nm).

Pseudomonas aeruginosa PA14 (DS), *Escherichia coli* K-12 (DS) *Haemophilus influenzae* (UHL), and *Moraxella catarrhalis* (UHL).

DNA Isolation

Mycobacterial growth indicator tube (MGIT) cultures were centrifuged at $755 \times g$ for 22 minutes, and the resulting pellets were resuspended in 0.2 ml of sterile water. Colonies were scrapped off Löwenstein-Jensen (LJ) slopes and resuspended in 0.2 ml of sterile water. Culture suspensions from MGIT and LJ were boiled at 100°C for 30 minutes, centrifuged at $16,100 \times g$ for 2 minutes, and the resulting supernatants used for PCR analyses.

Oligonucleotide Primer and Probe Design

The oligonucleotide primers and TaqMan probes used are listed in Table 1. Full-length 16S ribosomal DNA sequences representing mycobacteria isolated from humans were retrieved from The Institute for Genomic Research (TIGR) or the Ribosomal Database Project II.²² The sequences were aligned using the ClustalX program with default settings, and alignments were processed with a custom built algorithm. MYCO16SF and MYCO16SR primers were designed targeting 16S rDNA region conserved in all of the retrieved mycobacterial sequences. A BLASTN search with these primers reveals that several other actinobacteria may give amplicons with these primers. However, with the possible exception of *Nocardia* spp., none of these are acid fast. The MTBC-specific 16S TaqMan probe (MYCO16SPr) was that previously reported but with a different fluorophore-quencher pair to allow combination with SYBR Green.²³ The three RD750 primers were designed using Primer3 to give products of 126 and 193 bp with *M. tuberculosis* strains H37Rv and CH, respectively (J. Malkin, unpublished data). RD750 TaqMan probe (RD750Pr) was designed and synthesized by TIB MOLBIOL (Berlin, Germany). A BLASTN search demonstrated that none of the primers and TaqMan probes for both the 16S and RD assays shared significant similarity with other known nucleotide sequences or each other. Rv1519F and Rv1519R primers were from our previous study.¹⁰ All primers and MYCO16SPr were synthesized by MWG Biotech (Ebersberg, Germany).

Combined 16S-RD Assay

Each DNA lysate was tested operator-blinded in duplicate for both the 16S and RD assays. For the 16S assay, PCRs were performed in 25- μ l volumes containing 5 μ l of DNA lysate, 250 nmol/L of each primer MYCO16SF and MYCO16SR, 150 nmol/L MYCO16SPr, and 1 \times Absolute QPCR SYBR Green Mix (ABgene, Epsom, UK). For the RD assay, PCRs were performed in 25- μ l volumes containing 5 μ l of DNA lysate, 80 nmol/L of each primer RD750F, RD750R1, and RD750R2, 150 nmol/L RD750Pr, and 1 \times Absolute QPCR SYBR Green Mix. PCRs were performed in a Rotor-Gene machine (Qiagen, Valencia, CA) as follows: 95°C for 15 minutes to activate Taq polymerase, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, 72°C for 20 seconds, and 84°C for 20 seconds. ROX and Cy5 signals were acquired at 60°C, whereas SYBR Green signals were acquired at 84°C. When testing the effect of combining TaqMan and SYBR Green on assay performance, TaqMan only reactions were performed using Absolute QPCR Mix (ABgene), and all determinations were done in triplicate.

PCRs were repeated for all samples yielding no or anomalous signals with neat and diluted DNA lysates (1/10 and 1/100). Melt curves were examined for all assays. Mycobacterial 16S amplicons consistently yielded melt temperatures of $88.2 \pm 0.2^\circ\text{C}$. 16S PCR products for all reactions yielding SYBR Green signals only and randomly selected reactions yielding both TaqMan and SYBR Green signals were subjected to gel analysis, gel purification using QIAquick Gel Extraction Kit (Qiagen), and DNA sequencing (MWG Biotech). 16S rDNA sequences were processed and mycobacterial species were identified using the Ribosomal Differentiation of Medical Microorganisms database.²⁴

Rv1519 PCR

All samples were tested for the presence of intact or deleted *Rv1519* by our established conventional PCR assay as described previously.¹⁰ Each DNA lysate was tested operator-blinded. PCR was repeated for all samples yielding no bands or multiple bands in 25- μ l vol-

umes with 5 μl of neat and diluted DNA lysates (1/10 and 1/100).

Routine Testing of Clinical Mycobacterial Isolates at the Reference Laboratory

All clinical mycobacterial isolates were identified as part of the routine service by the Regional Centre for Mycobacteriology, Birmingham Heartlands Hospital, Birmingham, UK (reference laboratory) using the GenoType MTBC DNA strips for identification of MTBC and the GenoType *Mycobacterium* (consisting of two kits: Clinical Mycobacteria and Additional Species) for identification of NTM (Hain Lifescience, Nehren, Germany). Our 16S assay results were compared against these identification results as the gold standard. This laboratory also subjects all MTBC isolates to 15-locus VNTR-mycobacterial interspersed repetitive units genotyping. The five-locus VNTR analysis targets ETR A to ETR E as originally described by Frothingham and Meeker-O'Connell,²⁵ and mycobacterial interspersed repetitive units analysis follows the 10-locus method of Supply et al.²⁶

Simulated Mixed Infection Samples

For the mixed MTB-NTM infection tests, *M. smegmatis* was used to represent NTM. For the mixed MTBC strain studies, *M. tuberculosis* strains CDC 1551 (RD750⁺) and CH (RD750⁻) were used. Target DNA was diluted to 10⁵, 10⁴, and 10³ 16S rDNA copies/ μl . These preparations were mixed in a checkerboard format. PCRs were performed as above except that only 2 μl of mixed template DNA was used per reaction. All PCR assays were performed in duplicate.

Results

Effect of Combining TaqMan and SYBR Green Chemistries in Single Reactions

We tested the effects of combining TaqMan and SYBR Green chemistries in a single-reaction tube with *M. tuberculosis* CDC 1551 and *M. smegmatis* DNA for the 16S assay and *M. tuberculosis* CDC 1551 and CH DNA for the RD assay with target amounts in the range of 10³–10⁷ gene copies, and illustrative signal tracings are shown in Figure 3, A and B. Cycle threshold (Ct) values generated by TaqMan and SYBR Green for single and combined assays (six pairs of values for each chemistry) were compared by paired *t* tests. Although presence of the TaqMan probe had no measurable effect on SYBR Green Ct values, irrespective of the probe binding status of the reaction ($P > 0.1$), adding SYBR Green significantly increased the Ct for TaqMan signals ($P < 0.05$). Ct values were greater by approximately half a cycle and three cycles at high and at low target concentrations respectively (data not shown). The effect was more pronounced for the RD assay.

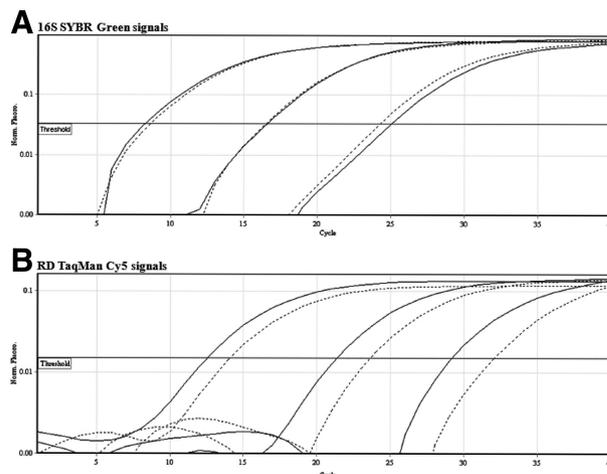


Figure 3. Effect of combining TaqMan and SYBR Green chemistries in single real-time PCRs. *M. tuberculosis* CDC 1551 target at 10⁷ gene copies and two 100-fold serial dilutions were tested. Dotted lines represent reactions containing both TaqMan and SYBR Green, whereas solid lines represent reactions containing either SYBR Green (A) or TaqMan (B) alone. A shows 16S SYBR Green signals, and B shows RD TaqMan Cy5 signals.

Specificity of the 16S-RD Assay

In view of the potential of the mycobacterial 16S primers to produce amplicons from nonmycobacterial actinobacteria, DNA extracts from *Corynebacterium glutamicum*, *Rhodococcus* sp., and *Streptomyces fradiae* were studied. Although the first two organisms yielded positive 16S SYBR Green signals, TaqMan signals were absent and the RD assay was negative for both signals (16S: T– S+; RD: T– S–). There were no significant signals detected for all other organisms tested (see *Materials and Methods* for details).

Investigation of Positive Cultures

A total of 88 cultures from different patients deemed probable mycobacteria-positive following Ziehl-Neelsen (ZN) acid fast staining obtained from our routine service between January and November 2007 were analyzed. All cultures except for one were confirmed to be positive for mycobacteria by the reference laboratory. This MGIT lysate gave no signal in either the 16S (T– S–) or RD assay (T– S–), consistent with the reference laboratory report of “no acid-fast bacilli seen.”

Of the 88 positive cultures, 42 were MGIT and 46 were LJ cultures. The combined 16S-RD assay results for analysis of these cultures are displayed in Table 2. The 16S assay and the RD assay identified 68 and 70 MTBC-positive cultures, respectively. Two MGIT lysates did not give an MTBC signal with the 16S TaqMan probe but were positive for 16S SYBR Green (T– S+) and for both RD TaqMan and SYBR Green signals (T+ S+). Direct sequencing of these two 16S amplicons yielded indeterminate results. Since both the RD750 primers and TaqMan probe used in this assay are MTBC specific, we concluded that the “false-negative” 16S TaqMan results associated with these two MGIT lysates probably may have been due to cross-reactive nonmycobacterial DNA

Table 2. Combined 16S-RD Assay Applied to 88 Consecutive Mycobacteria-Positive Cultures

	RD assay			Total
	T+ S+	T- S+	T- S-	
16S assay				
T+ S+	42	26	0	68
T- S+	2	0	17	19
T- S-	0	0	1	1
Total	44	26	18	88

T, TaqMan signal; S, SYBR Green signal. See Fig. 1 for further explanation.

present within these lysates. Reference laboratory results subsequently confirmed that the RD assay had correctly identified all 70 MTBC-positive cultures within the set of 88 cultures investigated. Seventeen 16S-RD results indicated NTM-positive cultures (16S T- S+ and RD T- S-); these again were subsequently confirmed by the reference laboratory.

16S Amplicon Sequencing and Identification of NTM

Comparisons of sequences of the 16S amplicons from the 17 probable NTM-positive cultures with those in the Ribosomal Differentiation of Medical Microorganisms database, yielded definitive results for 15 cultures (8 *M. avium* complex, 2 *M. gordonae*, 2 *M. kansasii*, 1 *M. abscessus*, 1 *M. peregrinum*, and 1 *M. malmoense*), which were also found to be concordant with those of the reference laboratory. 16S amplicons from two NTM-positive MGIT cultures failed to yield DNA sequence data; the two cultures were eventually identified as *M. gordonae* by the reference laboratory.

M. tuberculosis RD750 and VNTR Genotyping

Initial results indicated that 44 of the MTBC strains were RD750⁺ (RD T+ S+) and 26 were RD750⁻ (RD T- S+). These 70 lysates were subsequently analyzed by our established *Rv1519* PCR assay, and their VNTR profiles were received from the reference laboratory. Although the majority of our RD750⁻ isolates genotyped as VNTR 42234/5, a more extensive study has revealed that all can be encompassed within the profile x2234/5 (R.J.

Smith, unpublished data), and we have used this latter profile to recognize concordance or otherwise with our real-time RD assay. For 62 lysates, the results of all three genotyping assays (RD, *Rv1519* PCR, and VNTR) were concordant. In eight cases, no amplicons were obtained with the *Rv1519* PCR, but RD and VNTR results were concordant. Occasional *Rv1519* PCR failures are expected given the relatively large 1- to 3-kb amplicon sought and the crude DNA preparation used in this assay.

Potential to Detect Mixed Infections

To examine the capacity of our combined assay to detect mixed infections involving either a combination of MTBC and NTM or MTBC strains with intact and deleted RD750, we studied its performance using mixtures of purified DNA as detailed in Table 3. We note that it is only possible to reliably recognize the target detected solely by SYBR Green chemistry (NTM or RD750⁻ in the 16S and RD assays, respectively) when the copy number detected by the SYBR Green signal significantly exceeds that detected by the TaqMan probe in the same sample. Only in this situation can the higher SYBR Green signal be confidently interpreted to show the presence of the second TaqMan-negative target. This noted, it is apparent that the combined assay will not detect mixed infections when the SYBR Green signals are approximately equal to the TaqMan signals. The presence of NTM species in mixed MTBC-NTM samples did not affect the performance of the RD assay (data not shown). We emphasize that where signals suggestive of a mixed infection are obtained, further analyses such as cloning and sequencing in the case of the 16S assay and gel electrophoresis in the case of the RD assay could be applied to obtain further confirmation.

Discussion

We have developed a molecular assay capable of distinguishing MTBC and NTM when applied to positive LJ and MGIT cultures; further identification of NTM species is achieved by 16S rDNA sequencing. When positive for MTBC, the assay also determines whether the MTBC strain belongs to the EA-I lineage. Since roughly half of the MTBC isolates obtained by our service belong to this

Table 3. Potential of the Combined 16S-RD Assay to Detect Mixed Infections

	<i>M. smegmatis</i> (16S SYBR Green signal)			<i>M. tuberculosis</i> CH (RD SYBR Green signal)		
	10 ^{5*}	10 ⁴	10 ³	10 ⁵	10 ⁴	10 ³
<i>M. tuberculosis</i> CDC 1551 signals [†]						
10 ⁵	BOTH [‡]	CDC	CDC	BOTH	CDC	CDC
10 ⁴	BOTH	BOTH [§]	CDC	BOTH	BOTH	CDC
10 ³	MS	BOTH	CDC	BOTH	BOTH	BOTH

*Figures denote number of target DNA copies added.

[†]TaqMan signals from both 16S and RD assays.

[‡]BOTH, both targets detected; CDC, only *M. tuberculosis* CDC 1551 (RD750⁺) detected; MS, only *M. smegmatis* detected. No *M. tuberculosis* CH (RD750⁻) only signals were obtained.

[§]Borderline *M. smegmatis* signal.

lineage, the result provides an opportunity for preliminary epidemiological discrimination. As far as we are aware, the simultaneous use of TaqMan and SYBR Green chemistries within a single tube, as deployed in our assay, has not been reported previously.

The combination of the two signal generating chemistries had little consequence for the performance of the assay on positive mycobacterial cultures. Inclusion of TaqMan probes had no detectable effect on SYBR Green results. In contrast, inclusion of SYBR Green led to an increase in TaqMan Ct values, particularly at low target concentrations. This might reflect the impact of SYBR Green binding to the same region as the TaqMan probes and resultant lower probe cleavage rates. Although this had little consequence for the present study, it could lead to decreased assay sensitivity in clinical samples.

As expected, the combined assay gave 16S amplicons with some actinobacteria but was universally negative with the other bacterial targets tested. Since the assay is designed for use in conjunction with ZN-positive samples this should not lead to ambiguous results. Although *Nocardia* spp. are weakly ZN positive and can be isolated with both MGIT and LJ media, they will also stain Gram-positive. Thus, when applied to positive cultures, which are routinely ZN stained, additional consideration of Gram stain results and the results of conventional cultures should rule out any ambiguity in interpretation.

The 16S-RD assay correctly identified all 70 MTBC-positive cultures. Two MTBC-positive MGIT cultures failed to give TaqMan signals in the 16S assay. These results derived from MGIT culture lysates in which the possible presence of nonmycobacterial signals may have been a confounding factor. Interestingly the 16S melt temperature values obtained for these samples were more than 1°C lower than those obtained from confirmed mycobacterial amplicons and sequencing revealed amplicons with no clear identity (data not shown). It seems that occasionally the 16S assay produces anomalous amplicons that may be recognized by their low melt temperature values. MTBC DNA in these samples can be detected by the parallel RD assay. Thus, at the present time, there appear to be limitations to the negative predictive value of the 16S assay alone. We currently advocate use of the combined 16S-RD assay where our available data indicate all negative RD results are true negatives for MTBC.

Of the remaining 18 positive cultures analyzed, the combined assay correctly identified the presence of NTM sequence in all 17 reference laboratory-confirmed cases. Subsequent sequencing and Ribosomal Differentiation of Medical Microorganisms analysis produced NTM identities concordant with the reference laboratory in 15 cases. There were two primary sequencing failures, both from cultures yielding *M. goodii*. A final MGIT culture graded positive failed to yield any PCR signals and was subsequently determined to be negative for mycobacteria by the reference laboratory. This occurs in up to 5% of the MGIT cultures graded positive for acid-fast bacilli by our service. Application of the 16S-RD assay to such samples graded acid-fast bacilli-positive therefore has the potential to recognize such true negatives before forwarding for analysis by the reference laboratory. Since

a preliminary report indicating the likely isolation of mycobacteria is routinely issued to clinicians when cultures are forwarded to the reference laboratory, use of our assay to eliminate these preliminary false-positive results warrants further analysis.

Of 70 MTBC signals obtained, the RD assay assigned 44 isolates to RD750 intact and 26 to RD750 deleted. In each case where the conventional *Rv1519* PCR assay produced an *Rv1519*-related amplicon, the RD assay results were confirmed. As expected, all of the RD750⁻ strains fell into the VNTR x2234/5 group.

Our results demonstrate that within a total processing time of 4 hours the combined 16S-RD assay reliably identifies mycobacteria-positive cultures, differentiates between MTBC and NTM, and assigns the MTBC signals to within or outside the EA-I lineage. In our series of 88 positive cultures, this rapid assay gave primary results that were concordant with subsequent reference laboratory analysis in every case.

We also explored the potential of our combined assay to detect mixed infections. The results show that our assay has potential to detect mixed infections due to MTBC strains belonging to different lineages and mixed MTBC/NTM infections. This potential is limited to samples in which the SYBR Green signals generated by our assay indicate a significant excess of copy numbers over those attributable to the TaqMan probe in the same reaction. In this way, our study has demonstrated both the value and limitation of using a combination of TaqMan and SYBR Green signals in real-time assays.

Turning to the current clinical utility of the 16S-RD assay, early confirmation of MTBC or NTM infection is of established value and allows for appropriate clinical and public health management. Assignment of MTBC-positive cultures to within or outside a particular global lineage allows for recognition or exclusion of links between contemporary isolates and could therefore facilitate appropriate responses. Evaluation of the practical utility of such preliminary information is currently in progress.

Our assay could be adapted to target other lineage-defining RDs appropriate to different geographic locations. Indeed, one might even envisage applying an individually selected RD target analyses to different samples depending on the specific epidemiological question to be resolved. This assay can therefore be adapted to complement the local, routine mycobacterial service in specified populations.

We conclude that we have established a rapid molecular assay that confirms the presence of mycobacteria, differentiates between MTBC and NTM, and assigns MTBC signals to within or outside the EA-I lineage. The assay is reliable when applied to positive LJ and MGIT cultures, has potential to detect mixed infections, and is being further developed for direct application to clinical samples.

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