A Molecular Approach to the Diagnosis, Assessment, Monitoring and Treatment of Pulmonary Non-Tuberculous Mycobacterial Disease

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Abstract

Introduction: Non-Tuberculous Mycobacteria (NTM) can cause disease of the lungs and sinuses, lymph nodes, joints and central nervous system as well as disseminated infections in immunocompromised individuals. Efforts to tackle infections in NTM are hampered by a lack of reliable biomarkers for diagnosis, assessment of disease activity, and prognostication.

Aims: The broad aims of this thesis are:

1. to develop molecular assays capable of quantifying the 6 most common pathogenic mycobacteria (*M. abscessus, M. avium, M. intracellulare, M. malmoense, M. kansasii, M. xenopi*) and calculate comparative sensitivities and specificities for each assay.
2. to assess patients' clinical course over 12 – 18 months by performing the developed molecular assays against DNA extracted from sputum from patients with NTM infection.
3. to assess dynamic bacterial changes of the lung microbiome in patients on treatment for NTM disease and those who are treatment naïve.

Methods: DNA was extracted from a total of 410 sputum samples obtained from 38 patients who were either:

- commencing treatment for either *M. abscessus* or *Mycobacterium avium* complex.
- considered colonised with *M. abscessus* or *Mycobacterium avium* complex (i.e. cultured NTM but were not deemed to have infection as they did not meet ATS or BTS criteria for disease).
- Diagnosed with cystic fibrosis (CF) or non-CF bronchiectasis but had never cultured NTM.

For the development of quantitative molecular assays, NTM *hsp65* gene sequences were aligned and interrogated for areas of variability. These variable regions enabled the creation of species specific probes. *In vitro* sensitivity and specificity for each probe was determined by testing each probe against a panel of plasmids containing *hsp65*

gene inserts from different NTM species. Quantification accuracy was determined by using each assay against a mock community containing serial dilutions of target DNA.

Each sample was tested with the probes targeting: *M. abscessus, M. avium* and *M. intracellulare* producing a longitudinal assessment of NTM copy number during each patient's clinical course.

In addition, a total of 64 samples from 16 patients underwent 16S rRNA gene sequencing to characterise longitudinal changes in the microbiome of both NTM disease and controls.

Results: *In vitro* sensitivity for the custom assays were 100% and specificity ranged from 91.6% to 100%. In terms of quantification accuracy, there was no significant difference between the measured results of each assay and the expected values when performed in singleplex. The assays were able to accurately determine NTM copy number to a theoretical limit of 10 copies/µl.

When used against samples derived from human sputum and using culture results as a gold standard, the sensitivity of the assay for *M. abscessus* was found to be 0.87 and 0.86 for MAC. The specificity of the assay for *M. abscessus* was 0.95 and 0.62 for MAC. The negative predictive value of the assay for *M. abscessus* was 0.98 and 0.95 for MAC. This resulted in an AUC of 0.92 for *M. abscessus* and 0.74 for MAC.

Longitudinal analysis of the lung microbiome using 16SrRNA gene sequencing showed that bacterial burden initially decreases after initiation of antibiotic therapy but begins to return to normal levels over several months of antibiotic therapy. This effect is mirrored by changes in alpha diversity. The decrease in bacterial burden and loss of alpha diversity was found to be secondary to significant changes in specific genera such as *Veillonella* and *Streptococcus*. The abundance of other Proteobacteria such as *Pseudomonas* remain relatively constant.

Conclusion: The molecular assay has shown high *in vitro* sensitivity and specificity for the detection and accurate quantification of the 6 most commonly pathogenic NTM species. The assays successfully identified NTM DNA from human sputum samples.

A notable association between NTM copy number and the cessation of one or more antibiotics existed (i.e. when one antibiotic was stopped because of patient intolerance, NTM copy number increased, often having been unrecordable prior to this). The qPCR assays developed in this thesis provide an affordable, real time and rapid measurement of NTM burden allowing clinicians to act on problematic results sooner than currently possible.

There was no significant difference between the microbiome in bronchiectasis and cystic fibrosis nor was there a significant difference between the microbiome in patients requiring treatment for NTM and those who did not. Patients receiving treatment experienced an initial decrease in bacterial burden over the first weeks of treatment followed by a gradual increase towards baseline over the next weeks to months. This change was mirrored in measures of alpha diversity. Changes in abundance and diversity were accounted for by decreases in specific bacteria whilst the abundance of other bacteria increased, occupying the microbial niche created. These bacteria (for example *Pseudomonas spp*) are often associated with morbidity.

Declaration of originality

I declare that the body of work constituting this thesis is entirely my own original work, except where appropriately referenced. All experiments including data analysis were conducted by myself except where explicitly stated.

Copyright declaration

Acknowledgements

I commenced this thesis in 2016 and over the last five years have experienced several amazing highs and a low that has forced the whole world to pause. During the COVID-19 epidemic I have seen suffering and distress that has forced me to consider what I perceive as important and I owe a debt to many people for helping me navigate these times and this thesis.

I would like to start by thanking my academic supervisors Professors Miriam Moffatt and William Cookson for giving me the opportunity to pursue academia. It is a privilege for a clinician to be granted time to dip one's toes into the world that produces the evidence that we base our practice on. The work that is performed by the team has not only interested me over the last few years but has truly served to inspire me.

I first met my clinical supervisor, Professor Michael Loebinger, in 2013 and he has taken me under his wing for the past 8 years or so. He has been a fantastic primary supervisor who has pushed me to not only be a better clinician but has sparked my interest in research also.

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Over the last five years the number of people that I consider to be my family have grown and it is to these people that I dedicate this thesis.

To Helen and John thank you for being so welcoming and supporting Hayley and I in all our ambitions and endeavours.

To Auntie Katy and Mark, thank you for all you have done. The list is far too long to document here. You are remarkably generous and I am in your debt.

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List of Commonly Used Abbreviations

AFB - Acid fast bacilli
ATS - American Thoracic Society
AUC - Area under the [ROC] curve
BLAST - Basic local alignment search tool
BMI - Body mass index
bp - Base pairs
BTS - British Thoracic Society
^{o}C - Degrees Celcius
CF - Cystic fibrosis
COPD - Chronic obstructive pulmonary disease
cm - Centimetre
CT - Computed tomography
CTAB - Hexadecyl-trimethyl-ammonium bromide
dL - Decilitre
DNA - Deoxyribonucleic acid
dsDNA – Double stranded DNA
DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA - Ethylenediaminetetraacetic acid
FEV_1 - Forced expiratory volume in 1 second
FVC - Forced vital capacity
g - Gram
HIV - Human immunodeficiency virus
kg - Kilogram
L - Litre
LB - Lysogeny broth
m - Metre
MAC - Mycobacterium avium complex
mg - Milligram
ml – Millilitre
µl - Microlitre
mmol - Millimole
ng - Nanogram
NMDS - Non-metric multidimensional scaling
nmol - Nanomole
NTM - Nontuberculous mycobacteria
OTU - Operational taxonomic unit
PCR - Polymerase chain reaction
PEG - Polyethylene glycol
PERMANOVA - Permuted analysis of variance
qPCR - Quantitative PCR
RNA - Ribonucleic acid
ROC - Receiver operating curve
TE - Tris-EDTA
UV - Ultraviolet

Chapter 1: Introduction

Non-Tuberculous Mycobacteria (NTM) refer to all species belonging to the genus Mycobacterium but excluding those belonging to the tuberculosis complex (*Mycobaterium tuberculosis, M. bovis, M. africanum, M. microti, M. canetti, M. caprae, M. pinnipedii, M. suricattae* and *M. mungi*) and leprosy complex (*M. leprae* and *M. lepromatosis*). NTM can cause disease of the lungs and sinuses, lymph nodes, joints and central nervous system as well as causing disseminated infections in immunocompromised individuals [1].

When NTM species reside in the lungs and cause disease it is referred to as NTM pulmonary disease (NTM-PD). NTM can however reside benignly in the lungs and be detected by standard microbial culture but not appear to be an infective agent, due to the absence of symptoms and/or lack of destruction of the lung parenchyma. This causes considerable difficulty when assessing which patients require treatment for NTM disease and which are considered to be only NTM colonised [2].

1.1 Taxonomy

In 1959 Runyon categorised NTM according to their rate of growth, whether they produce pigment and finally whether this pigment is produced in the presence of UV light [3-5]. On the basis of these criteria, NTM are divided into four separate groups:

Group 1: Photochromogens - slow growing and produce a yellow-orange pigment only when exposed to light. Examples include *Mycobacterium kansasii.*

Group 2: Scotochromogens - slow-growing and produce a yellow-orange pigment regardless of whether they are grown in the dark or the light. Examples include *Mycobacterium gordonae.*

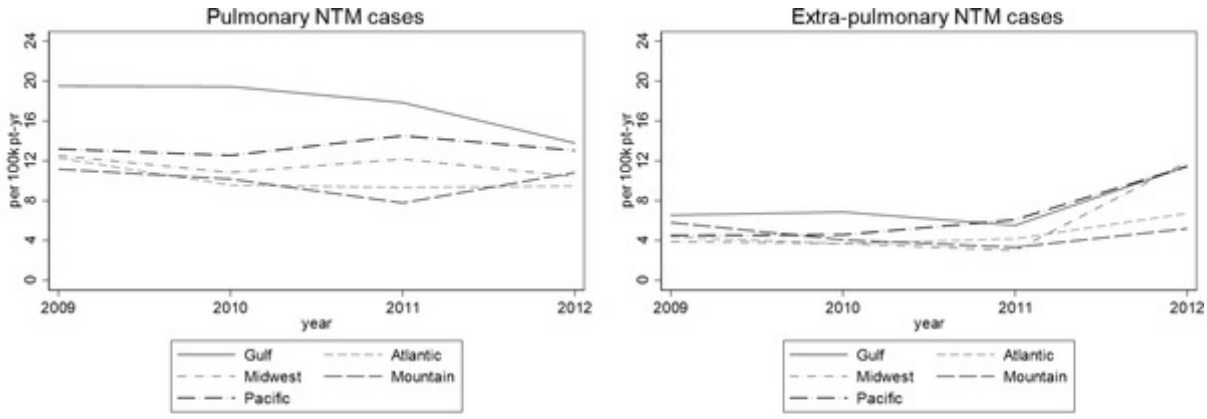
Group 3: Nonchromogens - slow-growing and never produce pigment. Examples include *Mycobacerium avium* and *Mycobacterium intracellulare* (these species form the Mycobacterium avium complex [MAC]).

Group 4: Fast growers – classified as fast growers based on forming colonies within 5 days of culture. Examples include *Mycobacterium abscessus*, *Mycobacterium bolletii* and *Mycobacterium massiliense.*

1.2 Epidemiology

The reported distribution of NTM disease varies greatly both within countries [6-13] and between countries [14] as well as by latitude [15].

Prevots *et al*. estimated the prevalence of NTM culture positivity in the USA in 2010 to be between 1.4 and 6.6 per 100,000 [16]. More recently, in 2018, Jones *et al*. noted the incidence of NTM disease to be 12.6/100,000. NTM incidence was highest in the Gulf States (South Eastern USA) illustrating the geographical differences in incidence within the country and also latitude [17].



Pulmonary	IRR	95% CI	P
Gulf	0.90	[0.85-0.95]	0.002
Atlantic	0.92	[0.86-0.98]	0.012
Midwest	0.96	[0.90-1.02]	0.182
Mountain	0.97	[0.85-1.10]	0.632
Pacific	1.01	[0.93-1.10]	0.795

Extra-Pulmonary	IRR	95% CI	P
Gulf	1.20	[1.10-1.31]	<0.001
Atlantic	1.18	[1.07-1.30]	0.001
Midwest	1.56	[1.41-1.72]	<0.001
Mountain	0.95	[0.78-1.15]	0.596
Pacific	1.42	[1.25-1.61]	<0.001

IRI: Incident rate ratio of 2012 compared to 2009; CI: Confidence interval

Figure 1.1 - Incidence rates of NTM cases per 100,000 patient-years receiving care over time. Reprinted from Jones *et al*. (2018) Epidemiology of Non-Tuberculous Mycobacterial Infections in the U.S. Veterans Health Administration. PLOS ONE.

The data from the Jones *et al.* study also illustrates the changing annual incidence of NTM disease. Interestingly the study found a significant overall downward trend in pulmonary incidence whilst there were increases in overall extra-pulmonary disease in all but the mountainous regions. Data obtained prior to this by Adjemian *et al*. (2012)

[8] does, however, seem to contradict this notion. The Adjemian *et al.* study found an increase in annual prevalence rising from 20 to 47 cases per 100,000 persons in the whole of the USA between 1997 and 2007, representing an 8.2% increase (Figure 1.2). The rate of increase was more marked in women compared to men (9.1% versus 6.4%) and in addition showed marked geographic variation with the highest period prevalence being observed in the West at 149 (95% CI, 136–163) cases/100,000 persons.

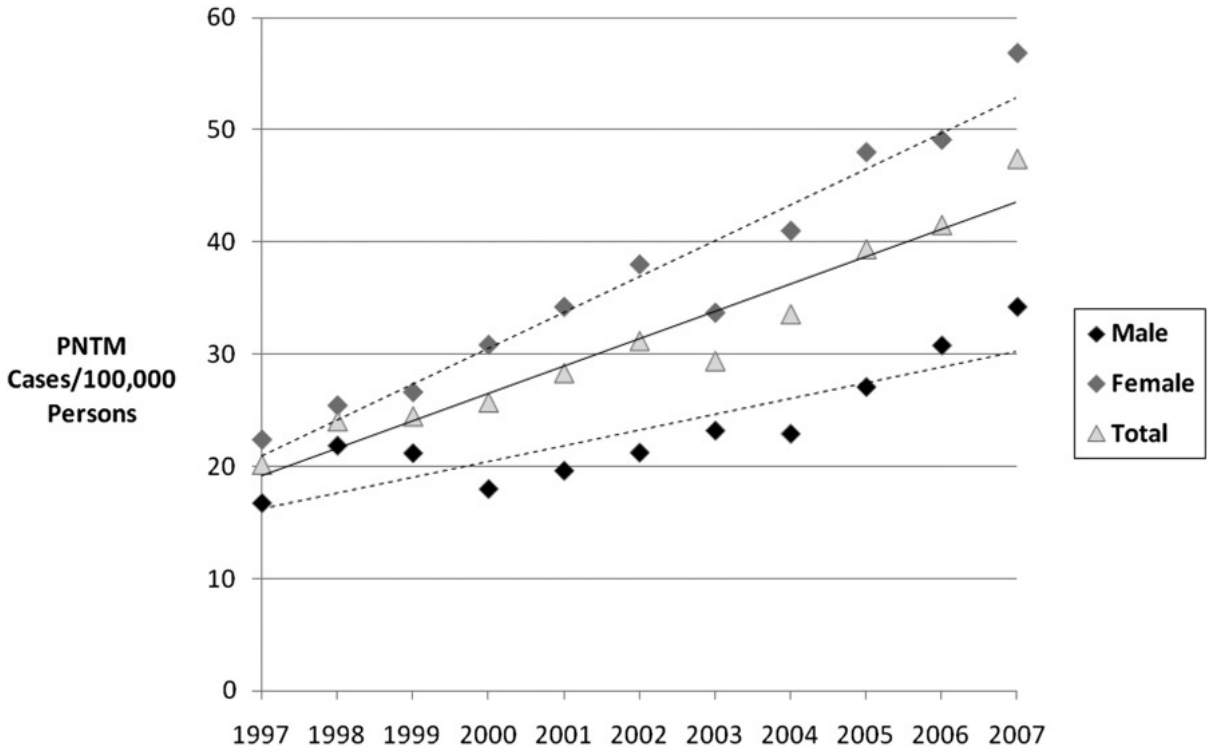


Figure 1.2 - Annual Prevalence of pulmonary NTM infection in USA 'Medicare' insurance scheme Beneficiaries. Reprinted with permission of the American Thoracic Society. Copyright © 2021. American Thoracic Society. All rights reserved. Cite: Adjemian J *et al*. (2012) Prevalence of Nontuberculous Mycobacterial Lung Disease in U.S. Medicare Beneficiaries. American Journal of Respiratory and Critical Care Medicine. 185 (8), 881-886. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society.

Perhaps the most compelling evidence of the variability of NTM prevalence comes from Hoefsloot *et al.* in 2013. In an effort to determine NTM species prevalence and variability, the study collected data from 62 centres from 30 countries across six continents (Figure 1.3). A total of 20,182 isolates were included in the study of which 91.3% were identified to the species level. The six most frequently isolated NTMs were

M. avium complex (9,421 isolates; 47%), *M. gordonae* (2,170 isolates; 11%), *M. xenopi* (1,605 isolates; 8%), *M. fortuitum* complex (1,322 isolates; 7%), *M. abscessus* (664 isolates; 3%) and *M. kansasii* (720 isolates; 4%). The study not only identified large intercontinental variability but variability between countries that often shared a border but with MAC predominating in most settings [18].

The most recent epidemiological data comes from Korea. Park *et al.* found age-adjusted prevalence of NTM infection rapidly increased from 2003 to 2016. The age-adjusted prevalence was 1.2 per 100,000 population in 2003 and 33.3 per 100,000 population in 2016 (Figure 1.4). The age-adjusted prevalence of NTM infection was higher in women with, in 2016, 45.1 per 100,000 for women compared to 20.8 per 100,000 population for men. In addition the prevalence of NTM infection was found to be increased with age [19].

Similar to prevalence, age-adjusted incidence of NTM infection was also increased from 2003 to 2016 (Figure 1.5). The age-adjusted incidence was 1.0 per 100,000 population in 2003 and 17.9 per 100,000 population in 2016. Women had about 2.5 times higher age-adjusted incidence than men (24.9 versus 10.4 per 100,000). Additionally, higher incidence was associated with advancing years.

Shah *et al.* reported the incidence of NTM isolation in the United Kingdom between 2007 and 2012 from both pulmonary and extra-pulmonary samples (the most common extra-pulmonary sites were: bone, lymph nodes, urine and bone). A total of 21,118 individuals had NTM culture positive isolates. Over the study period the incidence rose from 5.6/100,000 in 2007 to 7.6/100,000 in 2012 (P - value < 0.001). The incidence of culture positive pulmonary isolates increased from 4.0/100,000 to 6.1/100,000 (P - value < 0.001) whereas extra-pulmonary isolates fell from 0.6/100,000 to 0.4/100,000 (P - value < 0.001). The most frequently cultured organisms were MAC. The incidence of pulmonary MAC increased over the 5 years from 1.3/100,000 to 2.2/100,000 (P - value < 0.001). The majority of these individuals were over 60 years old [20]. It should be noted that this data concerns NTM culture positive isolates rather than individuals with disease caused by NTM. Similar findings were reported by Cowman *et al.* who described mycobacterial culture and drug sensitivity testing results over a period of 13 years in a major UK centre. Culture results were obtained from 109,311 samples of

which 5,960 samples isolated NTM species. NTM isolation increased year on year, driven by increases in *M. abscessus* and MAC [21].

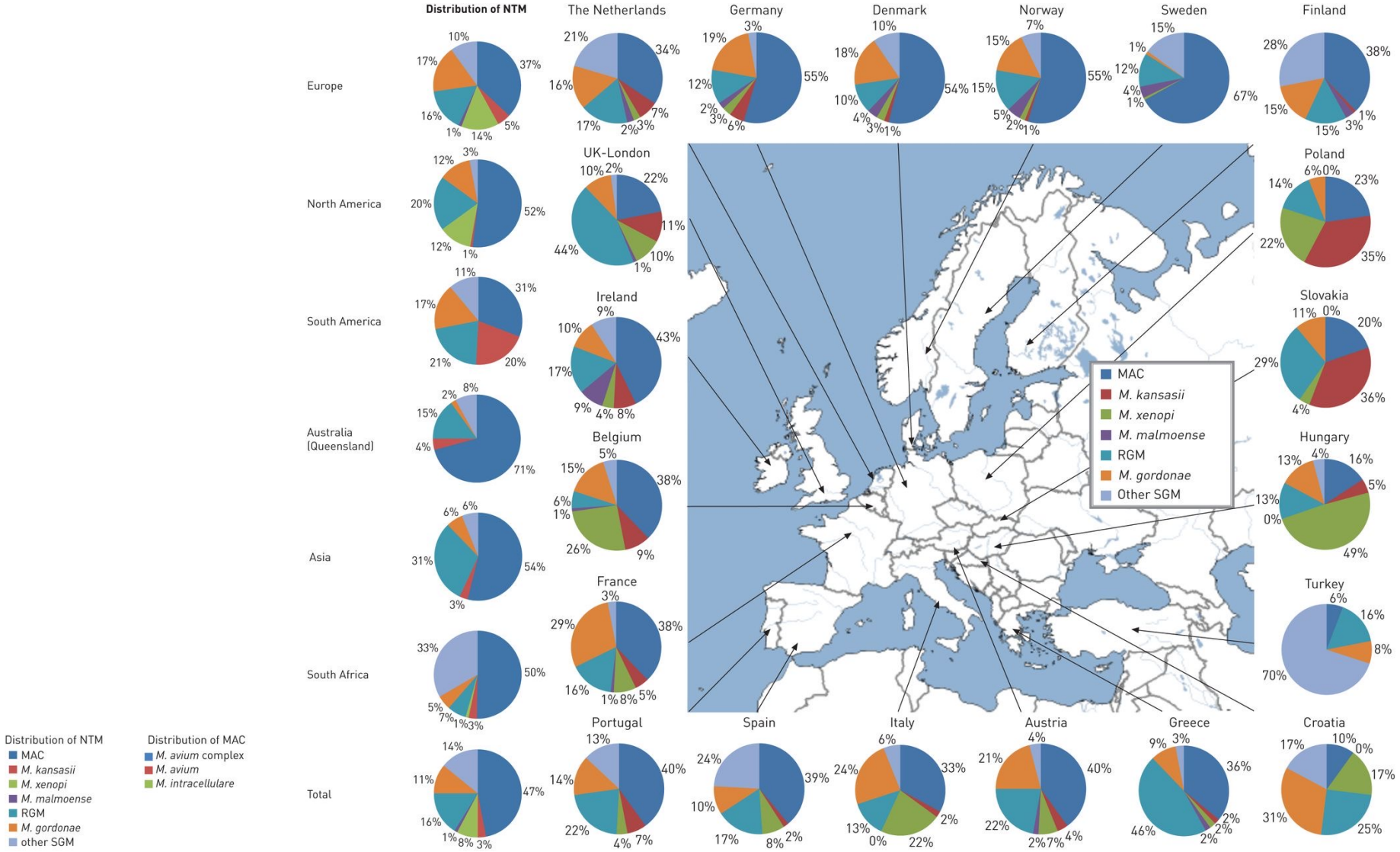


Figure 1.3 - Distribution of respiratory NTM isolates first by continent, then by European country. MAC: Mycobacterium Avium Complex; RGM: Rapid-Growing Mycobacteria; SGM: Slow-Growing Mycobacteria. Adapted with permission from Hoefsloot *et al*. (2013). The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. Eur Respir J.

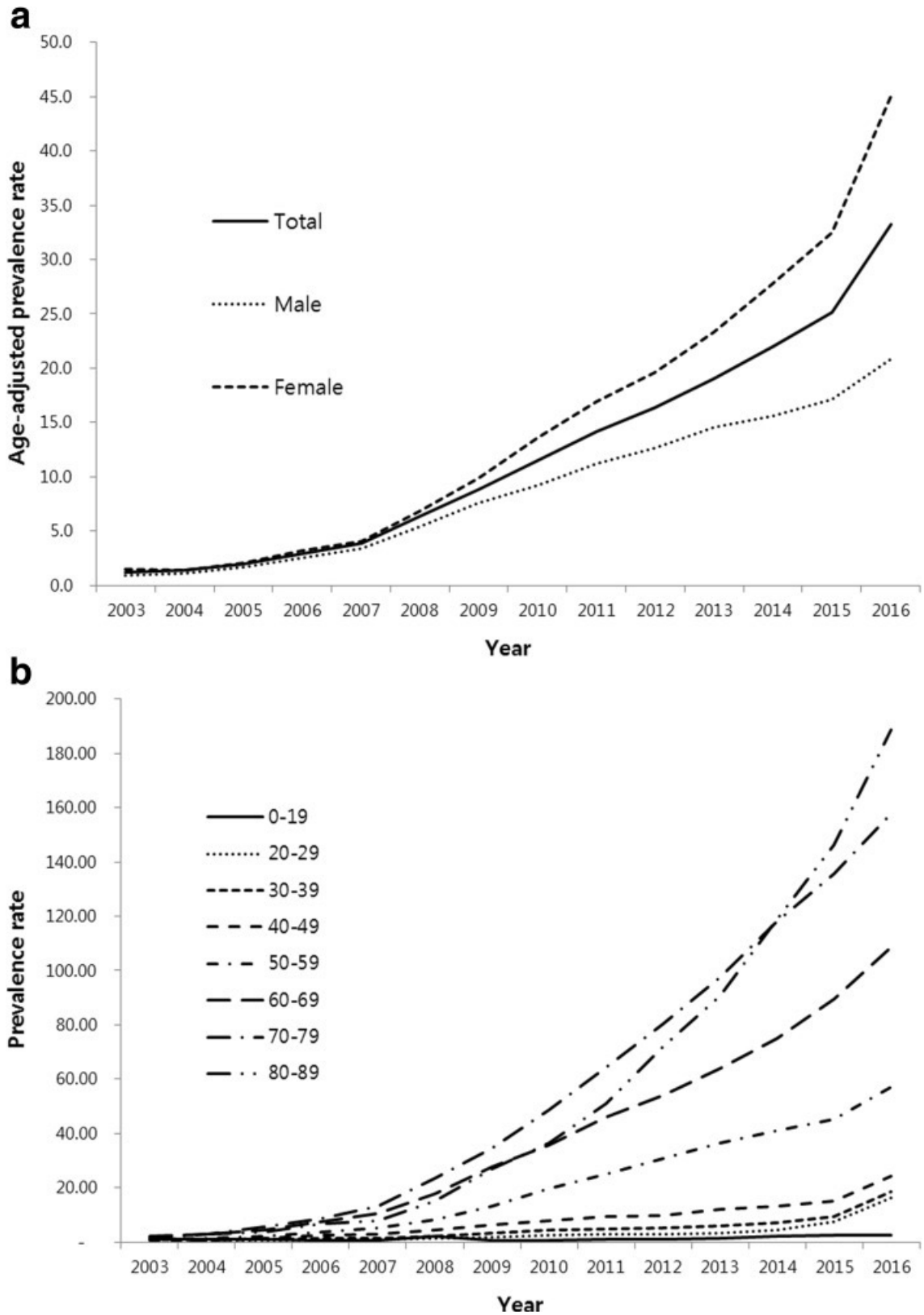


Figure 1.4 - Prevalence of NTM infection between 2003 and 2016 (**a**), and (**b**) split by age groups. The rates are per 100,000 population. Reprinted with permission from Park *et al.* (2019) Prevalence, Incidence, and Mortality of Non-Tuberculous Mycobacterial Infection in Korea: a Nationwide Population-based Study. BMC Pulm Med.

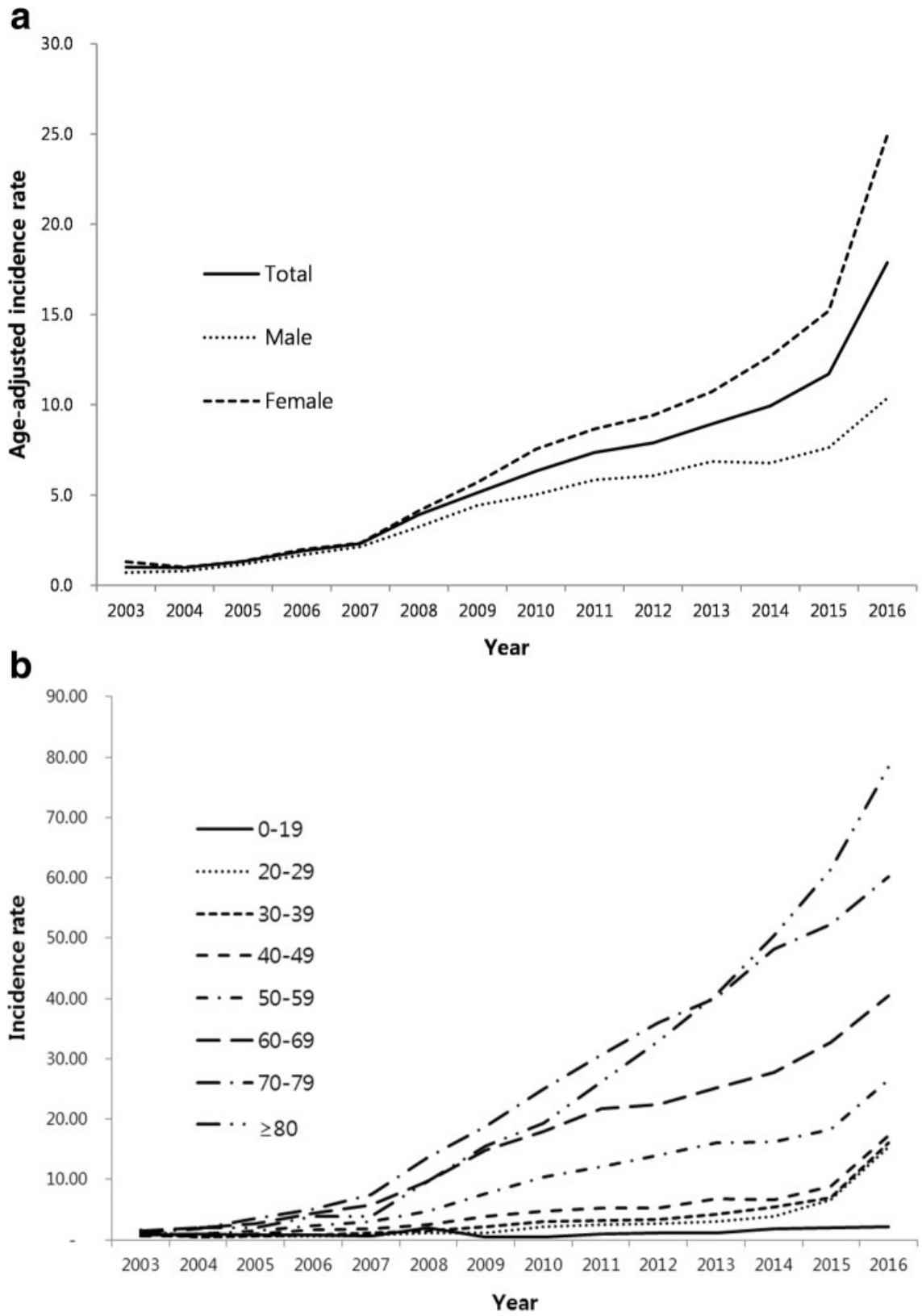


Figure 1.5 - Incidence of NTM infection between 2003 and 2016 (**a**) and by age groups (**b**). The rates are per 100,000 population. Reprinted with permission from Park *et al.* (2019) Prevalence, Incidence, and Mortality of Non-Tuberculous Mycobacterial Infection in Korea: a Nationwide Population-based Study. BMC Pulm Med.

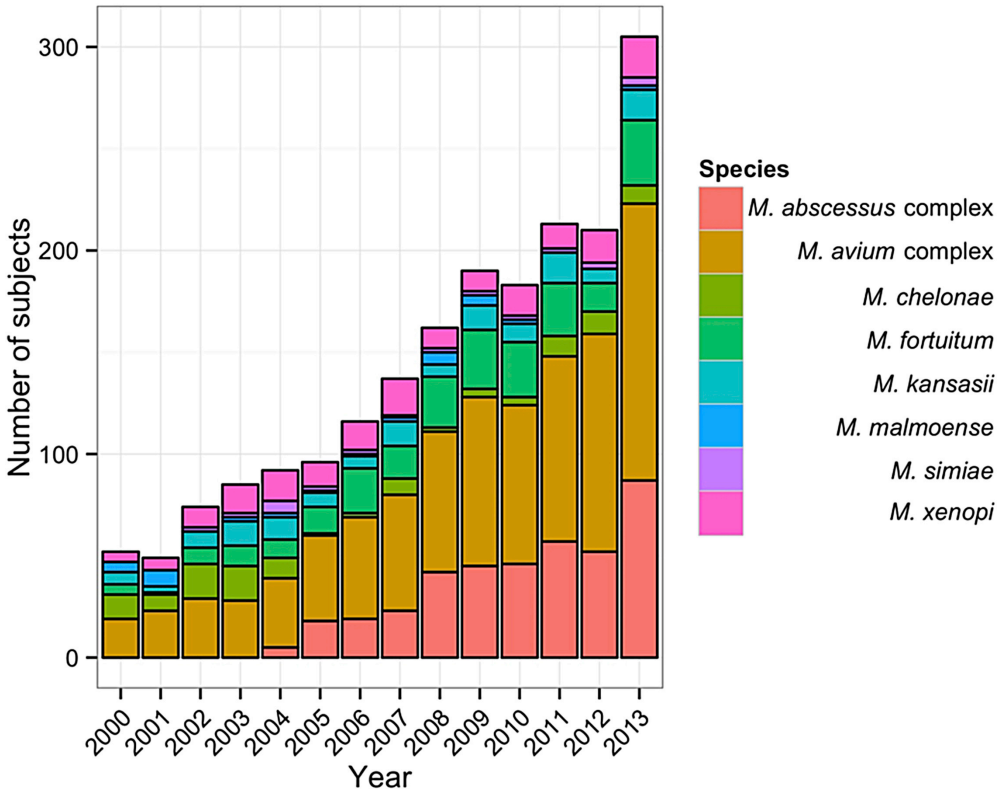


Figure 1.6 - Number of subjects isolating selected NTM species from a respiratory specimen (bronchoalveolar lavage or sputum) overtime. Reprinted with permission from Cowman *et al*. (2016) The Anti-microbial Susceptibility of Non-Tuberculous Mycobacteria. J Infect.

1.3 Pathogenesis

As NTM are ubiquitous bacteria, acquisition is a balance between patient exposure, pathogen virulence and host susceptibility to disease. The following forms of host susceptibility have been linked to NTM disease:

- Pre-existing lung disease
- Impaired host immunity
- Iatrogenic exposure

1.3.1 Pre-existing Lung Disease

Individuals at increased relative risk of developing NTM disease include those with an altered lung parenchyma, those with inflammatory airways disease and those with impaired mucociliary clearance. There is obviously a large overlap between all of these disease groupings but diseases associated with NTM infection include: asthma [22], chronic obstructive pulmonary disease (COPD) [23-25], alpha-1-antitrypsin deficiency [26],

pneumoconiosis [27], cystic fibrosis (CF) [6, 28], (non-cystic fibrosis) bronchiectasis [29-31], primary ciliary dyskinesia (PCD) [32] and allergic bronchopulmonary aspergillosis (ABPA) [33, 34]. Association between isolation of NTM species and more subtle changes on the spectrum of some of these diseases have also recently been reported. NTM isolation has been associated with heterozygous mutations associated with cystic fibrosis [35, 36] and even with mildly impaired ciliary function (without meeting the diagnostic criteria for primary ciliary dyskinesia) [37].

A number of other co-morbidities have additionally been associated with the isolation of NTM species. These include gastro-oesophageal reflux disease [38, 39], low Body Mass Index (BMI) [40, 41], low vitamin D concentration [42, 43] and rheumatoid disease [44-47].

1.3.2 Impaired Host Immunity

Disseminated mycobacterial infection has been noted in individuals harbouring mutations affecting the interferon (IFN)-γ – interleukin (IL)-12 pathway. To date, mutations have been identified affecting 11 genes (*IL12B*, *IL12RB1*, *ISG15*, *TYK2*, *IRF8*, *SPPL2A,CYBB*, *IFNGR1*, *IFNGR2*, *STAT1*, *NEMO*) [48]. Outside of this pathway, mutations in *GATA2* have been described that lead to defective haematopoesis and susceptibility to NTM [49, 50].

Several other cytokine pathways have been identified that are thought to predispose to NTM disease. TNF-α is known to play an essential role in the defence against *Mycobacterium tuberculosis* by activating macrophages and maintaining granuloma integrity. Several studies have reported impaired TNF-α release in subjects with NTM-Pulmonary Disease (NTM-PD) [51-57]. Other studies have shown reduced IL-12 [53-55] and IFN-γ [51, 52, 55, 58-60].

One study examined global transcriptional responses in NTM pulmonary disease through analysis of whole blood gene expression [61]. The *IFNG* gene that encodes interferon-γ was found to be downregulated.

It has been suggested that there may be a genetic contribution to disease susceptibility, supported by reports of familial clustering of NTM-PD [62]. The high incidence of NTM-PD seen in CF has led to the *CFTR* gene being proposed as a candidate gene for disease susceptibility. The incidence of *CFTR* mutations in patients with NTM-PD disease has been found to be significantly higher than the general population [51, 63-65]. Studies in Asian populations with a low prevalence of CF have also demonstrated an association between *CFTR* polymorphisms and NTM-PD [35, 66]. One retrospective study addressed the issue of whether *CFTR* mutation merely predisposes towards bronchiectasis or if it is independently associated with NTM-PD [63]. The study found a significantly higher incidence of *CFTR* mutation in patients with NTM-PD and bronchiectasis *versus* bronchiectasis alone (22% *versus* 5%).

The interpretation of these data is clouded by the potential immunomodulatory effect of mycobacterial infection on the immune response [67], as well as differences in study populations and the methodologies used. The weight of the evidence thus far suggests downregulation of the immune response in NTM-PD. Whether this downregulation is truly a predisposing factor or a result of disease, however, remains unresolved.

The major cause of acquired immunodeficiency (excluding iatrogenic sources) that gave rise to multiple cases of disseminated MAC infection in the 1980s was HIV infection. In the latter it was the reduced CD4+ counts that were found to be associated with occurrences of disseminated MAC infection [68, 69].

1.3.3 Iatrogenic

Many immunosuppressive or immunomodulatory drugs have been implicated in the development of NTM disease. These include anti-tumour necrosis factor alpha (TNF-α) therapy [70], immunosuppressive agents used in chemotherapy and for organ transplantation [68] and oral and inhaled corticosteroids [22, 71]. The association between long term use of Azithromycin and the development of NTM infection remains unclear. Mouse infection studies have shown that Azithromycin could block autophagic killing of NTM within macrophages [72] whilst other large retrospective studies have shown no such association [73-75].

1.3.4 Idiopathic Acquisition

A cohort of patients with no apparent predisposing conditions has also been described [76] and has since been labelled the 'Lady Windermere syndrome' [77]. Reich *et al.* proposed that chronic suppression of the normal cough reflex is responsible for the inability to clear the secretions from the right middle lobe and lingula. This habit results in a focus of inflammation in these areas, which in turn predisposes to MAC infection. Such patients are typically female, Caucasian, post-menopausal, taller and thinner than average with a higher than expected incidence of chest wall deformity [78]. Ninety per cent of patients had bronchiectasis in the right middle lobe [51]. These distinct characteristics has led to speculation that disease susceptibility may be related to hormonal differences and their influence over the immune response to NTM [58, 75, 79-82]. Further research is therefore warranted to explore the role of endocrine changes as independent susceptibility factors.

1.4 Transmission of NTM

Despite the fact that the *Mycobacterium tuberculosis* and *Mycobacterium leprosy* complexes are spread via aerosolisation of bacteria, traditionally it has been assumed that the acquisition of NTM is solely from environmental sources. Studies in the 1990s matched MAC isolates taken from patient samples to environmental point sources indicating environmental acquisition of MAC [83-85]. Other studies have taken MAC isolates from patients living in the same household and compared MAC genetic similarity. These studies have found that isolates were genetically dissimilar indicating that person to person admission had not occurred [86, 87].

More recently, in 2012, a report highlighted a likely outbreak of *M. abscessus* complex within a cystic fibrosis cohort in the USA. The study identified patient acquisition of the bacteria likely occurred during hospital visits when patients came in to contact with other patients with established *M. massiliense* colonisation [88]. Subsequent whole genome sequencing of this outbreak confirmed the presence of genetically similar isolates shared between patients raising the possibility of patient to patient spread [89]. In a related study, Bryant *et al.* conducted whole genome sequencing of 168 *M. abscessus* complex isolates from 31 patients and collected detailed corresponding epidemiological data of patients. The study revealed probable patient to patient spread of isolates [90]. The study was backed by a larger, multinational whole genome

sequence analysis of over 1,000 clinical isolates of *M. abscessus* complex. This study indicated widespread patient to patient transmission of *M. abscessus* complex with evidence of transcontinental spread of dominant circulating clones, which may be associated with worse clinical outcome [91]. Whilst recent evidence of patient to patient transmission has come to light it is yet to be substantiated whether this is due to direct inhalation of bacteria aerosolised by infected patients (as is the case with *Mycobacterium tuberculosis*) or whether it is secondary to environmental soiling via robust aerosolised bacteria which are then inhaled by susceptible patients.

1.5 NTM and Disease in Humans

The isolation of NTM has been shown to be associated with a decline in lung function in CF [92] and COPD [93] and with reduced quality of life in bronchiectasis [25, 94].

NTM are ubiquitous and as such a positive sputum culture may represent either contamination, a single isolate or colonisation with no evidence of organ damage or disease. Differentiating between these can be difficult, especially with the complications of comorbidities and have led to the development of guideline-based criteria.

1.5.1 Criteria for the Diagnosis of Non-Tuberculous Mycobacterial Lung Disease

In 2007, the American Thoracic Society produced guidelines for the diagnosis and treatment of pulmonary NTM disease [95]. These guidelines (Table 1.1) are based on culture results, clinical symptoms and radiological changes associated with the disease. The guidelines were adopted in 2017 by the British Thoracic Society (BTS) who produced their own version based on the 2007 criteria [2].

Table 1.1 – 2007 American Thoracic Society criteria for the diagnosis of NTM lung disease. Adapted from Griffith *et al.* (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med.

CLINICAL	MICROBIOLOGICAL
Pulmonary symptoms **OR** CT scan changes in keeping with NTM disease (see Section 1.5.3) **AND** Appropriate exclusion of other diagnoses	Positive culture results from 2 expectorated sputum samples **OR** Positive culture results from 1 bronchial wash **OR** Lung biopsy with histological features and 1 positive culture result

1.5.2 NTM Culture and Species Differentiation

Prior to the mycobacterial culture process, sputum samples must first undergo decontamination to prevent overgrowth by commensals [96]. The sample is then inoculated into a suitable growth medium. In the UK, the most common method is via an automated liquid culture system (e.g. Mycobacteria Growth Indicator Tube [MGIT]; BD Biosciences, Sparks, Maryland, USA) that utilises a Middlebrook 7H9 broth as a liquid growth medium.

Following successful mycobacterial culture, molecular techniques are typically employed to differentiate between species. Line probe assays are in widespread use across the UK and allow species differentiation as well as determination of selected antibiotic resistance. This technique is time consuming as it relies on mycobacterial culture that can take several weeks. Techniques to detect NTM directly from samples (i.e. bypassing the need for culture) have recently become commercially available. These techniques however are costly and clinical validation in different patient cohorts and across different species is incomplete and therefore their role in routine clinical practice remains to be determined.

If mycobacteria are successfully cultured from samples their clinical significance requires expert consideration. NTM are ubiquitous organisms that are resistant to decontamination. This feature has meant that they can be culpable for so called pseudo outbreaks [97]. NTM are also frequently isolated from sputum samples without any pathological signs or symptoms. The culture of NTM therefore does not

automatically indicate associated disease and it is essential to distinguish transient isolation from colonisation and critically from disease.

For a positive diagnosis of NTM pulmonary disease to be made, patients require 2 or more positive sputum culture results that identify the same mycobacterial species on each occasion. Alternatively, patients may have one positive bronchial wash/lavage or compatible histopathological findings with one positive culture result. These microbiological criteria for the diagnosis of the disease state are based on the findings of an observational study that involved patients with chronic respiratory disease and one or more sputum cultures positive for MAC [98]. The study found that 2% of patients with only one positive culture developed progressive radiological changes associated with NTM disease compared with 98% of patients with two or more positive cultures. Subsequent studies confirmed that the risk of developing NTM disease in the context of only one NTM positive culture is low (between 4% and 14%) [99, 100]. This risk is however likely to be higher in specific patient groups such as CF [101].

1.5.3 Radiological Changes

Radiological changes in NTM pulmonary disease include: nodules, cavities, the 'tree-in-bud' pattern, bronchiectasis and consolidation [102]. Two predominant patterns of disease have been described: 'fibrocavitary' disease and 'nodular-bronchiectatic' disease. Fibrocavitatory disease is characterised by cavitary lesions predominantly in the upper lobes and classically seen in men with COPD. Nodular-bronchiectatic disease presents as multifocal bronchiectasis with clusters of small nodules and branching linear structures that frequently involve the right middle lobe and the lingular segment of the left upper lobe. It is more common in women with bronchiectasis or without underlying lung disease (Figure 1.7) [103].

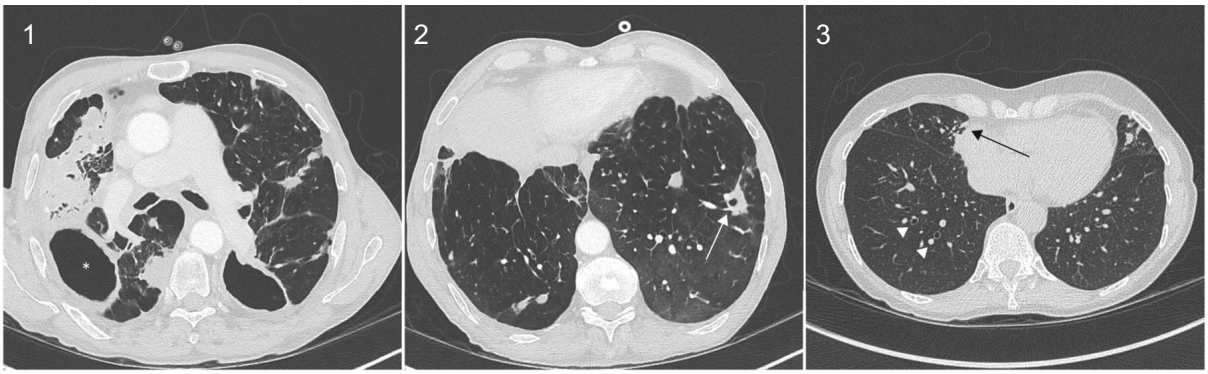


Figure 1.7 - High-resolution computed tomography imaging of pulmonary Non-Tuberculous Mycobacterial disease. Image 1 shows fibrocavitary disease. Image 2 shows cavitating nodules as indicated by the white arrow. Image 3 shows nodular bronchiectatic disease as indicated by the black arrow. Reprinted with permission from Cowman *et al.* (2019) Non-tuberculous mycobacterial pulmonary disease Eur Respir J. [67].

1.5.4 Symptoms Relating to NTM Infection

NTM infections develop slowly. Patients may have been symptomatic for several years prior to diagnosis. Common signs or symptoms include:

- Fever
- Weight loss
- Cough
- Lack of appetite
- Night sweats
- Haemoptysis
- Dyspnoea

1.6 Treatment

The choice of drug regimen for the treatment of NTM disease depends on species identification. Antibiotic selection is also influenced by *in vitro* drug susceptibility testing. This is however often a poor predictor of *in vivo* sensitivities and therefore a poor predictor of treatment outcomes [104]. There are exceptions to this, namely macrolide resistance in MAC and *M. abscessus*, aminoglycoside resistance in MAC and Rifampicin resistance in *M. kansasii*, which have been associated with higher treatment failure rates [105-107].

1.6.1 Mycobacerium avium complex

Mycobacterium avium and *Mycobacterium intracellulare* (MAC) are the species with the most evidence backing the recommended treatment regime. There have been no randomised controlled trials comparing treatment to placebo although the Research Committee of the BTS has compared the success of different drug regimens [104]. The study showed that a triple drug regimen including Rifampicin, Ethambutol and Isoniazid had a lower treatment failure rate than that of Rifampicin, Ethambutol and a placebo (16% versus 41%). The study however did not find any significant difference in all cause 5-year mortality. Triple therapy with the inclusion of macrolides has a reported sputum conversion rate of between 42 - 80% and has excellent *in vitro* activity against MAC [105, 108-112]. In 2014, Wallace *et al.* studied a retrospective case series involving 180 patients with nodular MAC-pulmonary disease on a three-drug regimen (Clarithromycin or azithromycin with Rifampicin and Ethambutol). No patient developed macrolide resistance with this regimen and there were no differences in outcome between patients treated with azithromycin (as part of a 3 times weekly regimen) versus Clarithromycin. While 86% of patients had culture-converted following 12 months of treatment, 14% suffered relapse during treatment and 48% after treatment had ceased [113].

Whilst intermittent regimens (i.e. regimens with Azithromycin 3 times weekly) appear equally effective and better tolerated than daily regimens in patients with nodular MAC-pulmonary disease, a large multicentre reported a culture conversion rate of only 4% after 12 months of treatment in patients with cavitary MAC-pulmonary disease using a 3 times weekly Azithromycin based regime [114]. BTS recommended drug regimens for the treatment of MAC disease are detailed in Table 1.2

Table 1.2 - Suggested antibiotic regimens for adults with M. avium complex-pulmonary disease. Adapted from Haworth *et al.* (2017) British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). Thorax [2].

***M. avium* complex pulmonary disease**	**Antibiotic regimen**
Non-severe MAC-pulmonary disease (i.e., AFB smear-negative respiratory tract samples, no radiological evidence of lung cavitation or severe infection, mild-moderate symptoms, no signs of systemic illness)	• Rifampicin 600 mg 3x per week • Ethambutol 25 mg/kg 3x per week • Azithromycin 500 mg 3x per week or clarithromycin 1 g in two divided doses 3x per week Antibiotic treatment should continue for a minimum of 12 months after culture conversion.
Severe MAC-pulmonary disease (i.e. AFB smear-positive respiratory tract samples, radiological evidence of lung cavitation/severe infection, or severe symptoms/signs of systemic illness)	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Azithromycin 250 mg daily or clarithromycin 500 mg twice daily and consider intravenous amikacin for up to 3 months or nebulised amikacin Antibiotic treatment should continue for a minimum of 12 months after culture conversion.
Clarithromycin-resistant MAC-pulmonary disease	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Isoniazid 300 mg (+pyridoxine 10 mg) daily or moxifloxacin 400 mg daily and consider intravenous amikacin for up to 3 months or nebulised amikacin. Antibiotic treatment should continue for a minimum of 12 months after culture conversion.

1.6.2 *Mycobacterium abscessus* treatment

A key determinant in the success of treatment of *Mycobacterium abscessus* complex is the actual species being treated. Jeon *et al.* reviewed a total of 65 patients with *M. abscessus* pulmonary disease on treatment with intravenous amikacin and cefoxitin for the first 4 weeks of treatment in combination with oral clarithromycin, ciprofloxacin and doxycycline for 12 months. Fifty-eight % of patients became and remained culture negative for at least 12 months. The study revealed long term culture negativity was significantly lower in patients with clarithromycin resistant isolates.

A subsequent analysis showed that culture conversion and maintenance of negative cultures occurred in 88% of patients with *M. massillense* compared with only 25% of patients with *M. abscessus* ($P < 0.001$). The difference in outcome is most likely explained by *M. abscessus* isolates having a functional *erm*(41) gene that confers inducible macrolide resistance [115, 116].

Wallace *et al.* used intravenous tigecycline as part of a multidrug treatment regime [117]. The majority of patients demonstrated clinical improvement by way of culture conversion, but the regime was subject to high rates of side effects (90% of patients developed side effects).

There have been a number of case series investigating the use of nebulised amikacin have been reported [118-121]. Although these studies contained only a few patients Jhun *et al.* found that at 12 months after amikacin inhalation therapy, 49% of patients had symptomatic improvement whereas 42% had radiological improvement. Conversion to a negative sputum culture occurred however in only 18% patients, and the culture conversion rate was higher in patients infected with macrolide-susceptible isolates (50%) than in those infected with macrolide-resistant isolates (11%) (P - value = 0.003). Caimmi *et al.* prescribed liposomal amikacin therapy to 5 patients. The 3 patients who completed the treatment did not have any respiratory exacerbation and were culture negative for *M. abscessus*. Overall nebulised amikacin is considered effective in a proportion of patients with refractory *M. abscessus.*

Several case series reports were identified that reviewed prolonged intravenous treatment and appeared to result in better clinical and microbiological outcomes, however drug toxicity and side effects were more common in these groups [122].

The treatment of *M. abscessus* utilises an initial phase antibiotic regimen (including intravenous and oral antibiotics) followed by a continuation phase antibiotic regimen (including inhaled and oral antibiotics). Treatment regimens depend on macrolide resistance [2]. BTS recommended drug regimens for the treatment of *M. abscessus* complex disease can be found in Table 1.3

Table 1.3 - Suggested antibiotic regimens for adults with *M. abscessus* complex-pulmonary disease. Adapted from Haworth *et al.* (2017) British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). Thorax [2].

***M. abscessus* complex pulmonary disease**	**Antibiotic regimen**
Clarithromycin sensitive isolates or inducible macrolide-resistant isolates	**Initiation phase:** • Intravenous amikacin 15 mg/kg daily or 3× per week and intravenous tigecycline 50 mg twice daily • Where tolerated intravenous imipenem 1g twice daily • Where tolerated oral clarithromycin 500 mg twice daily or oral azithromycin 250–500 mg daily **Continuation phase:** • Nebulised amikacin • Oral clarithromycin 500 mg twice daily or azithromycin 250–500 mg daily • 1–3 of the following antibiotics guided by drug susceptibility results and patient tolerance: oral clofazimine 50–100 mg daily§ oral linezolid 600 mg daily or twice daily oral minocycline 100 mg twice daily oral moxifloxacin 400 mg daily oral co-trimoxazole 960 mg twice daily
Constitutive macrolide-resistant isolates	**Initiation phase:** • Intravenous amikacin 15 mg/kg daily or 3x per week and intravenous tigecycline 50 mg twice daily • Intravenous tigecycline 50 mg twice daily • Where tolerated intravenous imipenem 1g twice daily **Continuation phase:** • Nebulised amikacin • 2–4 of the following antibiotics guided by drug susceptibility results and patient tolerance: oral clofazimine 50–100 mg daily§ oral linezolid 600 mg daily or twice daily oral minocycline 100 mg twice daily oral moxifloxacin 400 mg daily oral co-trimoxazole 960 mg twice daily

1.6.3 *Mycobacterium kansasii* Treatment

The largest study looking at treatment for *M. kansasii* was carried out by the Research Committee of the BTS and prospectively looked at treatment of 173 patients with rifampicin and ethambutol for 9 months of therapy. There was one treatment failure defined by positive sputum cultures at 7 and 8 months of treatment and relapse

occurred in 9.7% patients at a median of 23 months following treatment cessation. Poor adherence and reinfection were thought to be relevant in some of the cases that relapsed.

Other prospective studies that looked at 3 drug or more regimens looked at patients treated with isoniazid, rifampicin and ethambutol for 12 months with streptomycin twice weekly for the first 3 months [123]. There were no treatment failures and relapse occurred in only 1 of the 40 patients included. The third prospective study included 28 patients who were treated with rifampicin, isoniazid and ethambutol for either 12 or 18 months [124]. There were no treatment failures and one relapse in a patient treated for 12 months. The final study reviewed the outcomes of 18 patients treated with 3 times weekly clarithromycin, rifampicin and ethambutol for 12 months [125]. There were no treatment failures or relapse following treatment completion. BTS recommended drug regimens for the treatment of *M. kansasii* disease can be found in Table 1.4

Table 1.4 - Suggested antibiotic regimens for adults with *M. kansasii* pulmonary disease. Adapted from Haworth *et al.* (2017) British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). Thorax [2].

***M. kansasii* pulmonary disease**	**Antibiotic regimen**
Rifampicin sensitive *M. kansasii* pulmonary disease	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Isoniazid 300 mg (with pyridoxine 10 mg) daily or azithromycin 250 mg daily or clarithromycin 500 mg twice daily • Antibiotic treatment should continue for a minimum of 12 months after culture conversion.

1.6.4 *Mycobacterium malmoense* Treatment

There were two randomised controlled trials identified regarding treatment for *M. malmoense.* In 2001, the Research Committee of the BTS randomised 106 patients to receive a treatment regime of either rifampicin, ethambutol and isoniazid or rifampicin and ethambutol for a total of 2 years. The number of patients that completed treatment and were alive and cured at 5 years was only 38% - 44% [104].

In the second study, Jenkins *et al.* randomised 167 patients to receive rifampicin, ethambutol and clarithromycin or rifampicin, ethambutol and ciprofloxacin for 2 years

[126]. There were no differences in outcomes between the antibiotic treatment groups, with the relapse rate being only 5%. The number that completed the treatment regimens and were known to be alive and cured at 5 years, however, was only 38% in the clarithromycin group versus 20% in the ciprofloxacin group. BTS recommended drug regimens for the treatment of *M. malmoense* complex disease can be found in Table 1.5.

Table 1.5 - Suggested antibiotic regimens for adults with *M. malmoense* pulmonary disease. Adapted from Haworth *et al.* (2017) British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). Thorax [2].

***M. malmoense* pulmonary disease**	**Antibiotic regimen**
Non-severe *M. malmoense* pulmonary disease (i.e. AFB smear-negative respiratory tract samples, no radiological evidence of lung cavitation or severe infection, mild-moderate symptoms, no signs of systemic illness)	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Azithromycin 250 mg daily or Clarithromycin 500 mg twice daily • Antibiotic treatment should continue for a minimum of 12 months after culture conversion.
Severe *M. malmoense* pulmonary disease (i.e., AFB smear-positive respiratory tract samples, radiological evidence of lung cavitation/severe infection or severe symptoms/signs of systemic illness)	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Azithromycin 250 mg daily or clarithromycin 500 mg twice daily • consider intravenous amikacin for up to 3 months or nebulised Amikacin • Antibiotic treatment should continue for a minimum of 12 months after culture conversion.

1.6.5 *Mycobacterium xenopi* Treatment

The Research Committee of the BTS randomised 42 patients to receive rifampicin, ethambutol and isoniazid or rifampicin and ethambutol for 2 years [104]. The study experienced inconsistent results with a relapse rate of 18% for those on the two-drug regimen, and 5% for the three-drug regimen. Conversely the number of patients that completed treatment and were alive and cured at 5 years was higher in the two-drug regimen group.

Jenkins *et al.* randomised 34 patients to regimes containing either rifampicin, ethambutol and clarithromycin or rifampicin, ethambutol and ciprofloxacin. Relapse

rate was 24% with clarithromycin treated patients and 6% with ciprofloxacin treated patients. There was no significant difference between the number that completed the allocated treatment and were known to be alive and cured at 5 years (18% *versus* 12% respectively) [126]. BTS recommended drug regimens for the treatment of *M. xenopi* complex disease can be found in Table 1.6.

Table 1.6 - Suggested antibiotic regimens for adults with *M. xenopi* pulmonary disease. Adapted from Haworth *et al.* (2017) British Thoracic Society guidelines for the management of non-tuberculous mycobacterial pulmonary disease (NTM-PD). Thorax [2].

***M. xenopi* pulmonary disease**	**Antibiotic regimen**
Non-severe *M. xenopi* pulmonary disease (i.e. AFB smear-negative respiratory tract samples, no radiological evidence of lung cavitation or severe infection, mild-moderate symptoms, no signs of systemic illness)	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Azithromycin 250 mg daily or clarithromycin 500 mg twice daily • Moxifloxacin 400 mg daily or isoniazid 300 mg (+pyridoxine 10 mg) daily • Antibiotic treatment should continue for a minimum of 12 months after culture conversion.
Severe *M. xenopi* pulmonary disease (i.e. AFB smear-positive respiratory tract samples, radiological evidence of lung cavitation/severe infection or severe symptoms/signs of systemic illness)	• Rifampicin 600 mg daily • Ethambutol 15 mg/kg daily • Azithromycin 250 mg daily or clarithromycin 500 mg twice daily • Moxifloxacin 400 mg daily or isoniazid 300 mg (+pyridoxine 10 mg) daily • consider intravenous amikacin for up to 3 months or nebulised amikacin • Antibiotic treatment should continue for a minimum of 12 months after culture conversion.

1.7 Prognosis

The prognosis of NTM pulmonary disease has been shown to vary according to radiological characteristics, clinical features and the offending bacterial species.

In terms of radiology, Lam *et al.* showed that in a cohort receiving treatment for MAC, patients with cavitating disease had worse outcomes than those without [114]. Several case series have confirmed that disease associated with cavitation or consolidation have worse outcomes than nodular disease patterns [127-131]. Recently a study

investigating prognostic factors associated with long term mortality in NTM pulmonary disease found survival rates were higher in patients with non-cavitary disease compared to those with cavitating disease. The overall 5-, 10- and 15-year cumulative mortality rates for the non-cavitary nodular bronchiectasis (NB) form were 6.3%, 16.0% and 26.6%, respectively. This compares to 35.1%, 47.0% and 65.4% in patients with cavitating disease (Figure 1.8) [131].

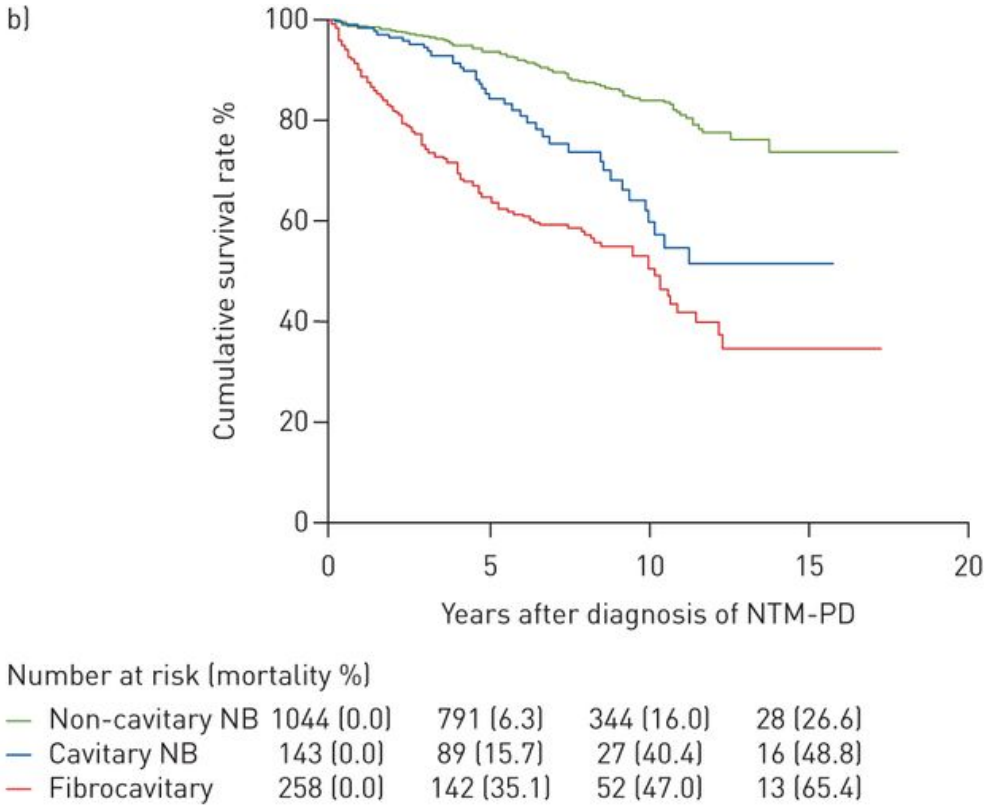


Figure 1.8 - Cumulative survival rates of patients with NTM-PD according to radiological form. Reprinted with permission from Jhun *et al*. (2020) Prognostic Factors Associated with Long-term Mortality in 1,445 patients with Non-Tuberculous Mycobacterial Pulmonary Disease: a 15-year Follow-up Study. Eur Respir J.

Clinical characteristics associated with worse prognosis include: male gender, increased age, cavitating disease, low BMI, anaemia, low albumin and a raised erythrocyte sedimentation rate [132]. Similar findings relating to low BMI [133], cavitating disease [134], aspergillus coinfection [135] and concomitant pulmonary hypertension [136] were reported in other studies.

Several studies examining outcomes in MAC pulmonary disease were identified in the literature. The Research Committee of the BTS found an all cause 5-year mortality of 36% [104]. Jenkins *et al.* found similar mortality figures of 39% [126]. Lower mortality rates however were reported in other studies. Gochi and Hayashi both reported a 5-year mortality of 12.5% and 23.9% respectively [132, 137]. Diel *et al.* carried out a systematic review of published mortality rates associated with MAC disease and reported a pooled estimate of five-year all-cause mortality was 27% (95% CI 21.3–37.8%) (Figure 1.9) [138].

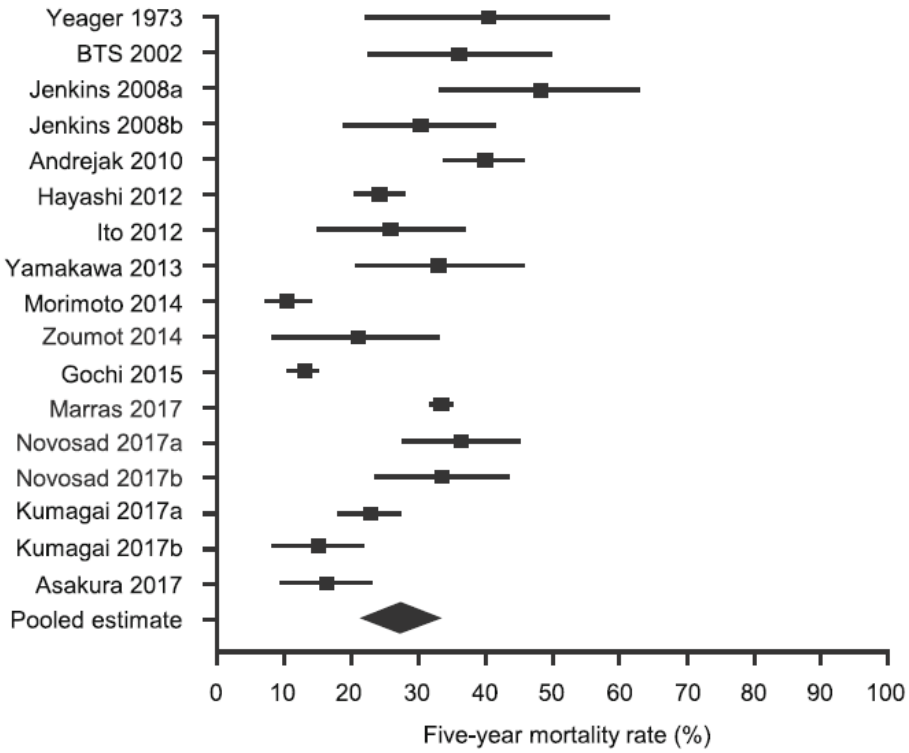


Figure 1.9 - Analysis of five-year all-cause mortality rates in selected data sets. Results are plotted ± 95% confidence interval (CI). Adapted from Diel *et al.* (2018) High Mortality in Patients with Mycobacterium avium complex Lung Disease: A Systematic Review. BMC Infectious Diseases.

In the case of other slow growing mycobacteria, *M. kansasii* is associated with a relatively favourable outcome. Several studies identified in the literature showed no deaths directly attributed to *M. kansasii* infection. The Research Committee of the BTS identified an all-cause mortality of 19% over the 4 years the study ran [139-141]. Jenkins *et al.* showed a 5-year all-cause mortality of 49% with a 4% mortality directly attributable to *M. malmoense* and a 38% 5 year all-cause mortality for *M. xenopi* (with 3% of deaths directly attributable to the bacteria).

In the case of mortality related to *M. abscessus* complex infection, the data is less clear and varies greatly according to clinical characteristics as well as radiological subtypes [136, 142]. Jhun *et al*. recently published 5-, 10- and 15-year cumulative mortality rates for patients with *M. abscessus* disease as 11.4%, 29.8% and 50.6%, respectively[131].

Two studies that compared several species in a single dataset were found in the literature. Marras *et al.* investigated mortality in Ontario between 2001 and 2013 (Table 1.7 and figure 1.10). This study included 9,681 patients with NTM pulmonary disease attributable to primarily 5 different species (MAC, *M. xenopi, M. fortuitum, M. abscessus, M. kansasii*). The study found a relatively low 5-year survival for patients with *M. xenopi* (adjusted hazard ratio = 1.39) whilst *M. abscessus* had the highest survival rate of any species of 72.8% (adjusted hazard ratio = 1.14) [143]

Table 1.7 – Survival data of different NTM species. Standardised mortality rates were standardised by sex and age in 5-y strata using data for the Ontario population. Hazard ratio was adjusted for sex, age, income quintile, location, Adjusted Clinical Group case mix system, baseline underlying conditions (asthma, chronic obstructive pulmonary disease, diabetes, HIV infection, rheumatoid arthritis, chronic kidney disease, gastroesophageal reflux disease, bronchiectasis, interstitial lung disease, cystic fibrosis, prior tuberculosis, lung cancer, solid organ transplantation or bone marrow transplantation), health use (number of hospitalizations and emergency department visits in year before index date), and NTM disease diagnosis during follow-up as time-varying covariate (for NTM isolation group only). Adapted from Marras *et al.* (2017), Pulmonary Non-tuberculous Mycobacteria–Associated Deaths, Ontario, Canada, 2001–2013, Emerg Infect Dis.

Species, Disease group	Number of Patients Included	% 5 Year Survival	Standardised Mortality Ratio (95% CI)	Adjusted Hazard Ratio (95% CI)
All NTM Species				
Disease	9,681	63.1	2.83 (2.74–2.92)	1.23 (1.17–1.28)
Isolation	10,936	73.4	2.30 (2.22–2.38)	1.00
MAC				
Disease	6,323	64.7	2.59 (2.49–2.69)	1.16 (1.09–1.24)
Isolation	5,756	73.2	2.27 (2.16–2.38)	1.00
M. xenopi				
Disease	2,263	56.8	3.49 (3.29–3.70)	1.39 (1.27–1.52)
Isolation	2,932	71.9	2.39 (2.23–2.55)	1.00
M. fortuitum				
Disease	265	64.8	2.70 (2.21–3.18)	1.17 (0.92–1.48)
Isolation	714	76.0	2.63 (2.27–2.99)	1.00
M. abscessus				
Disease	245	72.8	2.23 (1.71–2.74)	1.14 (0.76–1.72)
Isolation	185	77.1	2.10 (1.46–2.74)	1.00
M. kansasii				
Disease	158	62.3	4.37 (3.37–5.37)	1.15 (0.78–1.68)
Isolation	106	52.0	3.97 (2.89–5.05)	1.00
Other NTM Species				
Disease	427	66.7	3.08 (2.62–3.55)	1.27 (1.04–1.55)
Isolation	1,243	77.6	1.94 (1.72–2.15)	1.00

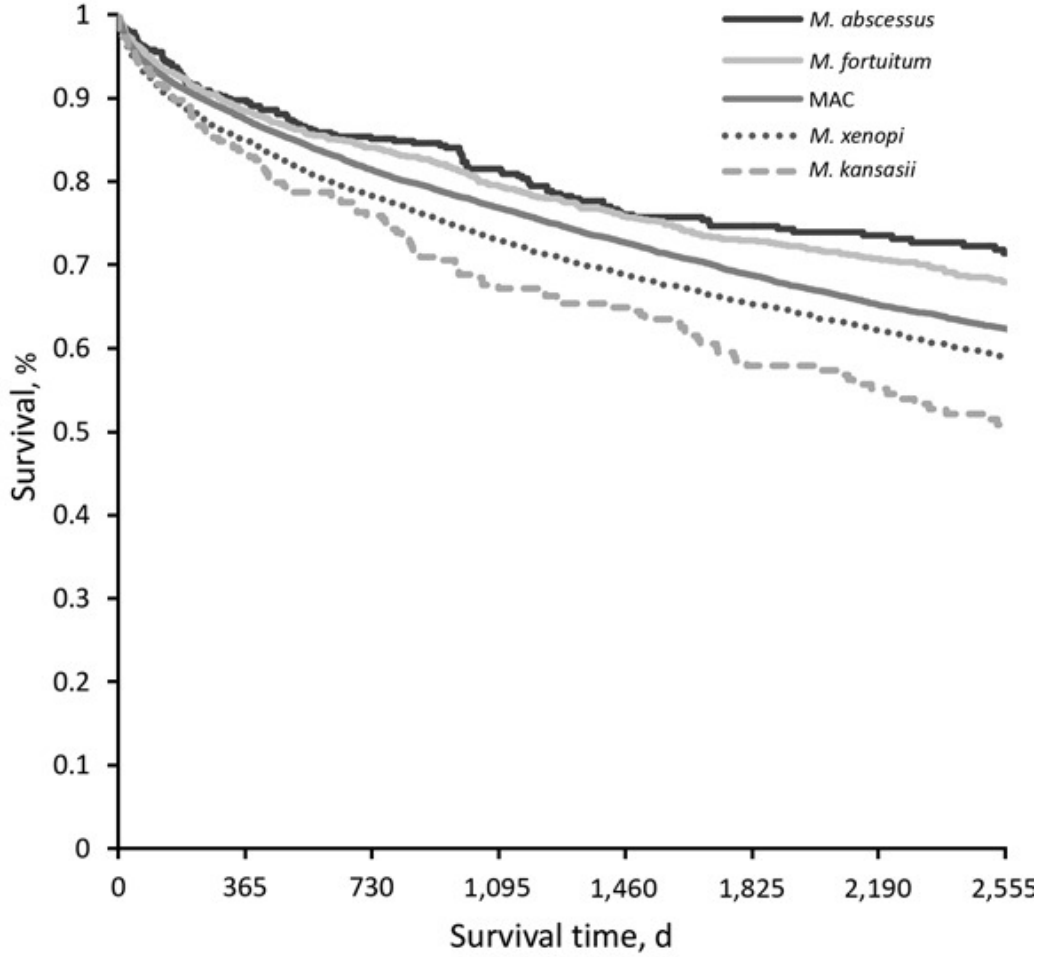
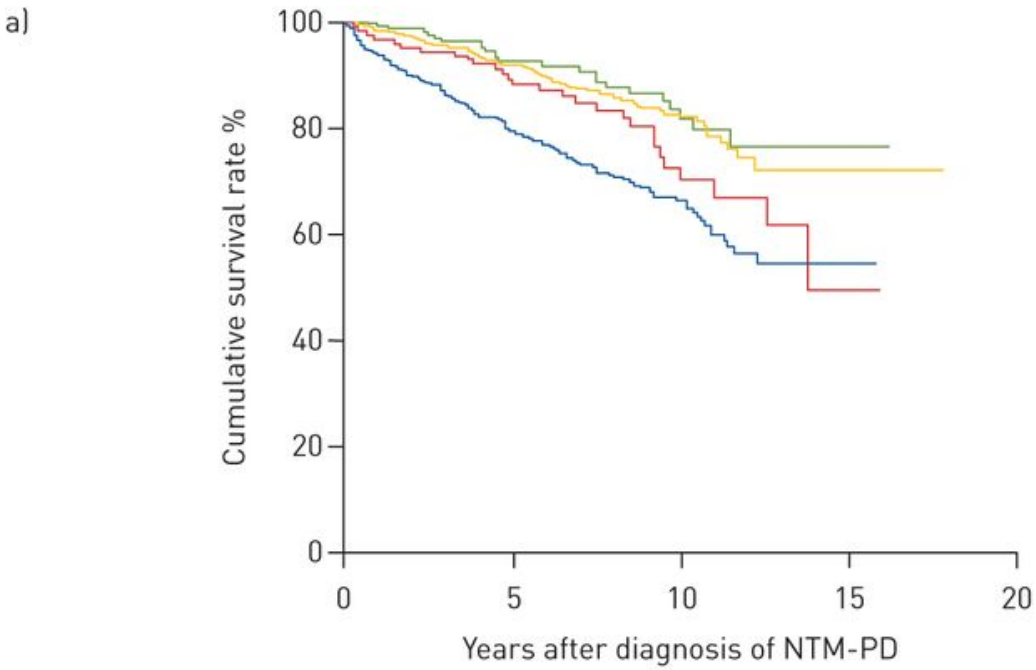


Figure 1.10 - Kaplan-Meier survival curves for any pulmonary NTM isolation, by species in Ontario, Canada, 2001–2013. Curve comprises all matched and unmatched patients identified during the study period. There is a statistically significant difference among curves (*P*<0.001, log-rank) in crude survival comparison, uncontrolled for any other variables. Differences between individual species pairs statistically significant (*P* < 0.00005) for all pairs except Mycobacterium abscessus versus M. fortuitum (*P*-value = 0.19), M. abscessus versus Mycobacterium avium complex (*P*-value = 0.14), and *M. fortuitum* versus MAC (*P*-value = 0.5). Reprinted from Marras *et al.* (2017), Pulmonary Non-tuberculous Mycobacteria–Associated Deaths, Ontario, Canada, 2001–2013. Emerg Infect Dis.

The second study found was conducted by Jhun *et al.* and retrospectively analysed mortality amongst 1,445 NTM patients from 1997 onwards, thus providing mortality data for 5-, 10- and 15-year intervals (figure 1.11). Overall, the survival rates were higher in patients with *M. massiliense* and *M. avium* compared to those with *M. abscessus* and *M. intracellulare*. The overall 5-, 10- and 15-year cumulative mortality rates for patients with *M. massiliense* disease were 7.3%, 18.1% and 23.5%, respectively, and were similar to those with *M. avium* disease (8.0%, 17.2% and 27.9%, respectively). The overall 5-, 10- and 15-year cumulative mortality rates for patients with *M. intracellulare* disease were 20.3%, 33.6% and 45.5%, respectively.

The overall 5-, 10- and 15-year cumulative mortality rates for patients with *M. abscessus* disease were 11.4%, 29.8% and 50.6%, respectively.



Number at risk (mortality %)				
M. massiliense	174 (0.0)	136 (7.3)	48 (18.1)	23 (23.5)
M. avium	655 (0.0)	481 (8.0)	146 (17.2)	31 (27.9)
M. intracellulare	487 (0.0)	318 (20.3)	94 (33.6)	28 (45.5)
M. abscessus	129 (0.0)	96 (11.4)	30 (29.8)	4 (50.6)

Figure 1.11 - Cumulative survival rates of patients with NTM-PD according to NTM species. Reprinted with permission from Jhun *et al.* (2020) Prognostic Factors Associated with Long-term Mortality in 1,445 patients with Non-Tuberculous Mycobacterial Pulmonary Disease: a 15-year Follow-up Study. Eur Respir J.

Despite the increased mortality rates associated with NTM disease it is noteworthy that NTM infection is rarely sited as the cause of patient death. This is likely due to patients suffering from a primary disease (e.g. cystic fibrosis) that paves the way for NTM infection. The study by Marras *et al.* is valuable as it attempts to adjust the data by underlying disease thus illustrating the true increased risk NTM disease poses.

1.8 Clinical Challenges

The global incidence of NTM isolation is increasing and its evolution from colonising agent to pathogenic bacteria results in deterioration in lung function, increased morbidity and mortality. Appropriate selection of patients suffering from disease (as opposed to patients who are simply NTM colonised) in a timely manner is therefore essential to prevent patient deterioration. The ATS guidelines were established to aid clinicians in these decisions. Microbiological confirmation of disease however is not entirely simple. Even with appropriate culture methods NTM often fail to grow and the fact that certain NTM culture more quickly than others raises the possibility that they will out compete slower growing NTM thus muddying the waters as to which NTM may be culpable for disease.

In order for ATS criteria to be fulfilled, patients should have either clinical or radiological signs of disease (see Figure 1.7). These signs are non-specific however and often mimic infections from other bacteria that co-exist with NTM. Clinicians must therefore decide whether an alternative diagnosis is more likely to be the causative agent of disease.

Even if the diagnosis of NTM is clear, the diagnosis of disease does not always necessitate treatment. In a study of patients with MAC disease, Hwang *et al.* found that over 20% of patients remained stable over a 3 year period [144]. A retrospective study, in which 80% of patients with MAC disease were observed rather than treated, found that an initial period of observation was not associated with adverse outcome [137]. Similarly, Park *et al.* found no deterioration in roughly 50% of patients who were observed after diagnosis of *M. abscessus* disease.

Decisions governing the commencement of treatment often lie in the radiological pattern of disease. In studies regarding MAC, pulmonary cavitation has been associated with worse prognosis [129, 130, 137]. Further reported multivariate predictors of disease progression requiring treatment in one study of MAC were increased age, lower body mass index (BMI), presence of systemic symptoms, positive sputum smear and the number of involved lobes [144].

Clinicians and patients should also decide upon a treatment goal. Treatment with curative intent (i.e. the resolution of symptoms and negative microbiology) is an obvious end goal but antibiotic regimes last for several months to years and are often accompanied by multiple side effects and intolerances. In these circumstances a cure may not be achievable, and treatment aimed at stabilising disease progression and controlling symptoms may be the best option. In this scenario a less efficacious but better-tolerated regimen may be selected [67].

In the case of cystic fibrosis, *M. abscessus* pulmonary infection has previously been recognised post lung transplant surgery [145-149]. Consequently *M. abscessus* eradication is attempted prior to listing patients for transplantation. Often these patients do not fulfil ATS guidelines for disease and the clinician must decide whether to treat based on whether the patient may be a future candidate for lung transplant.

Efforts to tackle infections in NTM are hampered by a lack of reliable biomarkers for diagnosis, assessment of disease activity, and prognostication [1]. Recently molecular methods have been applied to DNA extracted from respiratory samples to directly detect and identify NTM species without the need for culture. The reported sensitivity however is low with NTM detected in only 29–76% of culture positive samples [150-152]. No techniques have been developed to accurately quantify NTM burden. This is clearly unsatisfactory for a condition where the longitudinal monitoring of both disease and treatment response is crucial to patient management and wellbeing, and in the setting of CF may have important implications regarding isolation and even listing for transplantation.

1.9 Hypotheses

Assessment of NTM disease is reliant on the ability to culture culpable mycobacteria. The culture process is not reliable and suffers from a high false negative rate. In addition to this, growth of multiple NTM species may occur thus muddying the waters as to which species may be pathogenic. The sensitivity of culture as a diagnostic tool is also critical as treatment regimens are required to continue for months after the last positive culture result. False negative culture results may therefore have the unintended consequence of misleading clinicians and consequently treatment

regimens may be halted prematurely. This may in part account for high disease relapse rates. If a molecular test can be developed that has a lower diagnostic false negative rate than culture and if the test is also capable of quantifying genetic material, it will enable clinicians to assess response to treatment as well as reducing the false negative detection rate and therefore stopping the premature cessation of treatment.

I. The development of a quantitative PCR based NTM test will provide a faster and more accurate method of NTM species identification as well as providing information on bacterial DNA copy number. This will result in improved NTM detection and enable improved management decisions.

Disturbances in the lung microbiome are an established phenomenon in parenchymal lung disease [153-157]. Relatively few studies however have looked at the microbiome of the lung affected with NTM [158] and no studies have looked at longitudinal changes in the microbiome and the effects of anti-NTM treatment

II. There are interactions between the NTM and bacterial communities and the bacterial airway community will vary depending on the NTM status of the patient.

1.10 Study Aims

1. To develop molecular assays capable of quantifying the 6 most common pathogenic mycobacteria (*M. abscessus, M. avium, M. intracellulare, M. malmoense, M. kansasii* and *M. xenopi*) and calculate comparative sensitivities and specificities for each target species.
2. Produce a high resolution quantitative polymerase chain reaction (qPCR) readout of NTM patient's clinical course over 12-18 months based on respiratory samples with assessments of the impact of standard treatment.
3. To assess dynamic bacterial changes of the lung microbiome in patients on treatment for NTM disease and those who are treatment naïve.

Chapter 2: Materials and Methods

Table 2.1 – List of products (reagents and equipment) used and their suppliers.

Product	Manufacturer
Agarose	Bioline, London, UK
Agencourt AMPure XP beads	Beckman Coulter, Brea, USA
Allegra X-12R centrifuge	Beckman Coulter, Brea, USA
Agilent 2100 Bioanalyzer	Agilent Technologies, Santa Clara, USA
Chloroform:isoamyl alcohol 5:1	Sigma-Aldrich, St. Louis, USA
Ladder100bp DNA	New England BioLabs, Ipswich, USA
Elution Buffer with Tris (EBT)	Illumina, San Diego, CA, USA
FirstLight UV illuminator	UVP, Upland, USA
GelRed dye	Biotium, Hayward, USA
GenoType Mycobacterium CM VER.2.0	Hain Lifescience, Nehren, Germany
Monarch® DNA Gel Extraction Kit	New England BioLabs, Ipswich, USA
Heraeus LABOFUGE 400R centrifuge	Thermo Fisher Scientific, Waltham, USA
Heraeus Pico 21 microcentrifuge	Thermo Fisher Scientific, Waltham, USA
Hexadecyl-trimethyl-ammonium bromide (CTAB)	Sigma-Aldrich, St. Louis, USA
High Sensitivity DNA Kit	Agilent Technologies, Santa Clara, USA
HotStartTaq Plus Master Mix	QIAGEN, Hilden, Germany
HT1 hybridisation buffer	Illumina, San Diego, CA, USA
KAPA Library Quantification Kit (ROX Low) for Illumina	KAPA Biosystems, Wilmington, USA
KAPA SYBR FAST ROX Low 2X qPCR Master Mix	KAPA Biosystems, Wilmington, USA
LB culture broth with 100 µg/ml-1 ampicillin	Invitrogen/Thermo Fisher Scientific, Waltham, USA
LB solid media with 100 µg/ml-1 ampicillin	Invitrogen/Thermo Fisher Scientific, Waltham, USA
Linearised polyacrylamide (LPA)	Sigma-Aldrich, St. Louis, USA
Low-EDTA TE buffer	Invitrogen/Thermo Fisher Scientific, Waltham, USA
Lysing Matrix E tubes	MP Biomedicals, Solon, USA
Matt black 96-well microtitre plate	Corning Life Sciences, Tewksbury, USA

MicroAmp™ Optical 96-Well Reaction Plate with Barcode	Thermo Fisher Scientific, Waltham, USA
MiSeq benchtop sequencer	Illumina, San Diego, CA, USA
MiSeq v3 reagent kit	Illumina, San Diego, CA, USA
MP Bio FastPrep-24™ 5G beadbeating instrument	Thermo Fisher Scientific, Waltham, USA
Mycobacterial Growth Indicator Tube	Becton Dickinson, Franklin Lakes, New Jersey
Nanodrop 1000 spectrophotometer	Thermo Fisher Scientific, Waltham, USA
PCR grade water	
Peltier Thermal Cycler (Ptc-225)	MJ Research, Bio-Rad Laboratories, USA
Phase-lock gel tubes (2mls heavy)	5 PRIME, Hilden, Germany
Phenol:cholorform:isoamyl alcohol 25:24:1 pH 8.0	Sigma-Aldrich, St. Louis, USA
PhiX Control v3	Illumina, San Diego, CA, USA
Polyethylene glycol 6000 (PEG)	Sigma-Aldrich, St. Louis, USA
PureLink Quick Plasmid DNA Miniprep Kit	Invitrogen/Thermo Fisher Scientific, Waltham, USA
Q5 High-Fidelity 2X Master Mix	New England BioLabs, Ipswich, USA
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen/Thermo Fisher Scientific, Waltham, USA
QuantStudio™ 7 Flex Real-Time PCR System	Thermo Fisher Scientific, Waltham, USA
Sartorius Entris® Precision Balance	Sartorius, Göttingen, Germany
SOC medium	Sigma-Aldrich, St. Louis, USA
Sputasol (dithiothreitol 1.4%)	Thermo Fisher Scientific, Waltham, USA
TECAN Infinite 200 PRO microplate reader	Tecan Group, Männedorf, Switzerland
TOPO TA Cloning Kit for Sequencing	Invitrogen/Thermo Fisher Scientific, Waltham, USA

2.1 Study Design

2.1.1 Ethical Approval

Ethical approval was obtained through the North West - Liverpool Central Research Ethics Committee (reference 16/NW/0849).

2.1.2 Recruitment

A total of 38 patients with either cystic fibrosis (CF) or non-cystic fibrosis (non-CF) induced bronchiectasis were recruited to the study. Patients were recruited into three separate groups (see below) based on the inclusion and exclusion criteria detailed in Table 2.2.

Group 1. Patients due to start treatment for *Mycobacterium abscessus* or *Mycobacterium avium* complex (termed “abscessus treatment” and “avium treatment”).

Group 2. Patients who have cultured *Mycobacterium abscessus* or *Mycobacterium avium* complex on more than one occasion and are treatment naïve (termed “abscessus control” and “avium control”).

Group 3. Patients with bronchiectasis (CF and non-CF) but who have never cultured mycobacteria (termed “control”).

Table 2.2 – Study inclusion and exclusion criteria.

Inclusion Criteria	Exclusion Criteria
Age 16 years or older (no upper age limit) Able to give informed consent Regular sputum production Patients recruited to **Group 1** should be treatment naïve prior to recruitment and have cultured the same NTM on more than one occasion Patients recruited to **Group 2** should have cultured *Mycobacterium abscessus* or *Mycobacterium avium* complex on more than one occasion in the last six months but are treatment naïve Patients recruited to **Group 3** should have radiological evidence of bronchiectasis	Change in baseline bronchiectasis or NTM therapy between 28 and 2 days before recruitment

2.1.3 Longitudinal Assessment

Subjects provided sputum samples at regular intervals according to the group they were recruited to (for details of recruitment group see Section 2.1.2). Subjects recruited to the *abscessus* treatment and *avium* treatment groups (Group 1) provided a sputum sample at screening, then on a weekly basis for the first month followed by monthly samples to 12 months and then further samples at 15 and 18 months. Subjects recruited to Groups 2 and 3 provided a sputum sample at screening, then on a weekly basis for the first month followed by monthly samples to month three (Table 2.3).

Table 2.3 – Timings of receipt of samples by patient group.

Patient group	Study Procedures	Screening	Weeks				Months												
			1	2	3	4	2	3	4	5	6	7	8	9	10	11	12	15	18
Group 1	Sputum sample	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Group 2	Sputum sample	X	X	X	X	X	X	X											
Group 3	Sputum sample	X	X	X	X	X	X	X											

2.1.4 Sample Collection, Processing and Storage

Initial screening sputum samples (for participants in Group 1 these samples were obtained before treatment commenced) were divided into four equal aliquots using a Gilson pipette and sterile pipette tips. One aliquot was sent immediately for mycobacterial culture and one sample was frozen immediately at -80°C. The two other samples were kept at room temperature for 24 hours before being sent for culture and being frozen at -80°C respectively. A 24-hour freezing delay was performed to account for potential changes in microbiological communities that may occur between sputum expectoration and receipt of future samples (due to delays in delivery) in the laboratory.

Subsequent sputum samples were sent by study participants to the laboratory via first class postal delivery. Upon receipt samples were again divided in to two aliquots with one aliquot being sent for mycobacterial culture and the other frozen at -80°C.

All sputum samples were collected into and stored in Sterilin polypropylene 30ml universal containers.

2.1.5 Mycobacterial Culture and Identification

Sputum samples were processed for bacterial culture by the Royal Brompton Hospital Clinical Microbiology Department. The first stage of this process involves the removal of adherent saliva by adding an equal volume of Sputasol (Oxoid Ltd) to the sputum sample followed by homogenisation by incubation at 37°C and periodic shaking. Five hundred µl of the homogenised solution was then inoculated into a BD BACTEC™ Mycobacterial Growth Indicator Tube (MGIT™) containing a modified Middlebrook 7H9

broth base. Next the tubes were incubated at 37°C inside a BD BACTEC™ MGIT™ automated mycobacterial detection system until identified as positive or incubation time exceeded 6 weeks without growth. Species identification was subsequently performed from cultured samples using the GenoType Mycobacterium CM VER.2.0 (Hain Lifescience).

2.1.6 Clinical Data Collection

All clinical data was collected anonymously and stored on a secure university server before being processed using Microsoft Excel software (Microsoft, Redmond, Washington state, USA).

2.2 DNA Extraction

DNA was extracted from sputum samples for subsequent downstream analyses (Sections 2.3 and 2.4) using the phenol:chloroform method as follows.

Two litres of 1M sodium chloride (NaCl) solution was created by adding 58.44g NaCl to 1 litre de-ionised water. Fifty g hexadecyl-trimethylammonium bromide was next dissolved in 500ml 1M NaCl to produce a 10% CTAB solution.

A 1M monobasic phosphate (NaH_2PO_4) solution was made by adding 11.998g NaH_2PO_4 to 100ml 1M NaCl. A 1M dibasic phosphate (Na_2HPO_4) was made by adding 0.98g (Na_2HPO_4) to 500ml 1M NaCl.

One molar NaH_2PO_4 (15.9ml) was combined with 284.1ml 1M Na_2HPO_4 and 300ml 1M NaCl to produce a 0.5M phosphate buffer pH 8.0 solution. The 10% CTAB and 0.5M phosphate buffer solution were combined in a 1:1 ratio to produce a working CTAB buffer solution. The solution was sterilized by autoclaving.

A 30% w/v polyethylene glycol 6000 (PEG) solution was prepared by dissolving 60g PEG 6000 in 1.6M NaCl.

A 0.1M aluminium ammonium sulphate solution was prepared by the addition of 2.26g aluminium ammonium sulphate to 50ml of PCR grade water. The solution was filter sterilised by passing it through a 0.2µm filter.

All sputum samples were thawed in a dedicated Class 2 safety cabinet. Samples were weighed on a Sartorius Entris® Precision Balance and divided into approximate 300 microgram volumes. The exact sputum weights were recorded for later analysis. A working volume was placed into a Lysing Matrix E (LME) tube (MP Biomedicals, Solon, USA) and the remaining volumes were aliquoted and stored frozen at -80°C.

Five hundred µl of CTAB buffer solution was added to each LME tube in addition to 50µl of 0.1M aluminium ammonium sulphate solution. Samples were transferred to a fume cupboard where 500µl phenol:chloroform:isoamyl alcohol 25:24:1 was added. The LME tube was then transferred to the MP bio FastPrep-24™ 5G bead beating instrument and homogenised at 6,000rpm for 1 minute. The LME tube was subsequently centrifuged in a Heraeus Pico 21 microcentrifuge (Thermo Fisher Scientific, Waltham, USA) at 16,000g for 5 minutes.

At the same time a 2ml phase-lock gel tube (Quantabio 5PRIME) was centrifuged at 2,000g for 10 minutes to ensure the gel pelleted at the bottom of each tube. The aqueous phase from the centrifuged LME tube was transferred to the phase-lock tube. The phase-lock tube was centrifuged in an Eppendorf® centrifuge 5427R (Sigma-Aldrich, St. Louis, USA) at 16,000g for 10 minutes at 4°C to form a gel barrier between the aqueous and chloroform phase. Five hundred µl chloroform:isoamyl alcohol 5:1 was added to the aqueous phase and the solution mixed gently by shaking. The tubes were then centrifuged at 4°C for 10 minutes. The aqueous phase was next removed from the phase-lock tube and decanted into a 2 ml microcentrifuge tube containing 1µl linearised polyacrylamide (LPA) (Sigma-Aldrich, St. Louis, USA). Two volumes (compared to volume of aqueous phase) worth of 30% PEG solution was added to the microcentrifuge tube.

In order to maximise DNA yield, a second extraction was performed by adding 500µl CTAB buffer solution, 500µl phenol:chloroform:isoamyl alcohol 25:24:1and 50µl 0.1M

aluminium ammonium sulphate solution to the original LME tube. The tube was then transferred to the MP Bio FastPrep-24™ 5G bead beater and homogenised at 6,000rpm for 1 minute. Subsequently all stages as described above were followed. On this occasion, following centrifugation all phases were transferred to a new 2ml microcentrifuge tube containing 1µl LPA and 30% PEG added in the same manner.

All microcentrifuge tubes were gently mixed and stored at 4°C overnight. The following day the tubes were centrifuged at 16,000g for 20 minutes at 4°C. The 30% w/v PEG solution was aspirated and 500µl ice cold 70% ethanol added to remove precipitated salts before centrifugation at 16,000g for 5 minutes at 4°C. The ethanol was removed by pipetting. This procedure was performed twice more with 200µl ice cold 70% ethanol.

After the final ethanol wash pellets were air dried for 5 minutes before the addition of 50µl low EDTA TE (Invitrogen/Thermo Fisher Scientific, Waltham, USA). The suspended nucleic acid was then combined into a single 2ml tethered O-ring screw cap microcentrifuge tube (Star lab). Tubes were stored at -20°C until use.

2.3 Creation of *hsp65* Plasmid

2.3.1 Bacterial Strains

Lyophilised genomic DNA from NTM type strains were obtained from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkµlturen (DSMZ, Braunschweig, Germany). Bacterial strains were selected on the basis that they were common isolates from the respiratory tract or common respiratory pathogens. Lyophylised DNA was reconstituted with molecular grade water. A list of bacterial strains used can be found in Table 2.4.

Table 2.4 – List of bacterial strains obtained from DSMZ. Strains were used to create bacterial plasmids, as well as undergoing genomic amplification.

Species	DSMZ code
M. abscessus subsp. abscessus	44196
M. abscessus subsp. bolletii	45149
M. abscessus subsp. massiliense	45103
M. avium subsp. Avium	44156
M. avium subsp. intracellulare	43223
M. kansasii	44162
M. malmoense	44163
M. xenopi	43995
M. gordonae	44160
M. psychrotolerans	44697
M. chelonae subspecies chelonae	43804
M. fortuitum subspecies fortuitum	46621
M. szulgai	44166

2.3.2 Amplification

In order to generate a *hsp65* gene insert, the TB11 forward (5' – ACC AAC GAT GGT GTG TCC AT - 3') and TB12 reverse (5' – CTT GTC GAA CCG CAT ACC CT - 3') primers developed by Telenti *et al*. were used to generate a 441 base pair amplicon of the *hsp65* gene.

For a final volume of 25µl per reaction, 0.5µl TB11 primer and 0.5µl TB12 primer (both 10mM stock concentration), 2µl genomic template DNA, 12.5µl HotStartTaq Plus 2X Master Mix and 9.5µl PCR-grade water were combined in an eppendorf. PCR was performed on a Peltier thermocycler (model PTC-225) with cycling conditions of: 5 minutes at 95°C followed by 35 cycles of 95°C for 45 seconds, 53°C for 45 seconds and 72°C for 45 seconds; and a final step of 72°C for 10 minutes.

2.3.3 Gel Extraction

The amplicon generated from Section 2.3.2 was run against a 100bp DNA Ladder on a 150ml 1.2% agarose gel with 3µl GelRed dye at 120V for 60 minutes. The gel was prepared by dissolving 1.8 g of Bioline Agarose (Molecular Grade) in 150 ml of Tris Borate EDTA Buffer (1 X concentrate, pH 8.3). This solution was heated until

completely dissolved, resulting in a 1.2% Agarose gel solution. When the solution had cooled sufficiently, 3µl of Gel Red (Nucleic Acid Stain) was added (enabling visualisation of DNA under ultraviolet luminescence), the gel was poured and allowed to set.

The gel was subsequently viewed under a FirstLight UV illuminator. Appropriately sized amplicons were excised and underwent gel purification using the Monarch® DNA Gel Extraction Kit.

The excised agarose gel was weighed in a 1.5ml microcentrifuge tube. Four volumes (relative to the excised agarose) of Gel Dissolving Buffer were added to the microcentrifuge tube. The samples were incubated at 50°C and vortexed periodically until the gel had completely dissolved. The solution was then transferred to a spin column placed inside a collection tube. The column underwent centrifugation at 16,000g for 1 minute. The flow through was discarded.

Two hundred µl DNA Wash Buffer was added to the column and the centrifugation at 16,000g for 1 minute was repeated. This wash step of the column was repeated a further time. Next the column was transferred to a new 1.5ml microcentrifuge tube and 10µl DNA Elution Buffer was added to the centre of the column matrix. After incubating for a minute, the column was centrifuged for 1 minute at 16,000g to elute the DNA.

2.3.4 Amplicon Cloning

Plasmids were created using the TOPO® TA Cloning® Kit for Sequencing.

First, one vial of One Shot TOP10 *E. coli* cells was thawed on ice. Four µl of eluted DNA (as per above) was added to 1µl pCR-4 TOPO vector and 1µl salt solution (1.2M NaCl, 0.06M $MgCl_2$) in a 500µl microcentrifuge tube. The mixture was incubated at room temperature for 5 minutes then placed on ice. Two µl of the above reaction was added to the thawed One Shot TOP10 *E. coli* cells and incubated on ice for 30 minutes. The cells were then heat shocked in a water bath for 30 seconds at 42°C and placed on ice. Two hundred and 50µl of room temperature microbial growth medium (SOC)

(Sigma-Aldrich, St. Louis, USA) was added to the tube containing the cells and placed in a shaking-incubator at 37°C at 200rpm for 1 hour.

Fifty µl and 100µl aliquots were taken from the above reaction mix and spread onto plates of solid lysogeny broth (LB) media (Invitrogen/Thermo Fisher Scientific, Waltham, USA) containing 100 µg/ml ampicillin and incubated overnight at 37°C. Following overnight culture, colonies were selected from the plate and extracted using a sterile pipette tip. The tip was placed into a PCR mix containing: 0.5µl M13 forward (5' – GTA AAA CGA CGG CCA G - 3') and 0.5µl M13 reverse (5' – CAG GAA ACA GCTATG AC - 3') primers each at 10 mM, 12.5µl Q5 High-Fidelity 2X Master Mix and11.5µl PCR-grade water. PCR was performed on a Peltier Thermal Cycler (Ptc-225) with cycling conditions of: 98°C for 40 seconds followed by 30 cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 60 seconds; and a final step of 72°C for 7 minutes. PCR products were visualised on agarose gel as previously described (Chapter 2, Section 2.3.3) to confirm successful cloning of the 441bp amplicon.

Selected colonies were extracted again via pipette tip and transferred to a 13ml Sarstedt tube with assembled ventilation cap containing 5ml LB culture broth with 100 µg/ml ampicillin and incubated in a shaking-incubator at 37°C overnight.

2.3.5 Extraction of Cloned Plasmid DNA

Plasmid isolation was completed using the PureLink® Quick Plasmid Miniprep Kit as per manufacturer's guidelines.

After overnight culture (Chapter 2, Section 2.3.4), 2ml of LB culture broth was transferred to a 2ml microcentrifuge tube and centrifuged at 12,000g on a Heraeus Pico 21 microcentrifuge for 10 minutes. The supernatant was removed and discarded, and the pelleted cells resuspended in 250µl resuspension buffer (R3) containing RNAse A. Two hundred and 50µl lysis buffer (L7) was then added. The samples were inverted and incubated at room temperature for 5 minutes. Three hundred and 50µl precipitation buffer (N4) was then added to the suspension which was then inverted and centrifuged for 10 minutes in the same manner. The supernatant was transferred to a spin column sat within a 2ml microcentrifuge tube and centrifuged for 1 minute. Flow through was discarded and 500µl ethanol-based wash buffer (W10) added to the

column. The column was incubated at room temperature for 1 minute before the flow through was discarded and 700µl wash buffer (W9) with ethanol was added to the column. The column was again centrifuged for 1 minute and the flow through discarded. To remove any residual wash buffer, the sample was centrifuged for a further minute.

The column was then transferred to a new microcentrifuge tube and DNA eluted from the column by the addition of 75µl TE Buffer to the centre of the column. The column was incubated at room temperature for 1 minute. The column was then centrifuged for 2 minutes. Eluted plasmid DNA was then stored at -20°C until use.

2.3.6 Confirmation of Sequences

To confirm the presence and sequence of each *hsp65* insert, 15µl of each plasmid was added separately to 2µl of 10mM M13 forward primer (5'- GTA AAA CGA CGG CCA G -3') and 2µl of 10mM M13 reverse primer (5'- CAG GAA ACA GCT ATG ACC – 3'). The plasmid-primer mixes was sent externally for Sanger sequencing (Eurofins Genomics, Ebersberg, Germany). The generated sequences were then compared to sequences using the basic local alignment search tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

2.4 Quantification of Sample Bacterial Load

The 520F and 802R primers were used to quantify the 16S rRNA gene [159]. Quantitative PCR was performed using the QuantStudio™ 7 Flex Real-Time PCR System. Reactions were performed in triplicate on a 96 well MicroAmp™ Optical Reaction Plate with Barcode. Each reaction contained 7.5µl KAPA SYBR FAST ROX Low 2X qPCR Master Mix, 0.3µl forward primer, 0.3µl reverse primer (both stock concentration10µM), 1.9µl PCR-grade water and 5µl template DNA or DNA standard. Quantitative PCR cycling conditions were: 90°C for 3 minutes followed by 40 cycles of 95°C for 20 seconds, 50°C for 30 seconds and 72°C for 30 seconds then a melt curve consisting of 95°C for 15 seconds, 60°C for 1 minute, then an increase of 0.05°C/s to 95°C, followed by a final 95°C for 15 seconds.

16S rRNA standards were prepared from a clone of *Vibrio natriegens* that was serially diluted 1:10 with molecular-grade water to give concentrations ranging from 2 x 10^7 down to 2 x 10^2 copies/µl. Sample DNA was diluted 1:10 for testing.

2.5 DNA Sequencing

2.5.1 Primer Assembly

Targeted gene sequencing via the Illumina® MiSeq™ platform requires the use of barcoded primers. These primers consist of 5 regions with specific functions (Figure 2.1).

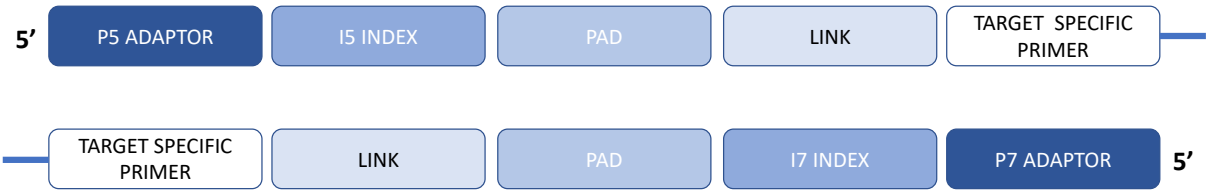


Figure 2.1 – Barcoded primer configuration for use with the Illumina® MiSeq™ platform.

P5 and P7 adaptor sequences are required for binding to the Illumina® flowcell and sequences were provided by the manufacturer. Index sequences were taken from the Nextera Index Kit (Illumina, San Diego, USA). A combination of eight i5 and twelve i7 sequences provides a barcode that is unique to each well on a 96 well plate. This means that when demultiplexing is carried out (see Section 2.3.4) reads can be traced back from the pooled library to their original sample. The pad region is a short sequence (typically 10 base pairs) designed to ensure that the sequencing primers have a melting temperature greater than 65°C. The link region is designed to reduce influence of the adaptors and pad sequences on the initial PCR. The 'target specific primer region' is engineered to amplify a specific sequence of the target genome. For analysis of communities based on the 16S rRNA gene the target specific primers were the 520F and 802R primers described by Claesson *et al.* [159]. Whole primer sequences are detailed in the Appendix (Table A1).

2.5.2 Quadruplicate PCR

A dedicated, template free UV hood was used to first UV irradiate four 96 well plates. A reaction cocktail was constructed so that each well contained 12.5µl Q5 High-Fidelity 2X Master mix and 6.5µl PCR-grade water. A 96 well plate containing the forward and reverse barcoded primers (at 1.5 µM) for each corresponding well had been pre-constructed (IDT®). Five µl of the primer mix was decanted to each corresponding well. Plates were then transferred to a UV irradiated hood where 1µl of each DNA template was transferred into an individual well (94 in total). For the last remaining two wells, a negative control was created by adding 1µl PCR-grade water to one well whilst in the last well a positive control was generated by the addition of 1µl of 10^8 genome copies/µl mock community. This was performed a further three times to produce quadruplicate plates.

16S rRNA PCR cycling was performed on a Peltier thermocycler. Conditions used were as follows: 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 50°C for 20 seconds and 72°C for 5 minutes. *Hsp65* PCR cycling conditions were: 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 55°C for 20 seconds and 72°C for 5 minutes.

To confirm no contamination had occurred during the PCR and successful amplification, amplicons from the wells containing negative and positive controls (mock community) were run on a 1.2% agarose gel against the four non-template controls (Chapter 2, Section 2.3.3 for gel preparation and use).

Each well from the four plates were subsequently pooled to a total volume of 100µl and successful amplification was confirmed by running a randomly selected row on a 1.2% agarose gel.

2.5.3 Paramagnetic Bead Based DNA Purification

Bioline JetSeq™ Clean solution was allowed to warm to room temperature for one hour prior to use. A uniform volume (70-80µl) was taken from each well and decanted into a round bottom 96 well plate. The Bioline JetSeq™ Clean solution was vortexed for 30 seconds to resuspend the paramagnetic beads. The solution was added to each well

in a 0.7:1 ratio (Bioline JetSeq™ Clean solution:sample) and mixed by pipetting. The plate was left at room temperature for 10 minutes before being placed on a magnetic plate for 5 minutes. The supernatant was then removed being careful not to disturb the paramagnetic beads. The plate was removed from the magnetic plate and 100µl 80% ethanol added to each well before resuspending the beads fully by pipette action. The plate was again placed onto the magnetic plate and left until the solution became clear. The liquid was removed before the addition of 100µl 80% ethanol and the above steps repeated.

After the final removal of the 80% ethanol, the pellets were allowed to air dry for approximately 5 minutes before the addition of 30µl low EDTA TE buffer. The pellets were then completely resuspended by pipetting. The plate was placed back on the magnetic plate and the suspensions containing the eluted DNAs carefully decanted into a new 96 well plate for the next steps.

2.5.4 Measurement of DNA Yield

Next the DNA products were quantified using the Quant-iT PicoGreen dsDNA Assay Kit on a TECAN Infinite 200 PRO microplate reader.

Reagents were first warmed to room temperature. A 20X TE solution was diluted to a working 1X TE using deionised water. A 1X PicoGreen working stock was created by diluting 200X PicoGreen reagent in a 1:200 ratio with the 1X TE solution. This solution was stored in the dark until use.

To quantify DNA yield, a DNA standard was created by adding 6µl lambda (λ) DNA standard (100ng/µl) to 594µl TE in a 1ml microcentrifuge tube. To ensure adequate mixing, the tube was vortexed. Serial dilutions were created by adding 300µl DNA standard from the previous aliquot to 300µl 1X TE in a 1ml microcentrifuge tube and vortexing for 10 seconds. A total of 7 serial dilutions were created with a final microcentrifuge tube consisting of 1X TE only. One hundred µl of each standard was transferred in duplicate to a black fluorimeter assay plate. Ninety 9µl of 1X TE was transferred to the remaining wells and 1µl of template DNA added to this. Finally, 100µl 1X PicoGreen reagent was added to all wells and well contents mixed by pipetting.

The plate was scanned using the following parameters: transmission wavelength 485nm, emission wavelength 535nm, 5 flashes per well using an extended dynamic range gain. DNA concentrations were calculated against the standard curve using a linear regression model on Excel software.

2.5.5 Equimolar Pooling

Having determined the DNA concentration of each sample, an equimolar pool of samples was created using the pre-determined DNA concentrations. Samples were pooled into a single 2ml microcentrifuge tube.

The equimolar pooled sample next underwent a further paramagnetic bead-based DNA purification step as detailed above (Chapter 2, Section 2.5.4) with the exception that a magnetic stand able to hold a microcentrifuge tube was used in place of the magnetic plate. The final library was resuspended in 30µl of low-EDTA TE in a 1.5ml microcentrifuge tube ready for gel extraction and purification.

2.5.6 Gel Extraction and Purification

The equimolar pooled sample was run against a 100bp DNA Ladder on a 150ml 1.5% agarose gel with 10µl GelRed dye run at 120V for 45 minutes. The gel was subsequently viewed under a FirstLight UV illuminator. The band of appropriate size (approximately 350 base pairs for the 16S rRNA gene and 441bp for the *hsp65* gene) was excised and underwent gel purification using the Monarch® DNA Gel Extraction Kit as detailed in Chapter2, Section 2.3.3.

2.5.7 High Sensitivity DNA Chip

To further check the pooled library size the Agilent 2100 High Sensitivity DNA chip was used. All kit components were warmed to room temperature for 30 minutes prior to use. The high sensitive DNA dye concentrate was vortexed for 10 seconds. Fifteen µl of the dye was decanted into the high sensitivity DNA gel matrix vial. This was subsequently stored at 4ºC in the dark. Prior to use the tube was vortexed for 10 seconds to mix the gel and dye. The gel dye mix was transferred to a spin filter. This was then placed in a microcentrifuge and spun at 2,240g for 10 minutes.

The high sensitivity DNA chip was placed on the chip priming station. Nine µl of the gel-dye mix was pipetted to the well labelled G (see Figure 2.2). With the syringe of the chip priming station set to 1ml, the station was closed and locked. The syringe was pressed and held in place by the clip for 60 seconds. The syringe was then released and reset to the 0.3ml mark. After 5 seconds the syringe was pulled back to 1 ml. The chip priming station was then pulled up and 9µl of the gel-dye mix were added to the wells marked **G** (Figure 2.2).

Five µl of high sensitivity DNA marker was loaded into the well labelled ladder and to each sample well. One µl of the high sensitivity DNA ladder was added to the well labelled with the ladder symbol (Figure 2.2).

One µl of sample was added to one sample well and 1µl of marker added to the unused sample wells.

The chip was placed horizontally in the adapter of the IKA vortex mixer and vortexed for 1 minute at 2,400rpm. Within 5 minutes of vortexing, the chip was placed in the Agilent 2100 Bioanalyser for analysis.

Figure 2.2 - Agilent 2100 High Sensitivity DNA chip illustrating different loading wells.

2.5.8 Library Quantitative PCR

The purified library was quantified using the Bioline JetSeq™Library Quantification Lo-ROX kit. DNA standards provided in the kit were as follows: 10pM, 1pM, 100fM, 10fM, 1fM, 100aM. In the first instance the library was diluted in the following manner: 1 in 1,000, 1 in 2,000, 1 in 4,000, 1 in 8,000. Per reaction 10µl 2X JetSeq Library Quantification Lo-ROX Mix and 5µl of the supplied primer mix were combined with 5µl of standard or the diluted library in a MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1mL (Applied Biosystems™).

Quantitative PCR was performed using the QuantStudio™ 7 Flex Real-Time PCR System. Cycling conditions were as follows: 95°C for 2 minutes followed by 40 cycles of 95°C for 5 seconds, 60°C for 45 seconds and then a melt curve consisting of 95°C for 15 seconds, 60°C for 1 minute, then an increase of 0.05°C/s up to 95°C for 15 seconds.

2.5.9 Final Library Preparation

To optimise cluster density and to act as a positive control, DNA from the bacteriophage Phi X 174 was added to the library. Two µl of 10nM PhiX was added to 3µl EBT buffer (Illumina, San Diego, USA). To denature the sample, 5µl 0.2N NaOH was added to the solution which was then incubated at room temperature for 5 minutes. Following this 990µl HT1 buffer (Illumina, San Diego, USA) was added to the solution. The PhiX was then further diluted to a concentration of 8 pM by adding 400µl of the PhiX to 600µl HT1 Buffer.

The library was denatured by adding 10µl 0.2N NaOH to 10µl of the original library. This was allowed to incubate at room temperature for 5 minutes, before the addition of 980µl HT1 buffer. A final library concentration of 8pm was achieved by diluting the denatured library with HT1 buffer.

A final library concentration of 8pM (with 20% PhiX spike) was prepared by adding 200µl of the 8pM PhiX with 800µl of the 8pM library.

2.5.10 MiSeq™ Sequencing

Prior to and after each sequencing run a maintenance wash of the MiSeq benchtop sequencer was performed according to the manufacturer's instructions.

An Illumina® MiSeq™ v3 cartridge was defrosted in a 20°C water bath and inverted to mix the reagents. Reservoir positions 12, 13 and 14 were pierced using a sterile pipette tip. Using an extra-long P200 pipette tip (VWR™), 3µl of read 1 primer mix was placed in reservoir 12, 3µl of index primer mix in reservoir 13 and 3µl of read 2 primer mix was placed in reservoir 14 (all primers at 100mM concentration) (see Appendix Table A1 for primer sequences). Each primer was mixed with cartridge reagent by pipetting. Lastly 600µl of the final denatured library was added to reservoir 17 (marked 'load sample' on the cartridge) and mixed by pipetting. The cartridge was immediately placed into the sequencer.

A MiSeq™ flowcell was concomitantly washed with deionised water ensuring all residual salt crystals had been removed prior to use. The flowcell was next dried with lint free optical paper before being cleaned with an optical lens wipe. The flowcell was then placed into the sequencer.

The wash bottle was removed from the sequencer and replaced with a bottle containing PR2 solution.

Sequencing was commenced and ran for 600 cycles with a run time of approximately 56 hours. During this time, a series of quality metrics were recorded. These included:

- cluster density - a measurement of clonal clusters density (K/mm^2).
- clusters passing filter - a measurement of clusters passing the internal MiSeq chastity filter (%PF). This serves as an indication of signal purity from each cluster.
- Quality Score (Q30) - the probability of an incorrect base call made during the sequencing process. A high Q-Score indicates that a base call is more reliable and less likely to be incorrect.

- Numbers of reads – the total number of reads produced at the end of the sequencing run.

2.6 QIIME Computational Analysis

FASTQ files generated by the MiSeq platform were imported to a working directory and processed with QIIME 1.9.1. Steps for data processing are outlined in Figure 2.3.

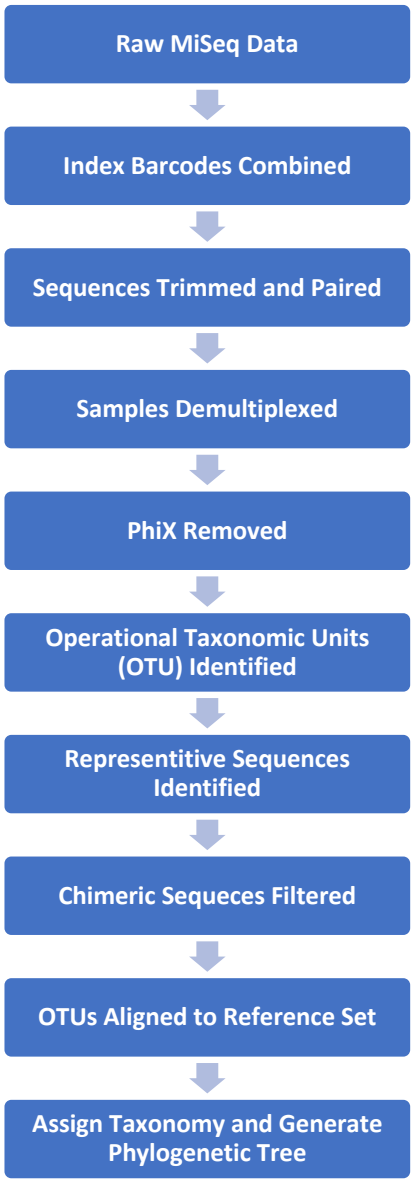


Figure 2.3 – QIIME data analysis pipeline.

2.6.1 Index Barcodes Combined

Each sample undergoes the PCR process with two primers. Each primer contains an eight base pair index read (Index 1 and Index 2 – see Chapter 2, Section 2.5.1). In this step each index read is combined to form a 16 base pair length barcode unique to each sample. This barcode can be used to subsequently demultiplex samples and assign OTUs to each sample.

2.6.2 Sequences Trimmed and Joined

Forward and reverse primers were trimmed using the trim_galore (Babraham Bioinformatics - http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) package and adaptor sequences removed. The minimum amplicon length required for trim_galore to function was set to 150 base pairs. Forward and reverse reads were then paired and those sequences with over 200bp overlap and fewer than 10% mismatches were retained.

2.6.3 Demultiplexing of Samples

Paired reads subsequently underwent the process of demultiplexing. During this process the transcribed primer index reads are used to map each read back to its original PCR well and therefore to the original sample.

2.6.4 Removal of PhiX

Sequences were next aligned to the PhiX genome and removed using the samtools and bamtools software [160] (https://github.com/pezmaster31/bamtools/wiki).

2.6.5 Identification of Operational Taxonomic Units (OTUs)

OTUs were identified using the SILVA rRNA reference gene database [161]. Sequences were clustered at a 97% similarity to the reference sequence and reads below this level were discarded.

A 10% subsample of these failed sequences were selected at random and clustered *de novo.* Each cluster centroid was chosen to represent a new reference sequence and sequences that were not identified against the SILVA rRNA reference gene database, were aligned against these *de novo* reference sequences.

2.6.6 Identification of Representative Sequences

After OTUs were identified the most abundant sequence within these clusters was picked to represent the OTU for taxonomic assignment.

2.6.7 Chimeric Sequences Filtered

The representative sequences were aligned against the SILVA database and the ChimeraSlayer algorithm [162] was used to identify chimeric sequences. Chimeric sequences with less than 60% similarity to reference sequences were filtered.

2.6.8 Alignment of OTUs to Reference Set

Representative sequences were then aligned using the PyNAST alignment method [163] to the SILVA rRNA gene database [161].

2.6.9 Assign Taxonomy and Generate Phylogenetic Tree

Using the uclust algorithm [164], representative sequences were aligned against the reference database. A minimum percent similarity of 90% between database and representative sequences was applied and taxonomy assigned on the basis of taxonomic similarity.

A maximum likelihood phylogenetic tree was constructed using the FastTree programme [165] from the representative sequences. Sequences within each OTU cluster were assigned the same taxonomy and a biom format OTU table was generated.

These files were imported into the R statistical analysis software environment (version 3.6.1) for further analysis.

2.7 Data Analysis Using R

All data was analysed using the 'The R Project for Statistical Computing' version 3.6.1 (R core team 2014). Packages used for downstream analysis included:

- Phyloseq (version 1.28.0) - a tool to analyse complex phylogenetic sequencing data
- ggplot2 (version 3.2.1) – a graphical analysis package
- stringr (version 1.4.0) - wrappers for common string operations
- grid (version 3.6.1)
- gridExtra (version 2.3) – both grid and the gridExtra packages are used for constructing grid-based plots
- plyr (version 1.8.4) – a tool for splitting, applying and combing data
- dplyr (version 0.8.3) – a grammar of data manipulation
- vegan (version 2.5.6 – a package for analyzing community ecology
- jpeg (version 0.1.8) – a package to read and write jpeg images
- decontam (version 1.4.0) – a package used to identify contaminants in marker gene and metagenomics sequencing data
- indicspecies (version 1.7.6) – a package to determine relationship between species and groups of sites
- ggdendro (version 0.1.20) – a package used to create dendrograms and tree diagrams using ggplot2
- RColorBrewer (version 1.1.2) - a package for generating colour palettes in R

2.7.1 Identifying Contaminants

The ‘decontam' package listed above was used to identify and filter potential contaminants. The decontam package utilises two methods to identify contaminants. The frequency method utilises the distribution of the frequency of each sequence serving as a function of DNA concentration obtained from sample quantitative PCR (i.e. if a sequence frequency is low in relation to the overall DNA concentration then the probability of it being a contaminant is higher). The prevalence method analyses the presence or absence of each sequence in negative controls is compared to the prevalence of sequences in samples.

Both methods generate a *P* value (default set to 0.1) to determine the probability of the sequence being a contaminant. Sequences identified as contaminants were then pruned from the phyloseq object.

2.7.2 Indicator Species Analysis

To assess for batch effect the indicspecies package was used for indicator species analysis was performed according to DNA extraction batch number. Significant indicator OTUs were compared to ensure that no OTUs appeared only in one extraction batch possibly indicating their role as contaminants.

2.7.3 Sample Rarefaction

OTUs with fewer than 10 reads were removed to account for spurious reads.

It is inevitable that sequencing depth will vary significantly between samples. There is a positive correlation between sequencing depth and the number of different observed OTUs. Consequently, samples with greater sequencing depth may have an artificially inflated diversity. To account for this potential bias data underwent rarefaction (random re-sampling) so that all samples contained the same number of reads.

The construction of rarefaction curves uses random resampling to estimate the number of generated OTUs (Y axis) against number of reads (X axis).

2.7.4 Measuring Diversity

Species diversity can be defined as the interplay between the number of different taxa present (richness) and the proportions of taxa present (evenness) within a given community. The terms alpha and beta diversity were coined by Whittaker [166, 167].

Alpha diversity refers to the diversity within a local habitat (for example a single sputum sample). In this thesis, alpha diversity was characterised using the following measurements:

- Observed Diversity – The number of different taxa within a sample.
- Shannon index – The Shannon index (or Shannon entropy) aims to quantify the degree of uncertainty associated with predicting the next correct OTU within a string of data.
- Simpson's index – Simpson's index of diversity measures the probability that two taxa randomly drawn from the same sample will be the same.
- Pielou's index – A measure of taxa evenness within a sample.

Beta diversity refers to the degree of taxonomic dissimilarity between samples in a regional habitat (for example multiple sputum samples). Beta diversity was measured by comparing the Bray Curtis dissimilarity between different sample groupings. This measure estimates the number of shared species between two groups and considers it as a proportion of total species number. Non-Metric multi-Dimensional Scaling (NMDS) was used to visualise trends in beta diversity. Statistical significance in beta diversity was assessed using the Adonis function in Vegan.

Chapter 3: Developing a Molecular Diagnostic Test

3.1 Introduction

3.1.1 How are Non-Tuberculous Mycobacteria (NTM) identified from patient samples?

Microbiological confirmation is an essential component of the diagnostic criteria for Non-Tuberculous Mycobacterial (NTM) disease [1, 2, 168]. Mycobacteria are generally acknowledged as being difficult to culture with slow culture times due to long reproductive cycles [169], leading to delayed diagnosis that may lead to progression of an insidious disease process.

The process of microscopy and culture still forms the bedrock of diagnosis of NTM infection. The sensitivity of this technique however, is greatly affected by sample quality as well as user experience [170]. Furthermore, microscopy and culture in isolation are unable to discriminate between mycobacterial species and further tests are subsequently required.

NTM culture requires the inoculation of samples into a culture medium. Prior to this, a decontamination procedure must be followed to prevent overgrowth of bacterial contaminants. These decontamination protocols can adversely affect NTM viability [171] thus reducing sensitivity. The most common culture technique is the use of Mycobacterial Growth Indicator Tubes (MGIT, BD Biosciences, Sparks, Maryland, USA) however, some NTM species require supplemented media or extended duration of incubation and despite this there remain species yet to be cultured [168, 172]. The vast majority of pathogenic NTM will grow within 6 weeks. The current UK recommendation is to culture for 8 weeks extending to 12 weeks if necessary. Culture techniques alone cannot be used to discriminate between different mycobacterial species.

Treatment of NTM disease requires accurate species identification because treatment regimens differ depending on the bacterial species isolated. Species identification is performed by molecular techniques. Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry has shown considerable promise in providing rapid speciation of NTM. The optimal method, however, for protein extraction

from mycobacteria and the exact discriminatory power of this method have yet to be fully established [173-175]. In the UK, commercially available kits utilising line probe assay technology are often employed for in house molecular identification of NTM species. Since 2018 the National Mycobacterial Reference Service has offered a species verification service, utilising whole genome sequencing techniques. This, however, confers an additional cost to the referrer and is not standard practice in UK clinical microbiology labs.

Although molecular techniques to detect NTM directly from respiratory specimens (without the need to culture samples) are now commercially available, they are costly and remain less sensitive than conventional acid-fast bacilli (AFB) culture. To date, clinical validation of these tests in different patient cohorts and for different NTM species is incomplete and therefore their role in routine clinical practice remains to be determined [2].

Considering the methods detailed above there is clearly a niche for an affordable method that, in a timely manner, could identify NTM to a species level.

3.1.2 What are the benefits of a direct, quantitative molecular test?

A further limitation of current NTM diagnostic techniques is that they provide a binary outcome (presence or absence of the isolate) and no information on quantity. Due to the challenges associated with mycobacterial culture false negatives are a constant risk leading to the potential for premature cessation of treatment. This potential is mitigated by the recommendation that therapy continues for at least one year after culture conversion (defined as three consecutive culture negative results over a period of at least three months) and so either treatment may be stopped prematurely leading to high rates of disease relapse or patients are exposed to medications with side effects for a longer period than required.

Direct molecular testing involves the analysis of samples without the need for culture. The result of this is far quicker as any delay incurred relates to the process of DNA extraction (approximately 48 hours as opposed to the potential 8 -12 weeks for culture recommended by the BTS guidelines). In the case of *Mycobacterium tuberculosis*

molecular testing based around the polymerase chain reaction (PCR) principle has been shown to have a similar specificity and predictive values to culture [176-178] and the addition of Taqman™ fluorescent probes can be used to quantify DNA copy number. This gives clinicians information on treatment efficacy and will allow clinicians to halt treatment with improved confidence.

A direct quantitative molecular assay has the potential to provide scientists and clinicians with a diagnostic test that is more sensitive, gives results in a matter of days and can quantify DNA copy number.

3.1.3 Hypothesis and Aims

Study hypotheses addressed in this chapter are as follows:

1. Molecular techniques will accurately identify mycobacterial DNA to a species level.
2. Molecular techniques can be used to quantify mycobacterial DNA copy numbers.

The aims of this chapter are as follows:

1. To develop a molecular technique that will allow identification of the six most commonly pathogenic mycobacterial species [18]:
    - *M. abscessus* complex
    - *M. avium*
    - *M. intracellulare*
    - *M. kansasii*
    - *M. malmoense*
    - *M. xenopi*
2. To develop a molecular technique that will accurately quantify DNA copy number of the above species.

3.2 Methods

3.2.1 Gene Selection

Genes that have been widely sequenced across mycobacterial species include the 16S rRNA, *rpoB* and *hsp65* gene. The *hsp65* gene has emerged as the favoured target

gene for species identification owing to its relatively large interspecies variability (allowing for species differentiation) and its preservation between isolates of the same species. Studies comparing the three genes to evaluate their performance as part of a multi-locus sequence analysis showed that the *hsp65* gene varied more amongst mycobacterial species (89.27% to 100% compared to 96.57% to 100% for 16S rRNA gene and 92.71% to 100% for *rpoB* gene) and that the rate of species level identification was highest among the *hsp65* gene (86.79% versus 71.3% versus 81.55%) [179, 180].

3.2.2 Primer and Probe Determination

Sequences for the *hsp65* gene from the 6 pathogenic mycobacteria described in Chapter 3, Section 3.1.3 were obtained from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov - accessed on 05/12/16) and aligned using the Geneious alignment software package (http://www.geneious.com/features/genome-alignment - accessed 01/02/17). Plasmid sequences obtained by Sanger-based sequencing (see Chapter 2, Section 2.2.2) were aligned against these sequences to confirm their identity.

Sequences were manually searched for areas of variation that would allow the design of probes to differentiate between species. Once these discriminatory areas were identified, primers were designed to generate a 140 base pair amplicon. An online tool was used to confirm that these custom primers would not generate amplicons from other bacterial species found in the respiratory tract (http://insilico.ehu.eus/PCR - accessed 05/02/17). Primers and probes were sourced from Eurofins (Eurofins Genomics, Ebersberg, Germany) with 'high purity salt free' (HPSF) purification. Primer and probe sequences are shown in Table 3.1.

Table 3.1 – Custom designed primers and probes used for identification and quantification of NTM species. Letters in red represent the names of each fluorophore (5' end) and the name of each quencher (3' end) used.

Sequence (5' to 3')	Function in Assay
CGAGACCAAGGASCAGATC	Forward primer
GCAGGCCGAAGGTGTTGG	Reverse primer
FAM TGCCACCGCGGCCATC BQ1	*M. avium* probe
HEX GATTTCGGCGGGCGACC BQ2	*M. intracellulare* probe
CY5 GGCCACGGCCGGTATCTCC BQ2	*M. abscessus* complex probe
FAM CGCGACCGCCGCGATCTCG BQ1	*M. malmoense* probe
HEX CATCTCCGCGGGTGACCAGG BQ2	*M. xenopi* probe
CY5 GGCGACCGCGGCCATCTCCGCC BQ2	*M. kansasii* probe

3.2.3 Establishing Annealing Temperatures

Theoretical melting temperatures for the designed primers were calculated using an online primer melting temperature calculator (https://tmcalculator.neb.com/#!/main - accessed 05/02/17). Based on these melting temperatures, a temperature gradient PCR ranging from 52°C to 70°C was conducted using a Peltier thermocycler (model PTC-225). Each reaction mix consisted of 12.5µl Q5 master mix, 0.5µl forward primer, 0.5µl reverse primer (both at 10 µM), 1µl plasmid DNA 10.5µl molecular grade water. Cycling conditions were 98°C for 30 seconds; followed by 40 cycles of 98°C for 10 seconds, variable temperature for 30 seconds, 72°C for 20 seconds before a final elongation step of 72°C for 2 minutes.

Amplification was confirmed on a 1.2% agarose gel with 3µl of GelRed dye with subsequent UV visualization on a FirstLight UV illuminator (Chapter 2, Section 2.3.3). Based on this an annealing temperature of 66°C was selected.

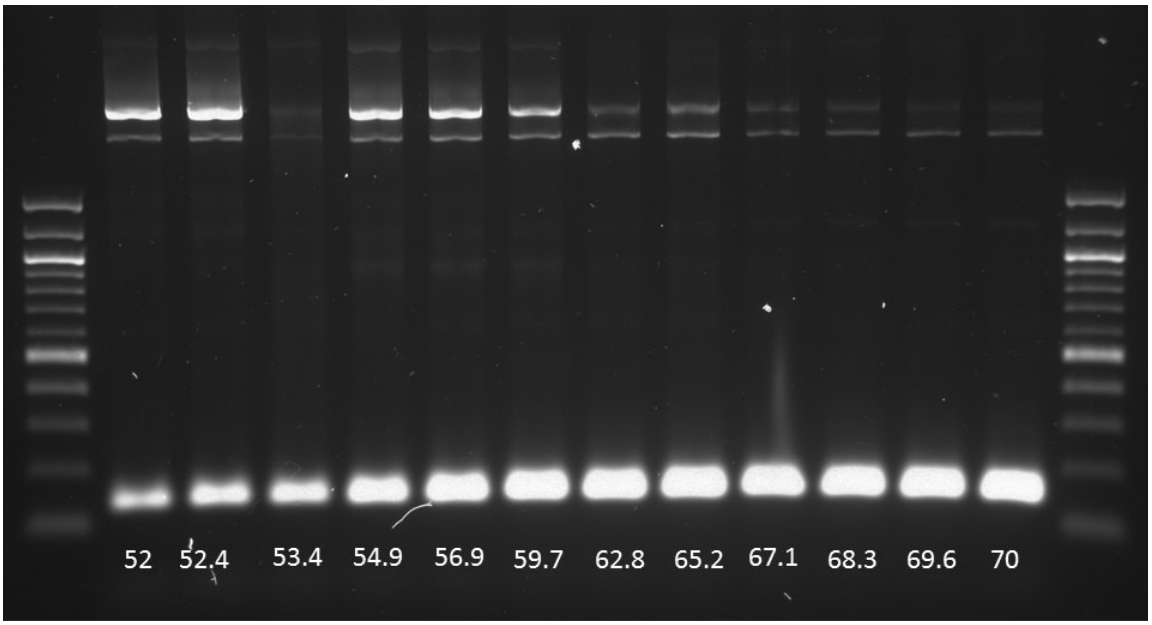


Figure 3.1 – Agarose gel showing PCR products from a temperature gradient PCR. Lanes from left to right were: 52°C, 52.4 °C, 53.4 °C, 54.9 °C, 56.9 °C, 59.7 °C, 62.8 °C, 65.2 °C, 67.1 °C, 68.3 °C, 69.6 °C and 70 °C. Temperatures between 65.2 °C and 67.1 °C resulted in little primer dimer formation with good visualisation of products.

3.3 Probe Specificity

3.3.1 Methods

To establish probe specificity, one probe assay was tested at a time in the following manner.

Serial dilutions of the reciprocal plasmid (to the probe being tested) were prepared in triplicate (concentrations ranged from 2 x10^8 to 2 x10^3 copies/µl) in a MicroAmp™ Optical Reaction with Barcode 96 well plate. These acted both as positive controls and as standards to assess assay efficiency.

In the remaining wells, plasmids containing the *hsp65* insert from different NTM species (see Chapter 2, Section 3) were individually tested in triplicate against each probe in turn. The identities of the 13 plasmids were initially concealed. Positive probe-plasmid reactions were noted with probe specificity subsequently calculated.

Each reaction mix contained: 10µl Luna Universal Probe qPCR Master Mix, 0.8µl forward primer, 0.8µl reverse primer (both at 10µM), 0.4µl probe (at 10µM), 3µl

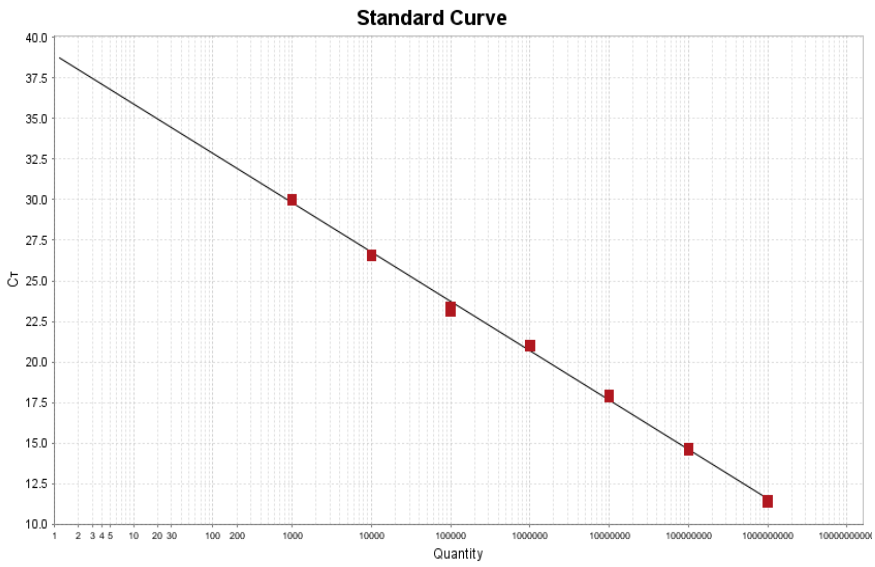
nuclease free water and 5ul template DNA. PCR was carried out using the QuantStudio 7 Flex Real-Time PCR System and analysed using the QuantStudio Software V1.3.

Cycling conditions were 98°C for 30 seconds; followed by 40 cycles of 98°C for 10 seconds, 66°C for 30 seconds, 72°C for 20 seconds before a final elongation step of 72°C for 2 minutes.

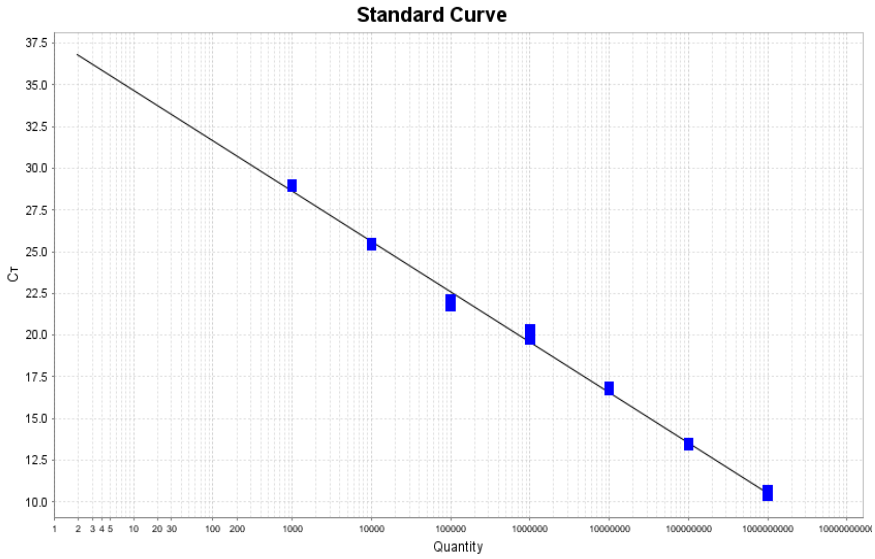
3.3.2 Results

3.3.2.1 Assay Efficiency

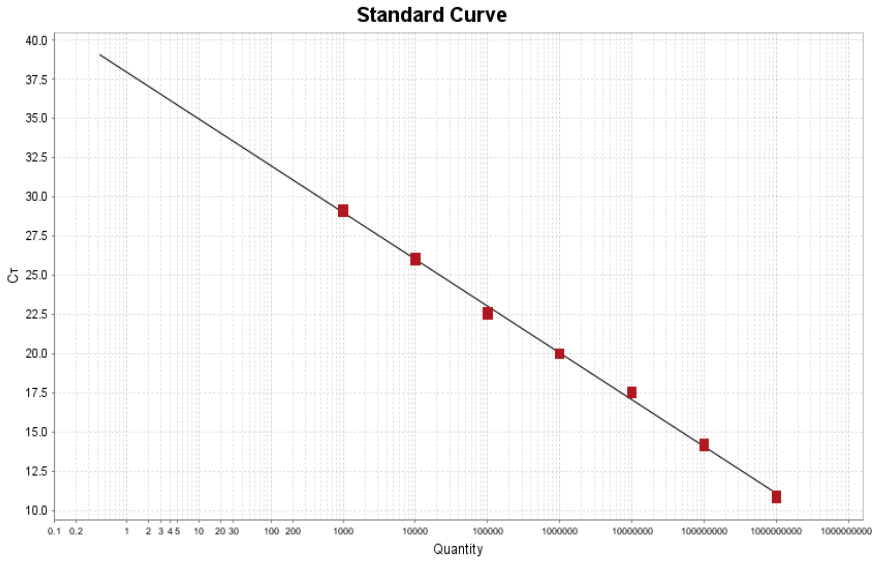
In an ideal reaction the number of amplicons of the target sequence should double during each replication cycle. This corresponds to a 100% amplification efficiency. Should the number of replicated molecules fail to double this is described as poor efficiency (below 100%). The most common reasons for lower efficiencies are poor primer design, non-optimised reagent concentrations or reaction conditions. Secondary structures (such as dimers and hairpins) or inappropriate melting temperatures can affect primer template annealing which results in poor amplification. The main reason for efficiency values greater than 100% is PCR inhibition. Inhibitors of the polymerase enzyme include excessive amounts of DNA/RNA or carry-over material in the sample. Desired PCR efficiencies range from 90 – 120% [181] [182]



Target: *M. abscessus* Slope: -3.041 Y– Inter 38.926 R^2 0.998 Efficiency 109.215 Error: 0.032

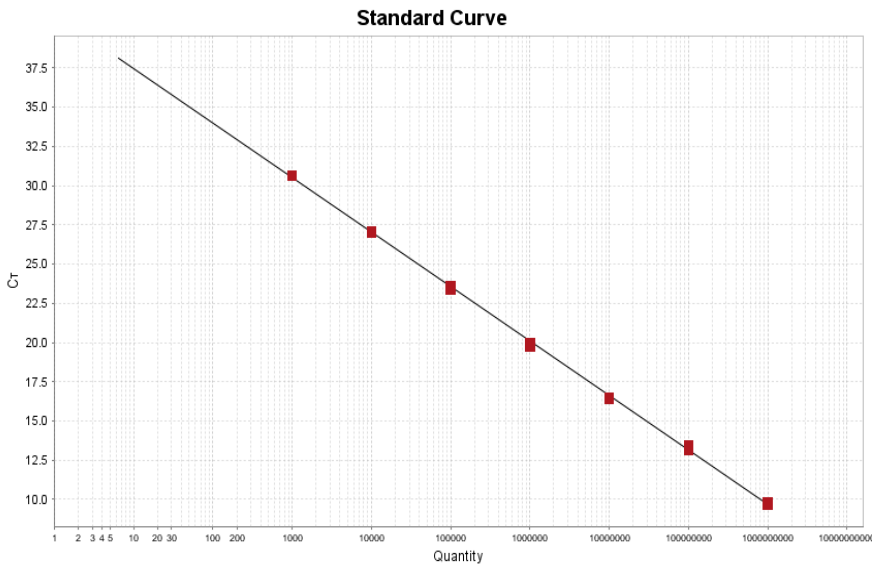


Target: *M. avium* Slope: -3.016 Y– Inter 37.673 R^2 0.996 Efficiency 110.59 Error: 0.043

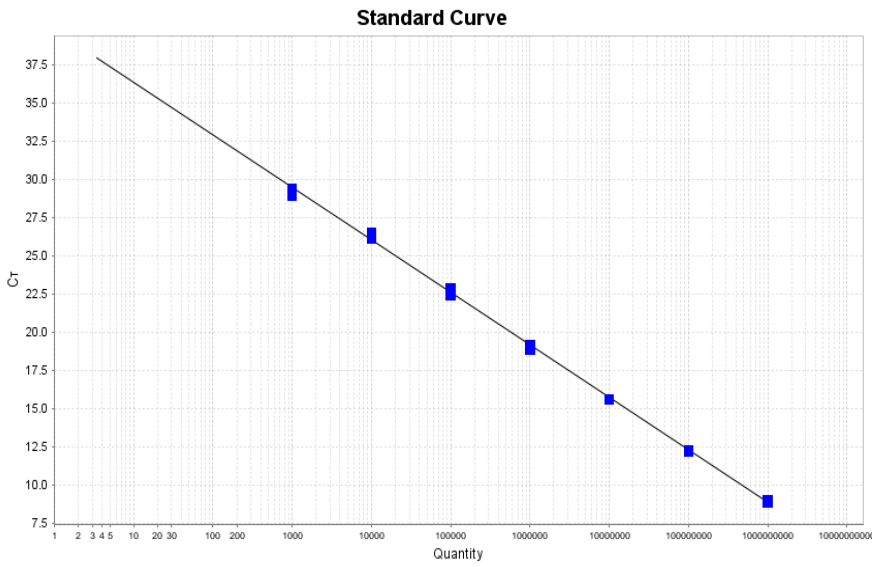


Target: *M. intracellulare* Slope: -2.984 Y– Inter 37.934 R^2 0.998 Efficiency 109.349 Error: 0.033

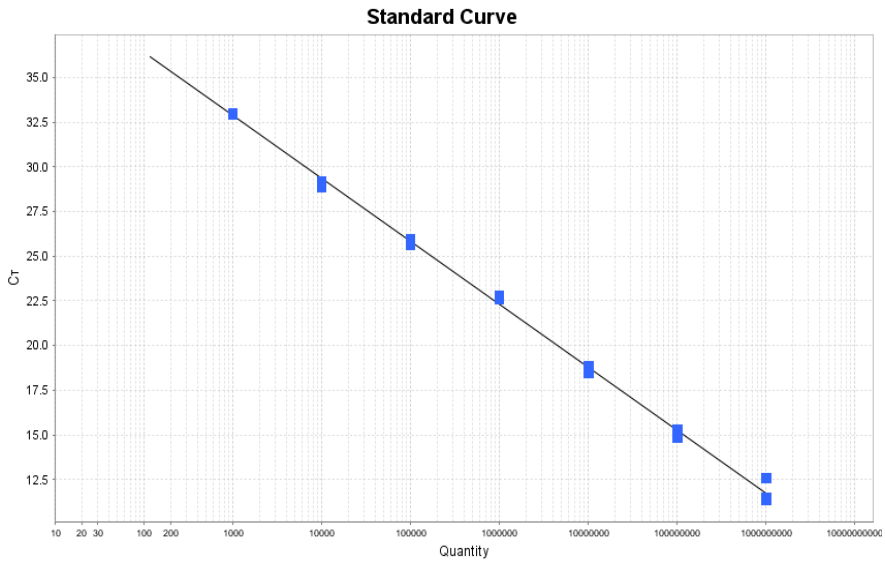
Figure 3.2 – Illustrative standard curves for custom primers and *M. abscessus* probe, *M. avium* probe, *M. intracellulare* probe. Reaction efficiencies were: 109.215%, 110.59%, 109.349% respectively with R^2 values of: 0.998, 0.996, 0.998 respectively with standards ranging from 10^8 to 10^2 copies/µl. X axis = copy number, Y axis = threshold cycle.



Target: *M. kansasii* Slope: -3.471 Y– Inter 40.898 R^2 0.999 Efficiency 94.115 Error: 0.018



Target: *M. malmoense* Slope: -3.429 Y– Inter 39.724 R^2 0.999 Efficiency 95.725 Error: 0.022



Target: *M. xenopi* Slope: -3.514 Y– Inter 43.385 R^2 0.998 Efficiency 92.561 Error: 0.035

Figure 3.3 – Illustrative standard curve for custom primers and *M. kansasii* probe, *M. malmoense* probe, *M. xenopi* probe. Reaction efficiency was: 94.115%, 95.725%, 92.561% respectively with R^2 values of: 0.999, 0.999, 0.998 respectively with standards ranging from 10^8 to 10^2 copies/µl. X axis = copy number, Y axis = threshold cycle.

3.3.2.2 Assay Specificity

After recording which plasmids reacted to the designed probes the plasmid identities were revealed. Probe specificity was defined as "the probe's ability to identify true negatives". Each probe's specificity was calculated using the following equation:

$$\frac{True\ Negatives}{True\ Negatives + False\ Positives}$$

Positive probe reactions are shown in Table 3.2 with calculated probe specificity detailed in Table 3.3.

Table 3.2 – Results of the customised probe reactions. A tick signifies positive annealing of the probe against the corresponding plasmid. A red tick indicates an appropriately positive reaction (true positive), a black tick an inappropriately positive reaction (false positive). The abscessus probe reacts appropriately with three different plasmids. These represent the *M. abscessus* complex.

		***hsp65* Mycobacterial Species Insert Identity**												
		psychrotolerans	*abscessus*	*bolletii*	*massiliense*	*avium*	*intracellulare*	*chelonae*	*fortuitum*	*kansasii*	*malmoense*	*szulgai*	*xenopi*	*gordonae*
Probe Identity	Abscessus		✓	✓	✓									
	Avium					✓						✓		
	Intracellulare						✓							
	Kansasii									✓			✓	
	Malmoense	✓									✓			
	Xenopi												✓	

Table 3.3 – Specificity of each custom probe based on the 13 different mycobacterial plasmids. Although specificity was only established using a selection of plasmids, an online tool confirmed that the custom primers do not amplify non-mycobacterial bacteria (http://insilico.ehu.eus/PCR - accessed 05/02/17).

Probe Identity	Specificity
Abscessus complex	100%
Avium	91.6%
Intracellulare	100%
Kansasii	91.6%
Malmoense	91.6%
Xenopi	100%

3.4 Probe Sensitivity

Probe Sensitivity was defined in two ways. As a diagnostic test, probe sensitivity was defined as "the probe's ability to identify true positives" (as above Section 3.3) using the equation:

$$\frac{True\ Positives}{True\ Positives + False\ Negatives}$$

On this basis each probe's *in vitro* sensitivity was calculated at 100%. Probe sensitivity was also defined as "the probe's ability to accurately quantify target DNA copy number". To do this, mock communities were created, and a known quantity of target DNA spiked into the assay.

3.5 Quantification of Mycobacterial DNA

3.5.1 Construction of a Mock Community

A mock community was created containing genomic DNA amplified using the Illustra™ Ready-To Go™ GenomiPhi™ HY DNA Amplification kit. The genomic DNA was obtained in lyophilised form from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The mock community composition was designed to reflect a simplified lung microbiome with common floral constituents (*Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae*), human DNA (selected as this is present in large quantities in sputum samples) and *M. gordonae* (selected as this is a common sputum

mycobacterial isolate that the customised primers will amplify but is thought to be non-pathogenic).

Genomic DNA was amplified in the following way: 25µl of the GenomiPhi kit denaturation buffer was added to 10ng of genomic DNA. PCR-grade water was then added to the mix to bring the total volume to 50µl. The mix was then heated to 95°C for 3 minutes to denature the DNA and then cooled to 4°C on ice. The 50µl of template was then added to the provided tubes containing the "cake" mix. The samples were then incubated at 30°C for 4 hours for DNA amplification before being heated to 65°C for 10 minutes to inactivate the Phi29 DNA polymerase enzyme before it was cooled to 4°C.

The resulting product was then quantified using the Quant-iT PicoGreen dsDNA Assay Kit (see Chapter 2, Section 2.5.4). DNA copy number was determined using the equation below and diluted to the concentrations specified in Table 3.4. Species were selected for the mock community on the basis that they are commonly found in the respiratory tract [183-185].

$$\text{number of DNA copies} = \frac{(\text{template amount (ng) x } 6.022\text{x}1023)}{(\text{template length (base pairs) x } 1\text{x}109 \text{ x } 650)}$$

Table 3.4 - Mock community constituents and their corresponding concentrations.

Species	DSMZ code	DNA Concentration (copies/µl)
S. pneumoniae	20566	2×10^5
S. aureus	20231	2×10^5
H. influenzae	4690	2×10^5
M. gordonae	44160	2×10^5
H. sapiens		2×10^3

3.5.2 Experimental Design

Quantitative PCR was performed as described in Section 3.3.1 using serial dilutions of the target plasmid as quantification standards ranging from 2×10^8 to 2×10^3 copies/µl. Template DNA consisted of 4µl of the mock community (Section 3.5.1) with the addition of 1µl target DNA. Measurement of target DNA was performed in triplicate with serial dilutions spiked into the mock community. Target DNA concentration

ranged from 1 x 10^6 copy/µl to 1 copy/µl. The resulting plate format is illustrated in Figure 3.4.

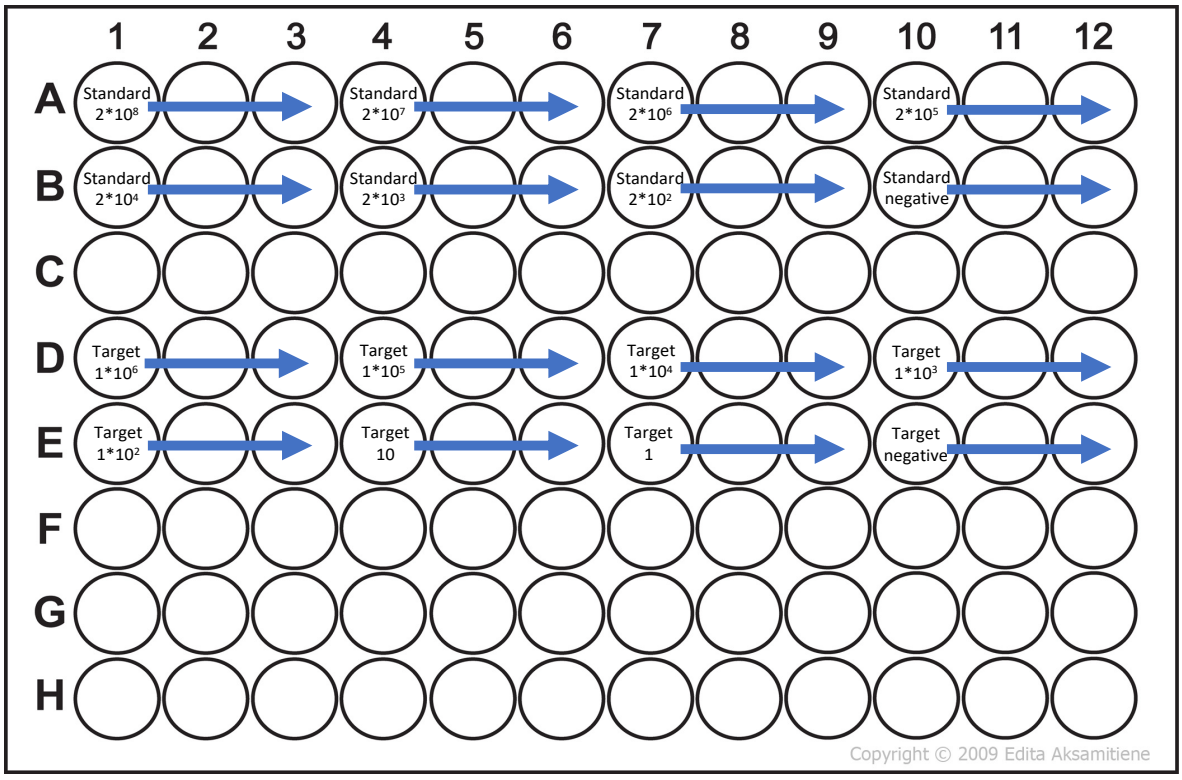


Figure 3.4 – Ninety-six well plate schematic illustrating the experimental set up detailed above. The first two rows were occupied with quantification standards ranging from 2 x 10^8 to 2 x 10^3 copies/µl. Target DNA concentration ranged from 1 x 10^6 copy/µl to 1 copy/µl.

In the first instance plasmids (see Chapter 2, Section 2.3) were used as an amplification target as they had undergone successful sequencing (Chapter 2, Section 2.3.6) thus confirming a single insert of each probes' target region. Accurate quantification of each plasmid therefore meant that any exaggeration in copy number when the assay was used with genomic DNA was either due to multiple *hsp65* gene inserts or "off target" amplification that did not occur against the plasmid.

The experiments were also designed to allow comparison of the accuracy of the probes when used in singleplex versus multiplex.

Singleplex reaction constituents were: 10µl Luna Universal Probe qPCR Master Mix, 0.8µl forward primer, 0.8µl reverse primer (both at 10µM), 0.4µl probe (at 10µM), 3µl nuclease free water and 5µl template DNA.

Multiplex reaction constituents were: 10µl Luna Universal Probe qPCR Master Mix, 0.8µl forward primer, 0.8µl reverse primer (both at 10µM), 0.4µl probe1, 0.4µl probe2, 0.4µl probe3 (all at 10µM), 3µl nuclease free water and 5ul template DNA. Multiplex reactions were limited to three probes owing to a potential for overlap of probe emission wavelengths which could have led to false positive results.

The combination of probes was also considered. It was felt that due to the *M. kansasii* probe's cross-reactivity with the *M. xenopi* plasmid (Table 3.2), the two probes should be placed together in combination so that this cross-reactivity bias could be compared directly alongside the behaviour of the *M. xenopi* probe.

The experimental design is illustrated in Figure 3.5.

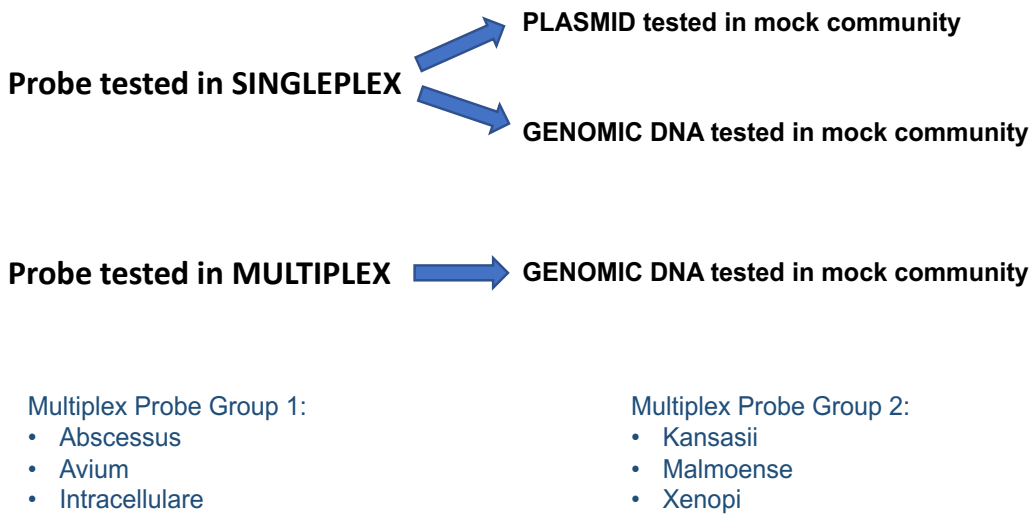


Figure 3.5 – Schematic illustrating experimental design. Probes were first tested in singleplex against their target plasmid before confirming that they worked against genomic DNA in the same manner. Following this, probes were tested in multiplex with three probes used each time. Probe groupings are detailed in blue.

3.5.3 Results

The assay was first tested with probes in singleplex against plasmids containing the target insert. The experiment was then repeated using probes in singleplex against genomic DNA before multiplex testing against genomic DNA (Figure 3.5).

3.5.3.1 Probes Tested in Singleplex against Plasmid

In the first set of experiments template DNA consisted of 4µl of mock community and 1µl of the target plasmid spiked in serial dilutions ranging from 1 x 10^6 copy/µl to 1 copy/µl. The figures below (Figures 3.6 to 3.11) give a graphical representation of each assays' ability to detect target plasmid DNA within a mock community. For details of the exact measured DNA quantity as well as Ct values for each experiment, please refer to Tables A1 to A6 inclusive of the appendix.

The distribution of all data sets was first assessed for normality using the Shapiro-Wilk normality test. This revealed a non-normal distribution and therefore the Wilcoxon rank sum test was used for comparisons between two groups.

M. abscessus Probe

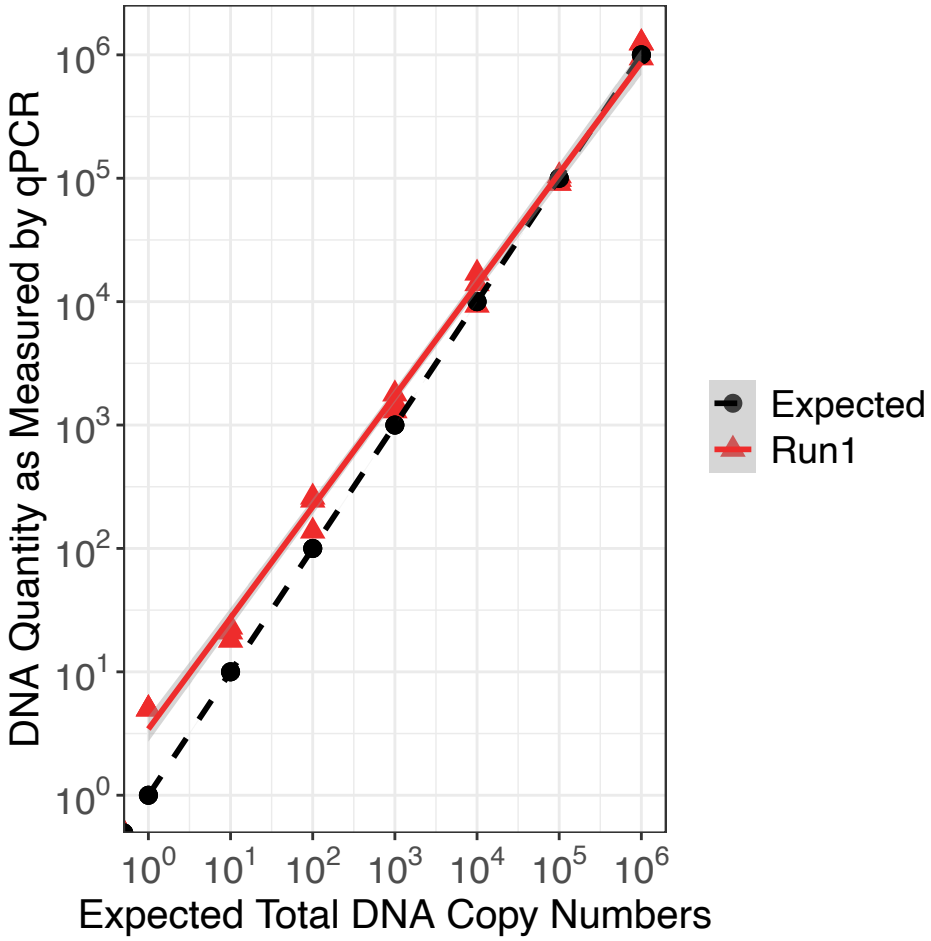


Figure 3.6 – Figure illustrating the assay's ability to detect the *M. abscessus* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.69).

The limit of detection is defined as "the lowest amount of analyte in a sample that can be detected with a 95% confidence that produces at least 95% positive replicates" [186]. In the case of the *M. abscessus* plasmid (Figure 3.6) the assay detected a minimal theoretical value of 1 as a mean of 4.673 target molecules. The limit of detection in this case ranges from 4.586 – 4.837 target molecules.

M. avium Probe

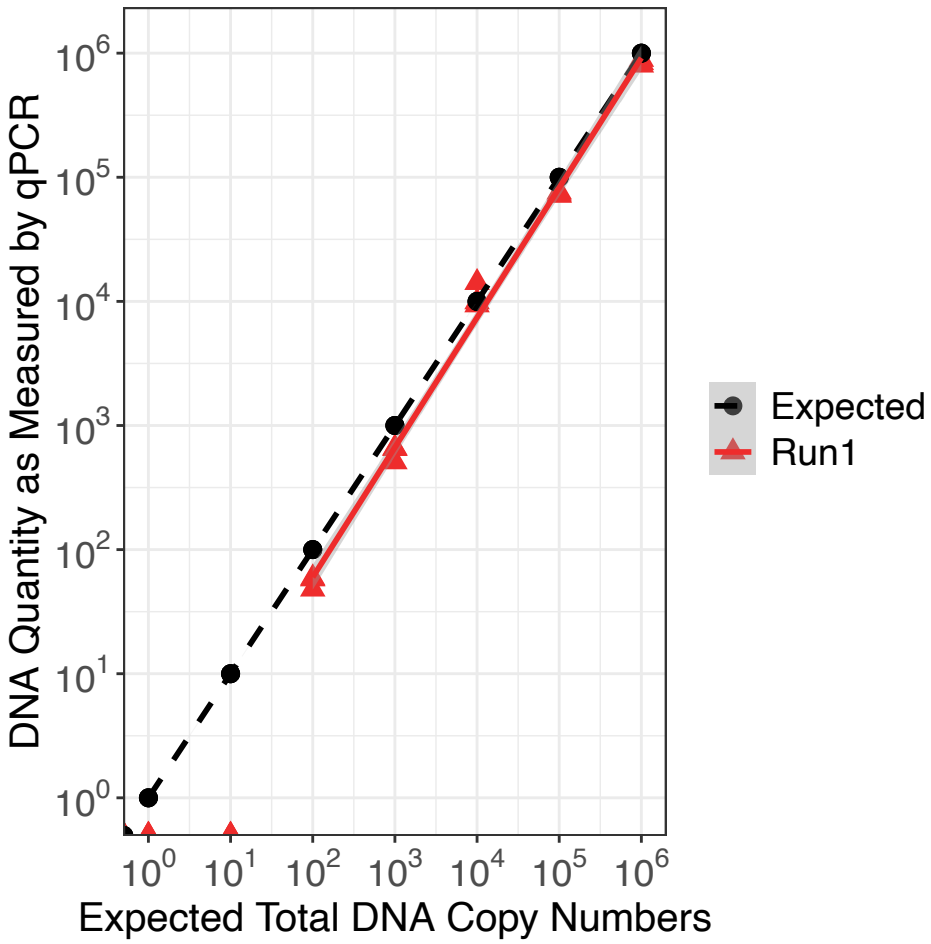


Figure 3.7 – Figure illustrating the assay's ability to detect the *M. avium* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.34).

In the case of the *M. avium* plasmid (Figure 3.7) the assay detected a minimal theoretical value of 100 as a mean of 54.914 target molecules. The limit of detection in this case ranges from 47.819 – 58.753 target molecules.

M. intracellulare Probe

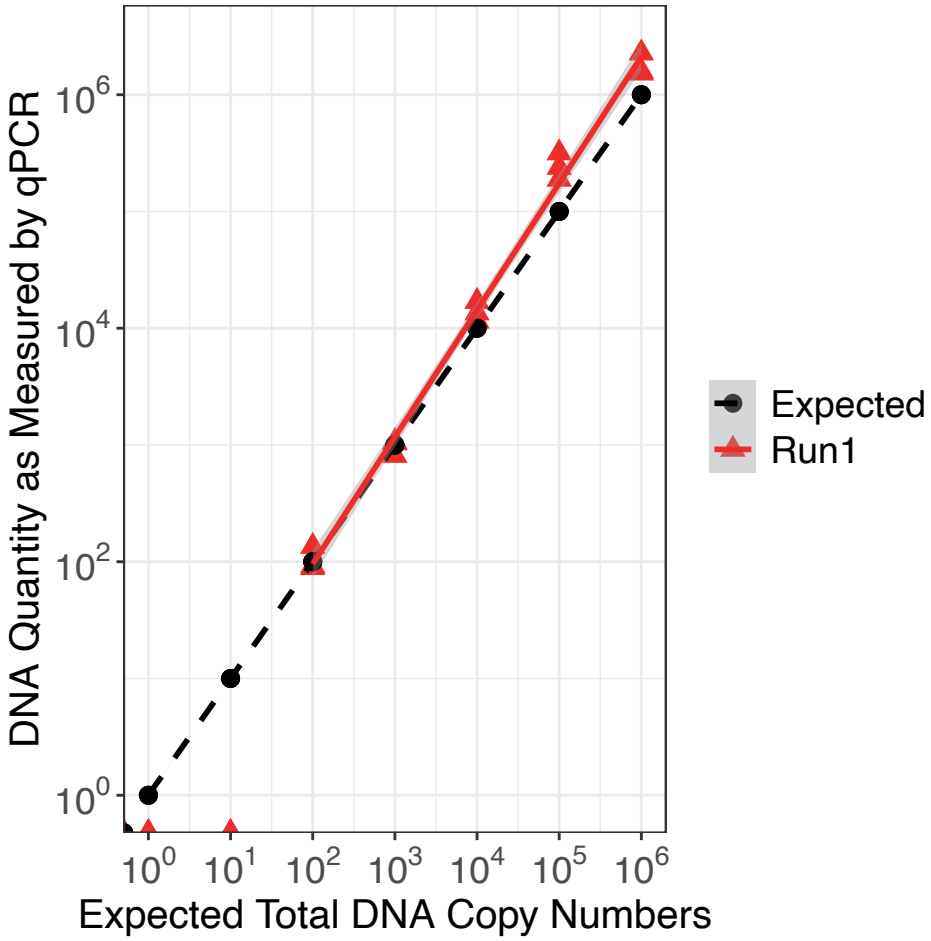


Figure 3.8 – Figure illustrating the assay's ability to detect the *M. intracellulare* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.79).

In the case of the *M. intracellulare* plasmid (Figure 3.8) the assay detected a minimal theoretical value of 100 as a mean of 105.11 target molecules. The limit of detection in this case ranges from 88.873 – 134.627 target molecules.

M. kansasii Probe

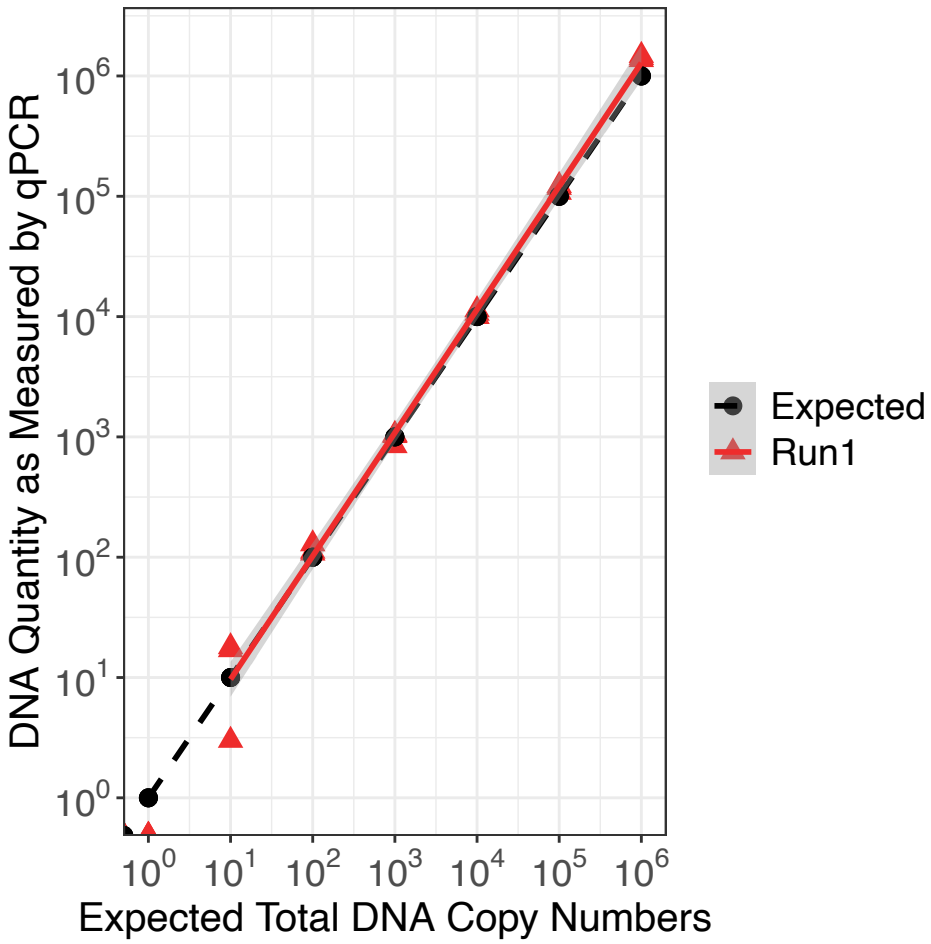


Figure 3.9 – Figure illustrating the assay's ability to detect the *M. kansasii* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.86).

In the case of the *M. kansasii* plasmid (Figure 3.9) the assay detected a minimal theoretical value of 10 as a mean of 12.61 target molecules. The limit of detection in this case ranges from 2.866 – 17.618 target molecules.

M. malmoense Probe

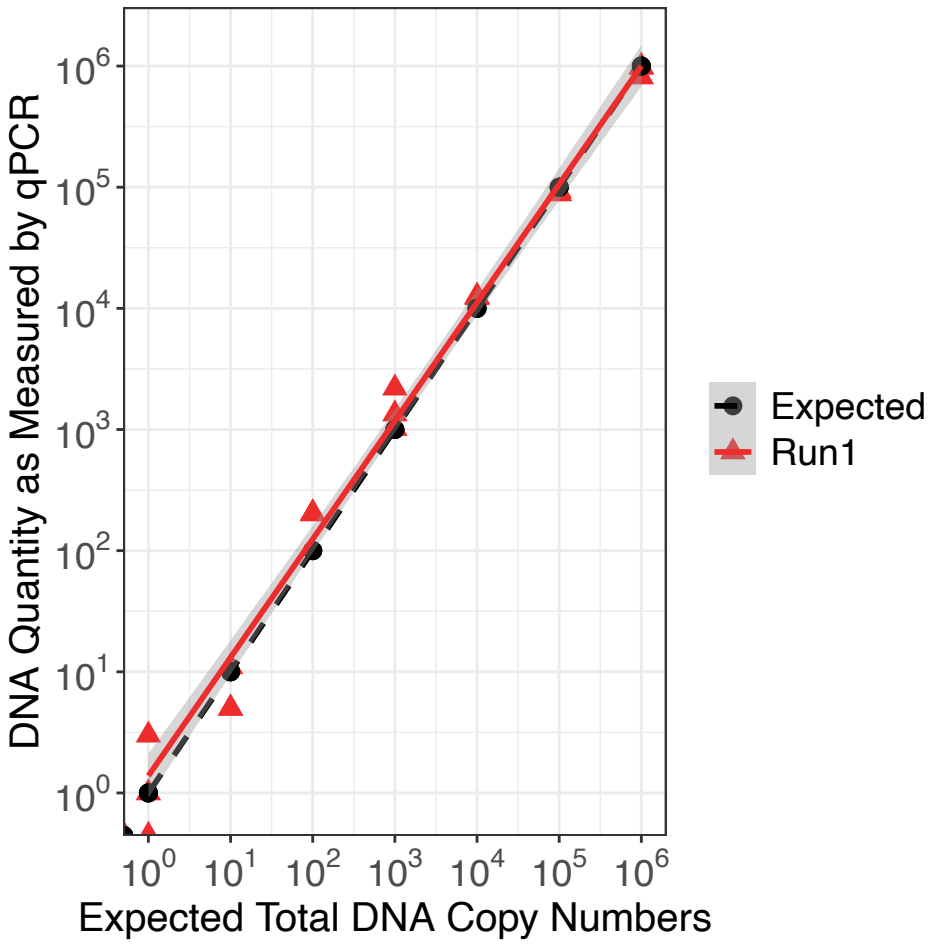


Figure 3.10 – Figure illustrating the assay's ability to detect the *M. malmoense* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.98).

In the case of the *M. malmoense* plasmid (Figure 3.10) the assay detected a minimal theoretical value of 1 as a mean of 1.3 target molecules. The limit of detection in this case ranges from 0.284 – 2.598 target molecules.

M. xenopi Probe

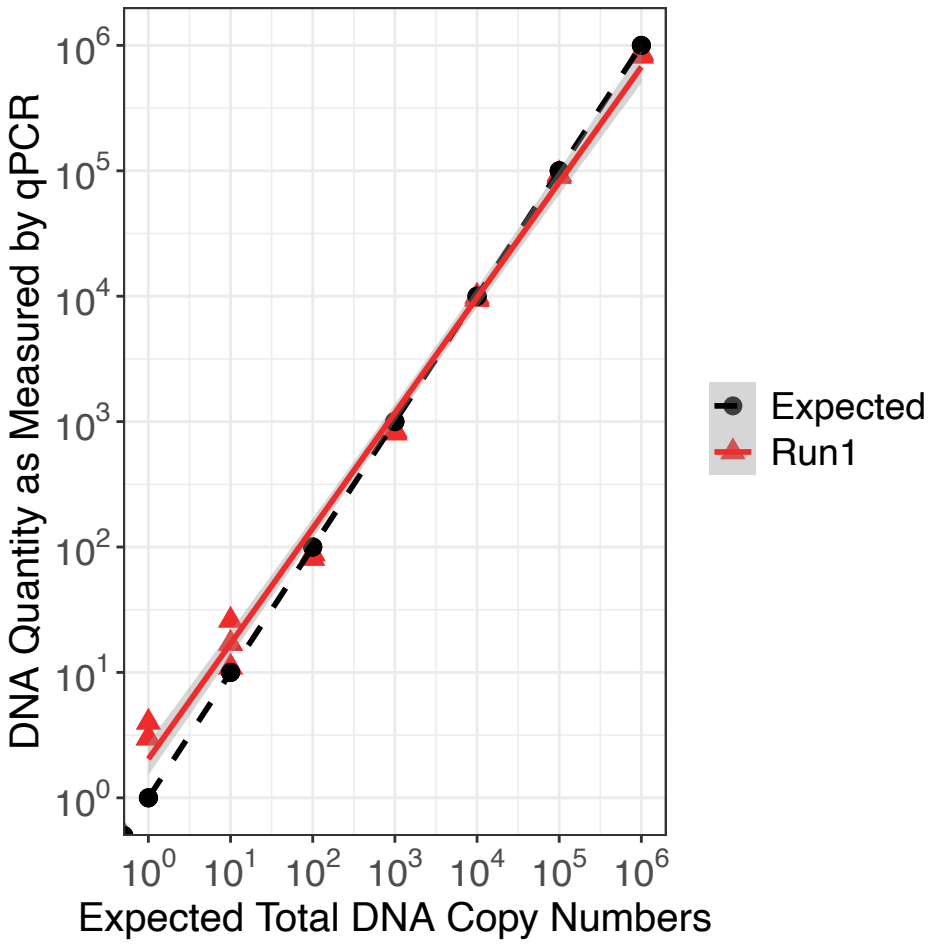


Figure 3.11 – Figure illustrating the assay's ability to detect the *M. xenopi* plasmid when present in a mock community. Measured DNA quantity within the assay is shown in red against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.79).

In the case of the *M. xenopi* plasmid (Figure 3.11) the assay detected a minimal theoretical value of 1 as a mean of 3.31 target molecules. The limit of detection in this case ranges from 2.908 – 3.387 target molecules.

3.5.3.2 Probes Tested in Singleplex against Genomic DNA

In the second set of experiments template DNA consisted of 4µl of mock community and 1µl of target genomic DNA spiked in serial dilutions ranging from 1 x 10^6 copy/µl to 1 copy/µl. To demonstrate the assay's reproducibility against genomic DNA, testing was performed in duplicate (Run 1 and Run 2 in each experiment). The figures below (Figures 3.12 to 3.17) give a graphical representation of each assay's ability to detect target DNA within a mock community. For details of the exact measured DNA quantity as well as Ct values for each experiment, please refer to Tables A7 to A12 inclusive of the appendices.

The distribution of all data sets was first assessed for normality using the Shapiro-Wilk normality test. This revealed a non-normal distribution and therefore the Kruskal-Wallis test was used for comparisons between more than two groups.

M. abscessus Probe

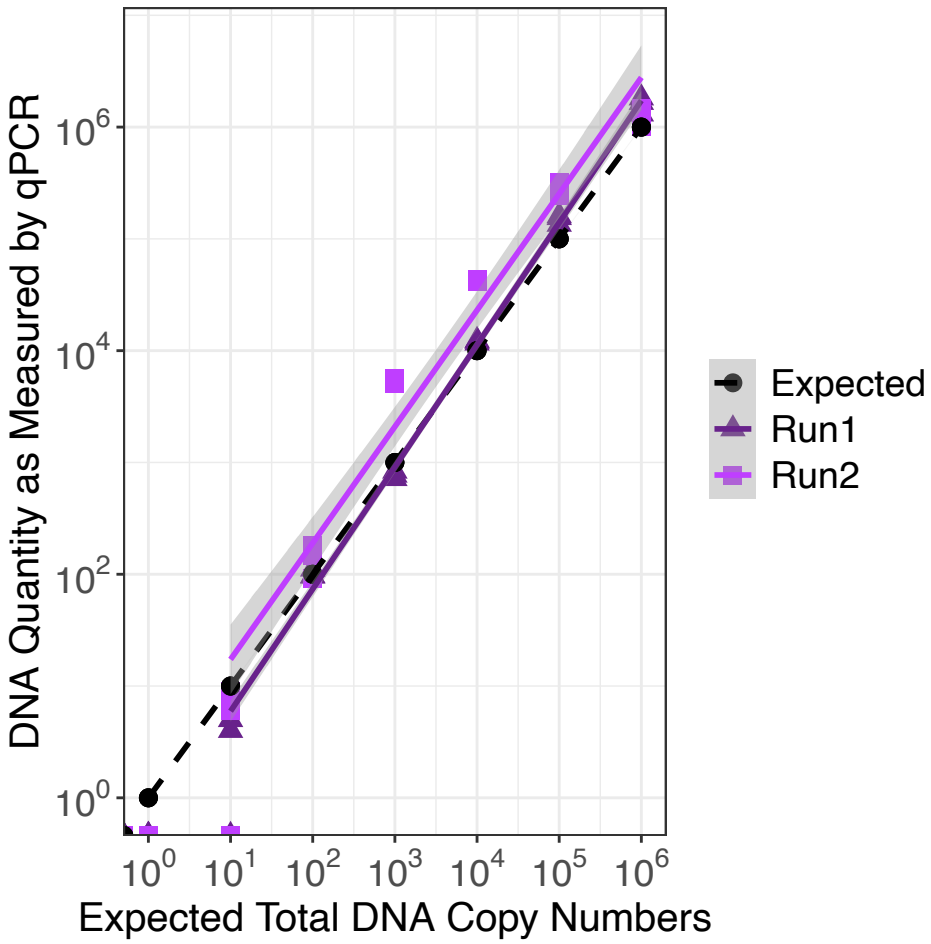


Figure 3.12 - Figure illustrating the assay's ability to detect *M. abscessus* when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.95).

In the case of genomic *M. abscessus* DNA (Figure 3.12), the assay detected a minimal theoretical value of 10 as a mean of 2.996 target molecules on 'Run 1' and 7.708 target molecules on 'Run 2'. The limit of detection in this case ranges from 4.368 – 9.128 target molecules.

M. avium Probe

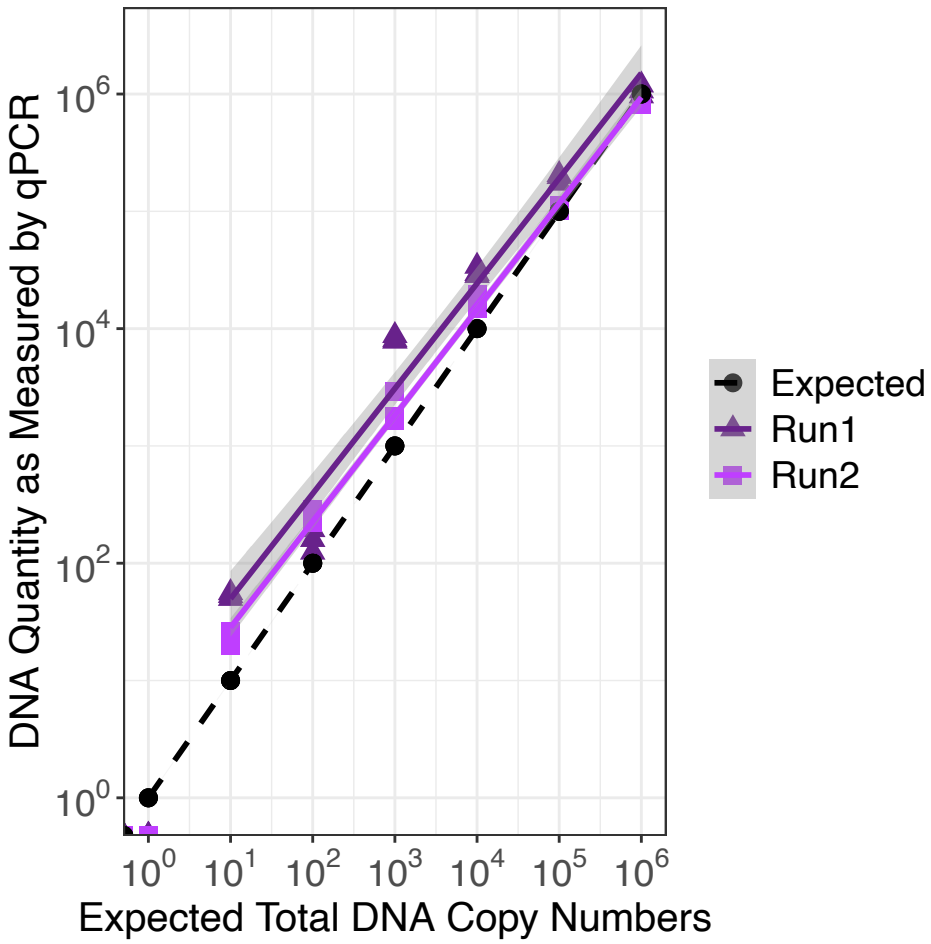


Figure 3.13 - Figure illustrating the assay's ability to detect *M. avium* when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.92).

In the case of genomic *M. avium* DNA (Figure 3.13), the assay detected a minimal theoretical value of 10 as a mean of 53.153 target molecules on 'Run 1' and 22.883 target molecules on 'Run 2'. The limit of detection in this case ranges from 20.48 – 56.13 target molecules.

M. intracellulare Probe

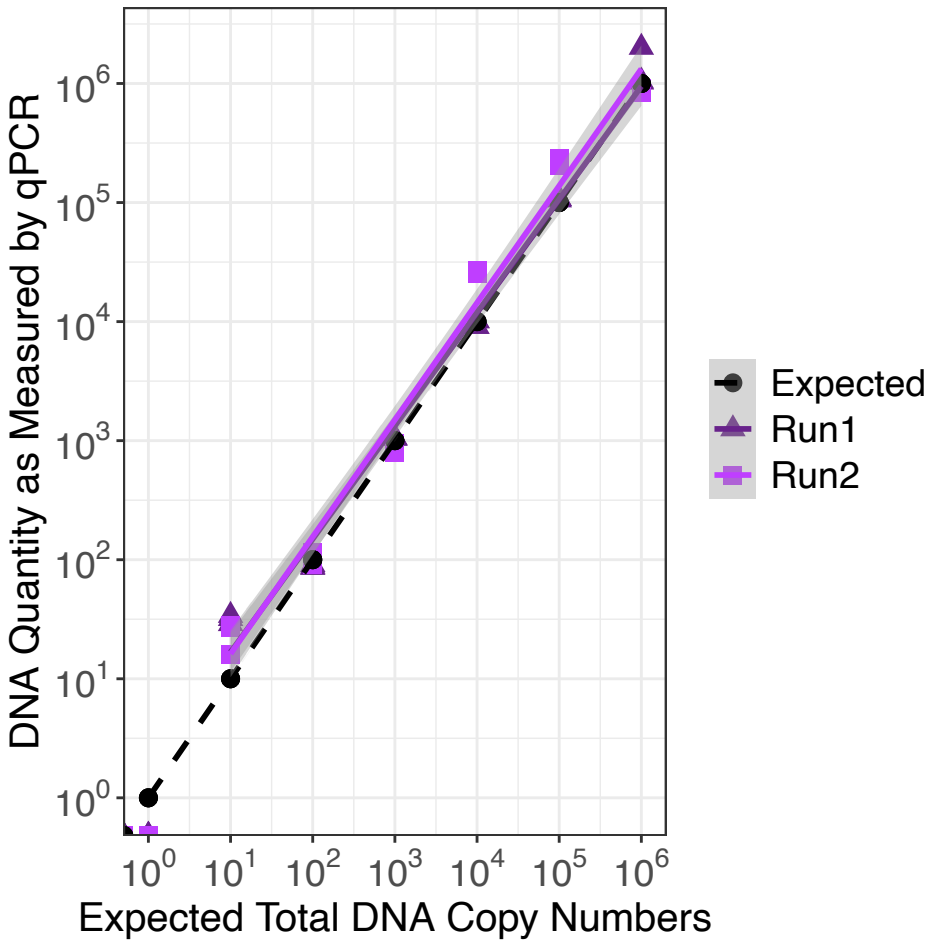


Figure 3.14 - Figure illustrating the assay's ability to detect *M. intracellulare* when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.99).

In the case of genomic *M. intracellulare* DNA (Figure 3.14), the assay detected a minimal theoretical value of 10 as a mean of 31.67 target molecules on 'Run 1' and 23.801 target molecules on 'Run 2'. The limit of detection in this case ranges from 16.113 – 34.21 target molecules.

M. kansasii Probe

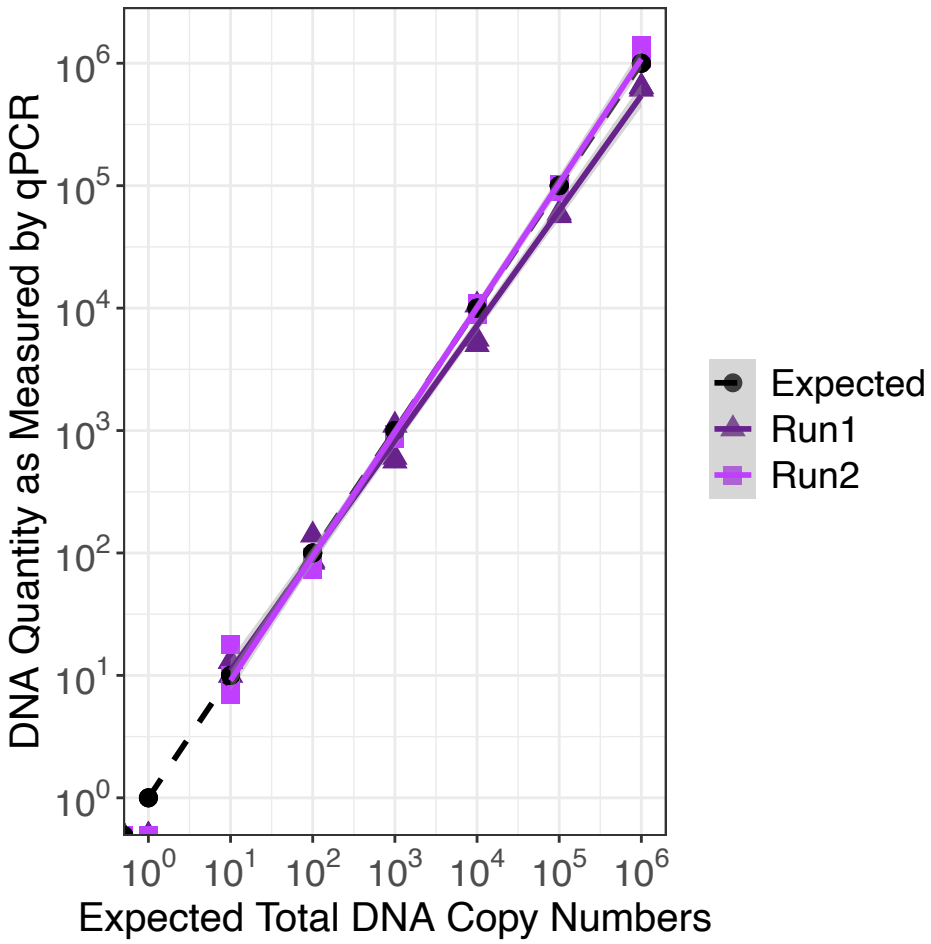


Figure 3.15 - Figure illustrating the assay's ability to detect *M. kansasii* when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.89).

In the case of genomic *M. kansasii* DNA (Figure 3.15), the assay detected a minimal theoretical value of 10 as a mean of 11.855 target molecules on 'Run 1' and 11.714 target molecules on 'Run 2'. The limit of detection in this case ranges from 7.181 – 18.073 target molecules.

M. malmoense Probe

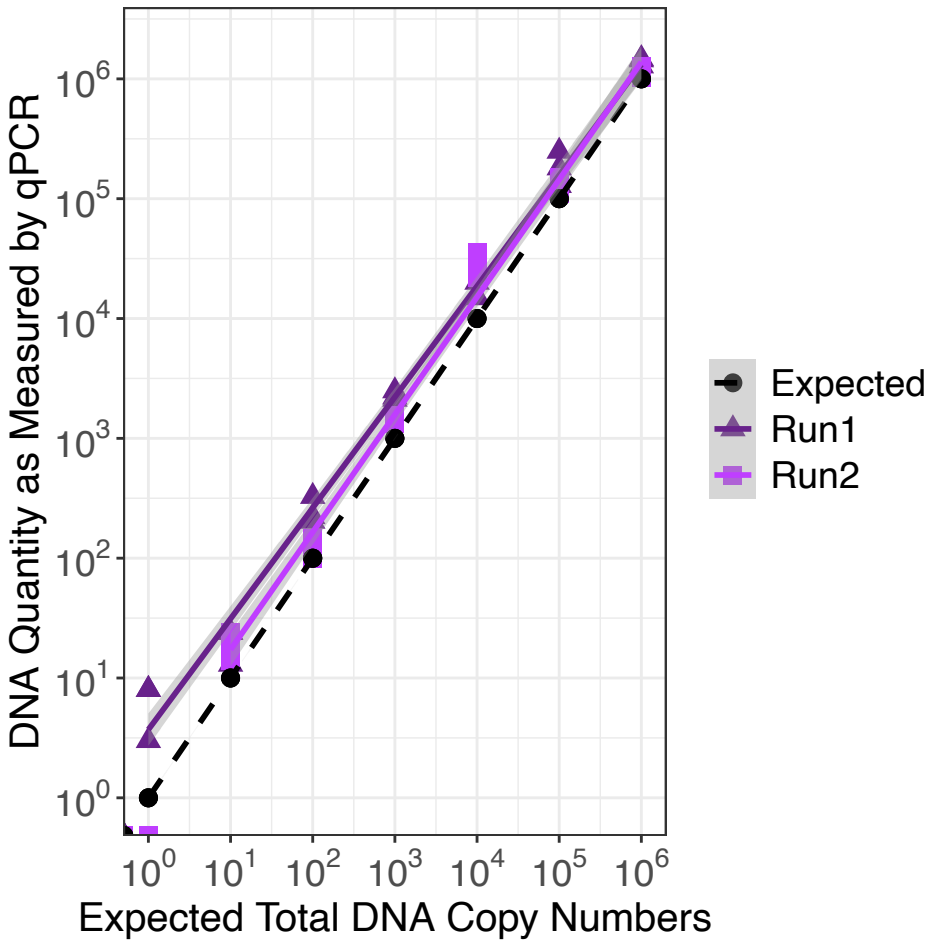


Figure 3.16 - Figure illustrating the assay's ability to detect *M. malmoense* when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.78).

In the case of genomic *M. malmoense* DNA (Figure 3.16), the assay detected a minimal theoretical value of 10 as a mean of 6.477 target molecules on 'Run 1' and 18.561 target molecules on 'Run 2'. The limit of detection in this case ranges from 6.477 – 18.561 target molecules.

M. xenopi Probe

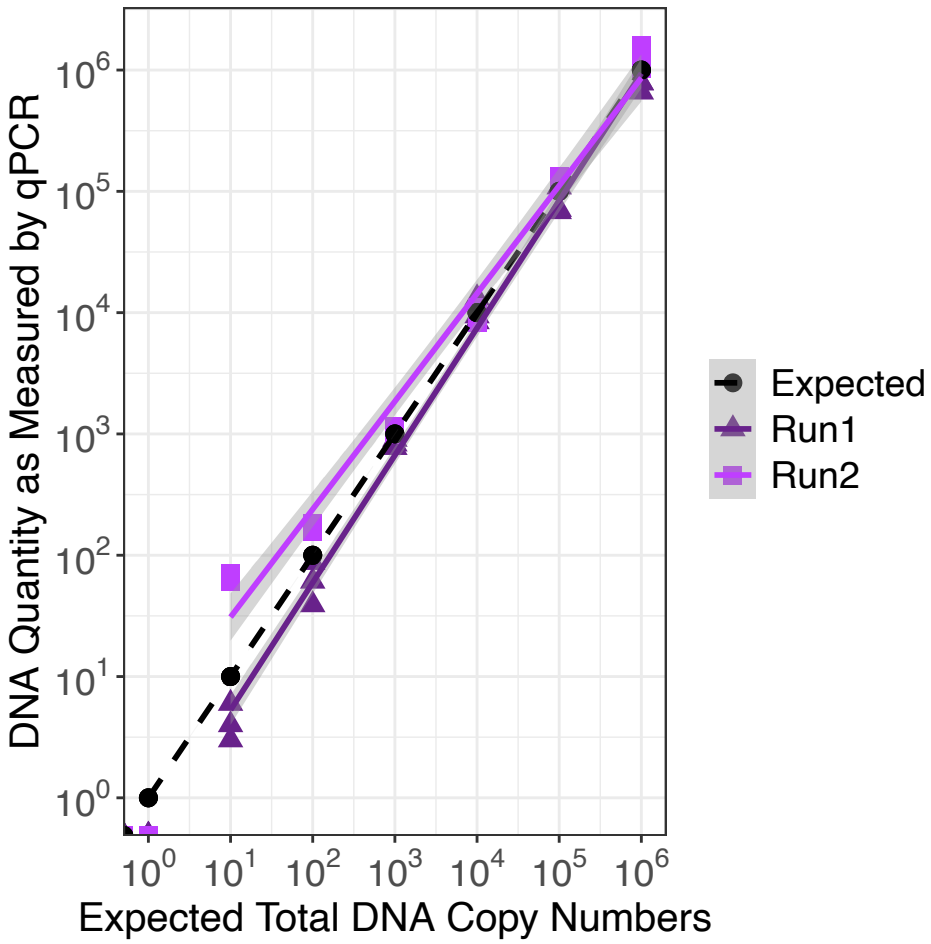


Figure 3.17 - Figure illustrating the assay's ability to detect *M. xenopi* when present in a mock community. Measured DNA quantity within the assay is shown (Run1). The experiment was repeated to show assay reproducibility (Run2). The black dashed line illustrates the ideal values.

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1' and 'Run 2') (*P*-value = 0.78).

In the case of genomic *M. xenopi* DNA (Figure 3.17), the assay detected a minimal theoretical value of 10 as a mean of 4.406 target molecules on 'Run 1' and 64.44 target molecules on 'Run 2'. The limit of detection in this case ranges from 3.235 – 71.02 target molecules.

3.5.3.3 Probes Tested in Multiplex against Genomic DNA

In the final set of experiments, template DNA consisted of 4µl of mock community and 1µl of target genomic DNA spiked in serial dilutions ranging from 1 x 10^6 copy/µl to 1 copy/µl. The figures below (Figures 3.18 to 3.23) give a graphical representation of each assays' ability to detect target DNA within a mock community. For details of the exact measured DNA quantity as well as Ct values for each experiment, please refer to Tables A13 to A18 inclusive of the appendices.

The distribution of all data sets was first assessed for normality using the Shapiro-Wilk normality test. This revealed a non-normal distribution and therefore the Wilcoxon rank sum test was used for comparisons between two groups.

M. abscessus Probe

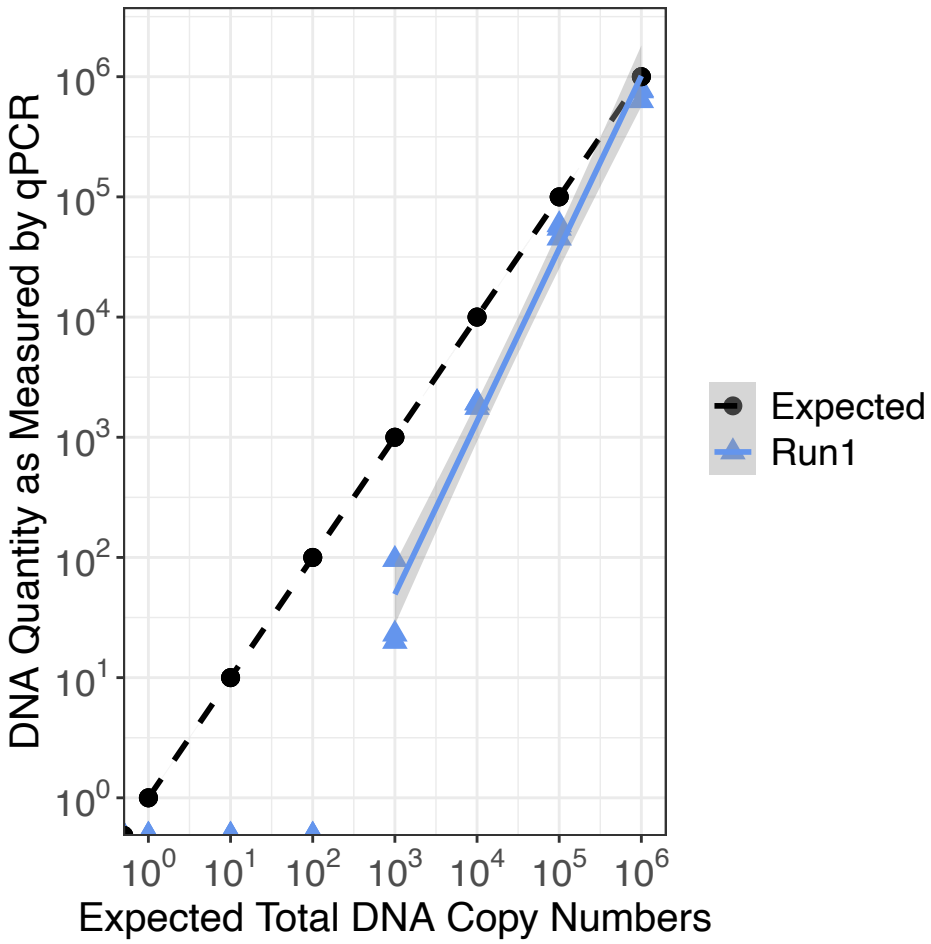


Figure 3.18 - Figure illustrating the assay's ability to detect *M. abscessus* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1') (*P*-value = 0.09).

In the case of genomic *M. abscessus* (Figure 3.18) the assay detected a minimal theoretical value of 1000 as a mean of 6.204 target molecules. The limit of detection in this case ranges from 19.816 – 96.195 target molecules.

M. avium Probe

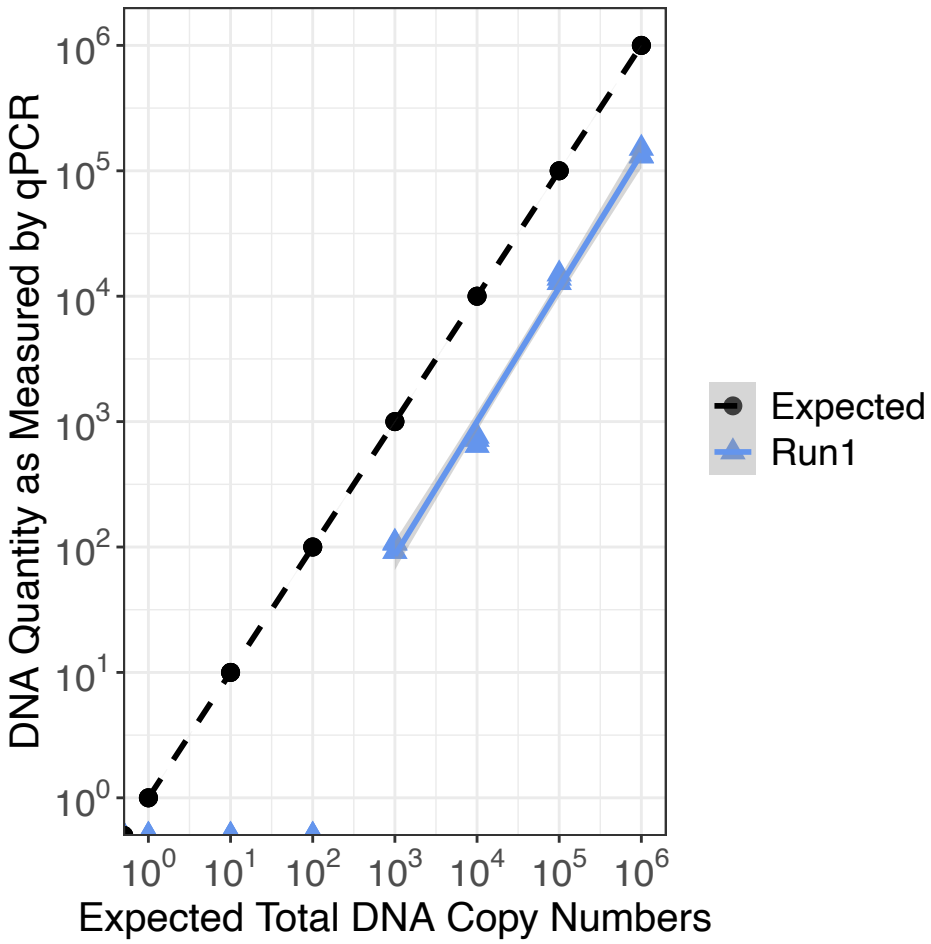


Figure 3.19 - Figure illustrating the assay's ability to detect *M. avium* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1') (*P*-value = 0.08).

In the case of genomic *M. avium* (Figure 3.19) the assay detected a minimal theoretical value of 1000 as a mean of 102.474 target molecules. The limit of detection in this case ranges from 91.995 – 108.607 target molecules.

M. intracellulare Probe

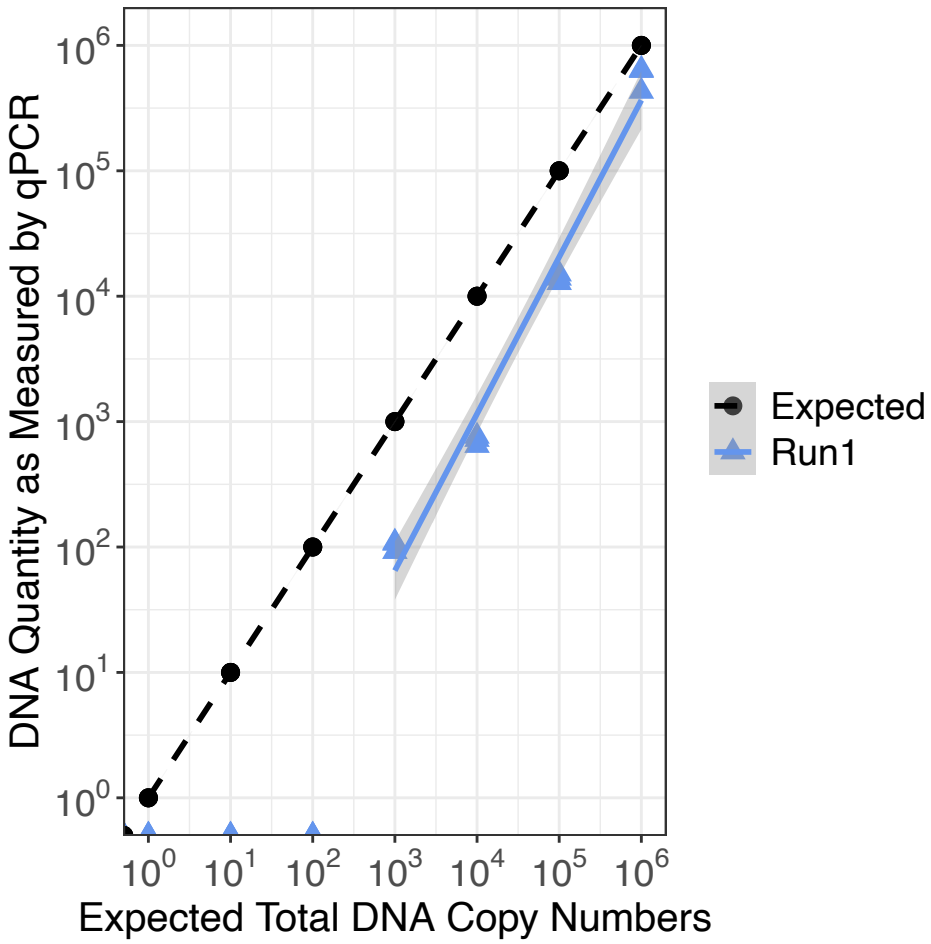


Figure 3.20 - Figure illustrating the assay's ability to detect *M. intracellulare* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1') (*P*-value = 0.08).

In the case of genomic *M. intracellulare* (Figure 3.20) the assay detected a minimal theoretical value of 1000 as a mean of 102.474 target molecules. The limit of detection in this case ranges from 91.995 – 108.607 target molecules.

M. kansasii Probe

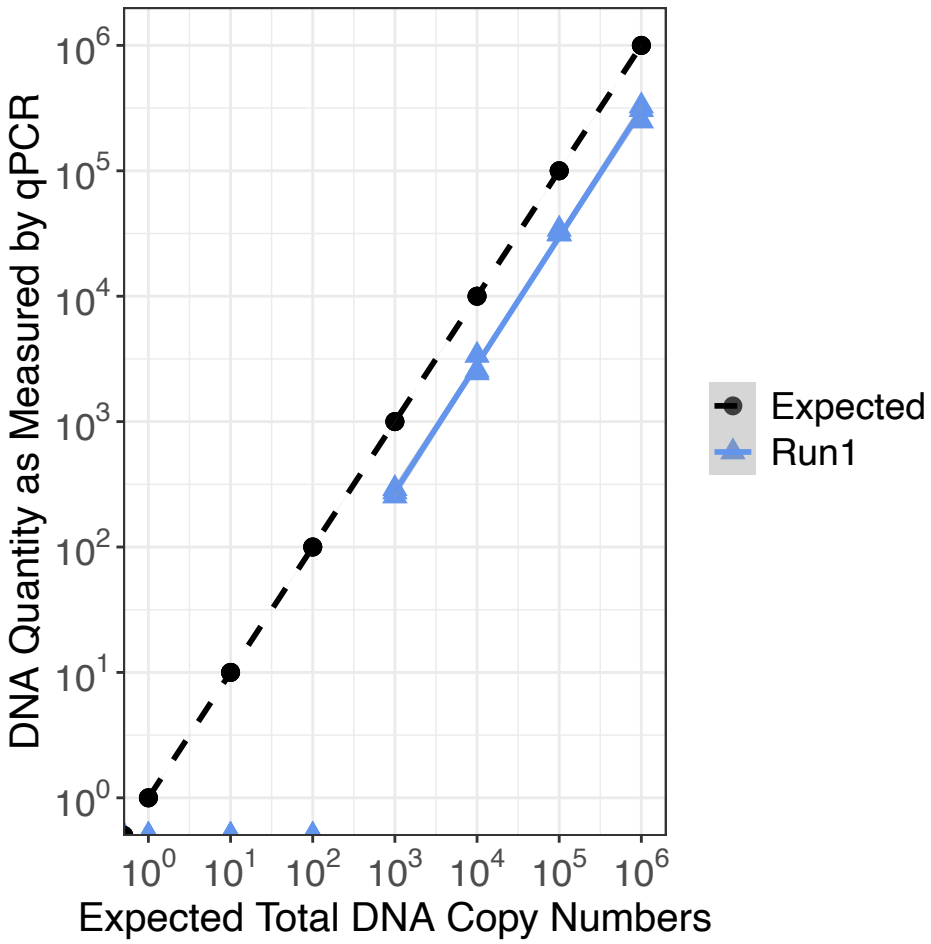


Figure 3.21 - Figure illustrating the assay's ability to detect *M. kansasii* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1') (*P*-value = 0.134).

In the case of genomic *M. kansasii* (Figure 3.21) the assay detected a minimal theoretical value of 1000 as a mean of 273.901 target molecules. The limit of detection in this case ranges from 255.18 – 291.649 target molecules.

M. malmoense Probe

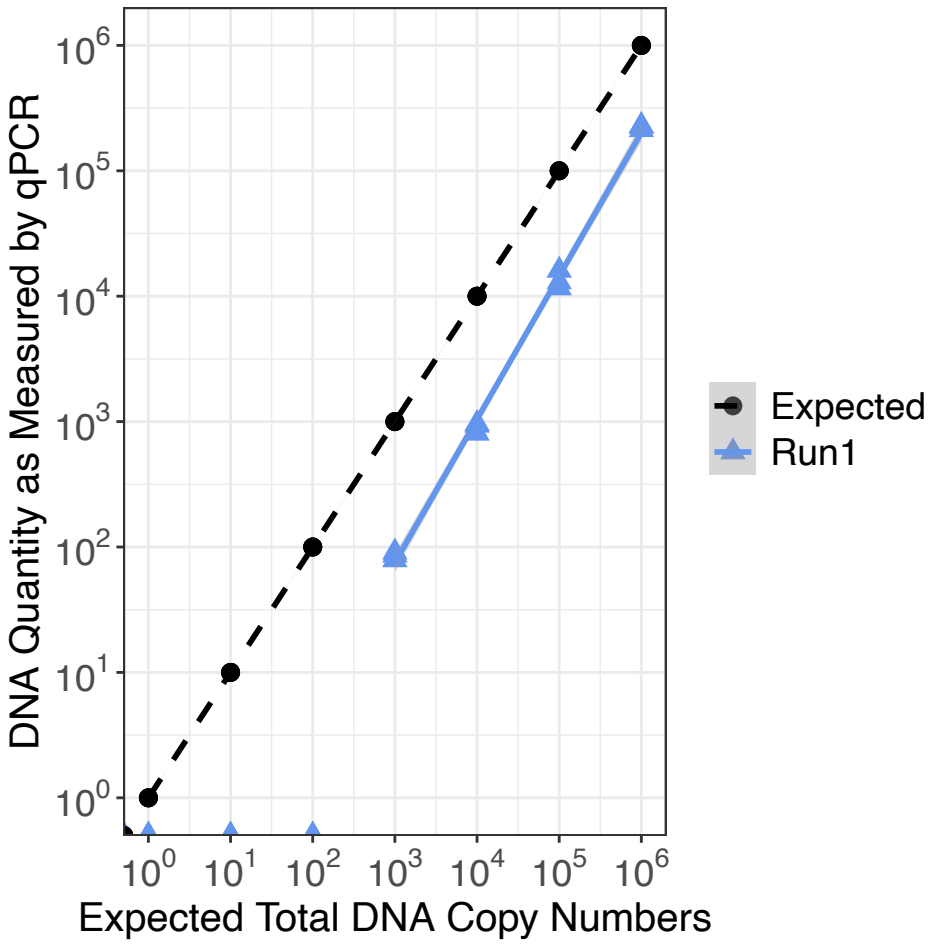


Figure 3.22 - Figure illustrating the assay's ability to detect *M. malmoense* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run1') (*P*-value = 0.06).

In the case of genomic *M. malmoense* (Figure 3.22) the assay detected a minimal theoretical value of 1000 as a mean of 85.07 target molecules. The limit of detection in this case ranges from 78.815 – 90.786 target molecules.

M. xenopi Probe

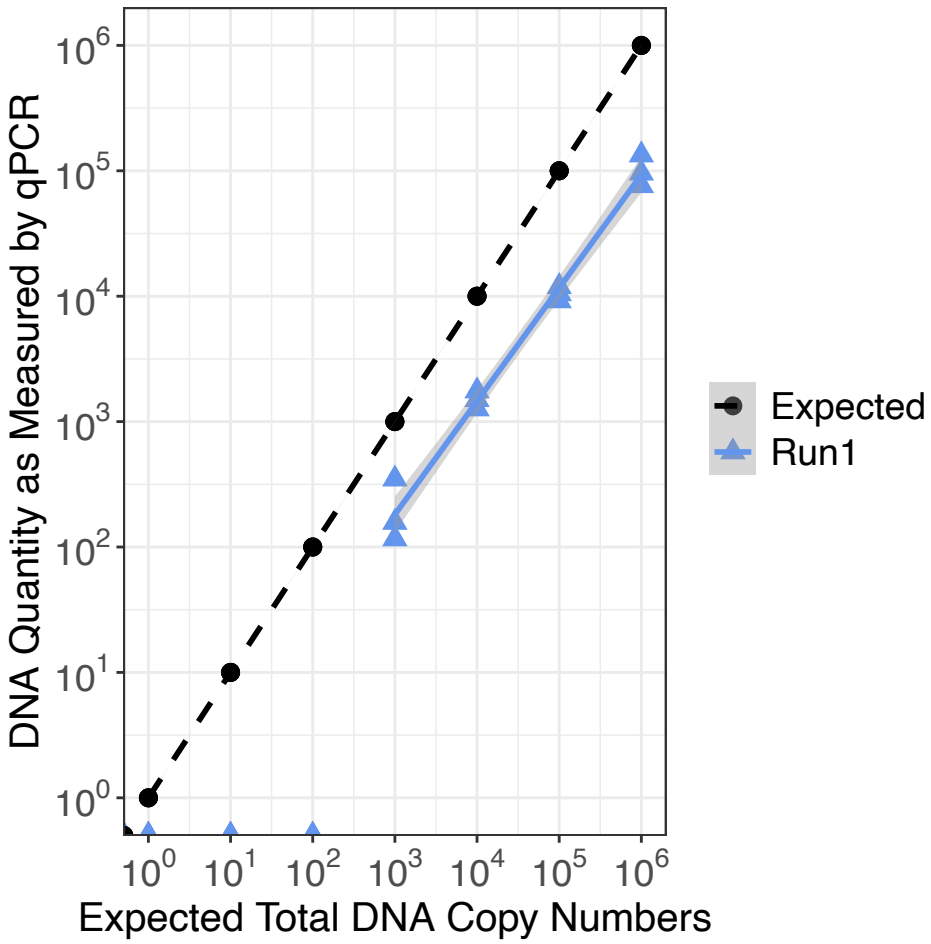


Figure 3.23 - Figure illustrating the assay's ability to detect *M. xenopi* when present in a mock community. Measured DNA quantity within the assay is shown in blue against the intended quantity (black dashed line).

There was no significant difference between the expected data set ('Expected') and the data generated from quantitative PCR ('Run 1') (*P*-value = 0.09).

In the case of genomic *M. xenopi* (Figure 3.23) the assay detected a minimal theoretical value of 1000 as a mean of 207.054 target molecules. The limit of detection in this case ranges from 116.309 – 347.626 target molecules.

3.6 Discussion

3.6.1 MIQE guidelines

In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published with the aim of "providing authors, reviewers, and editors specifications for the minimum information that must be reported for a qPCR experiment to ensure its relevance, accuracy, correct interpretation, and repeatability." [187, 188] The methodology and results laid out in this Chapter have adhered to the principle recommendations laid out in the guidelines including publication of:

- Experimental design
- Sample processing
- Nucleic acid extraction
- Reverse transcription reaction conditions and reactant concentrations
- qPCR target information

By adhering to the guidelines set out by MIQE, this study has been able to demonstrate experimental robustness thus ensuring its validity.

3.6.2 Assay Performance

This Chapter has described the development of a set of assays that can identify six pathogenic mycobacteria and differentiate between them at species level. In addition to this, the assay enables the accurate quantification of the molecular target to a theoretical level of 10 copies when in singleplex or 1,000 copies in multiplex.

To develop an assay considered robust enough to be utilised in clinical practice, it is imperative that the assay demonstrates a high specificity, sensitivity and reproducibility.

Specificity was first tested *in silico* to determine amplicon production when tested against other common respiratory tract commensals. It was found that the primers designed were specific only to targeted mycobacteria. Specificity was further improved by the custom design of Taqman™ probes which enabled species specific

differentiation amongst NTM. Owing to the high GC content and preservation of GC repeats amongst mycobacteria [189] probes often varied by only a few nucleotides. At lower annealing temperatures (below 66°C) a number of false positives were produced during specificity testing (Chapter 3, Section 3.3.2). It was found that by increasing the annealing temperature to 66°C, these false positives were largely eradicated without affecting primer binding (Chapter 3, Section 3.2.3).

Owing to the absence of false negatives during *in vitro* testing, sensitivity for each probe was calculated at 100% (Section 3.7). This was true when probes were tested solely against their target plasmid *in vitro* but is likely to be an exaggeration when compared to *in vivo* experimentation when the assay target is part of a complex micro-environment. Sensitivity will therefore be reassessed in Chapter 4 using microbiological culture as the gold standard.

With respect to *in vitro* testing, a more meaningful definition of sensitivity might be the assay's ability to detect and quantify target genetic material. NTM are pathogens that cause chronic infection and whose treatment requires several months of antibiotic therapy. Accurate quantification is therefore advantageous in that it provides an assessment of treatment efficacy. The assay's ability to quantify NTM was investigated by spiking a known amount of target DNA (as measured by PicoGreen quantitation) into a mock community consisting of genetic material from common respiratory tract commensals as well as human DNA.

In this experimental design DNA quantitation was first performed in singleplex. All singleplex assays demonstrated an excellent ability to detect and accurately quantify target genetic material. When genomic NTM DNA was quantified, statistical testing revealed no significant differences between expected DNA quantity and that measured by qPCR. To demonstrate reproducibility a second independent run was performed. There were no significant differences between runs and the expected values (*P*-values ranged from 0.78 to 0.99 using the Kruskal-Wallis rank test). The limit of detection was also noted for each run. Singleplex assays were able to consistently detect to a theoretical limit of 10 copies/µl with the lowest copy number accurately quantified as 3.235 copies.

Having established that these six singleplex qPCR assays produced accurate and reliable results, probes were combined to make two multiplex assays. Probes specific for *M. abscessus, M. avium* and *M. intracellulare* were combined in one assay and probes for *M. kansasii, M. malmoense* and *M. xenopi* formed the other.

Analysis of the multiplex data was performed using Wilcoxon rank sum test. The significance values ranged from 0.06 to 0.134 indicating that again there were no significant differences between expected values and the run itself. The performance of the assay in multiplex, however, repeatedly underestimated the quantity of target genetic material by a factor of roughly 10. This resulted in lower *P*-values indicating a higher level of dissimilarity between expected values and runs compared to those found in singleplex. In addition to this, the limit of detection was lower for each assay in singleplex.

A review of the literature revealed three previously published papers in which the authors developed assays for real time quantification of mycobacteria [190-192], but none that were aimed at direct quantification of specific mycobacterial species. The singleplex assays described in this chapter showed similar limits of detection compared to the latter publications but a superior limit of detection compared to the former (79–393, 16–79, 16-79 genome copies respectively). Sensitivity was defined as the percentage of Mycobacterium species which were detected, and specificity was defined as the percentage of non-target micro-organisms which were not detected according to the collection assessed. These published assays described demonstrated good sensitivity (77%, 100%, 100% respectively). Specificity was markedly reduced in the assay developed by Dutil *et al.* owing to the amplification of isolates of the *Staphylococcus* genus (100%, 44%, 91% respectively).

The singleplex assays developed in this chapter have demonstrated a consistently high specificity and sensitivity and a limit of quantification to a theoretical value of 10 target genome copies. These values in themselves are superior to those generated by other assays. The assays also hold the unique advantage of being able to quantify specific NTM species.

3.7 Conclusion

In conclusion, the work conducted in this Chapter has demonstrated six qPCR assays that, when used in singleplex reactions *in vitro*, can:

1. Identify the six most pathogenic NTM species.
2. Distinguish (to a species level) between the six most pathogenic NTM species.
3. Provide accurate quantification of NTM species copy number.

These assays do not rely on culture-based techniques and therefore can be performed in real time with results published in days rather than weeks. The assays have the theoretical potential to be used in clinical practice both as a rapid diagnostic technique and in the monitoring of response to treatment. In Chapter 5, these assays will be tested against DNA extracted from human sputum samples to assess each assay's ability to operate *in vivo*.

Chapter 4: Molecular Assessment of NTM Burden

4.1 Introduction

The advent of polymerase chain reaction (PCR) [193] radically changed biological sciences by allowing for the specific detection and production of large amounts of DNA. In 1993, Higuchi *et al*. recognised the process of PCR could be monitored by addition of a fluorescent label that binds to the accumulating PCR product [194]. As the concentration of PCR product increases, the intensity of the fluorescence signal also increases. This discovery paved the way for modern quantitative real-time PCR (qPCR).

Within the field of science, PCR is used in multiple applications:

- PCR can be used to detect variations in gene expression among cell types, tissues, and organisms.
- The process of genotyping utilises PCR to detect genetic variations in alleles in specific cells or organisms.
- PCR is used in gene sequencing by enriching template DNA.
- Fluorescent labelling has allowed accurate genetic quantitation that in turn has allowed for measurement of bacterial, fungal and viral burden from samples. This has applications in both science and medicine.

Within the sphere of clinical medicine, qPCR is used mostly in the detection of pathological viral material [195]. This is not unexpected as PCR assays were already in use as the method of choice for the detection of some viruses. The results of quantitation of a viral nucleic acid target are important when assessing the relationship between the viral load and the prediction of the progression of infection to clinical disease, for example in the case of HIV [196]. Quantitative test results for nucleic acid targets have become especially relevant with serial specimens from transplant patients to monitor for evolving infection or for assessing the effectiveness of antiviral therapy [197]. Commercially available assays for the quantification of viral load exist for: Cytomegalovirus [198], Epstein-Barr Virus [199], Hepatitis A, B, C, D and E viruses , Human Immunodeficiency Virus [200] to name a few.

The use of PCR for the detection of bacterial infections is less common in clinical practice owing to shorter infective periods that do not require monitoring. PCR techniques are, however, in common use for serious infections for example to detect and confirm presence of *Neisseria meningitidis* or *Streptococcus pneumoniae* in patients suspected of having meningitis, or in the detection and assessment of antibiotic sensitivity in *Mycobacteria tuberculosis*. At the time of writing, no studies were identified in the literature that used real time PCR in the identification and quantification of NTM species. The development of a real time PCR assay in this area has the potential to decrease time to diagnosis and allow for monitoring of NTM copy number during treatment.

4.2 Hypothesis and Aims

The hypotheses addressed in this Chapter are as follows:

1. The microbiome of the NTM patient may include more than one NTM species.
2. Sputum culture results underrepresent the presence of NTM species in the lungs of NTM patients.
3. The custom qPCR assay will detect the presence of NTM species that standard bacterial clinical culture failed to detect.
4. The custom qPCR assay can be used to map within patients changes in NTM copy number over time.

Consequently, the aims of this chapter are as follows:

1. To establish the specificity, sensitivity, positive and negative predictive values of the custom assay.
2. To explore changes in NTM DNA copy number over time (both in patients on treatment and those who are not).
3. To explore the potential for the lung microbiome to contain more than one NTM species.

4.3 Method

4.3.1 DNA Extraction

Sputum was obtained from recruited patients (Chapter 2, Section 2.1.2) over the course of 12 to 18 months (Chapter 2, Section 2.1.3). DNA was extracted from these samples as per the method detailed in Chapter 2, Section 2.2 and diluted in 100µl low TE.

For the purposes of this results chapter, labelling of extracted longitudinal sputum samples by time point is detailed in Table 4.1.

Table 4.1 – Table to illustrate timings of sample receipt to lab and their corresponding time point labelling that is subsequently used in the results section of this chapter. Immediate freezing indicates that samples were frozen immediately at -80°C. Delayed freezing indicates that samples were frozen at -80°C after storage at room temperature for 24 hours.

Time Received in Lab			Week				Month												
	Immediate freezing	Delayed freezing	1	2	3	4	2	3	4	5	6	7	8	9	10	11	12	15	18
Time Point	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

4.3.2 Quantitative PCR Assay

To establish optimal sample concentration for the assay, a pilot study was performed whereby different sample concentrations were used against the qPCR standards. A select group of samples were run: without dilution as well as with dilution 1 in 10, 1 in 50 and 1 in 100 (samples were diluted with PCR grade water). This was done as concentrated samples may lead to PCR inhibition. Conversely low template concentration may surpass the limit of assay detection [201, 202]. Base on the results of the pilot experiment a dilution factor of 1:50 was applied to all samples prior to analysis.

Extracted samples were diluted in a 1:50 ratio with PCR grade water. Quantitative PCR reactions were performed using a MicroAmp™ Optical Reaction with Barcode 96 well plate. For each qPCR reaction, 5µl of diluted sample was combined with 10µl 2 X Luna Universal Probe qPCR Master Mix, 0.8µl forward primer, 0.8µl reverse primer (both at 10µM), 0.4µl probe (at 10µM) and 3µl PCR grade water (total reaction volume 20 µl). For quantification standards, 5µl aliquots of serially

diluted plasmids were used (as per Section 4.7.1.2). Each standard and sample was tested in triplicate to ensure reliability of results. Details of the custom primers and probes used are detailed in Chapter 3 Section 3.2.2 Table 3.1. Each qPCR plate was run on the QuantStudio™ 7 Flex Real-Time PCR System (see Chapter 2, Section 2.4).

4.3.3 Data Analysis

Target DNA was quantified using the QuantStudio Software v1.3 before the results were exported into Microsoft® Excel for Mac 2011 software (Microsoft® Corporation, Redmond, USA). All further analysis was carried out in R version 3.6.1 analytical environment (see Chapter 2, Section 2.7).

Patient data was analysed according to recruitment group (see Chapter 2, Section 2.1.2) to allow direct longitudinal comparison between patients experiencing similar external pressures (i.e. anti-microbial therapy). This resulted in the following groups:

- Control group
- *M. abscessus* control group
- *M. avium* complex (MAC) control group
- *M. abscessus* treatment group
- *M avium* complex (MAC) treatment group

For changes based on individual patients please see Figures A1 to A34 inclusive in the appendices.

4.4 Clinical Characteristics of the Study Cohort

A total of 410 samples were obtained from 38 patients. Of the 38 patients, 15 patients had recurrent isolates of MAC (with 8 patients [53.3%] placed on treatment). A total of 18 patients recurrently isolated *M. abscessus* (with 14 patients [77.8%] placed on treatment). MAC treatment regimens were reasonably consistent with only one drug difference (Azithromycin versus Clarithromycin). With regards to the treatment of *M. abscessus*, the initiation phase again saw one drug difference (Azithromycin versus Clarithromycin) in contrast to the continuation phase that saw a total of 10 different treatment regimens used. These differed due to patient antibiotic intolerance. No significant differences were seen in clinical characteristics between microbiological subtypes apart from in the underlying disease group in which the majority of patients on treatment for *M. abscessus* had an underlying diagnosis of cystic fibrosis (Table 4.2).

Mycobacterial culture results are shown in Figure 4.1. Eight of the 14 patients commenced on treatment for *M. abscessus* were culture negative 12 months after initial culture conversion and were therefore deemed to be cured by BTS and ATS criteria [1, 2]. A total of 10 patients in this group cultured more than one mycobacterial species, illustrating the frequency of mixed isolates and the inherent difficulty faced when selecting treatment regimens. Four of the 8 patients commenced on treatment for MAC were culture negative 12 months after initial culture conversion and were therefore deemed to be cured by BTS and ATS criteria [1, 2].

Of the patients that cultured NTM but who were not receiving treatment (the *M. abscessus* controls and *M. avium* controls), all but one patient (patient 44) cultured their regular NTM species at a time point in the study period. NTM culture was inconsistent in this group, with 28 of the 70 samples failing to culture NTM. All of the patients recruited to the NTM culture negative group, remained negative for the duration of the study.

Table 4.2 – Clinical characteristics of the cohort. Table shows the breakdown of patients by gender, age, smoking status, underlying disease, medication implicated in the development of NTM disease, pre treatment pulmonary function results, BMI and antibiotics used to treat infection. *P*-values obtained using Kruskal – Wallis rank sum test.

		Mycobacterium avium		***Mycobacterium abscessus***		Control	***P*-value**
		Treatment	Control	Treatment	Control		
Gender	Male	7	3	7	3	2	0.9
	Female	1	4	7	1	3	
Mean Age at Recruitment (years)		56.81	49.86	30.43	47.75	44.4	0.7
Smoking History	Current	1	1	0	0	0	0.2
	Previous	3	0	0	0	0	
	Never	4	6	14	4	5	
Bronchiectasis (Bx)	Cylindrical	3	2	1	1	1	0.01
	Varicose	2	1	1	0	1	
	Cystic	2	1	0	0	0	
Cystic Fibrosis (CF)	ΔF508 mutation homozygous	1	0	7	0	3	
	ΔF508 mutation heterozygous	0	2	3	1	0	
	other	0	1	2	2	0	
Medications	Inhaled Corticosteroids	3	4	11	3	4	0.8
	Oral Corticosteroids	0	0	1	3	0	0.4
	Immunomodulation	1	0	0	0	0	0.09
Pulmonary Function	FEV1 % predicted	65.49	72.16	75.36	50.58	47.9	0.4
	FVC % predicted	88.89	91.73	89.98	79.4	66.58	0.3
Body Mass Index		21.46	21.13	20.68	22.26	22.11	0.4
Radiological Pattern of Disease	Cavitation	0	NA	4	NA	NA	0.2
	Nodular	8	NA	10	NA	NA	
***M. avium* Treatment**	Rif, Eth, AZT	6					
	Rif, Eth, Clari	2					
***M. abscessus* Initiation Treatment**	Clari, Amik(IV), Mero(IV), Tige			12			
	AZT, Amik(IV), Mero(IV), Tige			2			
***M. abscessus* Continuation Treatment**	Clari, Amik(neb), Mino, Cipro			5			
	Clari, Amik(neb), Mino, Clof			2			
	Clari, Amik(neb), Mino, Septrin			1			
	Clari, Amik(neb), Doxy, Cipro			1			
	Clari, Amik(neb), Mino, Clof			1			
	Clari, Mero(neb), Doxy, Clof			1			
	Clari, Amik(neb), Doxy, Moxi			1			
	AZT, Amik(neb), Mino, Cipro			1			
	AZT, Amik(neb), Doxy, Cipro			1			
	AZT, Colo(neb), Mino, Cipro, Septrin			1			

M. avium treatment regimen - consists of 3 drugs where: Rif=Rifampicin, Eth=Ethambutol, AZT=Azithromycin, Clari=Clarithromycin. *M. abscessus* treatment regimen - consists of an initiation phase where: Clari=Clarithromycin, Amik=Amikacin, Mero=Meropenem, Tige=Tigecycline, IV=intravenous. Also consists of a continuation phase where: Mino=Minocycline, Cipro=Ciprofloxacin, Clof=Clofazamine, Septrin=Septrin, Doxy=Doxycycline, AZT=Azithromycin, Colo=Colomycin, neb=nebulised.

	Sub. ID	Disease	Gender	Time Point 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
M.abscessus Treatment	2	CF	female	ABS	ABS	ABS												ABS			INT	INT
	4	CF	male				AVM															
	6	Bx	female		ABS	ABS	ABS															
	8	Bx	female	ABS	ABS	ABS	ABS	AVM														
	9	CF	female		XEN																	
	11	CF	male	ABS	ABS										XEN							
	14	CF	female	ABS	ABS		INT		INT													
	18	CF	male	ABS	ABS	ABS	ABS	ABS								ABS		KAN				
	19	CF	male	ABS	ABS	ABS								AVM		AVM	AVM		AVM		AVM	
	22	CF	female	ABS	ABS	ABS	ABS		ABS						ABS							
	23	CF	female	ABS	ABS	ABS			ABS	ABS	ABS		ABS		ABS	ABS						
	27	CF	male																			
	33	CF	male	ABS	ABS	AVM																
	35	CF	male	ABS	ABS					ABS				ABS	AVM		ABS	AVM		AVM		
M.avium Treatment	1	Bx	male	XEN	XEN		XEN															
	3	Bx	male																			
	7	Bx	female	AVM	AVM	AVM																
	17	Bx	male																			
	28	Bx	male	AVM	AVM					AVM							INT	INT				
	34	Bx	male			AVM	AVM			INT		INT		INT	INT	INT	INT		INT	INT	INT	
	38	Bx	male	AVM	AVM	AVM			AVM	AVM		AVM	AVM	AVM	AVM	AVM	AVM	AVM				
	39	CF	male	AVM	AVM	AVM					AVM		INT									
M.abs Control	13	Bx	male	ABS	ABS	ABS	ABS			ABS	ABS											
	37	CF	male						ABS	ABS	ABS											
	41	CF	female			ABS	ABS	ABS														
	44	CF	male																			
M.avium Control	5	Bx	female						FORT	INT												
	12	CF	female	INT	INT	INT		INT	AVM		INT											
	15	CF	male	AVM	AVM					ABS	ABS											
	21	CF	female	AVM	AVM		AVM	AVM		AVM	AVM											
	29	Bx	female				AVM															
	31	Bx	male	AVM	AVM	AVM			AVM		AVM											
	36	Bx	male	AVM	AVM		AVM	AVM		AVM	INT											
Control	42	CF	male																			
	45	Bx	male																			
	46	CF	female																			
	47	CF	female																			
	48	Bx	female																			

Figure 4.1 – Mycobacterial culture results by patient and time point. Blue square indicates a negative culture result whilst orange indicates positive. Name inside the orange box is NTM species (ABS = *M. abscessus,* AVM = *M. avium,* INT = *M. intracellulare,* XEN = *M. xenopi,* FORT = *M. fortuitum*). Hashed box indicates no sample was available for the patient. Time points 1&2=pre-initiation of treatment, 3-6=weeks1-4, 7-17=months2-12, 18=month15, 19=month18.

4.5 Comparing Time Points 1 and 2

Samples 1 and 2 were generated from the same divided sputum sample but a time delay of 24 hours was introduced between the divided sputums with one being placed immediately into -80°C storage and the other put into the -80°C storage 24 hours later (Chapter 2, Section 2.1.4). This time delay was performed to account for the delay between sputum production and receipt in the lab from later samples that were sent via postal service and therefore incurred a 24-hour delay from production to freezing.

One important phenomenon noted amongst the culture results was the failure of samples 1 and 2 (samples originating from the same sputum) from a couple of patients to culture NTM. The failure of samples at time points 1 and 2 from patients 6 and 9 to culture congruent NTM species illustrates the inconsistencies and shortfalls associated with standard clinical microbial culture as a means of diagnosis.

Amongst *M. abscessus* culture positive patients, treatment and control groups, 12 of the 18 experienced a rise in *M. abscessus* copy number between time points 1 and 2. The mean copy number at time point 1 was 1.39 x 10^9 copies/g sputum (SD = ±4.44x10^9) and 2.49 x 10^9 copies/g sputum (SD = ±5.79x10^9) at time point 2 (Figure 4.2). There was no significant difference between time points (Student's T test *P*-value = 0.53).

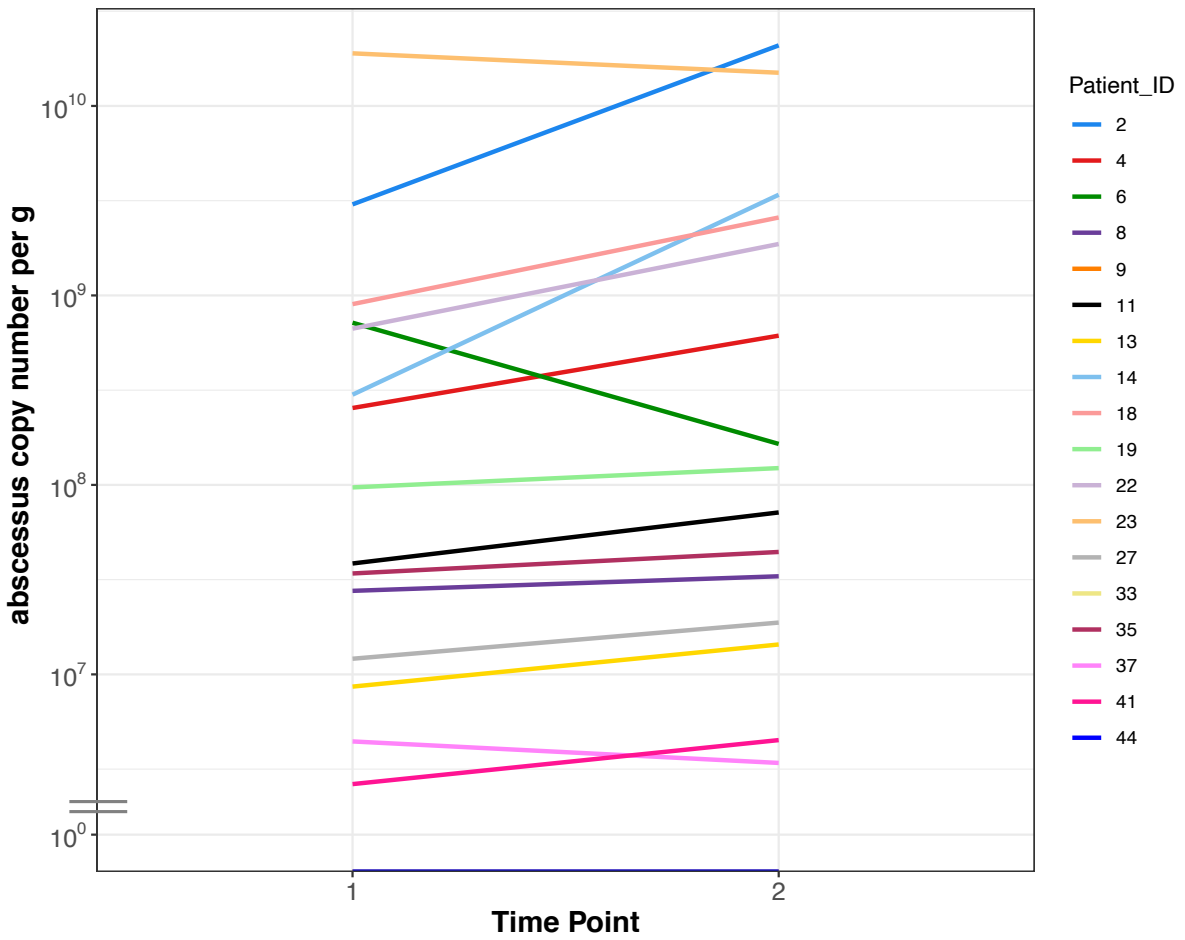


Figure 4.2 – *M. abscessus* copy number between time points 1 and 2. Samples were derived from the same sputum sample, but sample time point 2 incurred a 24-hour time delay before being processed. There were no significant differences in copy number (*P*-value = 0.53).

Amongst *M. avium* complex culture positive patients, treatment and control groups, 4 of the 15 experienced a rise in *M. avium* copy number between time points 1 and 2 (Patients 3, 12, 31, 38). The mean copy number at time point 1 was 8.2×10^{7} copies/g sputum (SD = $\pm 2.43 \times 10^{8}$) and 2.32×10^{8} copies/g sputum (SD = $\pm 7.78 \times 10^{8}$) at time point 2 (Figure 4.3). There was no significant difference between time points (Student's T test *P*-value = 0.49).

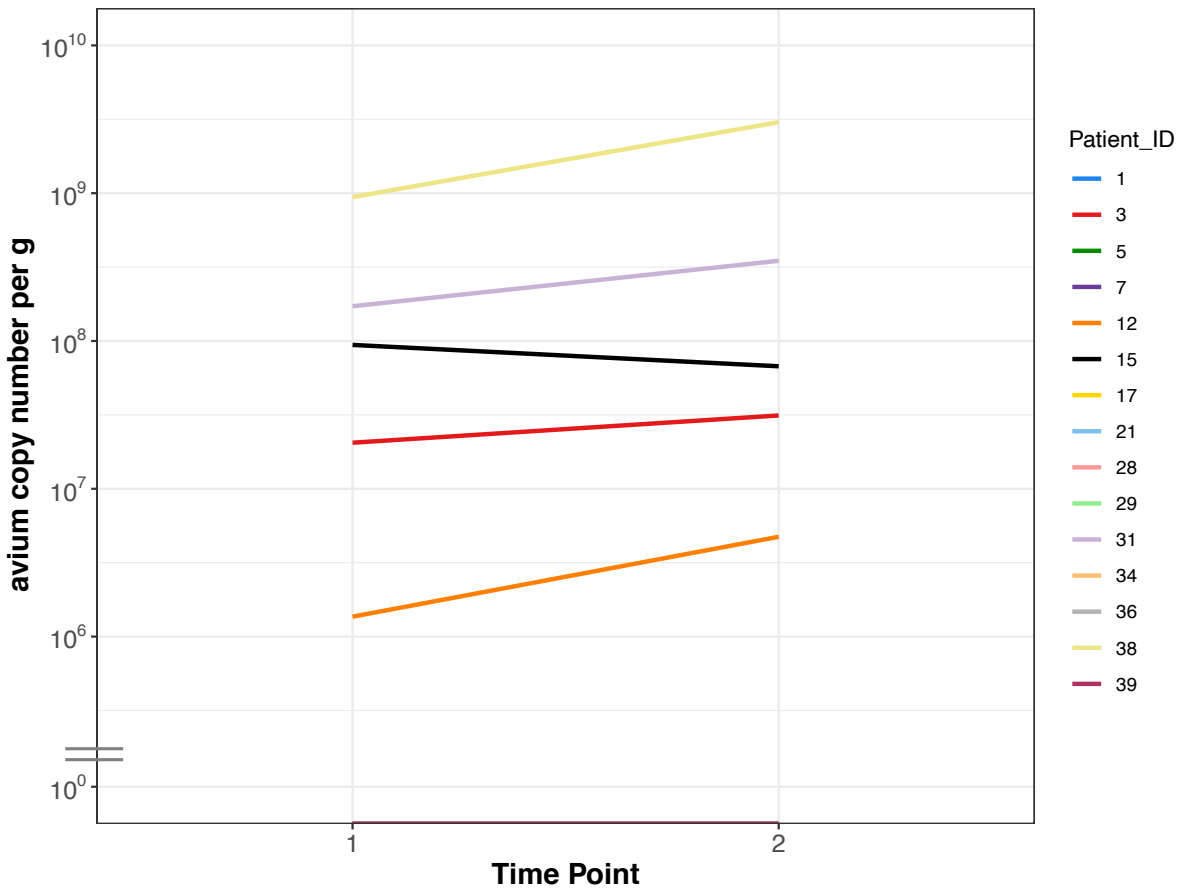


Figure 4.3 – *M. avium* copy number between time points 1 and 2. Samples were derived from the same sputum sample, but sample time point 2 incurred a 24-hour time delay before being processed. There were no significant differences in copy number (*P*-value = 0.49).

Amongst *M. avium* complex culture positive patients, treatment and control groups, 4 of the 15 experienced a rise in *M. intracellulare* copy number between time points 1 and 2 (patients 7, 21, 28, 36). The mean copy number at time point 1 was 5.01 x 10^7 copies/g sputum (SD = ±8.35 x 10^7) and 1.14x10^8 copies/g sputum (SD = ±2.44 x 10^8 at time point 2 (Figure 4.4). There was no significant difference between time points (Student's T test *P*-value = 0.35).

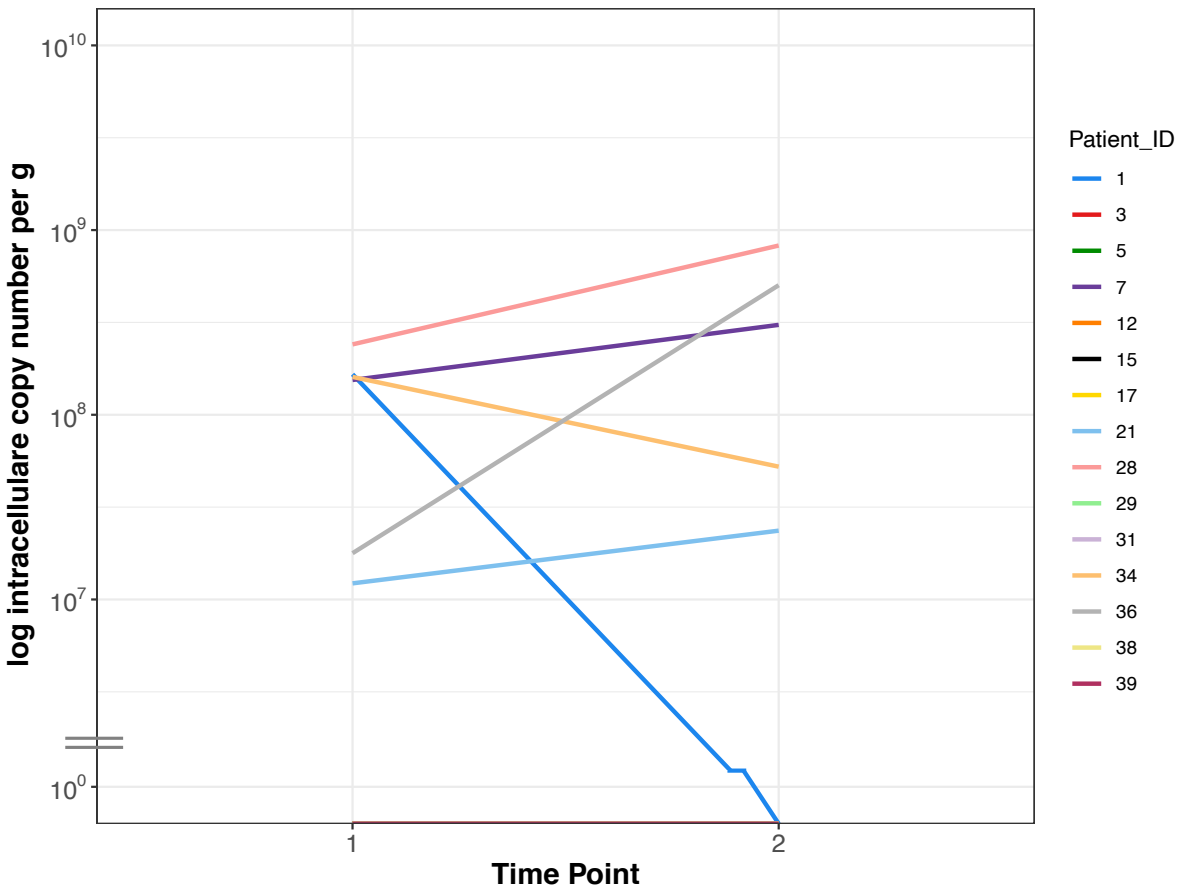


Figure 4.4 – *M. intracellulare* copy number between time points 1 and 2. Samples were derived from the same sputum sample, but sample time point 2 incurred a 24-hour time delay before being processed. There were no significant differences in copy number (*P*-value = 0.35).

4.6 Application of the Test to the Control Group

A total of 33 samples were analysed from the control cohort (7 samples not received in the lab). No samples were culture positive for NTM species.

The application of the qPCR assay targeting *M. abscessus* to patients in the control group revealed no positive results.

The application of the qPCR assay targeting *M. avium* to patients in the control group revealed positive results for a single patient, patient 42, at all time points apart from time point 1 (no sample was provided for time point 6). As shown in Figure 4.1, Patient 42 showed no positive mycobacterial culture results for the duration of the study and indeed had been historically mycobacteria culture naïve. All samples provided by Patients 45, 46,47 and 48 proved qPCR negative for *M. avium.* Statistical analysis by

Kruskall – Wallis one-way analysis of variance revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 4.5798, df = 7, *P*-value = 0.71) (Figure 4.5).

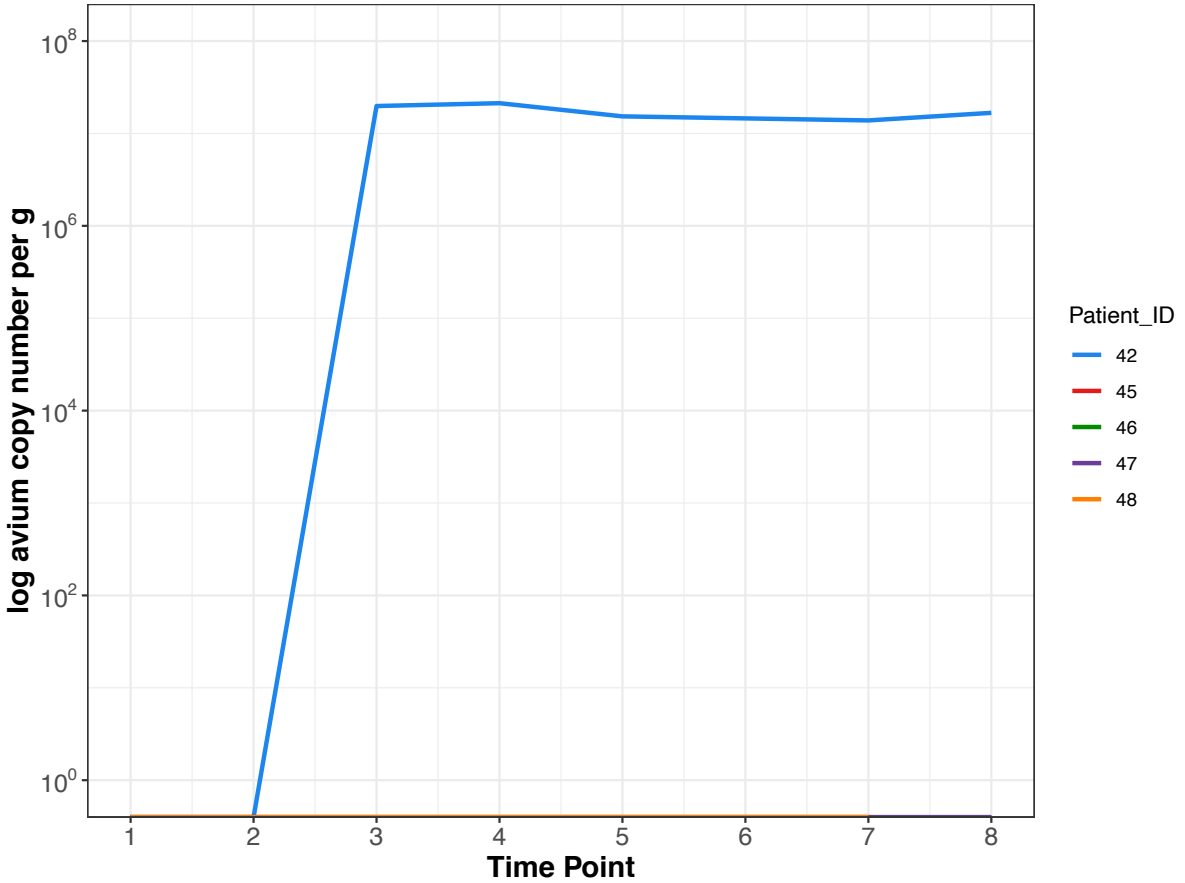


Figure 4.5 – Assay targeting *Mycobacterium avium* applied to samples from the control group (i.e. mycobacterial culture negative). Patient 42 showed positivity at all but one time point despite being culture negative. All results are expressed on a logarithmic scale.

The application of the qPCR assay targeting *M. intracellulare* to patients in the control group revealed positive results for two patients, Patients 45 and 46. Both patients were qPCR positive for *M. intracellulare* throughout all timepoints available, the time point 8 sample was missing for patient 45. As shown in Figure 4.4, Patients 45 and 46 were NTM culture negative during the study and had been historically culture negative also. All samples provided by Patients 42, 47 and 48 were qPCR negative for *M. intracellulare.* Statistical analysis by Kruskall – Wallis one-way analysis of variance revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 1.2279, df = 7, *P*-value = 0.99) (Figure 4.6).

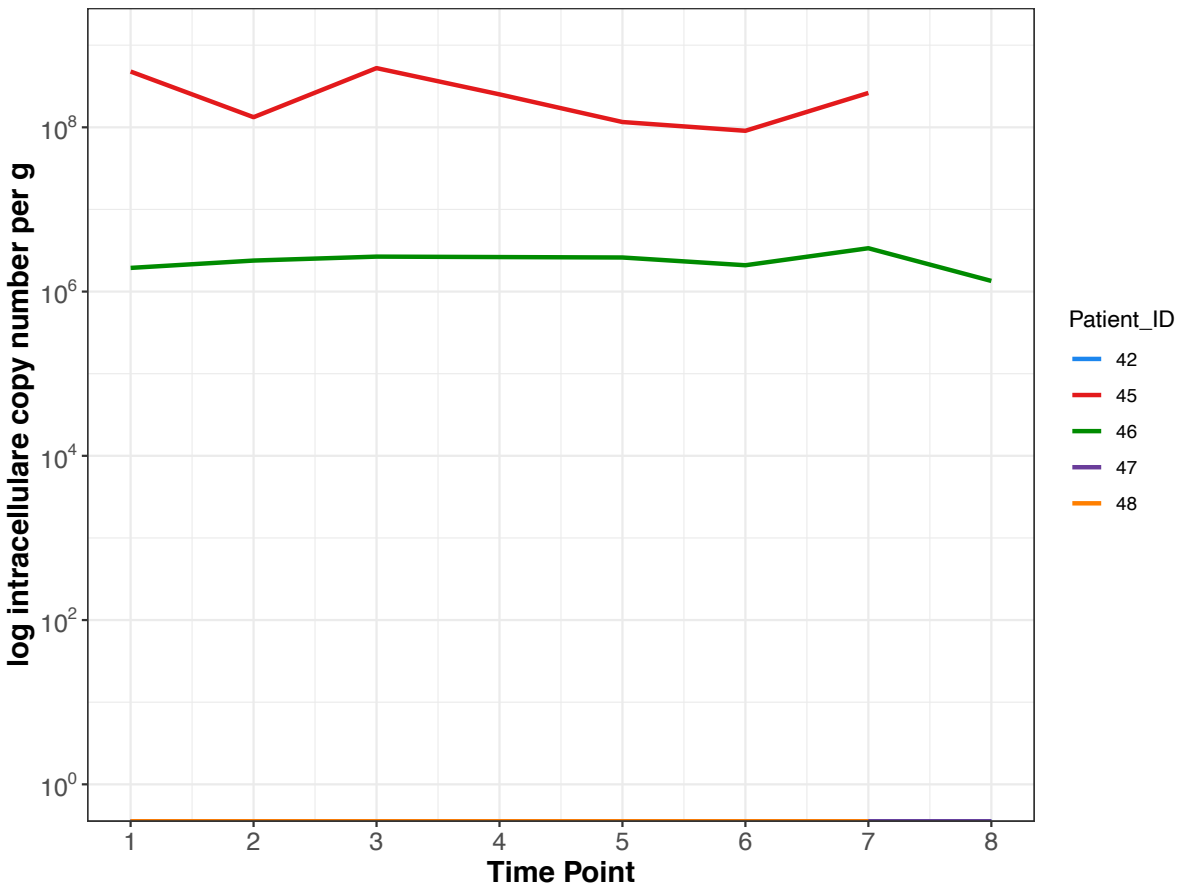


Figure 4.6 – Assay targeting *Mycobacterium intracellulare* applied to samples from the control group (i.e. mycobacterial culture negative). Samples from Patients 45 and 46 were both qPCR positive despite being negative by culture. All results are expressed on a logarithmic scale.

In summary, patients in the control group had been mycobacterial culture naïve prior to recruitment and remained mycobacterial culture negative for the duration of the study. When the samples they had provided underwent testing with the custom qPCR assays three of the five patients tested positive for the *Mycobacterium avium* complex (MAC). Quantification of *M. intracellulare* copy number remained relatively stable over time for Patients 45 and 46. Quantification of *M. avium* in Patient 42 was highly variable and may be explained by sputum being expectorated from a mixture of colonised and non-colonised pulmonary sites.

4.7 Application of the Test to the *M. abscessus* Control group

A total of 28 samples were analysed from the *M. abscessus* control cohort (4 samples not received in the lab). Twelve of these samples were culture positive for *M. abscessus*.

The application of the qPCR assay targeting *M. abscessus* to patients in the *M. abscessus* control group showed positive results in three of the four patients recruited to the group. Despite being *M. abscessus* culture positive prior to recruitment, Patient 44 failed to culture NTM during the study period. Although time points 3, 4, 5, 6 were unavailable the qPCR negative results are concordant with the sample culture results provided. Statistical analysis by Kruskall – Wallis one-way analysis of variance revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 3.5398, df = 7, *P*-value = 0.83) (Figure 4.7).

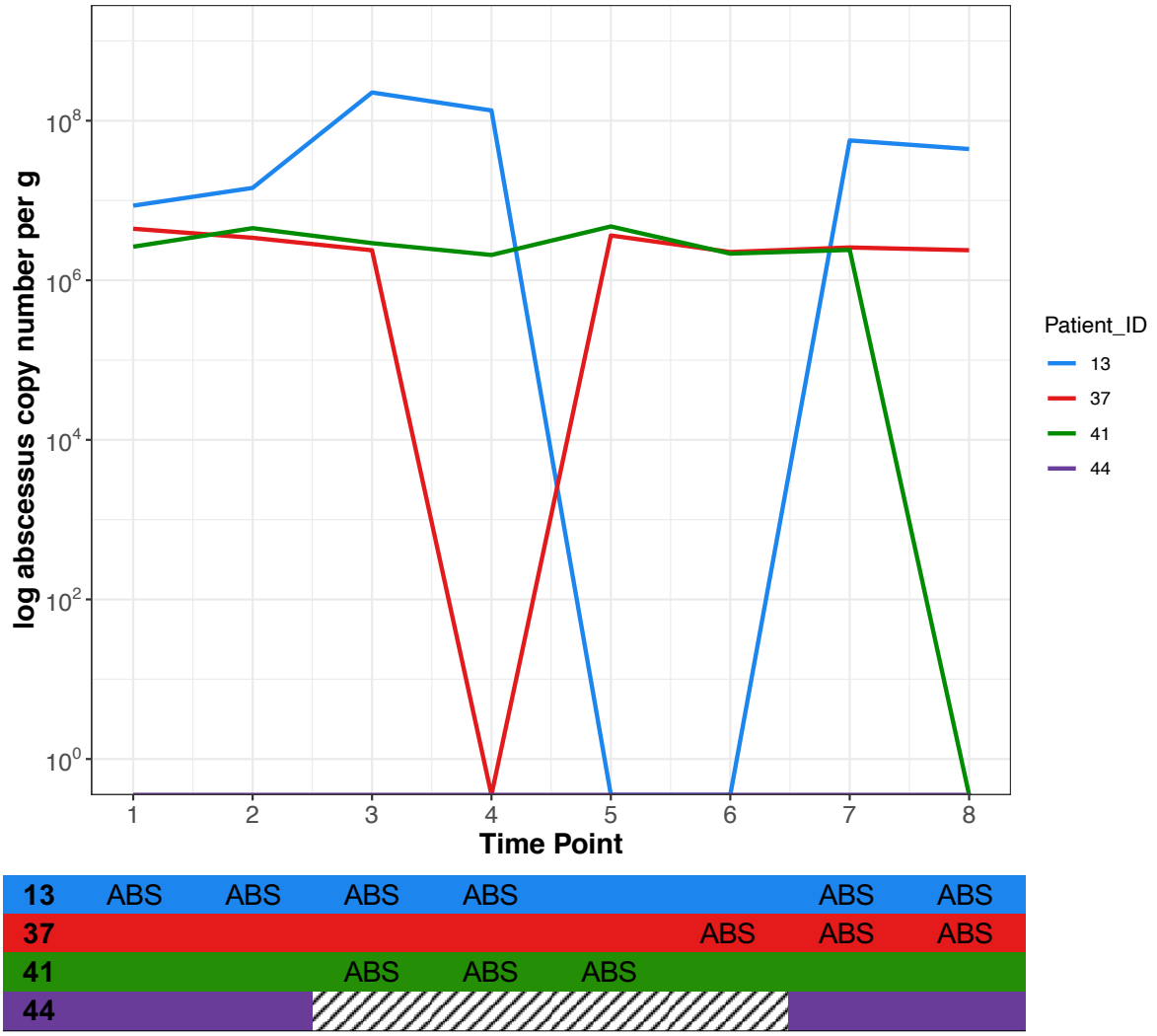


Figure 4.7 – Custom assay targeting *Mycobacterium abscessus* used with samples from the *M. abscessus* control group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. All patients had samples that were positive for *M. abscessus* apart from Patient 44. This was reflected in the culture results. All results are expressed on a logarithmic scale.

The application of the qPCR assay targeting *M. avium* to patients in the *M. abscessus* control group revealed no positive results. This was mirrored in the mycobacterial culture results.

The application of the qPCR assay targeting *M. intracellulare* to patients in the *M. abscessus* control group showed a total of three positive results. These results were not mirrored in the culture data suggesting a failure of culture to show mycobacterial presence. The positive results were for Patient 13 at time points 3 and 7 and Patient 44 at time point 8. Statistical analysis by Kruskall – Wallis one-way analysis of variance

revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 5.2898, df = 7, *P*-value = 0.62) (Figure 4.8).

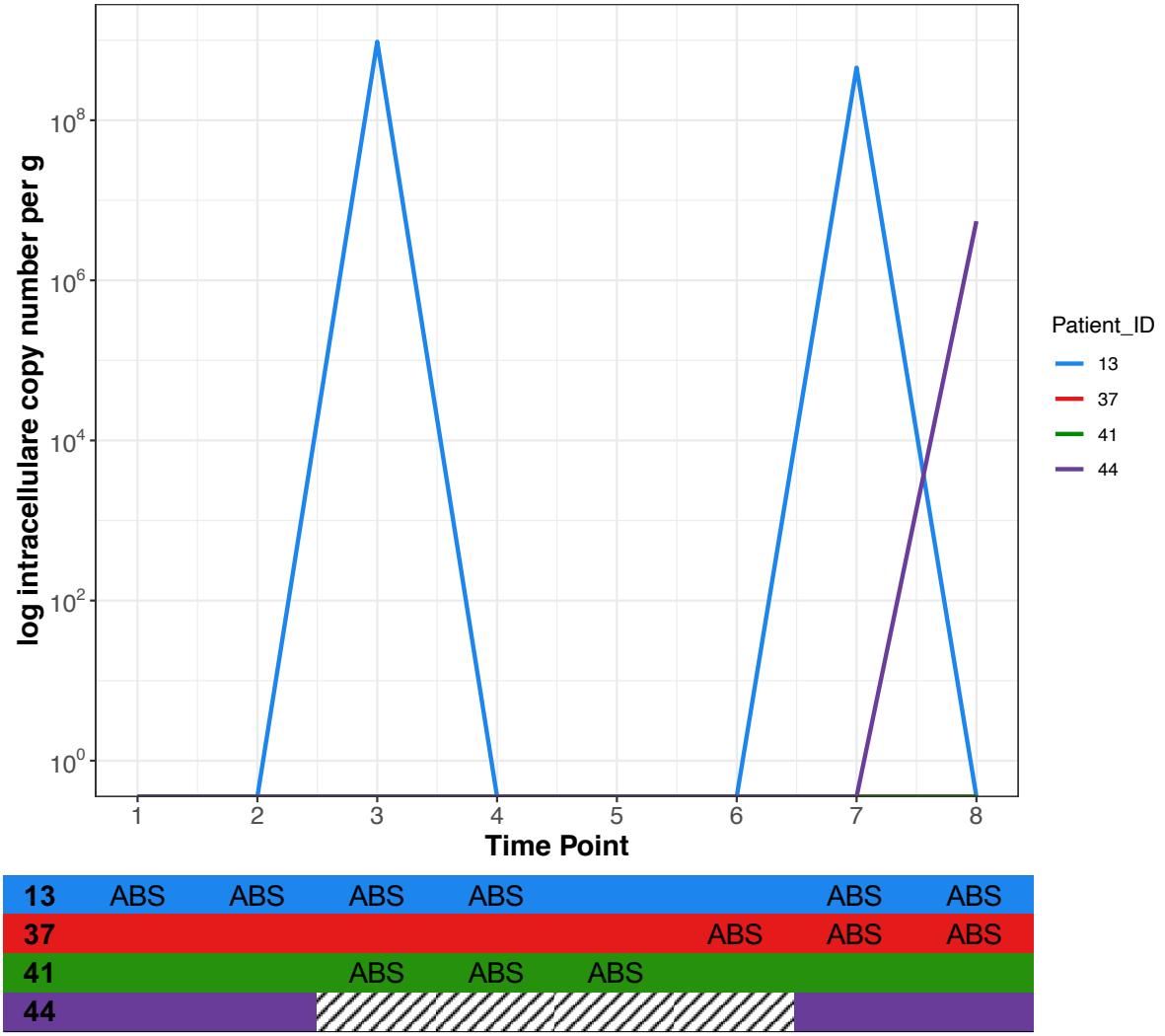


Figure 4.8 – Custom assay with *M. intracellulare* probe used with samples from the *M. abscessus* control group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. Patient 13 showed positive results at time point 3 and 7. Patient 44 showed a positive result at time point 8. Neither result was mirrored in the culture study suggesting a failure of culture to identify these bacteria. All results are expressed on a logarithmic scale.

4.8 Application of the Test to the *M. avium* Control group

A total of 42 samples were analysed from the *M. avium* control cohort (14 samples unavailable for analysis). Twenty-six of these samples were culture positive for MAC.

The application of the qPCR assay targeting *M. abscessus* to patients in the *M. avium* control group revealed positive results at time points 7 and 8 for Patient 15. Despite

never culturing *M. abscessus* prior to recruitment Patient 15 became culture positive at time points 7 and 8. These results were echoed by the custom assay. No significant difference was found between data points (Kruskal-Wallis chi-squared = 5.8093, df = 7, *P*-value = 0.56) (Figure 4.9).

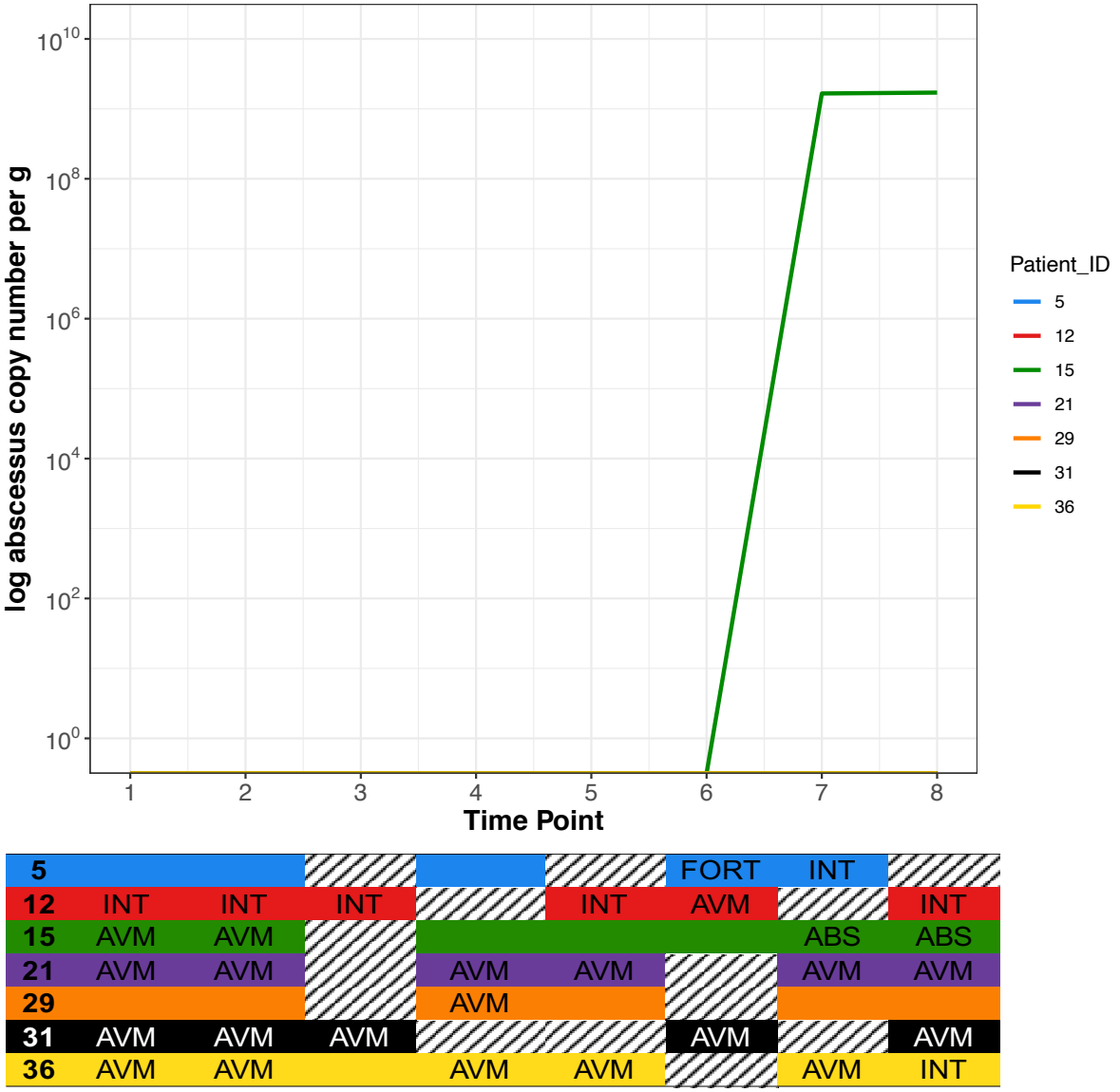
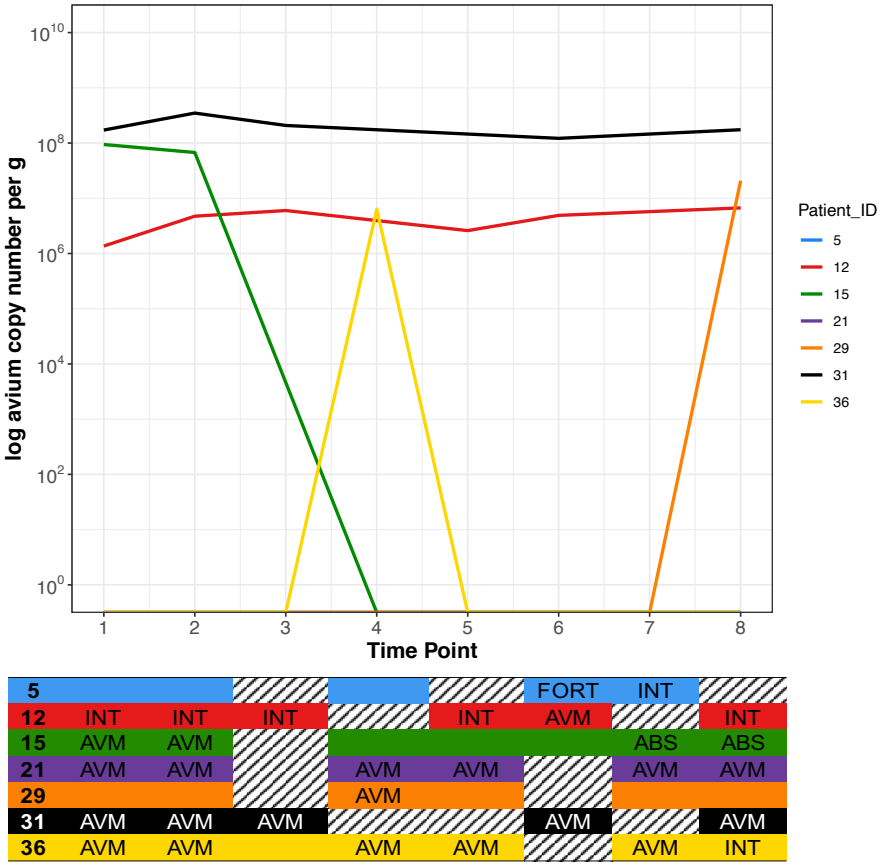


Figure 4.9 - Custom assay with *M. abscessus* probe used with samples from the *M. avium* control group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. Patient 15 became culture positive for *M. abscessus* at time points 7 and 8. These positive results were reflected in the qPCR assay. All results are expressed on a logarithmic scale.

The application of the qPCR assay targeting *M. avium* to patients in the *M. avium* control group showed positive results in 5 of the 9 patients, 12, 15, 29, 31 and 36 (Figure 4.10). Patient 12 culture was positive for the *Mycobacterium avium* complex at all time points tested (*M. avium* at time point 6 and *M. intracellulare* at all other

times). The qPCR assay showed PCR positive results for *M. avium* at all time points tested. Patient 15 showed culture positive results at time points 1 and 2 that were reflected by molecular testing. The qPCR assay was negative for Patient 21 throughout. This was not reflected by culture-based analysis which was positive for *M. avium* at all time points. The qPCR assay was however positive for *M. intracellulare* (part of the *M. avium* complex [Figure 4.11]). Patient 29 was culture positive for *M. avium* at time point 4 only. This was not reflected in the custom assay, but the assay did reveal a positive value at time point 8 that was not highlighted by culture-based analysis. Samples from Patient 31 were all culture positive and this was mirrored by the custom assay. Apart from time points 3 and 8, for all other times points tested Patient 36 was culture positive for *M. avium*. The *M. avium* qPCR assay showed positive results at time point 4 only but the *M. intracellulare* assay was positive at other time points. Statistical analysis by Kruskall – Wallis one-way analysis of variance revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 6.9611, df = 7, *P*-value = 0.43) (Figures 4.10 and 4.11).

a)



b)

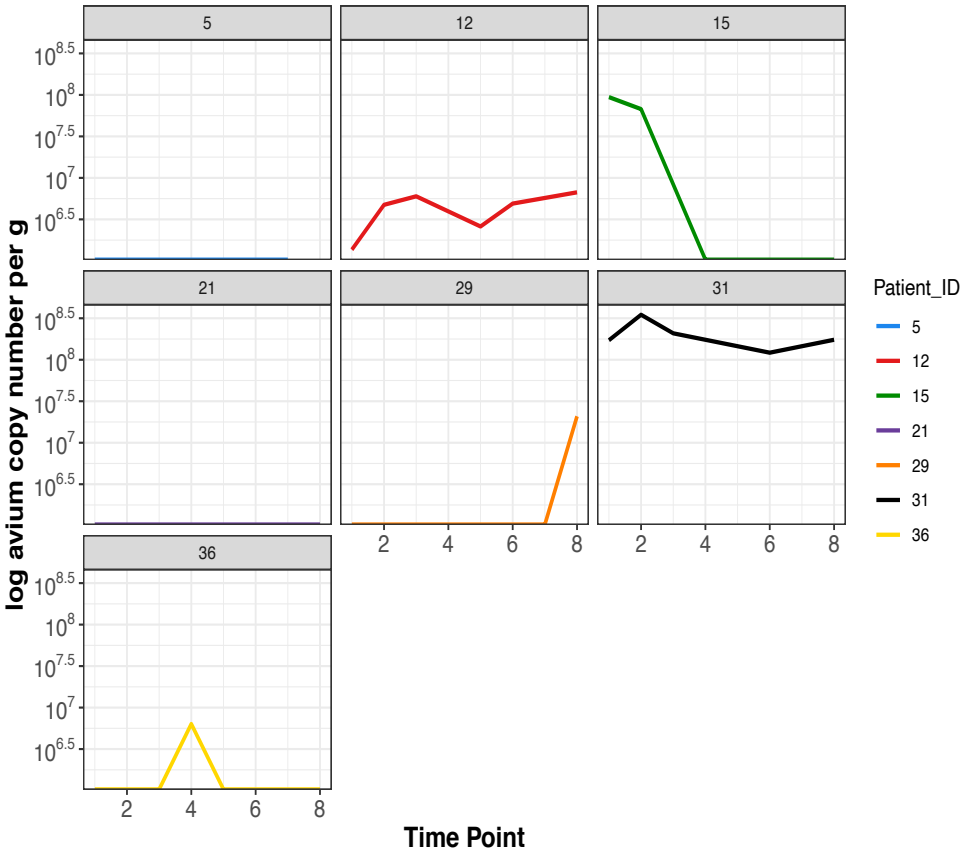


Figure 4.10 a) - Custom assay with *M. avium* probe used with samples from the *M. avium* control group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. Patients 5 and 21 were qPCR negative despite having at least one sample that was culture positive. The assay targeting *M. intracellulare* was however positive in these samples. All results are expressed on a logarithmic scale.

The application of the qPCR assay aimed at *M. intracellulare* showed positive results in Patient 5 at time point 7. This result was reflected by culture-based techniques. Patient 12 showed positive results at time points 5, 6 and 8. These results were reflected by culture-based analysis at time points 5 and 8. Time point 6 was reported as culture positive for *M. avium.* It is likely that Patient 12 has a microbiome consisting of both *M. avium* and *M. intracellulare.* This is confirmed by culture-based analysis and the qPCR assay. Patient 15 was negative for *M. intracellulare* on both culture and molecular detection. Patient 21 was identified as being culture positive for *M. avium* throughout the study. These results were not reflected in molecular studies however the samples were positive for *M. intracellulare* at all time points and showed a 100% concordance if culture reporting was aimed at identifying the *M. avium* complex. Samples from Patients 29 and 31 were both negative for culture and molecular testing. Patient 36 was reported as NTM culture positive for *M. intracellulare* at time point 8 only. At almost all other time points, with the exception of time point 3, the samples were culture positive for *M. avium.* The assay only showed positive results for *M. avium* at time point 4 but did however show positive results for *M. intracellulare* at time points 1, 2, 7 and 8. Statistical analysis by Kruskall – Wallis one-way analysis of variance revealed no significant differences between datasets (Kruskal-Wallis chi-squared = 4.1293, df = 7, *P*-value = 0.76) (Figure 4.11). Once more this illustrates shared microbial communities involving both species comprising the *M. avium* complex.

It is important to note that after combining this assay with that for *M. avium* there was 93% concordance between the custom assays and culture for MAC.

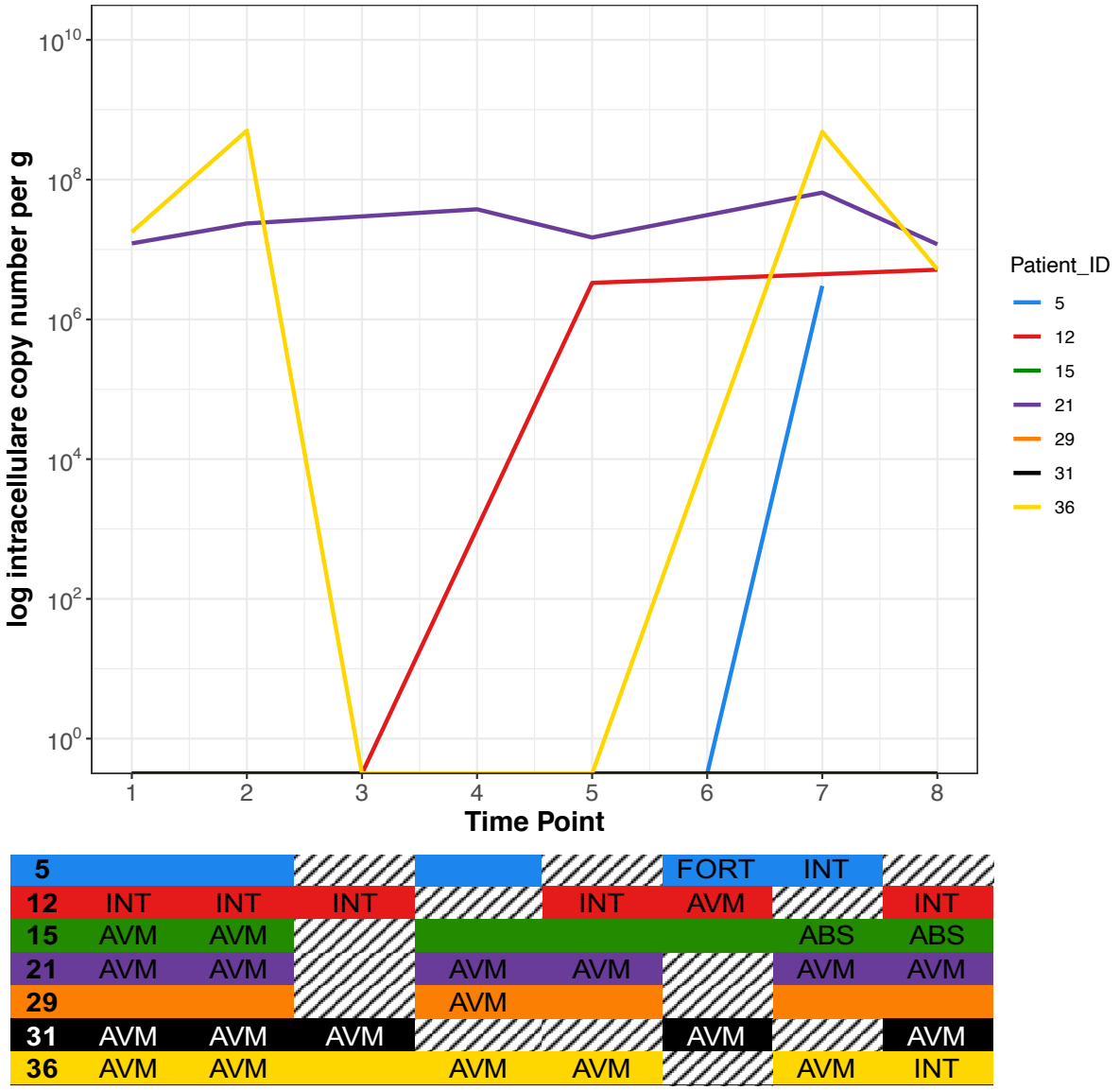


Figure 4.11 - Custom assay with *M. intracellulare* probe used with samples from the *M. avium* control group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. After combining this assay with that for *M. avium* there was 100% concordance between the custom assays and culture for MAC. All results are expressed on a logarithmic scale.

4.9 Application of the Test to the *M. abscessus* Treatment group

A total of 192 samples were received from patients from the *M. abscessus* treatment cohort (74 samples unavailable for analysis). Of the 192 samples, 46 were culture positive for *M. abscessus.* Of note within this cohort, 11 samples were culture positive for *M. avium*, 4 samples positive for *M. intracellulare*, 2 samples positive for *M. xenopi* and 1 positive for *M. kansasii*.

The application of the probe aimed at *M. abscessus* to the group of patients receiving treatment for *M. abscessus* is illustrated in Figure 4.12 and showed positive qPCR results for nearly all patients, the exception being Patient 33. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed a significant difference between datasets (Kruskal-Wallis chi-squared = 82.173, df = 18, *P*-value = 3.561e-10)

Patient 2 showed positive qPCR results at time points 1, 2, 3 and 15. These results were mirrored by culture showing 100% concordance between culture and the qPCR assay. Considering quantification, copy number increased from time point 1 to time point 2 owing to the intentional delay in sample freezing (sample 2 kept at room temperature for 24 hours prior to freezing). After a week of initiation therapy, *M. abscessus* copy number fell dramatically before being undetectable at time point 4 (the end of initiation therapy). Interestingly at time point 15 (a year of treatment), *M. abscessus* became detectable both with the qPCR assay and culture analysis. This followed a sustained period of patient intolerance to one antibiotic (Ciprofloxacin). After this antibiotic was replaced *M. abscessus* once again became undetectable.

In contrast, Patient 4 remained tolerant to all antibiotics (Table 4.2) during the treatment period and *M. abscessus* copy number became undetectable at time point 3 onwards. After 15 months of treatment, Patient 4 was deemed NTM free and treatment was ceased. After 18 months (time point 19), the patient remained NTM negative. These results were echoed in Patients 14, 19 and 22 and largely echoed in Patients 6 and 11. Interestingly with Patient 6, although from the same sputum, sample time point 1 failed to culture *M. abscessus* in contrast to sample time point 2. The qPCR assay identified *M. abscessus* in both samples 1 and 2. Samples time points 3 and 4 were both culture and qPCR assay positive for *M. abscessus.* Patient 11 showed good concordance between culture and PCR at time points 1 and 2 and remained both culture and qPCR negative for *M abscessus* but became culture positive for *M. xenopi* at time point 12.

Samples from Patient 8 were both culture and qPCR positive before treatment and became negative during the induction phase of treatment and subsequently remained negative thereafter. The dramatic increase in copy number from samples time points

3 to 4 cannot be explained but as *M. abscessus* copy number becomes unrecordable thereafter it is possible this was an anomalous result.

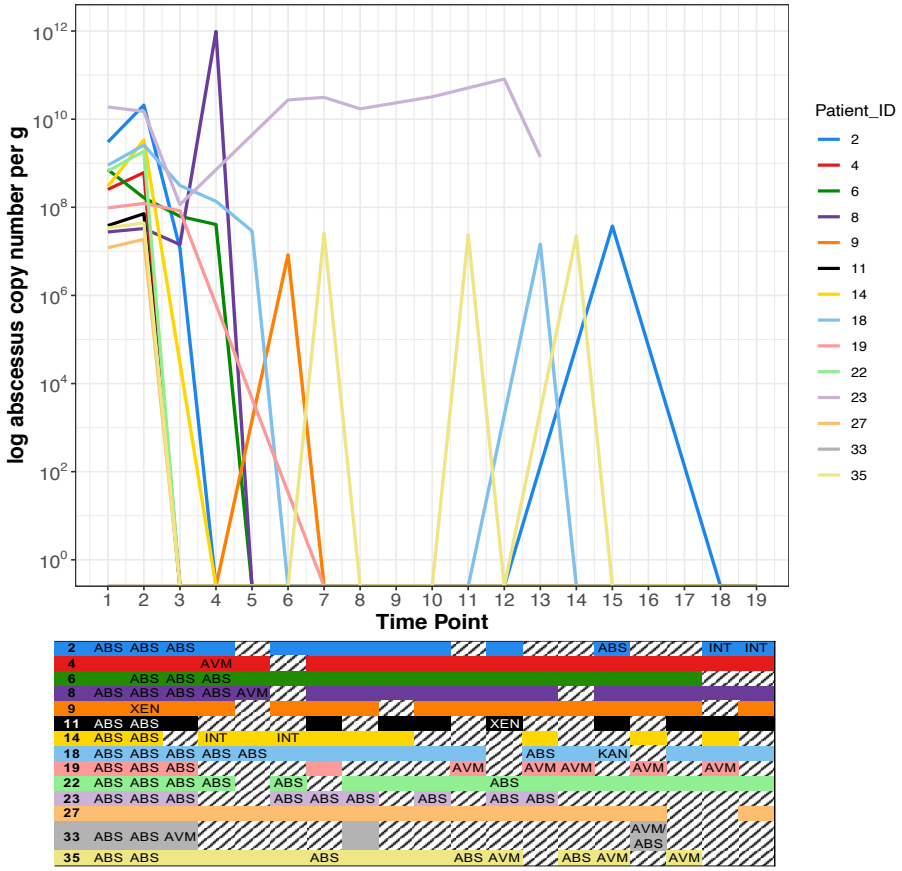
Patient 9 was culture negative throughout the study although sample time point 6 demonstrated positivity with the qPCR assay. Prior to the study, the patient had been inconsistently *M. abscessus* culture positive and therefore it is probable that sample time point 6 has demonstrated a failure of culture.

Patient 18 showed 100% concordance between culture and qPCR analysis becoming negative after 1 month of treatment. Patient 18 once again became culture and qPCR positive at time point 13. At this point nebulised Amikacin was temporarily stopped after the patient complained of shortness of breath.

Patient 27 was qPCR positive in samples time points 1 and 2 but this was not echoed by culture techniques. As the patient was culture positive prior to recruitment, this likely represents a failure of culture techniques to identify *M. abscessus* in this sample.

Patients 23 and 35 both showed recurrent isolates of *M. abscessus* during treatment. There was 100% concordance between culture and qPCR analysis in these patients. Both Patients 23 and 35 reported varied treatment compliance. During the initiation phase of treatment, qPCR copy number fell abruptly in samples from Patient 23 but intolerances to Ciprofloxacin and subsequently Moxifloxacin during the continuation phase meant that copy numbers returned to pre-treatment levels. Copy numbers decreased at time point 13 after the patient was admitted for intravenous therapy. No further samples were provided after this time point. Patient 35 suffered from side effects to nebulised Amikacin, Minocycline and Ciprofloxacin at various stages during their treatment regimen. These drug intolerances were reflected in the waxing and waning qPCR results shown below (Figure 4.12).

a)



b)

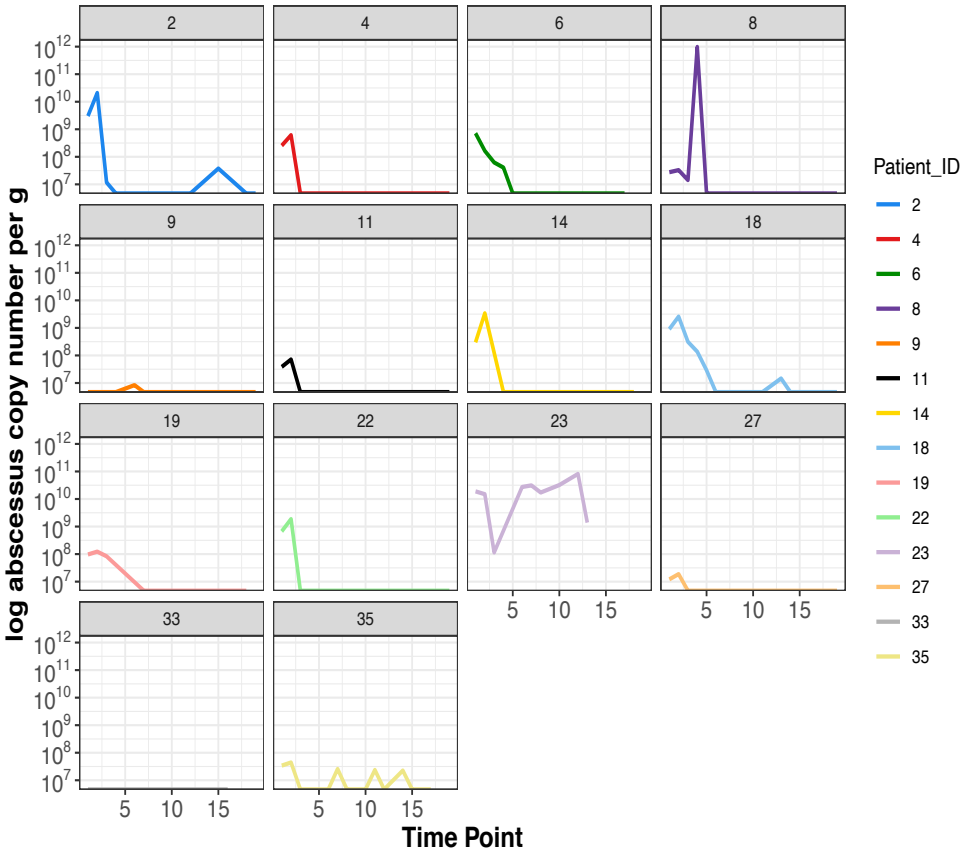
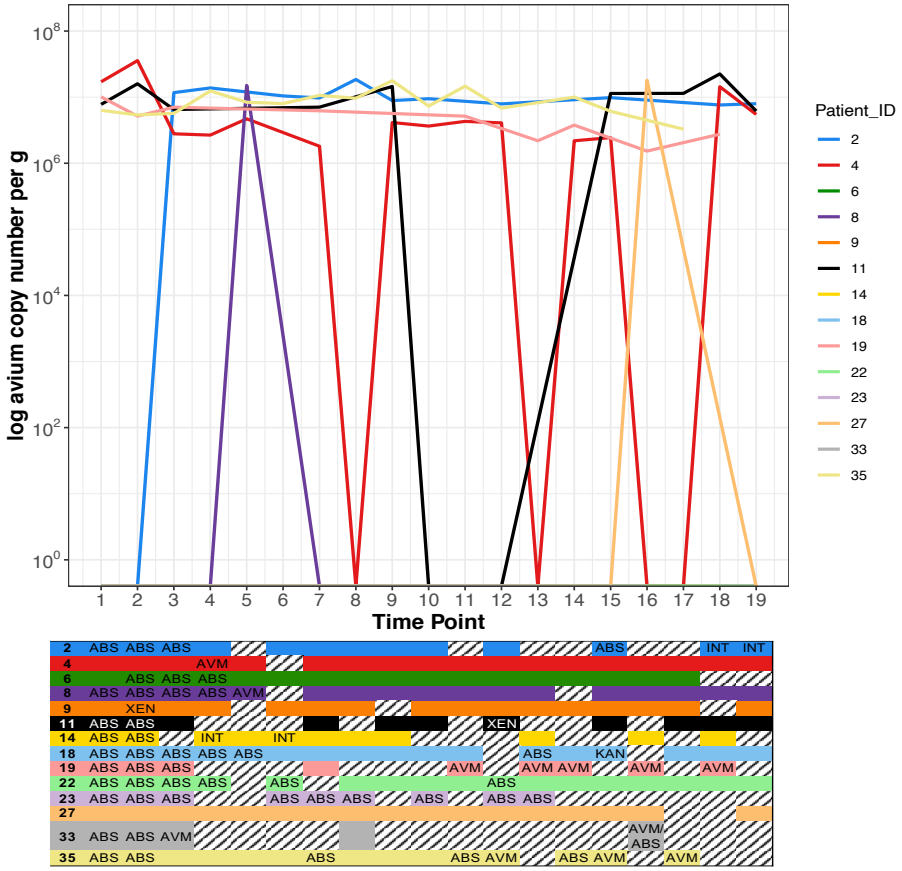


Figure 4.12 a) - Custom assay with *M. abscessus* probe used with samples from the *M. abscessus* treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. After initiation therapy copy number often fell to unrecordable levels and remained unrecordable as long as antibiotic therapy was adhered to. All results are expressed on a logarithmic scale.

The application of the probe aimed at *M. avium* to the group of patients receiving treatment for *M. abscessus* is shown in Figure 4.13. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 8.0016, df = 18, *P*-value = 0.98).

Samples from Patients 2, 4, 8, 11, 19, 27 and 35 all gave positive results. Patients 2, 4 and 11 exhibited positive qPCR results at regular time points. This was not reflected by culture-based analysis which remained negative throughout indicating a failure of culture-based analysis to identify NTM. Patient 8 was culture and qPCR positive for *M. avium* at time point 5. Patient 19 was qPCR positive for *M. avium* at all time points although this was only reflected by culture analysis for 5 (11, 13, 14, 16 and 18) of the nine time points. A similar pattern was seen for Patient 35 who was qPCR positive throughout (total of 16 time points) but only culture positive at time points 12, 15 and 17. The qPCR assay was negative throughout (5 time points) for Patient 33 despite positive culture results at time points 3 and 16.

a)



b)

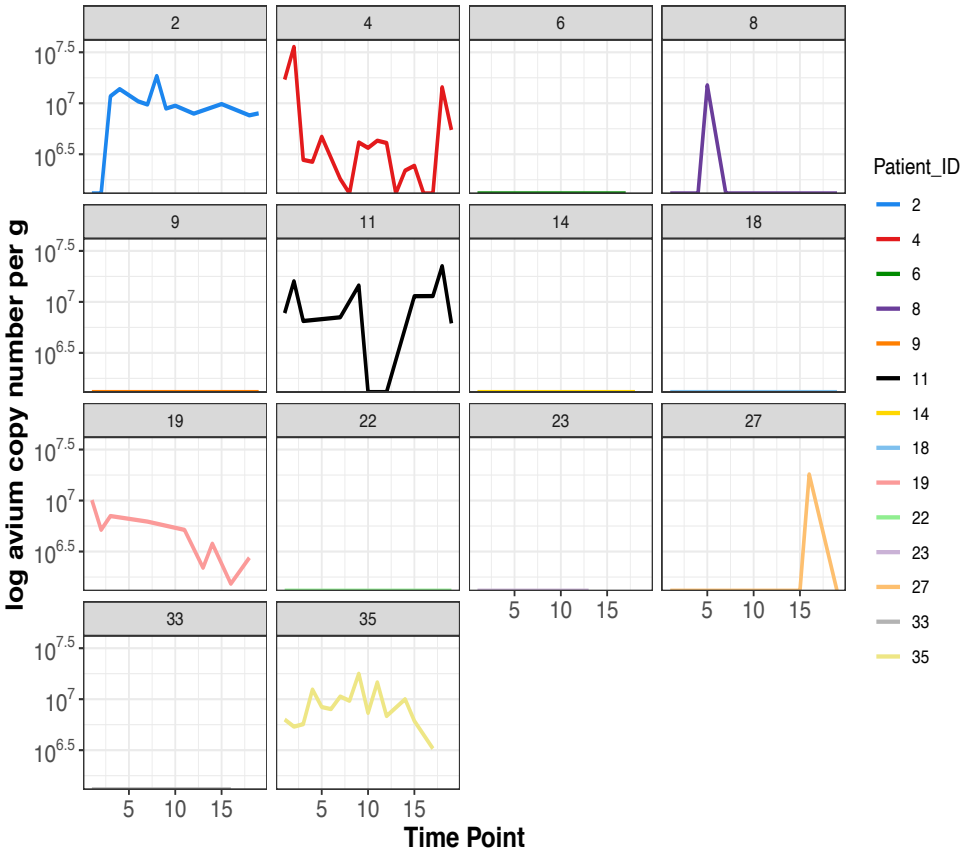
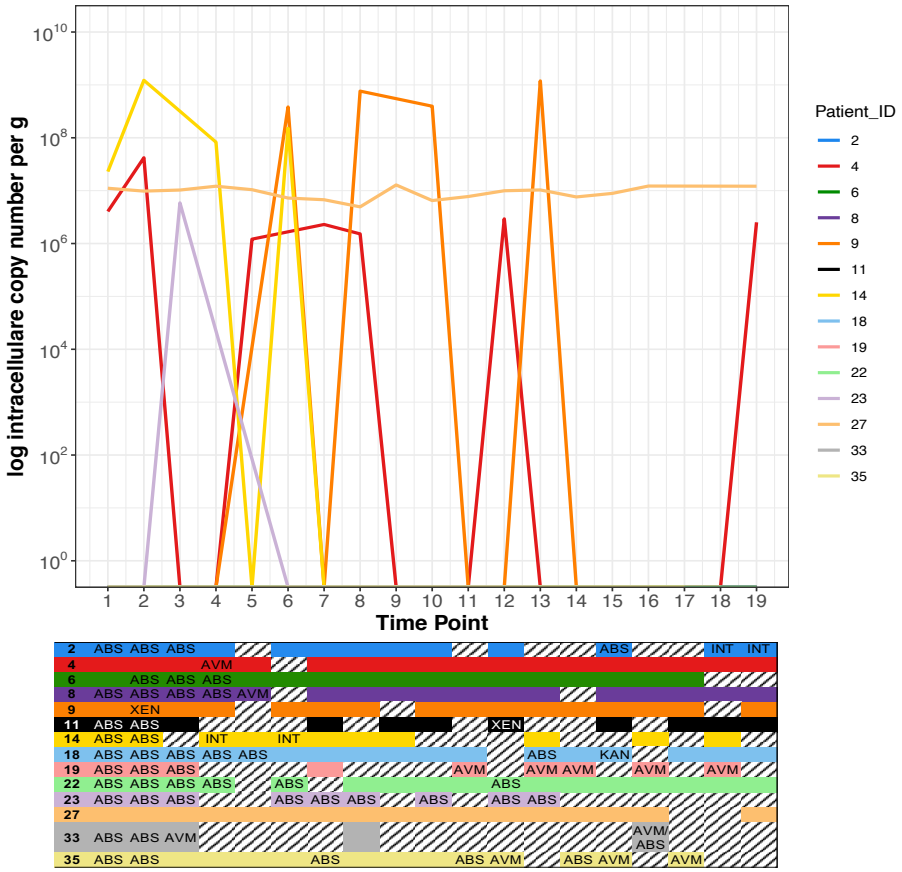


Figure 4.13 a) - Custom assay with *M. avium* probe used with samples from the *M. abscessus* treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. Ten of the 14 patients receiving treatment for *M. abscessus* tested positive for MAC at some stage during treatment. Many of these samples were culture negative suggesting a failure of culture to recognise mixed mycobacterial samples. All results are expressed on a logarithmic scale.

The application of the probe aimed at *M. intracellulare* to the group of patients receiving treatment for *M. abscessus* is illustrated in Figure 4.14. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 8.557, df = 18, *P*-value = 0.97).

Positive results for Patients 4, 9, 14, 23 and 27 were found. Patients 9, 23 and 27 were culture negative for *M. intracellulare* for the duration of the study raising the possibility that culture failed to identify the species. Patient 14 was culture positive at time points 4 and 6 only. This is in contrast to the qPCR assay that showed positive results at time points 1, 2, 4 and 6. Patient 4 showed positive qPCR results for *M. intracellulare* at regular time points although this was never reciprocated by culture analysis. Indeed Patient 4 showed positive qPCR results for *M. abscessus. M. avium* and *M. intracellulare* confirming the presence of a mixed NTM microbiome that is underrepresented by culture alone.

a)



b)

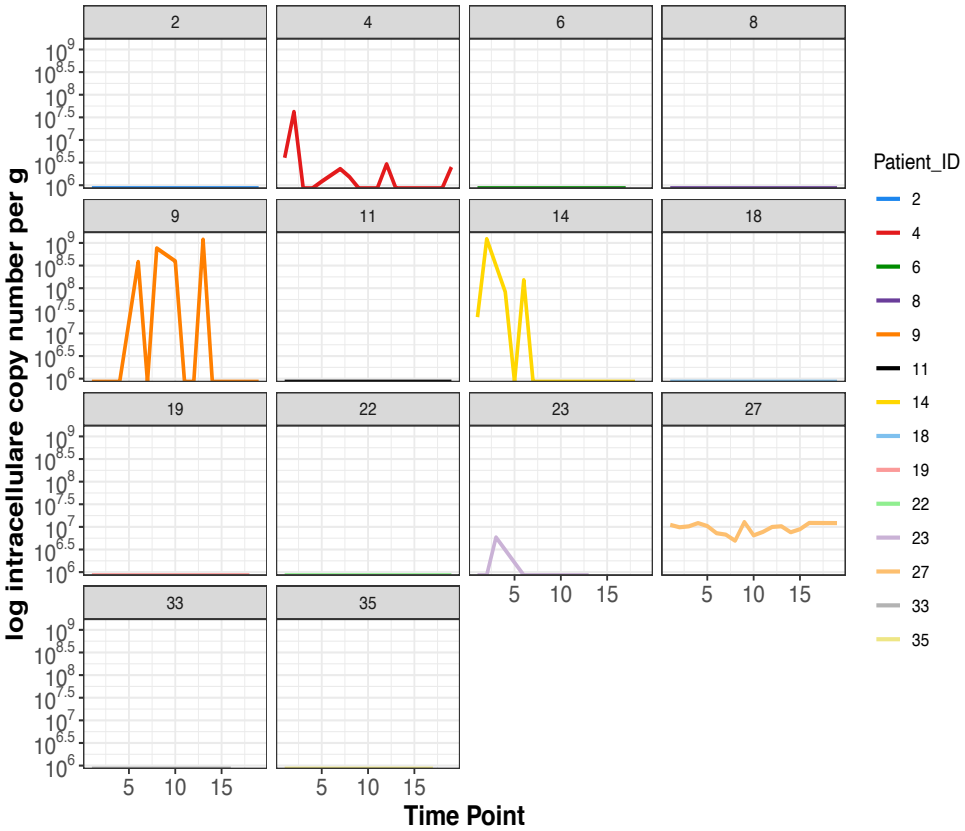


Figure 4.14 a) - Custom assay with *M. intracellulare* probe used with samples from the *M. abscessus* treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. Ten of the 14 patients receiving treatment for *M. abscessus* tested positive for MAC at some stage during treatment. Many of these samples were culture negative suggesting a failure of culture to recognise mixed mycobacterial samples. All results are expressed on a logarithmic scale.

4.10 Application of the Test to the *M. avium* Complex Treatment group

A total of 117 samples were received from patients from the MAC treatment cohort (35 samples unavailable for analysis). Of the 117 samples, 24 were culture positive for *M. avium* and 12 for *M.intracellulare.* Of note within this cohort, 3 samples were culture positive for *M. xenopi*.

The application of the probe aimed at *M. abscessus* to the group of patients receiving treatment for *M. avium* is shown in Figure 4.15 with positive results obtained for Patients 7 and 39 at time points 15 and 7 respectively. Culture was negative at these time points. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 17.074, df = 18, *P*-value = 0.52).

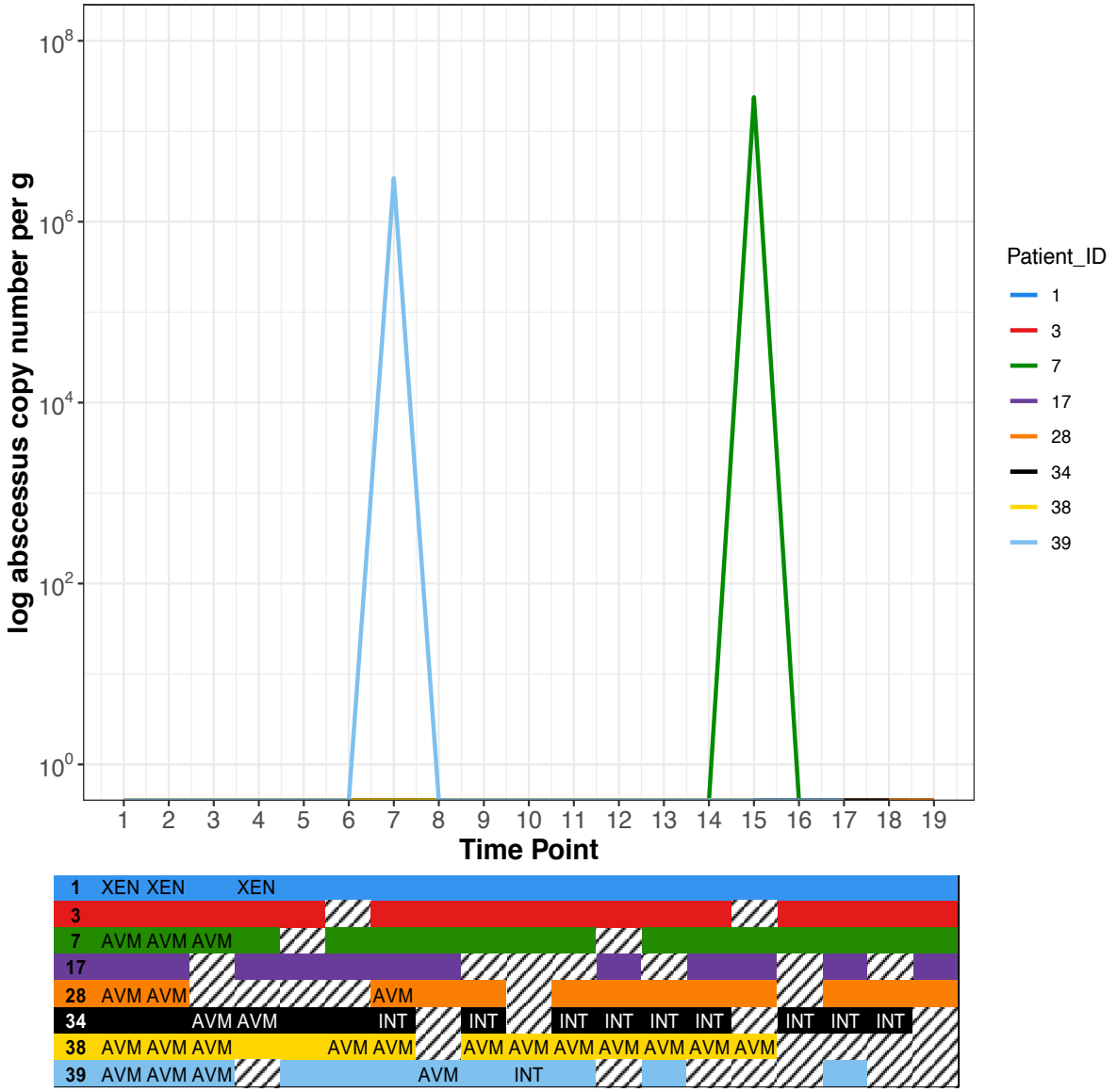


Figure 4.15 - Custom assay with *M. abscessus* probe used with samples from the *M. avium* complex treatment group. Patient culture results by time point are displayed below qPCR data. Hashed box indicates unavailable samples. All samples in this group were culture negative for *M. abscessus* suggesting a failure of culture techniques to identify this species at these time points. All results are expressed on a logarithmic scale.

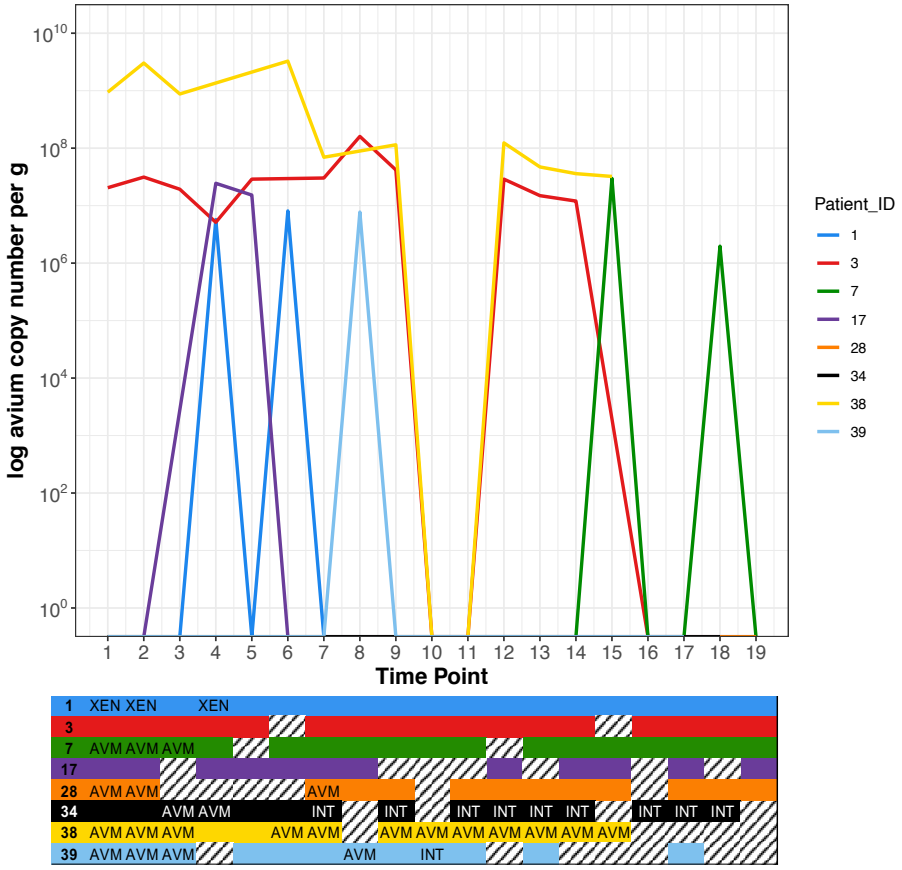
Results of application of the *M. avium* specific qPCR assay to the group receiving treatment for *M. avium* complex are shown in Figure 4.16. Positive results were seen for Patients: 1, 3, 7, 17, 38 and 39. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 13.944, df = 18, *P*-value = 0.73).

Interestingly Patients 28 and 34 remained *M. avium* qPCR assay negative throughout the study period despite culturing a combination of *M. avium* and *M. intracellulare.* The samples did, however, return positive *M. intracellulare* qPCR assay results illustrating the importance of using both assays in identification of MAC.

Patients 1, 7, 17 and 39 all had a maximum of two isolates that were qPCR assay positive for *M. avium.* This is noteworthy as Patients 1 and 17 failed to culture *M. avium* at any point during their treatment course. It could be postulated that again, qPCR has identified the presence of NTM species where culture has failed.

Patients 7 and 39 both cultured *M. avium* on occasions where the qPCR assay failed to identify *M. avium*. Patient 3 was culture negative throughout their treatment course but (apart from time points 10 and 11) was qPCR assay positive until time point 16. These results are in marked contrast to culture-based results that were negative throughout treatment. Patient 38 was culture positive at all time points for *M. avium*. These times were qPCR assay positive for *M. avium.* Time points 10 and 11 were qPCR assay negative for *M. avium* despite being culture positive. The positive results for Patient 38 cannot be fully explained as the patient was reportedly fully compliant with medication throughout the treatment period.

a)



b)

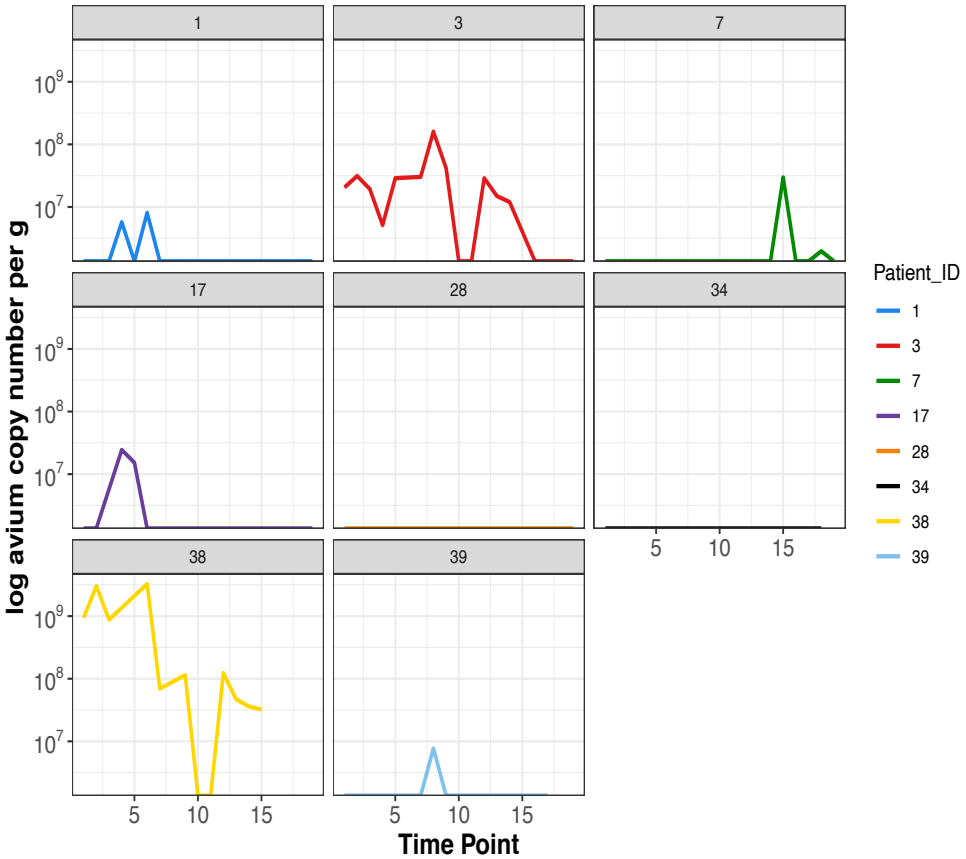


Figure 4.16 a) - Custom assay with *M. avium* probe used with samples from the *M. avium* complex treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. All results are expressed on a logarithmic scale.

Results of the application of the *M. intracellulare* specific qPCR assay to the group receiving treatment for *M. avium* complex are shown in Figure 4.17 and positive results were obtained for Patients 1, 7, 28 and 34. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 8.381, df = 18, *P*-value = 0.97).

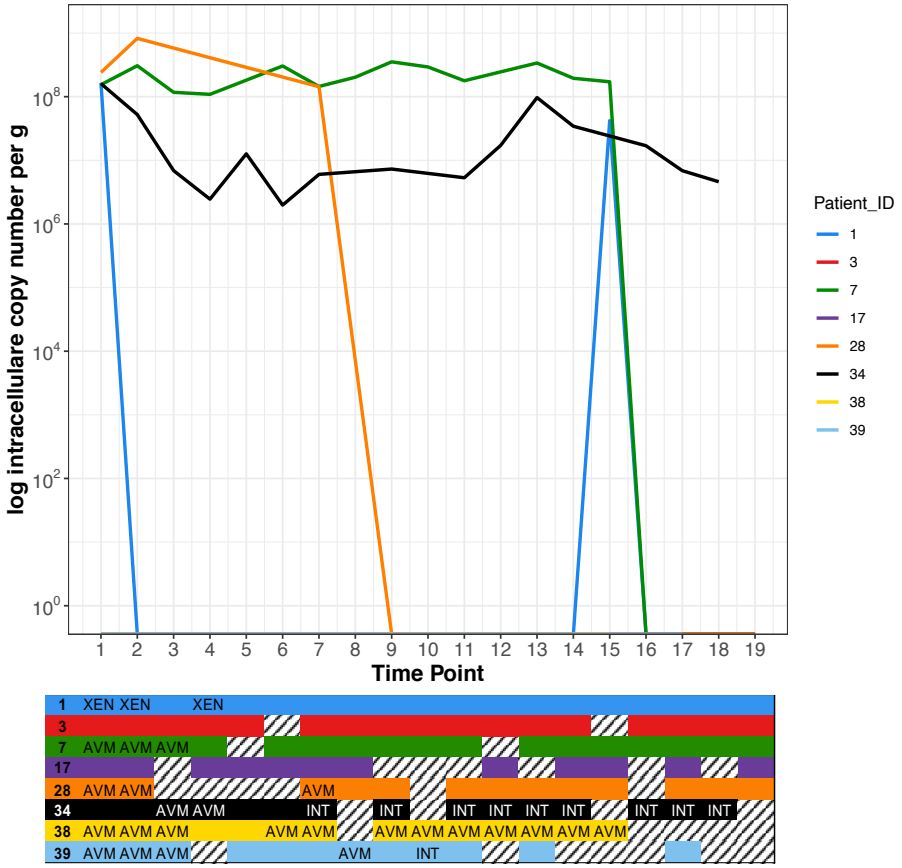
Patient 1 was positive for *M. intracellulare* at time points 1 and 15. In combination with the qPCR assay targeting *M. avium* (Figure 4.16) positive results for either *M.intracellulare* or *M. avium* (the two species that make up the *Mycobacterium avium* complex) were found at time points 1, 4, 6 and 15. This is in contrast to culture-based techniques that failed to detect MAC at any point.

In Patient 7 the qPCR assay directed at *M. intracellulare* was positive until month 10 (time point 15) of treatment. It was at month 10 that the qPCR assay aimed at *M. avium* also became positive (Figure 4.16). In contrast, culture-based techniques only detected the presence of MAC at time points 1, 2 and 3.

Patient 28 tested positive for MAC based on culture techniques at time points 1, 2 and 7. This was mirrored in the qPCR results after positive results for *M. intracellulare* at these time points. The assay became and remained negative after 2 months (time point 7) of anti-microbial therapy.

Patient 34 was culture positive for MAC at all time points apart from at 1, 2, 5 and 6. Interestingly the qPCR assay shows a spike in *M. intracellulare* copy number between months 6 and 8 of treatment (time points 11 and 13). At this time the patient reported side effects of their medication and Azithromycin was temporarily stopped.

a)



b)

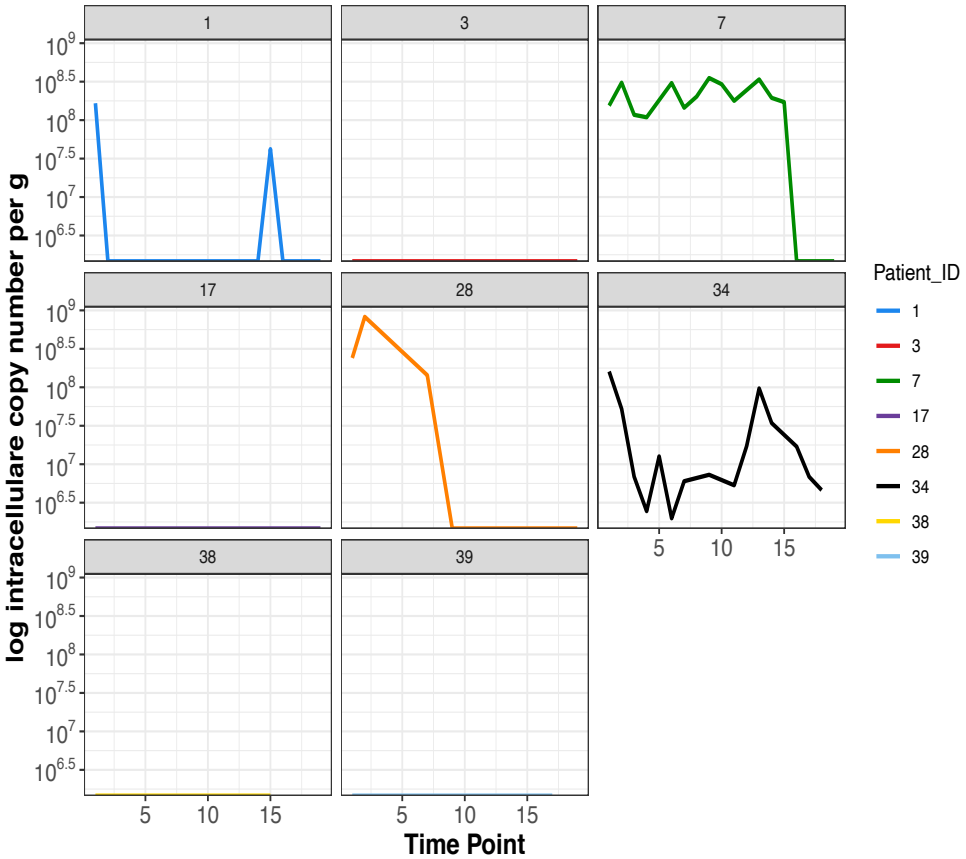
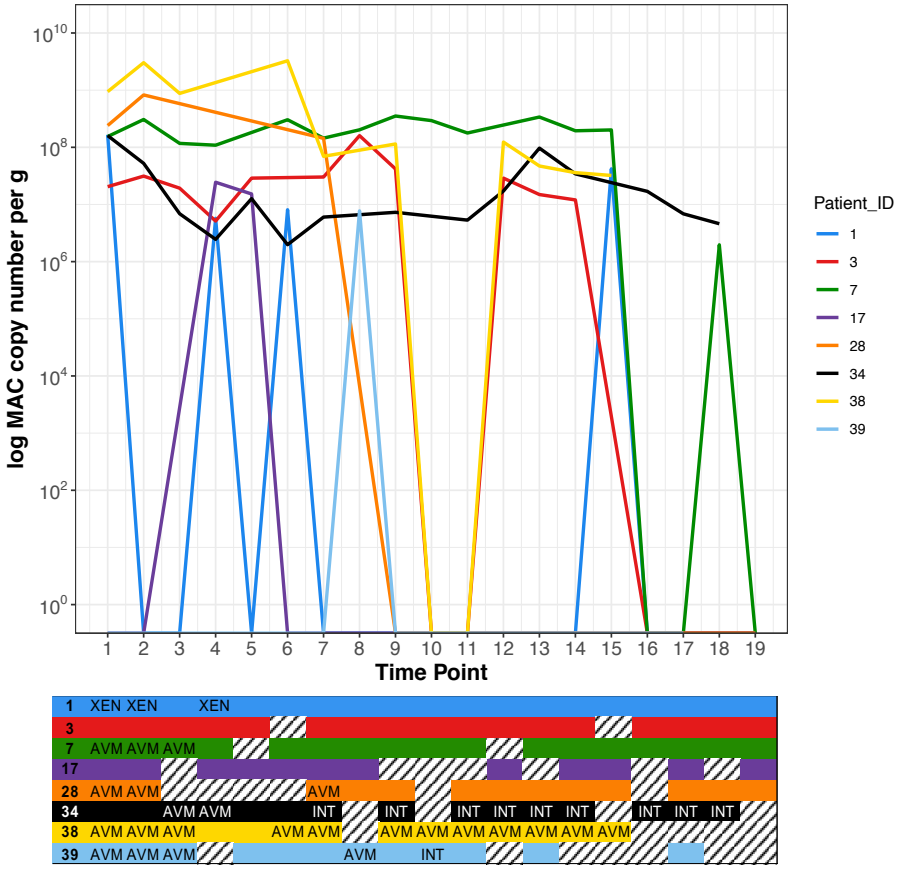


Figure 4.17 a) - Custom assay with *M. intracellulare* probe used with samples from the *M. avium* complex treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. All results are expressed on a logarithmic scale.

Owing to incongruity between culture-based results and the qPCR assays targeting *M. avium* and *M. intracellulare*, it was decided to combine both assays to form a “MAC qPCR assay” whereby MAC copy number is equal to the sum of the *M. avium* and *M. intracellulare* qPCR assays (Figure 4.18). When the *M. avium* and *M. intracellulare* assays were combined, the assays showed 78% concordance with positive culture results. Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant difference between datasets (Kruskal-Wallis chi-squared = 23.541, df = 18, *P*-value = 0.15).

a)



b)

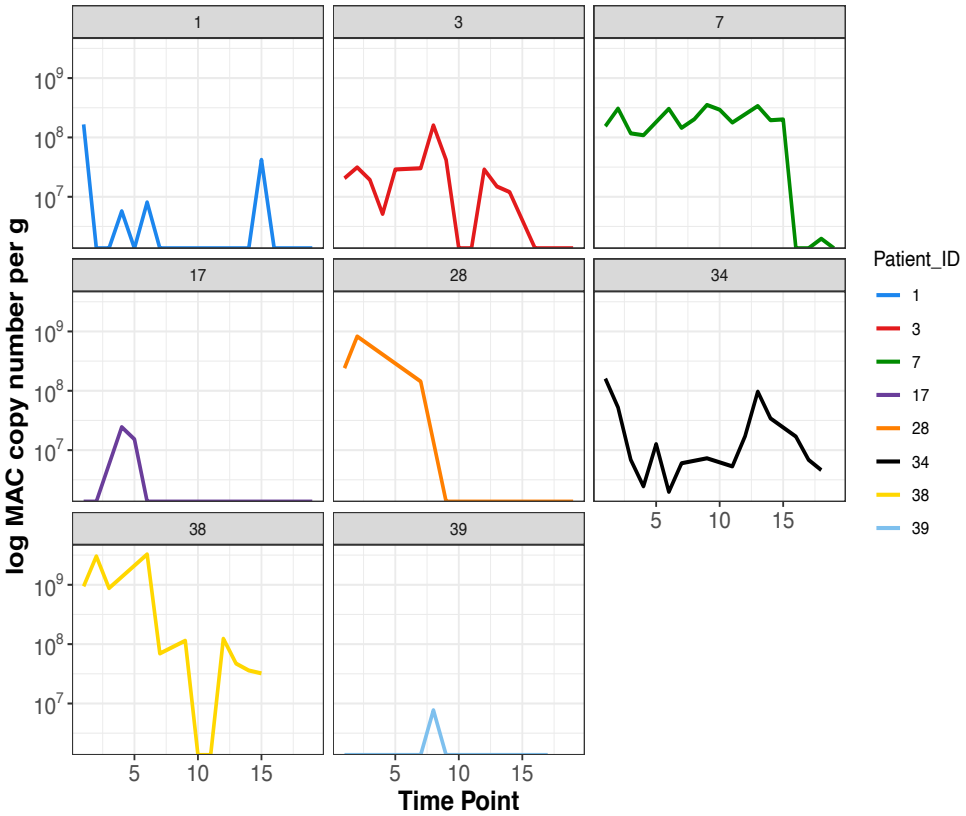


Figure 4.18 a) - Custom assay generated by combining the qPCR assays targeting *M. avium* and *M. intracellulare* used with samples from the *M. avium* complex treatment group. Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **b)** - results by individual patients. When the *M. avium* and *M.*

intracellulare assays were combined, the assays showed 78% concordance with positive culture results. All results are expressed on a logarithmic scale.

4.11 Assessment of Test Characteristics

Using culture-based techniques as a gold standard test, the sensitivity, specificity, positive and negative predictive value of each assay was calculated and is shown in Table 4.3.

Table 4.3 – Individual probe characteristics based on culture-based results sourced from clinical sputum samples. Each probe result was compared to the corresponding sample culture result which was used as the gold standard test. From this the: true positives, false positives, true negatives and false positives are listed. Under this the sensitivity, specificity, positive predictive value and negative predictive value for each probe are listed.

	Probe			
	abscessus	**avium**	**intracellulare**	**MAC**
True positive	52	30	15	67
False positive	16	78	82	127
True negative	334	276	305	205
False negative	8	25	8	11
Sensitivity	0.87	0.58	0.65	0.86
Specificity	0.95	0.78	0.79	0.62
PPV[1]	0.76	0.28	0.15	0.35
NPV[2]	0.98	0.92	0.97	0.95

[1]PPV=Positive Predictive Value
[2]NPV=Negative Predictive Value

4.11.1 Assessment of the *M. abscessus* Assay

The assay targeting *M. abscessus* performed well, demonstrating a high specificity (0.95) (Table 4.3). The least accurate diagnostic characteristic was the positive predictive value (0.76) owing to the false positive number (N=16). The number of false positives is discussed in Chapter 4, Section 4.12.1.1 below.

The assay's predictive power was assessed by the construction of a receiver operator characteristic (ROC) curve (Figure 4.19) and the calculation of the area under the curve (AUC) using the ROCR package [203]. This demonstrated an AUC of 0.923.

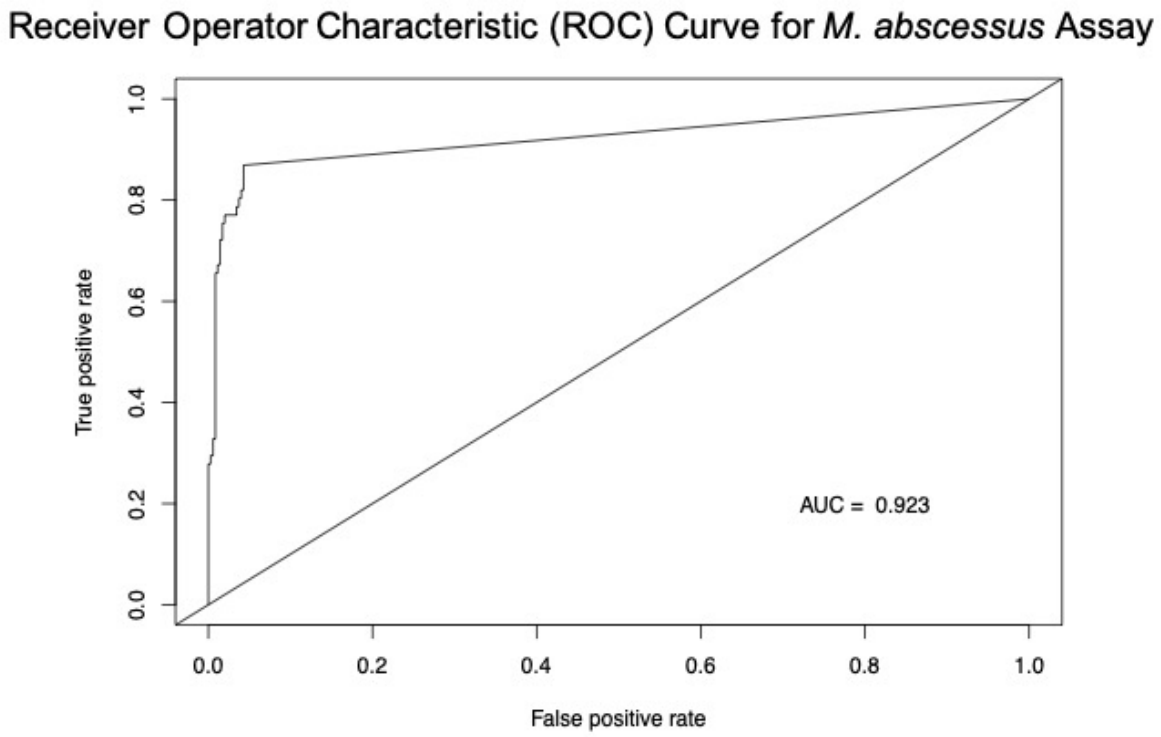


Figure 4.19 – Receiver Operator Characteristic (ROC) Curve for the *M. abscessus* assay. The ROC curve illustrates the diagnostic ability of a test by plotting the true positive rate against the false positive rate. The Area Under the Curve (AUC) can be interpreted as the probability that a randomly chosen diseased subject is rated as more likely to be diseased than a randomly chosen non-diseased subject. A diagnostic test with no false positives scores 1. *M. abscessus* assay AUC = 0.923.

4.11.2 Assessment of the *M. avium* Assay

The probe targeting *M. avium* performed less favorably with a lower specificity (0.78). This was again in large part due to the high number of false positives in the group (N=78) (Table 4.3). This resulted accounted for the poor positive predictive value of the test (0.28). Unfortunately, the assay suffered a relatively high number of false negatives (N=22) which also accounted for the relatively low sensitivity (0.58) of the assay.

The assay's predictive power was again assessed by the construction of a ROC curve (Figure 4.20). This demonstrated an AUC of 0.673.

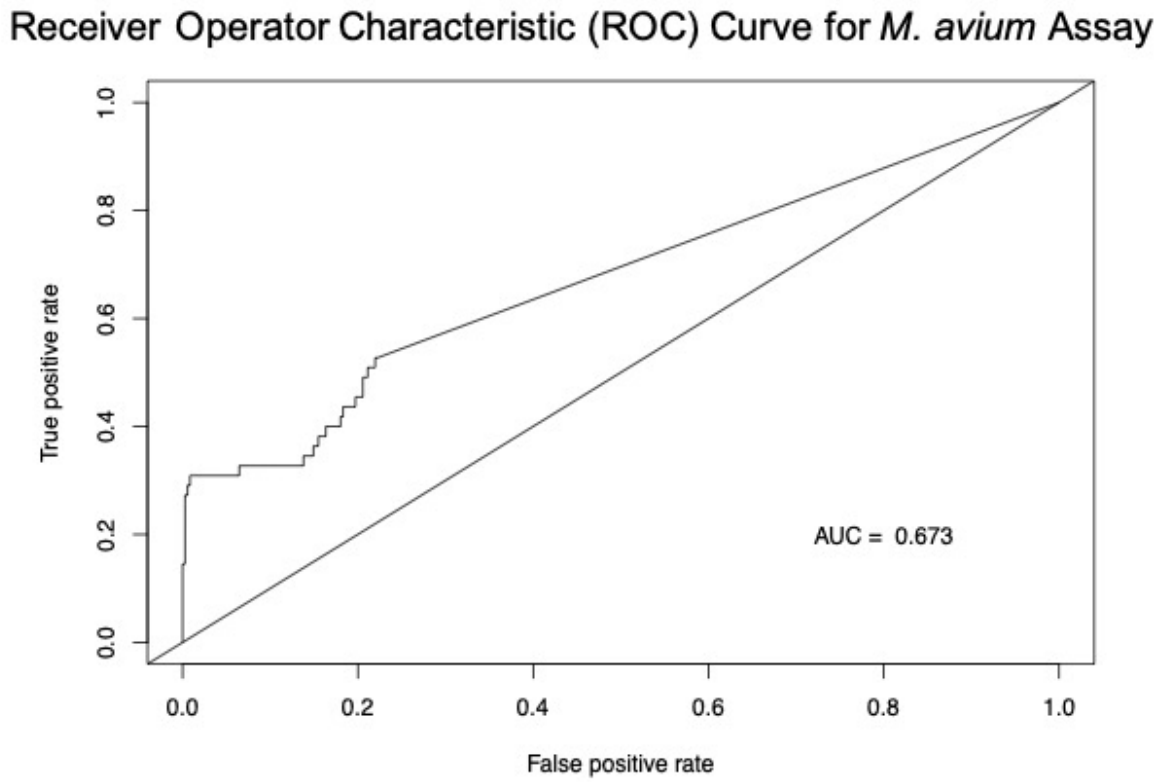


Figure 4.20 – Receiver Operator Characteristic (ROC) Curve for the *M. avium* assay. The ROC curve illustrates the diagnostic ability of a test by plotting the true positive rate against the false positive rate. The Area Under the Curve (AUC) can be interpreted as the probability that a randomly chosen diseased subject is rated as more likely to be diseased than a randomly chosen non-diseased subject. A diagnostic test with no false positives scores 1. *M. avium assay* Area Under the Curve (AUC) = 0.923.

4.11.3 Assessment of the *M. intracellulare* Assay

Similar results were noted with the assay targeting *M. intracellulare.* The assay found a high number of false positive results (N=83) that accounted for a specificity of 0.79 and a positive predictive value of 0.15. Again, the assay suffered a relatively low sensitivity of 0.65. This was due to a combination of small true positive and false negative numbers (Ns 15 and 8 respectively). As for all assays the negative predictive value was nonetheless good (0.97) due to the low number of false negatives (N=8).

The assay's predictive power was again assessed by the construction of a ROC curve (Figure 4.21). This demonstrated an AUC of 0.301.

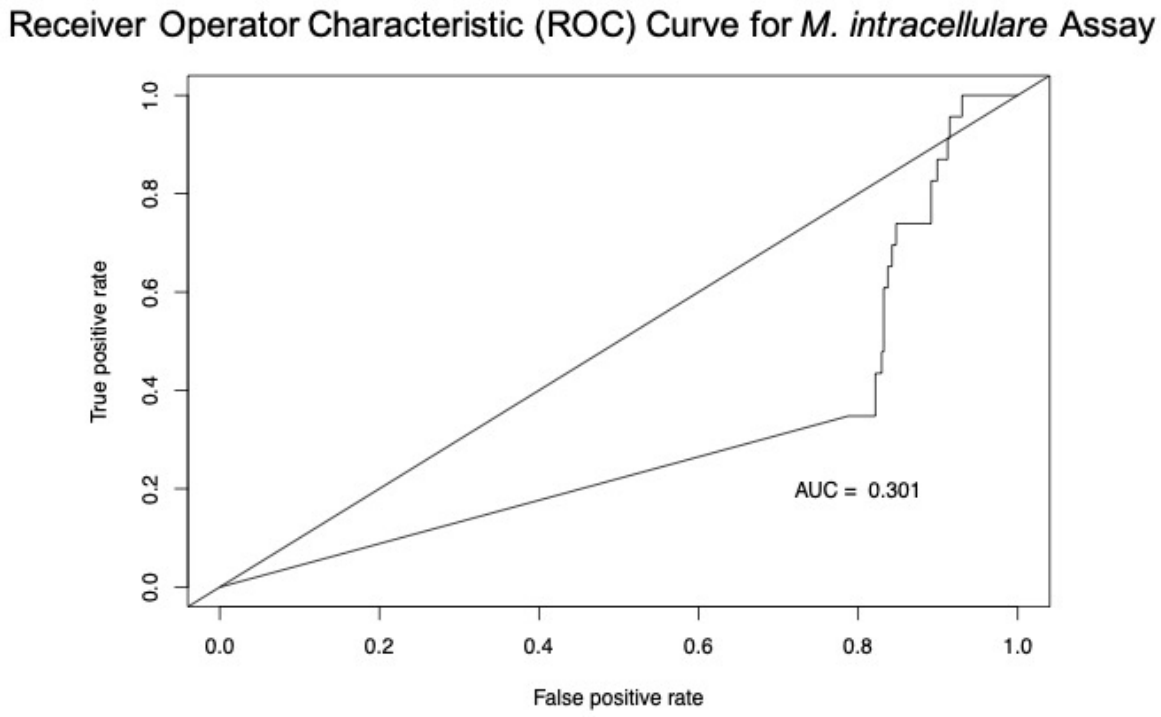


Figure 4. 21 – Receiver Operator Characteristic (ROC) Curve for the *M. intracellulare* assay. The ROC curve illustrates the diagnostic ability of a test by plotting the true positive rate against the false positive rate. The Area Under the Curve (AUC) can be interpreted as the probability that a randomly chosen diseased subject is rated as more likely to be diseased than a randomly chosen non-diseased subject. A diagnostic test with no false positives scores 1. *M. intracellulare* Area Under the Curve (AUC) = 0.301.

4.11.4 Assessment of the MAC Assay

As results from the assay targeting *M. avium* and *M. intracellulare* showed relatively high numbers of false positives, it was postulated that the terms *M. avium* and *M. intracellulare* may have been used interchangeably and that samples growing *M. intracellulare* may have been reported as *M. avium* and vice versa. Consequently, the results for the *M. avium* and *M. intracellulare* probes were considered as one (MAC). This resulted in a marked improvement in sensitivity of 0.86 owing to relatively reduced false negative numbers (N = 25 for *M. avium*, 8 for *M. intracellulare* and 11 for MAC). The specificity of the combined assays was 0.62.

Construction of a ROC curve (Figure 4.22) demonstrated an AUC of 0.741.

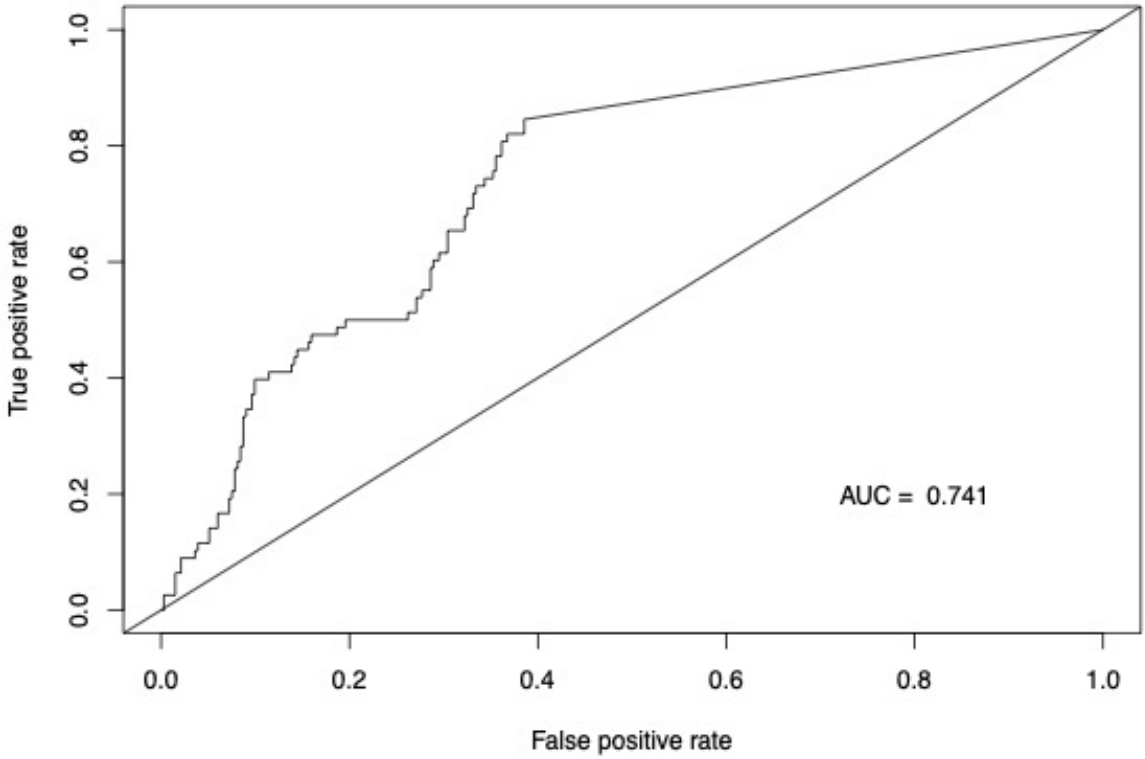


Figure 4. 22 – Receiver Operator Characteristic (ROC) Curve for the combined *M. avium* and *M. intracellulare* (MAC) assay. The ROC curve illustrates the diagnostic ability of a test by plotting the true positive rate against the false positive rate. The Area Under the Curve (AUC) can be interpreted as the probability that a randomly chosen diseased subject is rated as more likely to be diseased than a randomly chosen non-diseased subject. A diagnostic test with no false positives scores 1. MAC Area Under the Curve (AUC) = 0.741.

4.12 Discussion

This chapter has described the application of a custom qPCR assay (outlined in Chapter 3) to a total of 410 samples from: patients known to isolate either *M. abscessus* or MAC and on treatment, patients isolating those bacteria but not on treatment and finally patients who were NTM culture negative.

In Chapter 3 Section 3.5, the assay was shown to be successful at accurately determining DNA copy number within a mock community. Within this chapter (Sections 4.5 - 4.9), the assay's ability to detect NTM DNA copy number in samples derived from human sputum has been demonstrated. The dynamic nature of changes in target DNA have been successfully mapped to each patient's clinical picture so that a successful treatment regime has been shown to reduce target DNA to undetectable

levels. Conversely, lapses in treatment compliance has been shown to lead to a resurgence in NTM copy number.

4.12.1 Consideration of Assay Characteristics

The assay used to detect *M. abscessus* performed exceptionally well achieving an AUC value of 0.923. The test suffered a small number of false positives (N = 16) that accounted for a slightly diminished effectiveness of the assay. In particular, the assays used for the identification of *M. avium* and *M. intracellulare* performed less favorably (AUCs of 0.673 and 0.301 respectively). This was accounted for by the high false positive rates observed for each assay. As discussed in Chapter 4, Section 4.10.4 above, it was felt that a confused nomenclature involving the *M. avium* complex may account for the number of false positives and therefore the two assays were considered together. By doing this the AUC was improved to 0.741 implying that confused naming of culture results may, in part, account for the lower AUC values that were seen.

It is generally acknowledged that a test with an AUC of above 0.9 offers excellent discriminatory value whilst values of 0.5 or lower offer no discriminatory value [204-206]. By this metric, the assays for the detection of *M. abscessus* and those for the detection of MAC offer an excellent and a fair discriminatory value respectively. Possible reasons for false positives (and therefore lower AUC values) as well as reasons for false negatives are discussed below.

4.12.1.1 False Positives

The qPCR assays targeting *M. avium* and *M. intracellulare* had a high number of false positives. This accounted for a lower specificity and a lower positive predictive value than expected. There are two possible explanations for the number of false positives. Firstly, that the assay is non-specific and produced an inappropriate amplicon and secondly that traditional culture techniques have a relatively high failure rate and therefore a high false negative rate of its own. Assay specificity was carefully reviewed in Chapter 3 Section 3.3, where *in silico* testing first showed the custom primers were unlikely to produce off target amplification.

Next the entire assay was tested *in vitro* against plasmids containing inserts from different NTM species (Chapter 3 Section 3.3.2.2). These inserts did, however, all generate an amplicon courtesy of the custom primers but each assay was able to correctly identify its target plasmid owing to the specificity of the probe. It could be said therefore that this assay has two levels of discrimination owing to the high level of specificity of both the primers as well as to the probes. Finally, to test each assays' ability to quantify NTM copy number, each assay was tested against a mock community containing common respiratory pathogens as well as human DNA. The assay performed well and was able to accurately determine a predetermined amount of target DNA in the context of the mock community. This would imply that no off-target amplification occurred *in vitro*.

The possibility that culture techniques have a high number of false negatives and therefore may not be an ideal gold standard test to compare the assay to is supported by data in this experiment. As stated in Chapter 2, Section 2.1.4, samples at time points 1 and 2 originated from the same sputum sample and therefore it is assumed that both samples would culture the same NTM. This was the case for the most part but the sample from Patient 6 at time point 1 and Patient 9 at time point 1 failed to culture NTM. Samples at time point 2, from the same patients, cultured *M. abscessus* and *M. xenopi* respectively.

Other studies have found mixed success when comparing PCR assays to NTM culture. Writing in 1994, Beige *et al*. compared PCR techniques to mycobacterial culture for the detection of *Mycobacteria tuberculosis.* The PCR assay detected 48 out of 49 culture positive samples but identified DNA from *M. tuberculosis* in 16 out of 54 culture negative samples [176]. More recently, studies exploring the relationship between the lung microbiome and NTM using gene sequencing techniques have revealed a far wider ranging and more complex microbiome than culture alone can describe [158, 207]. It is therefore likely that when used in isolation mycobacterial culture misses the presence of NTM and that the "false positives" this present study has identified are actually true positives missed by culture analysis.

Anecdotal evidence from this present study exists that may support such an assertion and can be found amongst samples in the *M. abscessus* control group. Patients 37

and 41 both started as NTM culture negative despite regularly being positive prior to recruitment. Both patients subsequently became culture positive during the study. Molecular analysis revealed that samples from both patients were positive at the start of the study (despite being culture negative) and remained consistently positive. Within the *M. avium* complex treatment group, patients 1 and 3 were culture negative for MAC during their entire treatment (despite being positive prior to recruitment). Both patients tested PCR positive for either *M. avium or M. intracellulare* at regular time points until months 9 and 10 of treatment. These results were classified as false positives but, owing to the poor sensitivity associated with culture techniques used in isolation, may indeed be true positives.

If we accept the assertion that many of the qPCR assay false positive results are indeed true positives, this has implications particularly for the *M. abscessus* control and treatment groups. Within these groups 89 samples were qPCR assay positive for MAC but only 13 samples were both culture and qPCR assay positive. It is possible therefore that there is far more MAC (and possibly other mycobacteria) in circulation than previously estimated and this may in turn have implications in the choice of target NTM when initiating treatment. Patients 2, 4 and 19 for example were all placed on treatment for *M. abscessus* but had also cultured MAC before and during the study period. Samples from these patients were also regularly qPCR assay positive for MAC raising the possibility of a mixed NTM infection or even infection caused singularly by MAC.

4.12.1.2 False Negatives

Each assay suffered a number of false negatives after testing against samples (i.e. samples were culture positive but assay negative). These false negatives undermine each assay's sensitivity and negative predictive value. There are three possible explanations to account for the false negatives.

The first broad explanation can be described as assay failure i.e. target DNA was present in the correct volume and concentration, but a problem existed with one or more of the assay constituents (forward and reverse primers, probe, master mix and PCR gradient water). This is an improbable solution as samples were run on plates

with a dilution series of plasmids that acted both as quantification standards but also as positive controls. These positive controls were subject to the same assay (as well as reaction conditions) as samples and no failed positive controls were noted.
The second relates to failure of the DNA being tested. Template DNA can be affected, for a number of reasons, prior to testing with qPCR. In the first instance, human error can account for either a poor total DNA yield or poor DNA quality during the extraction process. The reason why the presence of low DNA concentration would lead to a false negative is self-explanatory. An extracted sample containing impurities can lead to PCR inhibition and therefore on occasion, although the template may contain DNA, the qPCR process returns negative results [208-210]. In the second instance, template DNA may become disrupted during handling. Repeated freeze-thaw cycles as well as prolonged exposure to UV light have both been shown to have a damaging effect on DNA [211-214].

The third and final possibility, and perhaps the most likely, is that an inappropriate DNA concentration was used during testing. Prior to running all samples, a pilot study was performed whereby different sample concentrations were used against the qPCR standards (Chapter 4, Section 4.3.2). A select group of samples were run without dilution as well as in a 1 in 10, 1 in 50 and 1 in 100 dilutions (samples were diluted with PCR grade water). This was conducted to establish optimal sample concentration for the assay, as concentrated samples may lead to PCR inhibition either by high levels of PCR inhibitors or by binding of magnesium (necessary for polymerase function) to template DNA. Conversely low template concentration may surpass the limit of assay detection [201, 202]. After the pilot experiment, a dilution factor of 1:50 was decided upon and applied to all samples prior to analysis. This may however have resulted in the over dilution of some samples or conversely the sample may still not have been sufficiently diluted.

4.12.2 Consideration of the Assay as a Tool for Quantification

Assessment of the assay's ability to detect and accurately quantify NTM copy number was investigated in Chapter 3, Section 3.7.2. In this chapter longitudinal changes in NTM copy number have been considered.

NTM copy number was first analysed after sputum samples were exposed to a 24-hour delay in the freezing process. This was done to control for later samples incurring a 24-hour delay whilst they were sent to the lab prior to being placed in the freezer. The results showed no significant changes in NTM copy number between time points indicating that this delay does not affect NTM burden.

Cuthbertson *et al.* have assessed previously the impact of delayed freezing of human sputum samples, from patients with cystic fibrosis, on culture-independent microbiological analyses. They found that the percentage abundance of anaerobes significantly decreased after 12 h at room temperature [215]. NTM are aerobic bacteria but have notoriously slow growth rates setting them apart from other aerobic bacteria. This may account for the different findings between the present study and the study of Cuthersbertson *et al*.

The finding that NTM copy number does not change significantly over 24 hours is important as it would allow patients to send regular samples from home for qPCR analysis without fear of any detrimental impact on the results and thereby patient treatment management. This is especially important in the 'COVID era' where hospital visits should be limited.

Following this, Chapter 4 Section 4.8 considered the effect of treatment directed at *M. abscessus.* Aside from Patients 9 and 33, a commonality shared by each patient within the study was a high NTM copy number at the pre-treatment time points, followed by a rapid decrease (often to undetectable levels) during the initiation phase of anti-microbial therapy. Indeed analysis by Kruskal – Wallis one-way analysis of variance found changes in *M abscessus* copy number during treatment to be highly significant (chi-squared = 82.173, df = 18, *P*-value = 3.561e-10) (section 4.9). This was in contrast to *M abscessus* copy number in the non treatment group (section 4.7).

A further point of interest was observed in patients who were non-compliant with antibiotic therapy, often because of side effects. Even when *M. abscessus* copy number was unrecordably low for a period of months, an interruption of one or more antibiotics for a week or longer resulted in *M. abscessus* being recognisable by qPCR. This is illustrated in Patient 18 who, after 8 months of antibiotic therapy, ceased

nebulised Amikacin therapy due to shortness of breath. After the drug was held for 2 weeks, the patient became culture and qPCR positive for *M. abscessus.* Similarly, Patients 23 and 35 both showed recurrent isolates of *M. abscessus* during treatment. There was 100% concordance between culture and molecular analysis in these patients. Both Patients 23 and 35 reported varied treatment compliance, Patient 23 to Ciprofloxacin and Patient 35 to Amikacin, Minocycline and Ciprofloxacin (all nebulized) at various stages. In both patients, however, there was an initial drop in DNA copy number during the initiation phase and it was only during the continuation phase when the patients reported poor treatment compliance that copy number became highly variable. As mycobacterial DNA copy numbers became resurgent at the point where either Amikacin or Ciprofloxacin were temporarily held, it could be postulated that treatment with bacteriostatic antibiotics (such as tetracyclines or macrolides) in combination with only one bactericidal antibiotic (such as Ciprofloxacin and Amikacin) results in NTM resurgence.

During treatment, it appears that *Mycobacterium abscessus* DNA copy numbers fall rapidly to levels below this assay's ability to detect the bacteria (theoretical limit of detection calculated previously 10 copies/µl [Chapter 3, Section 3.5.3.2]). The presence of the bacteria remains at low levels for months but if treatment is stopped prematurely either by the clinician or, more likely, because of treatment side effects then copy number again increases to become recordable. This phenomenon is well documented in the treatment of mycobacterial disease owing to slow replication rates. The recalcitrance of mycobacteria to eradication is thought to result from its achieving a non-replicating (dormant) state in the host. As virtually all classes of antibiotics require bacterial replication for their action, the non-replicating state is thought to render mycobacteria phenotypically resistant to otherwise bactericidal antibiotics [216-219]. Premature cessation of antibiotic therapy therefore allows these previously 'dormant' bacteria to become detectable in a relatively short time period.

In Section 4.10 above, the effect of treatment directed at the *M. avium* complex was considered. Treatment regimens differ significantly between *M. abscessus* and *M. avium* complex not just in terms of differing antibiotics but in that there is no initiation phase of treatment and consequently there is no initial rapid decline seen in *M. avium* complex copy number. A typical decline seen in MAC copy number can be seen in

Patients 28, 34 and 38 (Figures 4.16, 4.17, 4.18) all of whom experienced an overall decline over several months (unless patients suffered from antibiotic side effects). The fall in MAC copy number did not reach significance (Kruskal-Wallis chi-squared = 23.541, df = 18, *P*-value = 0.15) in contrast to patients on treatment for *M. abscessus*.

A concerning observation was noted in Patient 7. Patient 7 became culture negative after 1 week of anti MAC treatment but was qPCR positive up until 10 months of treatment (time point 15) (Figure 4.17). In these circumstances it is likely that the patient's medication will have been stopped prematurely and therefore may have resulted in an early relapse of disease. A similar situation was noted in Patient 1, who was never found to be culture positive for MAC during the study period but was intermittently qPCR positive up until month 10 (time point 15) of treatment (Figures 4.16 and 4.17).

4.12.3 Future Improvements

The largest cause for concern in this study was the small but significant number of false negatives. This was partially addressed in the *M. avium* and *M. intracellulare* groups by combining the results of both assays after it was noted that a variable nomenclature had been adopted when reporting the culture results. Even after accounting for this, the *M. abscessus* assay suffered 8 false negatives and the MAC assay 11. Possible reasons for this have been discussed earlier in Chapter 4, Section 4.12.1.2 but these could be remedied in two possible ways.

In the first instance, the cell lysis methodology discussed in Chapter 2, Section 2.2 could be adapted so that a non-mechanical cell lysis step is introduced. An osmotic shock lysis step involves a sudden change in osmotic gradient surrounding a cell creating a concentration difference between the inside and outside of the cell. The cell membrane becomes permeable to water due to osmosis. If the concentration of salt is lower in the surrounding solution, water enters the cell and the cell swells up and subsequently bursts. This technique is suitable for mammalian cell lysis due to the fragile structure of the membrane [220]. Mycobacteria species are however largely resistant to this form of lysis and mechanical breakage is typically used for the extraction of nucleic acids and protein [221]. By inducing eukaryotic cell lysis prior to

using the protocol described in Chapter 2, Section 2.2, human cell material can largely be removed from the sample. This has the effect of removing unwanted DNA that may otherwise have caused PCR inhibition. Consequently, samples are likely to behave in a more uniform manner.

The second recommendation is that when samples are tested in a clinical setting, each sample should be tested in multiple dilutions. This is so that samples cannot be under diluted (so that PCR inhibitors are carried over and excess DNA inhibits PCR) nor that they can be over diluted as to be undetectable.

Future studies applying this extraction method and performing sample quantification with serial dilutions are needed to fully validate this methodology and prove that it provides a low false negative rate.

4.12.4 Potential Use in Clinical Practice

The treatment success rate for NTM lung disease is unsatisfactory. Macrolide-containing regimens have been found to successfully eradicate MAC lung disease in only 60 to 80% of patients, with 20 to 40% failing to respond to treatment [222-224]. In addition, a significant proportion of successfully treated patients experience disease recurrence [225]. Premature cessation of treatment is believed to be linked to relapse of disease.

This study revealed a number of samples that were culture negative but PCR positive, indicating that due to the failure of NTM culture, patient treatment has the potential to be stopped prematurely. Although the assays described require some degree of modification before the realisation of no false negatives, they are clearly able to detect NTM in samples that conventional culture techniques miss. The use of the assay in clinical practice may therefore stop premature cessation of treatment leading to lower relapse rates.

The assays have also demonstrated an ability to detect mixed samples where multiple NTM species exist. These mixed growth samples are often missed due to competition of NTM colony forming units to acquire growth substrates and it is believed that slower

growing NTM may miss out to the faster growers under such conditions [226-229]. The relevance of mixed samples is not clear, but it could be postulated that mixed NTM infections exist and that in this scenario isolation of only one NTM species by traditional culture techniques may result in an inadequate antibiotic regimen.

Using this molecular approach additionally has the benefit of testing direct from sputum and thus does not incur the delay that techniques, dependent on mycobacterial culture, do. This means that sample assessment can be completed in approximately 48 hours rather than the weeks culture-based techniques can take. The result of this is that the diagnosis of NTM infection can be made faster, clinicians can see in real time that treatment regimens are working and, if drug intolerance is observed, clinicians can quickly substantiate whether this has resulted in NTM copy number resurgence and therefore can intervene in a timely manner.

The assays have shown the ability to quantify NTM copy number *in vivo*. Although it is difficult to substantiate such claims (as no existing quantification method exists for comparison) the assays previously proved their accuracy *in vitro* (Chapter 3 Section 3.8.3).

It is therefore foreseeable that after some modification, the assays could be used in clinical practice alongside conventional culture techniques for the identification and quantification of NTM. Culture still has a vital role in that it allows antibiotic sensitivities to be determined as well as providing a relatively cheap way of screening for NTM disease. After the decision to commence treatment is made, quantitative PCR may then find its niche due to its sensitivity, ability to detect multiple organisms (and therefore identify mixed infections) as well as its ability to monitor response to mycobacterial treatment.

4.13 Conclusion

In conclusion, the work conducted in this chapter has shown:

- A 24-hour delay in sputum processing does not affect NTM bacterial burden and therefore samples sent via postal service are not subject to bias.

- The custom qPCR assay for *M abscessus* displayed excellent test characteristics (sensitivity = 0.87, specificity = 0.95, positive predictive value = 0.76, negative predictive value = 0.98) resulting in an AUC of 0.923.
- The custom qPCR assay for MAC displayed mixed test characteristics (sensitivity = 0.86, specificity = 0.62, positive predictive value = 0.35, negative predictive value = 0.95) resulting in an AUC of 0.741.
- It is likely that these poorer test characteristics are at least in part due to the use of a poor gold standard, in the form of culture-based techniques, leading to a higher number of false positive results.
- Because of this, it is likely that NTM is under represented in the lung and the qPCR assays developed here may provide a truer representation of the NTM microbiome.
- The assays can be used to rapidly (within 48 hours) quantify NTM species and this in turn gives information pertaining to antibiotic response and culture conversion.
- In future, the assays could be used on sputum from patients who have recently isolated NTM for the first time or those being observed to see if they meet ATS criteria for treatment. The assays could then be used longitudinally to see if NTM burden can be used to predict need for treatment.

Chapter 5: The Lung Microbiome and Non-Tuberculous Mycobacteria (NTM)

5.1 Introduction

It has been proposed that microbiome composition is determined by the interplay of three constantly changing factors: microbial immigration from the pharynx, elimination of microbes from the airways by the host defence, and reproduction rates of individual members of the microbiota as determined by host growth conditions (i.e. presence or absence of disease) [154]. In the diseased lung, growth conditions change dramatically, creating favourable conditions for the reproduction of selective bacteria [230]. Studies have identified that the diseased lung is associated with an alteration in community richness (number of species) with a shift in community composition away from the Bacteroidetes phylum (a phylum that dominates the healthy lung microbiome) towards Proteobacteria [231, 232]. Differences in lung microbiota have been associated with important clinical features of chronic lung disease including: frequency of infections in bronchiectasis [233], mortality in idiopathic pulmonary fibrosis [232], and responsiveness to corticosteroids and antibiotics in asthma [234, 235]. A comprehensive understanding of the changes that occur in the airway microbiome may in future allow clinicians to tailor treatment regimes, predict treatment response and to understand factors contributing to mortality.

5.1.1 Aims and Hypothesis

The aim of this chapter therefore is to perform 16S rRNA gene sequencing of DNA extracted from sputum samples in order to characterise differences in the bacterial lung microbiome between patients: requiring treatment for NTM; those that culture NTM but do not require treatment and NTM naive individuals. Furthermore, the evolving microbiome of all patient groups will be characterised over a 3-month period. Quantitative PCR of the 16S rRNA gene will be used to quantify bacterial burden in all three patient groups.

The hypotheses addressed in this chapter are:

1. The pulmonary microbiome in cystic fibrosis will differ from that seen in bronchiectasis.

2. The pulmonary microbiome will differ between individuals: requiring treatment for NTM, those that culture NTM but do not require treatment, and those who are NTM culture naïve.
3. Treatment of pulmonary NTM alters the lung microbiome.

5.2 Methods

5.2.1 Sample Collection and Selection

Patients were recruited to the study as detailed in Chapter 2, Section 2.1.2 and provided sputum samples longitudinally (Chapter 2, Section 2.1.3). Upon receipt, sputum samples were processed (Chapter 2, Section 2.1.4) before DNA was extracted (Chapter 2, Section 2.2). Patient clinical data (including corresponding sample culture results were recorded as per Chapter 2, Section 2.1.6.

To ensure no bias and representation of all subgroups, baseline samples were selected for 16S rRNA sequencing analysis (illustrated in Table 5.1). The spectrum of samples represented: underlying disease (cystic fibrosis or non-cystic fibrosis bronchiectasis) by microbiological subtype (*M. abscessus, M. avium* or control) and whether patients were receiving treatment. Samples were analysed to account for changes over a 3-month period. The number of patients by group as well as the total number of samples analysed is detailed in Table 5.1.

In addition to the 64 samples used, a further 2 samples underwent sequencing. These were a mock community and a negative control.

Table 5.1 – Number of samples (and patients) analysed by microbiological subtype and disease. Treatment groups consist of patients receiving antibiotic therapy for the Mycobacterium illustrated in the table. Control groups consist of patients who are not on antibiotic therapy.

	TREATMENT		CONTROL		
	abscessus	*avium*	*abscessus*	*avium*	Control
Cystic Fibrosis	28	0	8	4	4
Bronchiectasis	4	12	4	0	0
Total Samples	**32**	**12**	**12**	**4**	**4**
Total Patients	**8**	**3**	**3**	**1**	**1**

5.2.2 16S Quantitative PCR

Quantification of bacterial burden was performed by targeting the 16S rRNA gene with the 520F and 802R primers as described by Claesson *et al.* [159] (see Chapter 2, Section 2.4 and Appendix Table A1). Samples were analysed randomly and in triplicate.

5.2.3 16S rRNA Gene Sequencing including Primer Design

Sequencing was performed using the Illumina MiSeq platform (see Chapter 2, Section 2.5.10). FASTQ files generated by the MiSeq platform were imported to a working directory and processed with QIIME 1.9.1. Raw sequence data underwent quality control and processing to generate a final operational taxonomic unit (OTU) table as well as a maximum likelihood phylogenetic tree (see Chapter 2, Section 2.6). These files were imported to the R Project for Statistical Computing software for analysis (Chapter 2, Section 2.7).

5.2.4 Data Analysis

Data was analysed as per Chapter 2, Section 2.7. The phyloseq package (version 1.30.0) was used to construct estimates in alpha diversity (Simpson, Shannon, total richness and Pielou's evenness index). Either Student's t-Test or Wilcoxon rank-sum test was used to look for significance between two groups of data. Kruskal - Wallis test was used to look for significance between more than two groups of data. Kendall rank correlation was used to compare continuous variables for longitudinal data (e.g. changes in diversity over time). A P-value of < 0.05 was considered significant.

Beta diversity was measured using Bray-Curtis dissimilarity index. Non-metric multi-dimensional scaling (NMDS) plots were used to visualize trends in beta-diversity. Statistical comparisons of beta diversity between samples were performed using the adonis function (PERMANOA) in the vegan package.

Similarity of Percentages (SIMPER) analysis was used to determine the contribution of each OTU to the observed Bray-Curtis dissimilarity between sample variables. The Kruskal - Wallis test was then utilised to determine the significance between relative abundances of each OTU across variables. Simper analysis was conducted using the simper function in the vegan package.

5.3 Study Cohort

Demographics of the 16 patients are detailed below in Table 5.2. Numbers relating to NTM culture conversion for patients receiving treatment are also detailed in the bottom section of the table.

Table 5.2 – Patient demographics according to microbiological subtype. Culture conversion is defined as 3 consecutive culture negative sputum samples over a period of 3 months as per BTS guidelines [2].

		TREATMENT		CONTROL			*P*-value
		abscessus	*avium*	*abscessus*	avium	Control	
Gender	Male	3	2	2	1	0	0.9
	Female	5	1	1	0	1	
	Mean Age at recruitment (yrs)	32.1	55.7	46.7	18	30	0.7
	Standard Deviation Age at recruitment	9.4	7.6	22	0	0	
Lung Function	Mean FEV1 (%predicted) at recruitment	82.9	54.8	46.4	93	67.1	0.4
	Standard Deviation FEV1 at recruitment	19	44.3	13.9	0	0	
	Mean FVC (%predicted) at recruitment	104.5	91.2	65.8	105	92.4	0.3
	Standard Deviation FVC at recruitment	11.2	40.3	9.3	0	0	
Body Mass Index		20.68	21.46	22.26	21.13	22.11	0.4
Bronchiectasis (Bx) pattern of disease	Cylindrical	1	2	0	0	0	0.01
	Varicose	0	1	1	0	0	
	Cystic	0	0	0	0	0	
Cystic Fibrosis (CF) gene status	ΔF508 mutation homozygous	5	0	1	1	0	
	ΔF508 mutation heterozygous	2	0	1	0	1	
	other	0	0	0	0	0	
Radiological Pattern of Disease	Cavitation	3	0	NA	NA	NA	0.2
	Nodular	5	3	NA	NA	NA	
Microbiological Evolution	Culture conversion at 1 year	5	3	NA	NA	NA	
	No culture conversion at 1 year	3	0	NA	NA	NA	

5.4 Results

5.4.1 Sample NTM Culture Results

In this study only 1 of the 3 patients included in the *M. avium* treatment group cultured *M. avium* within the study timescale and this was before the initiation of any treatment. All patients initiated on treatment for *M. avium* became and remained culture negative after 2 weeks of anti-microbial therapy (Table 5.3).

Over the study period all patients on treatment for *M. abscessus* became NTM culture negative at 3 months. This did not however remain the case with three patients who became culture positive beyond 3 months (Patients 2, 18 and 35) (Table 5.3).

Patient 15 was included on the basis that he or she cultured *M. avium* but was not on treatment (avium control group). The patient isolated *M. avium* before the initiation of treatment but remained NTM negative until month 3 when *M. abscessus* was isolated.

Patients 13, 37 and 41 were included as they regularly isolated *M. abscessus* but were not on treatment during the study period (abscessus control). Each patient isolated *M. abscessus* at least once during the 3-month period. Patient 15 isolated *M. avium* but was not on treatment (avium control). This patient isolated both *M. avium* and *M. abscessus* over the 3-month period. Patient 46 had never isolated any NTM species prior to recruitment and this remained the case during the study (control).

Table 5.3 – Longitudinal assessment of culture results by patient. Columns left to right show Patient ID; Disease; Description=microbiological subgroup and whether patient is on treatment or not; Antibiotic Therapy=combination of antibiotics used in treatment, where two sets of antibiotics are shown for *M. abscessus* treatment the first group are the initiation phase followed by the continuation phase (Rif=Rifampicin, Eth=Ethambutol, AZT=Azithromycin, Amik=Amikacin, Mero=Meropenem, Tig=Tigecycline, Doxy=Doxycyline, Cipro=Ciprofloxacin, Clari=Clarithromycin, Mino=Minocycline, Clof=Clofazamine); orange box=NTM culture positive, blue box=NTM culture negative. White box indicates no sample collected at this time point.

Patient ID	Disease	Description	Antibiotic Therapy	Week 0 (tp1)	Week 2 (tp2)	Week 4 (tp3)	Month 3 (tp4)	Month 6	Month 12	Month 12+
1	Bronchiectasis	*Avium* Treatment	Rif, Eth, AZT	*XENOPI*	*XENOPI*					
2	Cystic Fibrosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Tig AZT, Amik(neb), Doxy, Cipro	*ABSCESSUS*					*ABSCESSUS*	*INTRA*
6	Bronchiectasis	*Abscessus* Treatment	Amik(IV), Mero(IV),Clari Mino, Clof, Amik(neb)	*ABSCESSUS*	*ABSCESSUS*	*ABSCESSUS*				
7	Bronchiectasis	*Avium* Treatment	Rif, Eth, AZT	*AVIUM*						
9	Cystic Fibrosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Tig Clari, Doxy, Cipro, Clof, Mero(neb)	*XENOPI*						
13	Bronchiectasis	*Abscessus* Control		*ABSCESSUS*	*ABSCESSUS*		*ABSCESSUS*			
14	Cystic Fibrosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Clari Clari, Doxy, Moxi, Tig, Amik(neb)	*ABSCESSUS*	*INTRA*	*INTRA*				
15	Cystic Fibrosis	Avium Control	Septrin prophylaxis	*AVIUM*			*ABSCESSUS*			
17	Bronchiectasis	Avium Treatment	Rif, Eth, AZT							
18	Cystic Fibrosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Tig Clari, Doxy, Mino, Septrin, Amik(neb)	*ABSCESSUS*	*ABSCESSUS*				*ABSCESSUS*	*ABSCESSUS*
22	Cystic Firosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Clari Mino, Cipro, Amik(neb)	*ABSCESSUS*	*ABSCESSUS*	*ABSCESSUS*				
27	Cystic Fibrosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Clari Mino, Cipro, Amik(neb)							
35	Cystic Firosis	*Abscessus* Treatment	Amik(IV), Mero(IV),Tig Mino, Cipro, Amik(neb), AZT	*ABSCESSUS*				*ABSCESSUS*	*AVIUM*	*ABSCESSUS*
37	Cystic Fibrosis	*Abscessus* Control				*ABSCESSUS*	*ABSCESSUS*			
41	Cystic Fibrosis	*Abscessus* Control			*ABSCESSUS*					
46	Cystic Fibrosis	Control								

5.4.2 Sequencing Quality

Sequencing was performed on a total of 64 samples from 16 patients. One mock community and 1 negative control sample were also included in the sequencing run. After combining the forward and reverse reads, de-multiplexing, decontamination and quality filtering a total of 4,645,956 reads were left with an average of 70,393.27 reads per sample (range = 275 – 180,243).

5.4.3 Sample Controls

The mock community used for the 16S analysis was comprised of 27 different bacterial genera. Bacterial strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). The constituents of the 16S mock community are detailed in Table 5.4. All 27 genera constituting the mock community were identified during sequencing in their expected proportions (Figure 5.1).

Table 5.4 - Breakdown of the mock community members. Individual type strains community with the DSMZ Strain numbers.

Full Name	Strain Number
Actinomyces odontolyticus	DSM-19120
Bifidobacterium dentium	DSM-20436
Burkholderia cepacia	DSM-7288
Chlamydophila pneumoniae	DSM-19748
Corynebacterium pseudodiphtheriticum	DSM-44287
Enterococcus faecalis	DSM-20478
Escherichia coli	DSM-30083
Fusobacterium nucelatum subsp. nucleatum	DSM-15643
Granuclicatella adiacens	DSM-9848
Haemophilus influenzae	DSM-4690
Haemophilus parainfluenzae	DSM-8978
Leptotrichia buccalis	DSM-1135
Moraxella catarrhalis	DSM-9143
Mycobacterium bovis	DSM-43990
Mycobacterium psychrotolerans	DSM-44697
Mycoplasma penumoniae	DSM-22911
Neisseria flavescens	DSM-17633
Neisseria meningitidis	DSM-10036
Nocardia farcinica	DSM-43665
Pasteurella multocida subsp. multocida	DSM-16031
Pseudomonas fluorescens	DSM-50090
Rothia mucilaginosa	DSM-20746
Sallmonella enterica subsp. enterica	DSM-17058
Staphylococcus aureus subsp. aureus	DSM-20231
Streptococcus agalactiae	DSM-2134
Streptococcus mitis	DSM-12643
Streptococcus pneumoniae	DSM-20566
Streptococcus pyogenes	DSM-20565
Streptococcus pseudopneumoniae	DSM-18670
Streptococcus constellatus subsp. constellatus	DSM-20575
Streptococcus infantis	DSM-12492
Streptococcus parasanguinus	DSM-6778
Streptococcus sanguinis	DSM-20567
Treponema denticola	DSM-14222
Veillonella dispar	DSM-20735
Vibrio natriegens	DSM-759

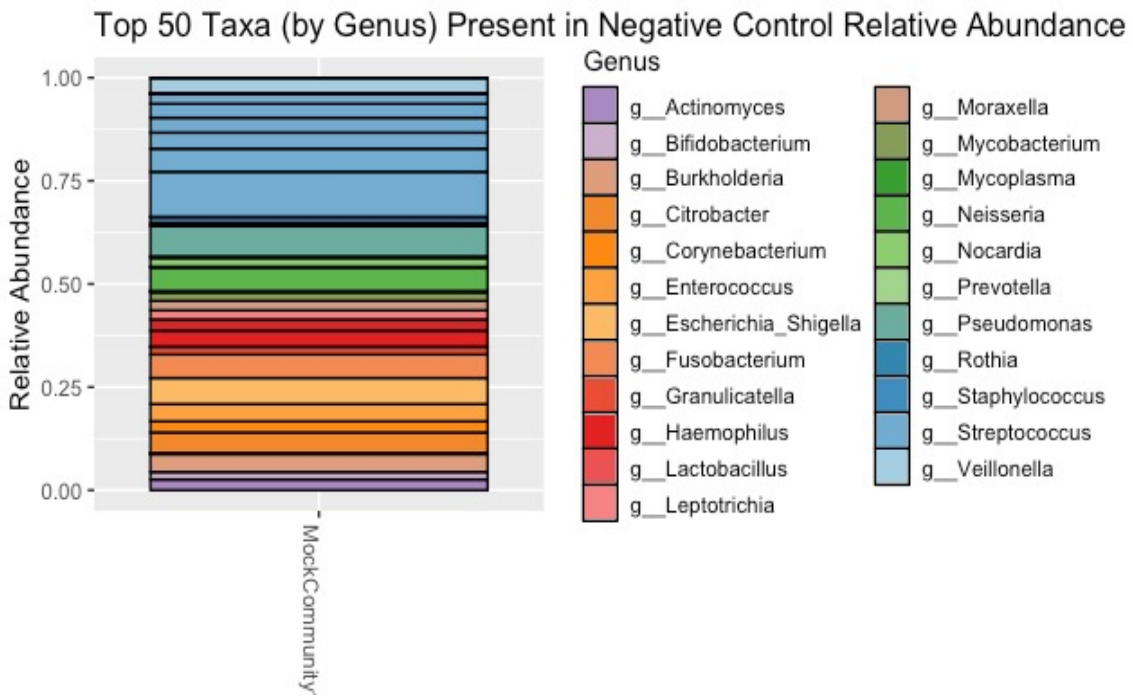


Figure 5.1 – Relative abundance of the mock community structure by genus for the 16SrRNA sequencing run. All expected genera were identified in expected abundances.

5.5 Decontamination

To identify OTUs associated with a specific extraction batch, indicator species analysis was performed using the 'indicspecies' R package. Analysis was performed using extraction batch number as a variable to assess whether any OTUs were significantly associated with one extraction batch which would be an indication of them being potential contaminants. No OTUs were found to be significantly associated with a single extraction batch (Figure 5.2).

After indicator species analysis, the decontam package in R was used to aid in identification of other potential contaminants. The package utilises two methods to identify OTUs from the phyloseq object. The first method is the "frequency" method. In this method, the distribution of the frequency of each sequence feature as a function of sample DNA concentration (sample DNA concentration obtained via qPCR analysis) is used to identify contaminants. The second method is the "prevalence" method. This method compares the prevalence of OTUs (presence/absence) across samples and compares the data to the prevalence in negative controls. After OTUs were identified as contaminants they were pruned from the phyloseq object.

After decontamination, OTUs with fewer than 10 reads were removed. OTUs of the phylum Cyanobacteria, order Rhodobacterales, Rhizobiales and Methylophilales, the family Oxalobacteraceae, the genus Derxia and Rhodococcus were also removed as these have been identified and previously reported as recognised common contaminants [236].

Samples were statistically interrogated by extraction batch number using a Bray-Curtis dissimilarity distance matrix. Significance was determined using the adonis function (PERMANOVA) No significant differences in community composition were seen between batch number indicating no significant biases or contamination between batches (R^2 = 0.28).

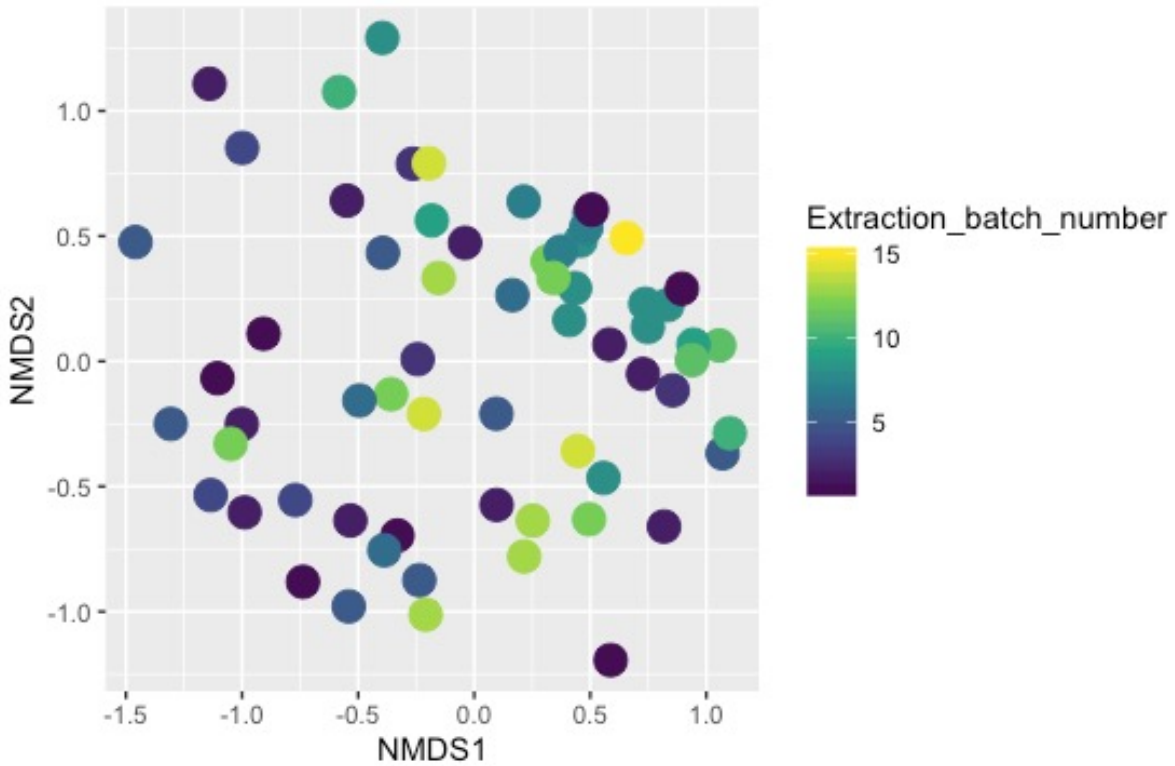


Figure 5.2 - Non-metric multidimensional scaling (NMDS) of all samples by extraction batch number. Samples were statistically interrogated using a Bray-Curtis dissimilarity distance matrix. No significant differences between batch number were observed indicating no significant contamination during DNA extraction.

After decontamination and removal of OTUs with fewer than 10 reads, a total of 1,390,004 reads remained comprising 1,011 different OTUs from 64 samples obtained from 16 patients. The sequencing depth varied greatly between samples however hence the next step was to perform rarefaction.

5.6 Rarefaction

In 1971 Hurlbert criticised traditional methods of ecological measurement [237] and suggested the use of rarefaction methods designed by Sanders [238]. Hurlbert suggested that if raw data were to be compared directly between samples, variations in sequencing depth could misrepresent sample diversity by conferring bias against samples with low sequence depth. To negate this bias, samples were standardised by rarefying to an even sequencing depth. Rarefaction performs random subsampling of a population without replacement. It is the most commonly reported method of standardisation but can result in sample drop out depending on the chosen rarefaction level.

In general terms, the higher the chosen rarefaction level the more OTUs are included in analysis but the greater the potential for bias and the greater the number of samples omitted. If a lower rarefaction level is selected more OTUs are omitted. The rarefaction level was therefore chosen by the generation of rarefaction curves (Figure 5.3) to determine the number of reads at which each sample curve begins to plateau. The majority of OTUs are captured prior to the plateau and further sampling is unlikely to capture additional OTUs. Any sample with a total number of reads below the rarefaction level is omitted.

After consideration, it was decided to rarefy data to a depth of 1,000 reads. This resulted in the loss of 2 samples and 4,910 OTUs.

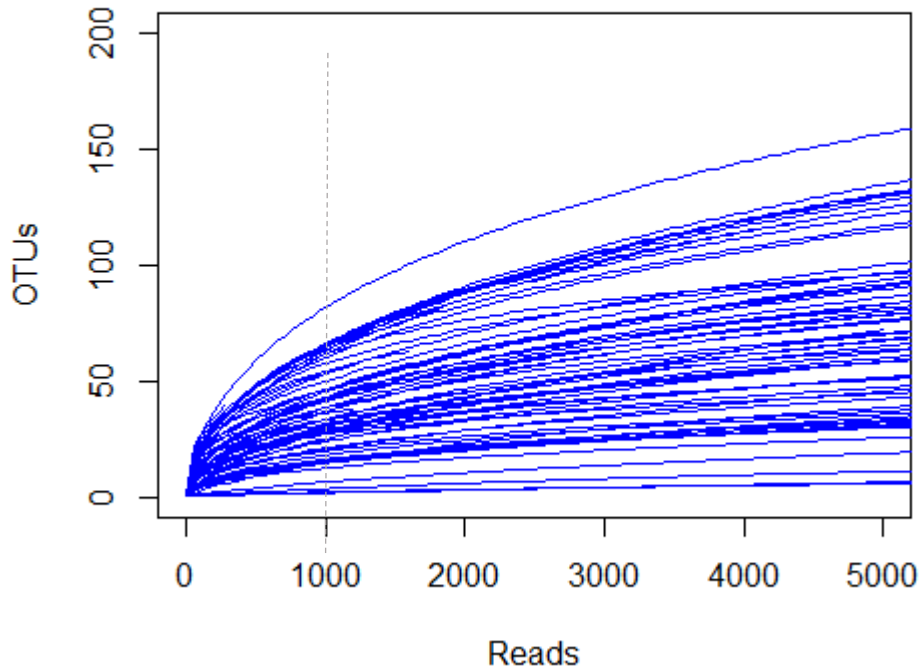


Figure 5.3 – Rarefaction curve for each sequenced sample. Each blue line represents a sequenced sample. The Y axis illustrates the number of different OTUs identified and the X axis the total number of reads assigned to those OTUs. The grey dotted line indicates the point at which samples were rarefied.

5.7 Exploring Differences between (non-cystic fibrosis induced) Bronchiectasis and Cystic Fibrosis

To assess for differences between underlying disease aetiology (i.e. bronchiectasis or cystic fibrosis), samples at time point 1 (pre-treatment) were compared based on

whether they were from patients with bronchiectasis (n = 5) or patients with cystic fibrosis (n = 11).

5.7.1 16S rRNA Quantitative PCR Copy Number

The mean 16S copy number per sample in bronchiectasis cases was 6.97 x 10^9 copies/g sputum (median = 3.4 x 10^9 copies/g sputum, standard deviation = 7.84 x 10^9 copies/g sputum). The mean 16S copy number per sample in cystic fibrosis cases was 1.02 x 10^{10} copies/g sputum (median = 5.14 x 10^9 copies/g sputum, standard deviation = 1.31 x 10^{10} copies/g sputum).

There was no significant difference in bacterial abundance between patients with bronchiectasis compared to cystic fibrosis (Student's t-Test *P*-value = 0.47) (Figure 5.4).

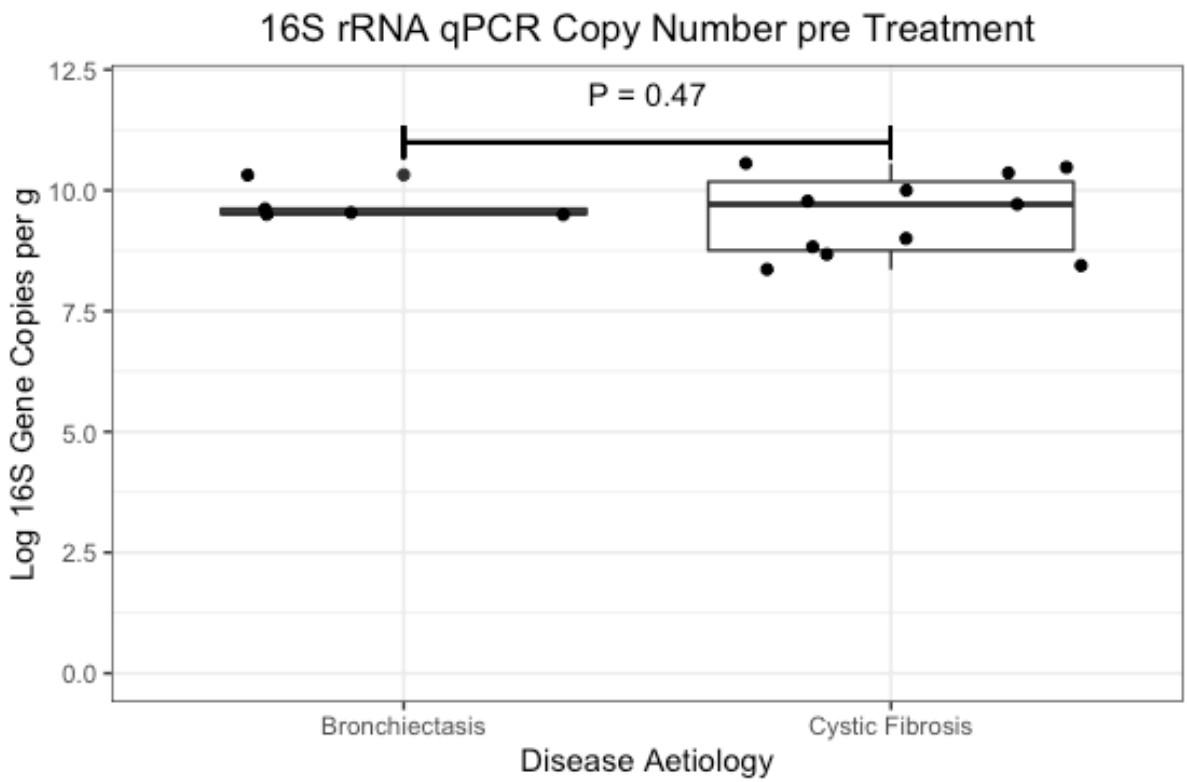


Figure 5.4 – Comparison of 16S *rRNA* gene copy number (as determined by qPCR) between non-cystic fibrosis bronchiectasis and cystic fibrosis induced bronchiectasis before treatment initiated. The data consists of samples from patients in both the treatment and non-treatment groups.

5.7.2 Differences in Alpha Diversity

There were no significant differences in measures of alpha diversity between samples taken from patients with bronchiectasis and those with cystic fibrosis before the initiation of treatment (time point 1) (Figure 5.5). All *P*-values obtained using Wilcoxon rank-sum test (Simpson's diversity; W = 34, *P*- value = 0.51. Shannon diversity; W = 35, *P*-value = 0.44. Richness; W = 35, *P*-value = 0.44. Pielou's evenness; W = 35, *P*-value = 0.44.).

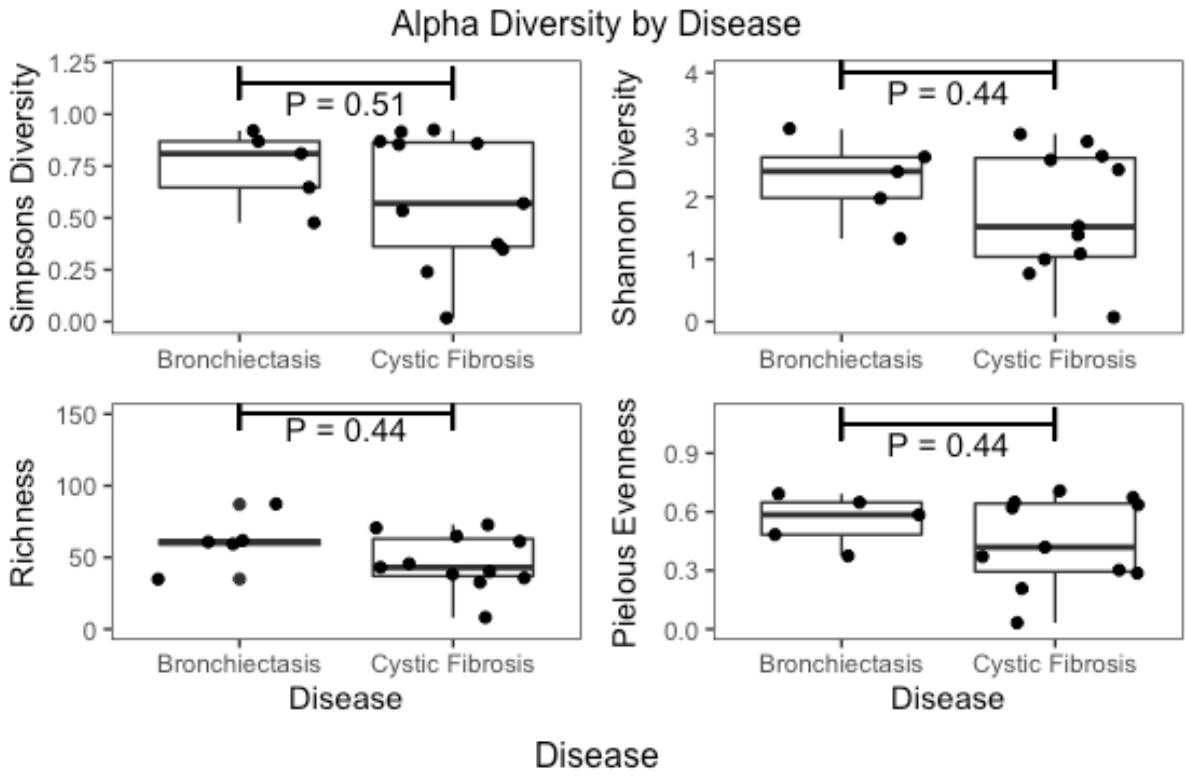


Figure 5.5 – Comparison of alpha diversity between non-cystic fibrosis bronchiectasis and cystic fibrosis induced bronchiectasis before treatment initiated. The data consists of samples from patients in both the treatment and non-treatment groups. The mean Simpson diversity index for bronchiectasis was 0.74 (range = 0.48 – 0.92) and 0.59 (range = 0.018 – 0.92) for patients with cystic fibrosis. The mean Shannon diversity index for bronchiectasis was 2.29 (range = 1.33 – 3.09) and 1.77 (range = 0.068 – 3.017) for patients with cystic fibrosis. The mean observed richness for bronchiectasis was 60.8 (range = 35 – 87) and 46.73 (range = 8 – 73) for patients with cystic fibrosis. The mean Pielou's evenness index for bronchiectasis was 0.56 (range = 0.37 – 0.69) and 0.45 (range = 0.033 – 0.71) for patients with cystic fibrosis.

5.7.3 Differences in Community Composition

Beta diversity was assessed using adonis PERMOANOVA using Bray-Curtis distance matrix. The mean Bray-Curtis dissimilarity between bronchiectasis samples at time point 1 was 0.72 and 0.73 for cystic fibrosis. There was no significant difference in beta diversity (R^2 = 0.06, *P*- value = 0.38).

5.7.4 Differences in Specific Bacterial Abundances

Before the commencement of any treatment the most common genera identified in patients with bronchiectasis were: *Streptococcus*, *Pseudomonas* and *Veillonella* (33%, 20% and 9.2% respectively). The most common genera in patients with cystic fibrosis were *Pseudomonas*, *Streptococcus* and *Staphylococcus* (39.4%, 11.1% and 9.1% respectively) (Table 5.5).

Table 5.5 – The 20 most common genera seen in samples from bronchiectasis patients and in samples from patients with cystic fibrosis samples. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

Bronchiectasis (n=5)				Cystic Fibrosis (n=11)			
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Genus**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Streptococcus	0.8	1648	33	*Pseudomonas*	0.8	4334	39.4
Pseudomonas	0.6	999	20	*Streptococcus*	1.2	1219	11.1
Veillonella	0.6	461	9.2	*Staphylococcus*	0.8	996	9.1
Haemophilus	0.7	267	5.3	*Veillonella*	1.2	573	5.2
Actinomyces	1.2	257	5.1	*Actinomyces*	1.5	384	3.5
Neisseria	0.7	245	4.9	*Granulicatella*	2.1	383	3.5
Granulicatella	0.8	163	3.3	*Mycobacterium*	1	315	2.9
Gemella	1.4	160	3.2	*Stenotrophomonas*	1.1	295	2.7
Leptotrichia	1.5	153	3.1	*Haemophilus*	0.8	290	2.6
Rothia	0.6	101	2	*Stomatobaculum*	1.4	287	2.6
Prevotella	0.5	94	1.9	*Neisseria*	1.2	256	2.3
Fusobacterium	1	82	1.6	*Prevotella*	0.9	232	2.1
Dolosigranulum	0.8	74	1.5	*Rothia*	1.3	167	1.5
Capnocytophaga	0.7	45	0.9	*Fusobacterium*	1.8	143	1.3
Selenomonas	0.8	43	0.9	*Gemella*	1.4	132	1.2
Atopobium	1	29	0.6	*Bifidobacterium*	0.7	113	1
Parvimonas	2	21	0.4	*Solobacterium*	5	112	1
Corynebacterium	0.6	14	0.3	*Enterococcus*	1.8	91	0.8
Stomatobaculum	0.7	14	0.3	*Atopobium*	1.3	72	0.7
Solobacterium	4	12	0.2	*Leptotrichia*	0.8	70	0.6

Similarity percentage (simper) analysis was conducted using bronchiectasis and cystic fibrosis as variable groups. The simper function performs pairwise comparisons of groups of sampling units and finds the contribution of each OTU to the overall between group dissimilarity (dissimilarity measured using Bray Curtis distance matrix). To test

significance, the relative abundance of each OTU across the two groups was compared using the Kruskal-Wallis rank sum test.

OTUs assigned to the *Pseudomonas, Streptococcus* and *Staphylococcus* generas contributed the most to average dissimilarity between bronchiectasis and cystic fibrosis groups. *Staphylococcus_3526* and *Gemella_152* OTUs showed the greatest differences between groups although these differences were not significant (*P*-value = 0.1) (Table 5.6).

Table 5.6 – Summary of simper analysis between: bronchiectasis and cystic fibrosis groups. Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity, all OTUs together add up to 100 (shown here are OTUs contributing to 77.63% of total dissimilarity); *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between groups.

OTU identity	Species Contribution to Average Dissimilarity	Average Abundance Bronchiectasis	Average Abundance CF	% Contribution to Dissimilarity	% cumulative contribution	*P*-value
Pseudomonas_2114	0.19	194.8	393.18	26.6	26.58	0.21
Streptococcus_574	0.1	223.6	71.91	13.58	40.16	0.15
Staphylococcus_3526	0.04	0.4	89	6.17	46.33	0.1
Veillonella_3368	0.04	82.8	39.64	5.16	51.49	0.11
Streptococcus_487	0.03	78.4	29.82	4.8	56.29	0.23
Haemophilus_1826	0.02	44.6	14.91	3.37	59.66	0.29
Neisseria_1599	0.02	41.6	19.55	3.11	62.77	0.6
Actinomyces_778	0.02	32.4	29.36	2.46	65.23	0.78
Granulicatella_3662	0.02	30	30.45	2.17	67.4	0.86
Mycobacterium_949	0.01	0	28.27	1.96	69.36	0.32
Gemella_152	0.01	30.8	11.64	1.96	71.32	0.1
Stenotrophomonas_2039	0.01	0	26.45	1.84	73.16	0.14
Stomatobaculum_3032	0.01	1.8	22.55	1.62	74.78	0.49
Prevotella_2474	0.01	16.8	16.82	1.58	76.36	0.73
Fusobacterium_2663	0.01	16.4	13	1.27	77.63	0.3

For non-cystic fibrosis induced bronchiectasis the most common specific OTUs were: *Streptococcus* spp., *Pseudomonas* spp. and *Veillonella* spp. (22.4%, 19.5% and 8.3% respectively). These three OTUs were present in 100% of samples in the bronchiectasis group. A total of 10 different OTUs were identified in the baseline

bronchiectasis group that were not found in the baseline cystic fibrosis group. The genera that these OTUs belong to were however found in the cystic fibrosis group. The most common OTUs in the cystic fibrosis group were: *Pseudomonas* spp., *Staphylococcus* spp. and *Streptococcus* spp. (39.3%, 8.9% and 7.2% respectively). These OTUs were present in 90.1%, 60.4% and 60.4% of samples respectively. A total of 8 different OTUs identified in the baseline cystic fibrosis group were not present in the baseline bronchiectasis group. Two genera were found in the cystic fibrosis group but not in the bronchiectasis group. These were: *Mycobacterium* spp. and *Stenotrophomonas* spp. (Figure 5.6)

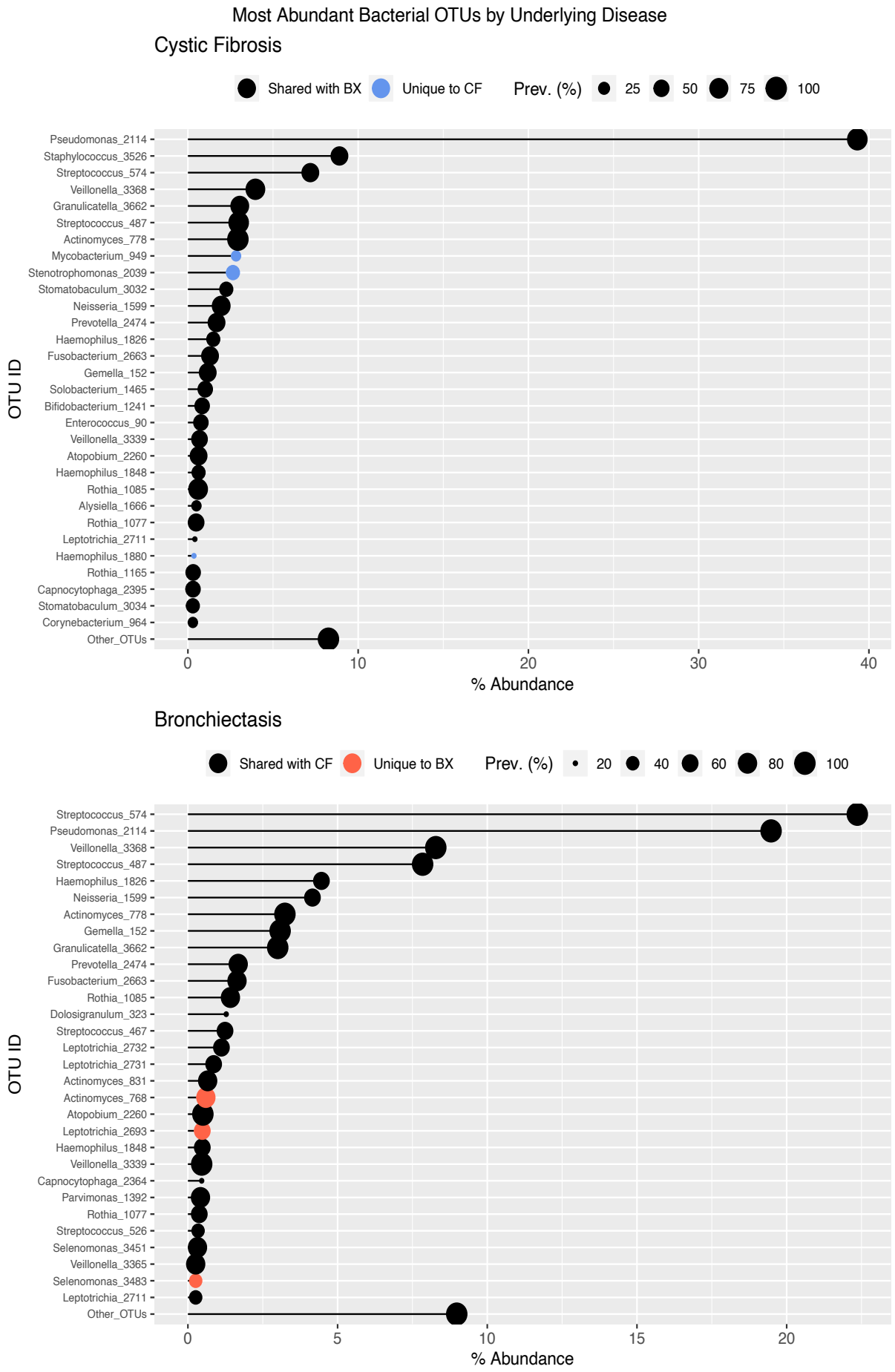


Figure 5.6 – The 50 most abundant bacterial OTUs in the baseline bronchiectasis group. X axis = percentage total abundance across all samples. Y axis = operational taxonomic unit identity (OTU ID). Marker size is indicative of OTU prevalence across the samples. An orange marker colour signifies that the OTU is unique to bronchiectasis.

5.8 Exploring Differences in Patients Requiring Treatment

In order to explore differences between patients who required treatment for NTM (abscessus treatment [abstx, n=8] and avium treatment [avtx, n=3]) and those who cultured NTM but did not require treatment [abscessus control [absctrl, n=3]) and avium control [avctrl, n=1]), pretreatment samples (time point 1) from each group were compared to those from NTM culture naïve patients (control [ctrl, n=1]).

5.8.1 16S rRNA Quantitative PCR Copy Number

Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant differences between all groups (chi-squared = 2.5385, df = 2, *P*-value = 0.28). A significant difference between the abscessus treatment group and the control group was found (*P*-value = 0.05) although only one sample from the control group was analysed (Figure 5.7).

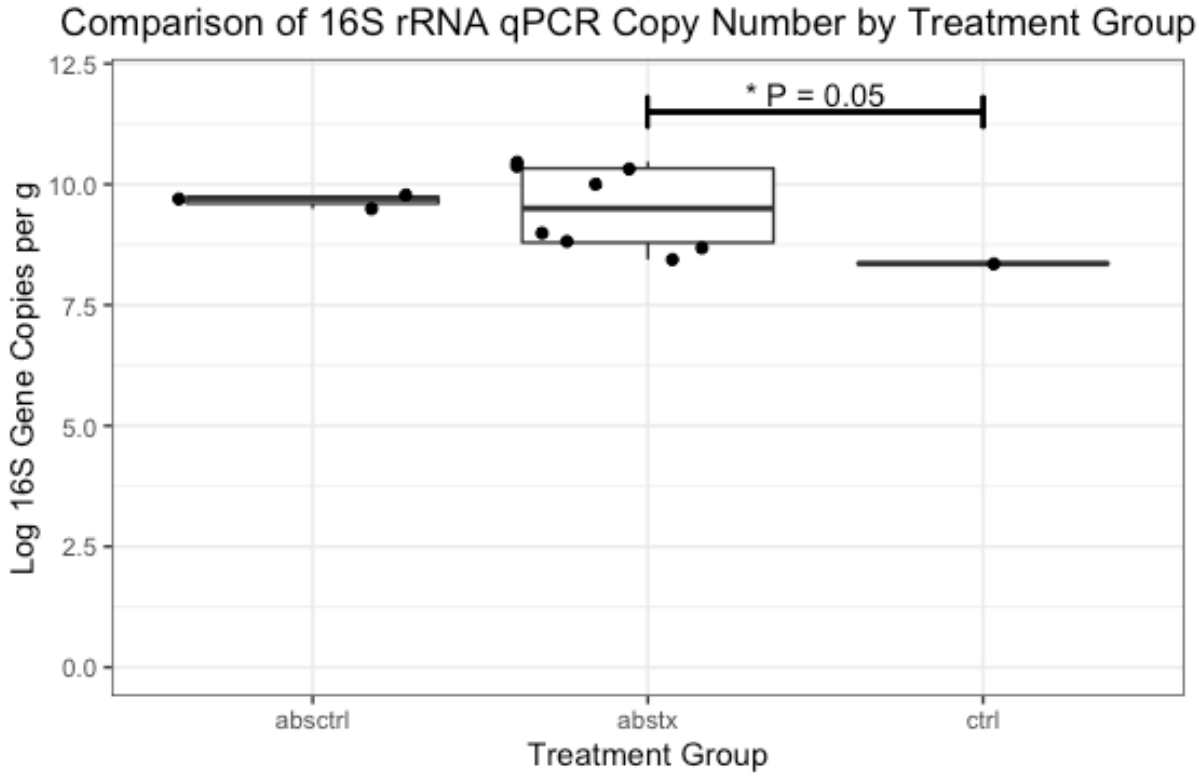


Figure 5.7 - Comparison of 16S *rRNA* gene copy number (as determined by qPCR) between *M. abscessus* control, *M. abscessus* treatment (samples are pre-initiation of treatment) and control group. The mean 16S copy number per sample in the abscessus control group at time point 1 was 4.74 x 10^9 copies/g sputum (median = 5.14 x 10^9 copies/g sputum, standard deviation = 1.47 x 10^9 copies/g sputum). The mean 16S copy number per sample in abscessus treatment group at time point 1 was 1.07 x 10^{10} copies/g sputum (median = 5.5 x 10^9 copies/g sputum, standard deviation = 1.21 x 10^{10} copies/g sputum). 16S copy number in the control group at time point 1 was 2.29 x 10^8 (one sample analysed).

Statistical analysis by Kruskal – Wallis one-way analysis of variance revealed no significant differences between all groups (chi-squared = 3.2, df = 2, *P*-value = 0.2). A significant difference between the avium control group and the control group was found (*P*-value = 0.04) but only one sample from each group was compared (Figure 5.8).

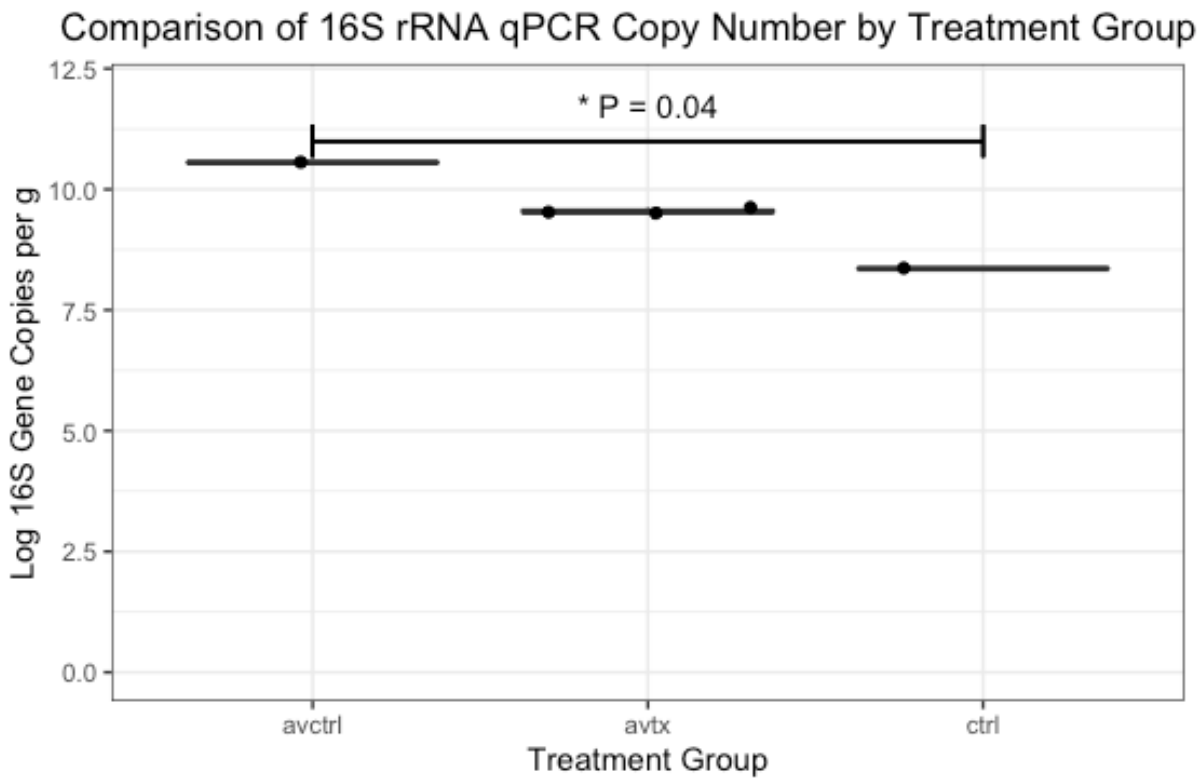


Figure 5.8 - Comparison of 16S rRNA gene copy number (as determined by qPCR) between *M. avium* control, *M. avium* treatment (samples are pre-initiation of treatment) and control group. The 16S copy number in the avium control group at time point 1 was 3.6 x 10^{10} copies/g sputum (one sample analysed). The mean 16S copy number per sample in avium treatment group was 3.59 x 10^{9} copies/g sputum (median = 3.4 x 10^{9} copies/g sputum, standard deviation = 5.1 x 10^{8} copies/g sputum). 16S copy number in the control group was 2.29 x 10^{8} (one sample analysed).

5.8.2 Differences in Alpha Diversity

5.8.2.1 Alpha Diversity between Abscessus Groups

There were no significant differences in alpha diversity measures between abscessus control, abscessus treatment and control samples at time point 1 (Figure 5.9) (Simpson's diversity index = chi-squared = 2.4872, df = 2, *P*-value = 0.29, Shannon's diversity index = Kruskal-Wallis chi-squared = 2.4872, df = 2, *P*-value = 0.29, observed

OTU richness = Kruskal-Wallis chi-squared = 0.2597, df = 2, *P*-value = 0.88, Pielou's evenness index = Kruskal-Wallis chi-squared = 2.4872, df = 2, *P*-value = 0.29).

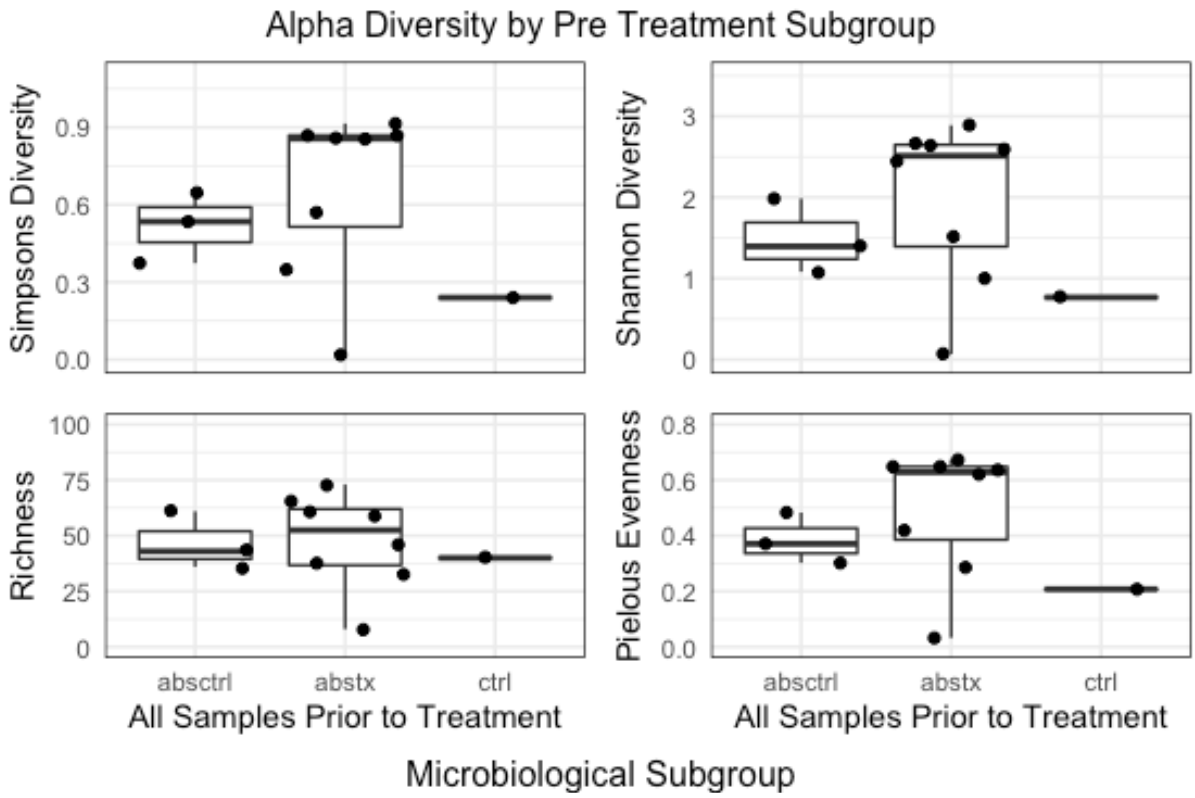


Figure 5.9 – Differences in alpha diversity between patient groups at time point 1. Patients were divided based on whether they: cultured *M. abscessus* but did not require treatment (absctrl), cultured *M. abscessus* and required treatment (abstx), were NTM culture naïve (ctrl). The mean Simpson diversity index for the abscessus control group at time point 1 was 0.52 (range = 0.37 – 0.65). For patients in the abscessus treatment group the mean Simpson diversity index was 0.66 (range = 0.018 – 0.91). For patients in the control group the Simpson diversity index was 0.24. The mean Shannon diversity index for the abscessus control group at time point 1 was 1.49 (range = 1.083 – 1.99). For patients in the abscessus treatment group the mean Shannon diversity index was 1.98 (range = 0.068 – 2.89). For patients in the control group the Shannon diversity index was 0.77. The mean observed richness for the abscessus control group at time point 1 was 46.67 (range = 36.0 - 61.0). The mean observed richness for the abscessus treatment group at time point 1 was 47.88 (range = 8.00 - 73.00). The observed richness for the control group was 40. The mean Pielou's evenness index for the abscessus control group at time point 1 was 0.39 (range = 0.3 – 0.48). The mean mean Pielou's evenness index for the abscessus treatment group at time point 1 was 0.5 (range = 0.033 – 0.67). The Pielou's evenness index for the control group was 0.21.

5.8.2.2 Alpha Diversity between Avium Groups

There were no significant differences in alpha diversity measures between avium control, avium treatment and control samples taken from patients before the initiation of treatment (Figure 5.10) (Simpson's diversity index = Kruskal-Wallis chi-squared = 3.2, df = 2, *P*-value = 0.2, Shannon's diversity index = Kruskal-Wallis chi-squared =

2.1333, df = 2, *P*-value = 0.34, observed OTU richness = Kruskal-Wallis chi-squared = 0.8, df = 2, *P*-value = 0.67, Pielou's evenness index = Kruskal-Wallis chi-squared = 3.2, df = 2, *P*-value = 0.2).

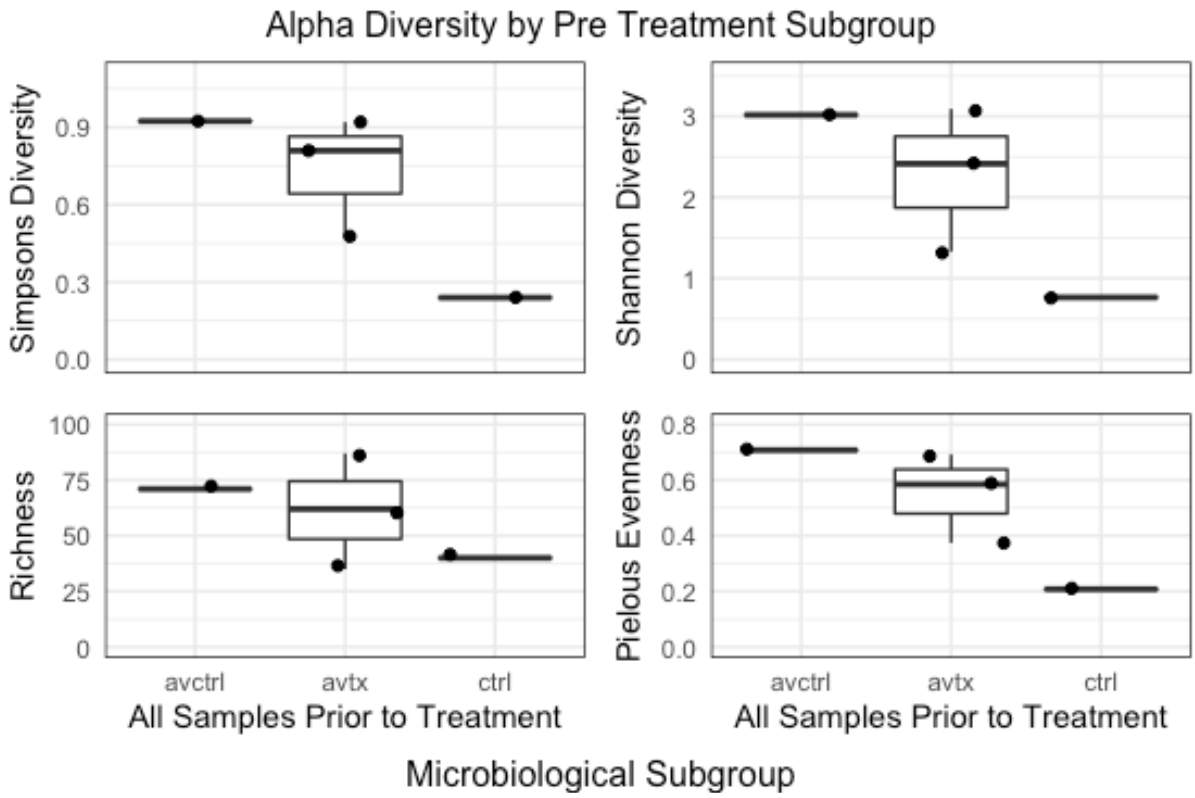


Figure 5.10 - Differences in alpha diversity between patient groups at time point 1. Patients were divided based on whether they: cultured *M. avium* but did not require treatment (avctrl), cultured *M. avium* and required treatment (avtx), were NTM culture naïve (ctrl). The Simpson diversity index for the avium control group at time point 1 was 0.92. For patients in the avium treatment group the mean Simpson diversity index was 0.74 (range = 0.48 – 0.92). For patients in the control group the Simpson diversity index was 0.24. The Shannon diversity index for the avium control group at time point 1 was 3.02. For patients in the avium treatment group the mean Shannon diversity index was 2.28 (range = 1.33 – 3.092). For patients in the control group the Shannon diversity index was 0.77. The observed richness for the avium control group at time point 1 was 71. The mean observed richness for the avium treatment group at time point 1 was 61.33 (range = 35 - 87). The observed richness for the control group was 40. The Pielou's evenness index for the avium control group at time point 1 was 0.71. The mean Pielou's evenness index for the avium treatment group at time point 1 was 0.55 (range = 0.37 – 0.69). The Pielou's evenness index for the control group was 0.21.

5.8.3 Differences in Beta Diversity

The mean Bray-Curtis dissimilarity across all pairs of samples was 0.26 for the abscessus control group and 0.82 for the abscessus treatment group. The difference between all groups was not significant however (R^2 = 0.2, *P*-value = 0.38).

The mean Bray-Curtis dissimilarity across all pairs of samples was 0.7 for the avium treatment group. The difference between all groups was not significant (R^2 = 0.58, *P*-value = 0.3).

5.8.4 Differences in Specific Bacterial Abundances

5.8.4.1 Differences Between *M. abscessus* Groups

In all three groups, *Pseudomonas* was the most abundant genus before the commencement of treatment - 25.3% for *M. abscessus* treatment group, 68% for the *M, abscessus* control group and 87.2% for the NTM culture naive group. *Streptococcus* was the next most abundant genus in both the treatment group and the *M. abscessus* control group (12.9% and 12.2% respectively) but was less dominant than both *Haemophilus* and *Lautropia* in the NTM culture naïve group (3.5% and 1.7%). *Mycobacterium* was only detected in the *M. abscessus* treatment group contributing to 3.9% of total abundance (Table 5.7).

Table 5.7 – The 20 most common genera seen in samples from: *M. abscessus* treatment, *M. abscessus* control and the control group. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

	Abscessus Treatment			**Abscessus Control**			**Control**		
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Pseudomonas	0.5	2023.0	25.3	0.5	2041.0	68.0	0.1	872.0	87.2
Streptococcus	0.9	1035.0	12.9	0.3	365.0	12.2	0.1	13.0	1.3
Staphylococcus	0.6	927.0	11.6	0.2	69.0	2.3	0.0	0.0	0.0
Veillonella	0.7	478.0	6.0	0.5	122.0	4.1	0.1	2.0	0.2
Actinomyces	1.3	387.0	4.8	0.4	47.0	1.6	0.1	10.0	1.0
Mycobacterium	1.0	315.0	3.9	0.0	0.0	0.0	0.0	0.0	0.0
Granulicatella	1.5	312.0	3.9	0.4	43.0	1.4	0.0	0.0	0.0
Stomatobaculum	1.3	289.0	3.6	0.7	10.0	0.3	0.0	0.0	0.0
Stenotrophomonas	1.0	288.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
Haemophilus	0.7	255.0	3.2	0.1	24.0	0.8	0.1	35.0	3.5
Neisseria	0.8	246.0	3.1	0.2	15.0	0.5	0.2	4.0	0.4
Prevotella	0.6	222.0	2.8	0.2	23.0	0.8	0.0	0.0	0.0
Gemella	1.0	173.0	2.2	0.6	15.0	0.5	0.1	1.0	0.1
Rothia	1.0	155.0	1.9	0.1	8.0	0.3	0.2	12.0	1.2
Solobacterium	4.0	107.0	1.3	2.0	10.0	0.3	0.0	0.0	0.0
Bifidobacterium	0.6	106.0	1.3	0.0	0.0	0.0	0.1	5.0	0.5
Enterococcus	1.6	90.0	1.1	0.2	1.0	0.0	0.0	0.0	0.0
Fusobacterium	1.5	82.0	1.0	0.3	17.0	0.6	0.3	10.0	1.0
Lautropia	2.0	13.0	0.2	0.0	0.0	0.0	0.5	17.0	1.7
Dolosigranulum	0.2	2.0	0.0	0.8	74.0	2.5	0.0	0.0	0.0

Similarity percentage (simper) analysis was conducted by comparing:

1. *M. abscessus* treatment group and the *M. abscessus* control group
2. *M. abscessus* treatment group and the control group
3. *M. abscessus* control group and the control group

The simper function performs pairwise comparisons of groups of sampling units and finds the contribution of each OTU to the overall between group dissimilarity (dissimilarity measured using Bray Curtis distance matrix). To test significance, the relative abundance of each OTU across the two groups was compared using the Kruskal-Wallis rank sum test (Table 5.8).

OTUs assigned to the *Pseudomonas, Staphylococcus and Streptococcus* generas contributed the most to average dissimilarity between the *M. abscessus* treatment and *M. abscessus* control groups. *Pseudomonas 2114* OTU showed the greatest differences between groups although these differences were not significant *(P*-value = 0.1).

The same OTUs contributed the most to average dissimilarity between the *M. abscessus* treatment and control group. *Lautropia 1746* OTU showed the greatest difference between groups although these differences were not significant (*P*-value = 0.09).

OTUs assigned to the *Pseudomonas, Streptococcus* and *Haemophilus* generas contributed the most to average dissimilarity between the *M. abscessus* control and control groups. The *Lautropia 1746* OTU again showed the greatest difference between groups although these differences were not significant (*P*-value = 0.08).

Table 5.8 – Summary of simper analysis between: 1. *M. abscessus* treatment group and *M. abscessus* control group; 2. *M. abscessus* treatment group and the control group; 3. *M. abscessus* control group and the control group. Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity; *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between groups.

OTU identity	Contribution to Average Dissimilarity	Average Abundance Abscessus Treatment	Average Abundance Abscessus Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Pseudomonas_2114	0.2545208	250.875	676.3333	37.5	37.5	0.1
Staphylococcus_3526	0.0606042	114.375	21.3333	8.93	46.4	0.92
Streptococcus_574	0.04275	62.5	94.6667	6.3	52.7	0.22
Veillonella_3368	0.028125	56	29	4.2	56.9	0.68
Streptococcus_487	0.0245833	57	14	3.6	60.5	0.41
Mycobacterium_949	0.0194375	38.875	0	2.8	63.3	0.36
Stenotrophomonas_2039	0.01775	35.5	0	2.7	66	0.24
Actinomyces_778	0.0167708	38.875	11.6667	2.4	68.4	0.22
Stomatobaculum_3032	0.0157917	31.5	1	2.3	70.7	0.91
Granulicatella_3662	0.0142292	33.625	13.6667	2.1	72.8	0.54

OTU identity	Contribution to Average Dissimilarity	Average Abundance Abscessus Treatment	Average Abundance Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Pseudomonas_2114	0.3250625	250.875	871	44.4	44.4	0.25
Staphylococcus_3526	0.0571875	114.375	0	7.8	52.2	0.31
Streptococcus_574	0.03125	62.5	0	4.3	56.5	0.31
Streptococcus_487	0.02775	57	2	3.8	60.3	0.25
Veillonella_3368	0.02775	56	1	3.8	64.1	0.44
Haemophilus_1826	0.02	16.5	32	2.7	66.8	0.2
Mycobacterium_949	0.0194375	38.875	0	2.7	69.5	0.6
Actinomyces_778	0.0185625	38.875	2	2.5	72	0.25
Stenotrophomonas_2039	0.01775	35.5	0	2.4	74.4	0.49
Granulicatella_3662	0.0168125	33.625	0	2.3	76.7	0.24

OTU identity	Contribution to Average Dissimilarity	Average Abundance Abscessus Control	Average Abundance Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Pseudomonas_2114	0.0973333	676.3333	871	32.2	32.2	0.18
Streptococcus_574	0.0473333	94.6667	0	15.67	47.9	0.18
Haemophilus_1826	0.0146667	2.6667	32	4.86	52.8	0.16
Veillonella_3368	0.014	29	1	4.64	57.4	0.18
Dolosigranulum_323	0.0106667	21.3333	0	3.53	60.9	0.56
Staphylococcus_3526	0.0106667	21.3333	0	3.53	64.5	0.35
Lautropia_1746	0.0085	0	17	2.81	67.3	0.08
Granulicatella_3662	0.0068333	13.6667	0	2.27	69.5	0.18
Streptococcus_487	0.006	14	2	1.98	71.5	0.18
Actinomyces_778	0.0048333	11.6667	2	1.6	73.1	0.18

5.8.4.2 Differences Between *M. avium* Groups

In the *M. avium* treatment group, *Streptococcus* was the most abundant genera before the commencement of treatment (41.1% in *M. avium* treatment group, 23.1% for the *M. avium* control group and 1.3% for the NTM culture naive group). *Pseudomonas* was the next most abundant genera in the treatment group (13.2%) but only contributed to 0.1% of the relative abundance of the *M. avium* control group. The most abundant genera in the control group was *Pseudomonas* (88.1%). The most abundant genera in the *M. avium* control group was *Veillonella* which contributed to 25.1% of the relative abundance. *Mycobacterium* was not detected in any group (table 5.9).

Table 5.9 – The most common genera seen in samples from: *M. avium* treatment, *M. avium* control and the control group. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

	Avium Treatment			Avium Control			Control		
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Streptococcus	0.5	1234.0	41.1	0.2	220.0	23.1	0.1	13.0	1.3
Pseudomonas	0.3	396.0	13.2	0.0	1.0	0.1	0.1	872.0	88.1
Haemophilus	0.6	243.0	8.1	0.0	0.0	0.0	0.1	35.0	3.5
Neisseria	0.6	234.0	7.8	0.1	2.0	0.2	0.2	4.0	0.4
Veillonella	0.3	193.0	6.4	0.2	239.0	25.1	0.1	2.0	0.2
Granulicatella	0.5	113.0	3.8	0.4	78.0	8.2	0.0	0.0	0.0
Actinomyces	0.7	101.0	3.4	0.3	96.0	10.1	0.1	10.0	1.0
Gemella	1.0	93.0	3.1	0.1	10.0	1.0	0.1	1.0	0.1
Leptotrichia	0.7	62.0	2.1	0.4	58.0	6.1	0.2	4.0	0.4
Rothia	0.3	59.0	2.0	0.3	34.0	3.6	0.2	12.0	1.2
Capnocytophaga	0.7	45.0	1.5	0.2	16.0	1.7	0.1	4.0	0.4
Prevotella	0.3	41.0	1.4	0.2	40.0	4.2	0.0	0.0	0.0
Fusobacterium	0.5	40.0	1.3	0.3	76.0	8.0	0.3	10.0	1.0
Selenomonas	0.4	22.0	0.7	0.3	30.0	3.1	0.2	5.0	0.5
Parvimonas	1.5	20.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0
Lautropia	1.0	11.0	0.4	0.0	0.0	0.0	0.5	17.0	1.7
Leucobacter	1.0	9.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Porphyromonas	0.5	9.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Alysiella	2.0	6.0	0.2	1.0	54.0	5.7	1.0	1.0	0.1
Mycobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Similarity percentage (simper) analysis was conducted by comparing:

1. *M. avium* treatment group and the *M. avium* control group
2. *M. avium* treatment group and the control group
3. *M. avium* control group and the control group

OTUs assigned to the *Streptococcus, Pseudomonas* and *Veillonella* generas contributed the most to average dissimilarity between the *M. avium* treatment and *M. avium* control groups (Table 5.10). The *Leptotrichia 2711* and *Veillonella 3365* OTU showed the greatest difference between groups although these differences were not significant (*P*-value = 0.16).

There were no significant differences between the *M. avium* treatment, *M. avium* control and control group. The same OTUs contributed the most to average dissimilarity between the *M. avium* treatment and control group with *Pseudomonas 2114* OTU showing the greatest difference between groups. OTUs assigned to the *Pseudomonas* and *Veillonella* generas contributed the most to average dissimilarity between the *M. avium* control and control groups.

Table 5.10 – Summary of simper analysis between: 1. *M. avium* treatment group and *M. avium* control group; 2. *M. avium* treatment group and the control group; 3. *M. avium* control group and the control group. Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity; *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between groups.

OTU identity	Contribution to Average Dissimilarity	Average Abundance Avium Treatment	Average Abundance Avium Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Streptococcus_574	0.1101667	336	117	16.5	16.5	0.65
Pseudomonas_2114	0.0646667	130.3333	1	9.7	26.2	0.56
Veillonella_3368	0.049	54	152	7.3	33.5	0.18
Actinomyces_778	0.0365	16	89	5.4	38.9	0.18
Haemophilus_1826	0.0358333	71.6667	0	5.4	44.3	0.35
Neisseria_1599	0.0325	65.6667	2	4.9	49.2	0.65
Fusobacterium_2663	0.0313333	13.3333	76	4.6	53.8	0.18
Veillonella_3339	0.027	7	61	4.1	57.9	0.18
Alysiella_1666	0.026	2	54	3.9	61.8	0.18
Streptococcus_487	0.0253333	42.3333	93	3.8	65.6	0.18

OTU identity	Contribution to Average Dissimilarity	Average Abundance Avium Treatment	Average Abundance Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Pseudomonas_2114	0.3703333	130.3333	871	45.6	45.6	0.16
Streptococcus_574	0.168	336	0	20.6	66.2	0.18
Neisseria_1599	0.0326667	65.6667	1	4	70.2	0.65
Haemophilus_1826	0.0305	71.6667	32	3.8	74	0.65
Veillonella_3368	0.0265	54	1	3.2	77.2	0.18
Streptococcus_487	0.0201667	42.3333	2	2.8	80	0.18
Granulicatella_3662	0.017	34	0	1.8	81.8	0.18
Gemella_152	0.0143333	29.6667	1	1.8	83.6	0.18
Streptococcus_467	0.0081667	17	2	1	84.6	0.65
Rothia_1085	0.0076667	16	4	0.9	85.5	0.65

OTU identity	Contribution to Average Dissimilarity	Average Abundance Avium Control	Average Abundance Control	% Contribution to Dissimilarity	% Cumulative Contribution	*P*-value
Pseudomonas_2114	0.435	1	871	45.3	45.3	0.32
Veillonella_3368	0.0755	152	1	7.9	53.2	0.32
Streptococcus_574	0.0585	117	0	6.1	59.3	0.32
Streptococcus_487	0.0455	93	2	4.7	64	0.32
Actinomyces_778	0.0435	89	2	4.5	68.5	0.32
Granulicatella_3662	0.0365	73	0	3.8	72.3	0.32
Fusobacterium_2663	0.033	76	10	3.5	75.8	0.32
Veillonella_3339	0.03	61	1	3.1	78.9	0.32
Alysiella_1666	0.0265	54	1	2.8	81.7	0.32
Leptotrichia_2711	0.0225	45	0	2.3	84	0.32

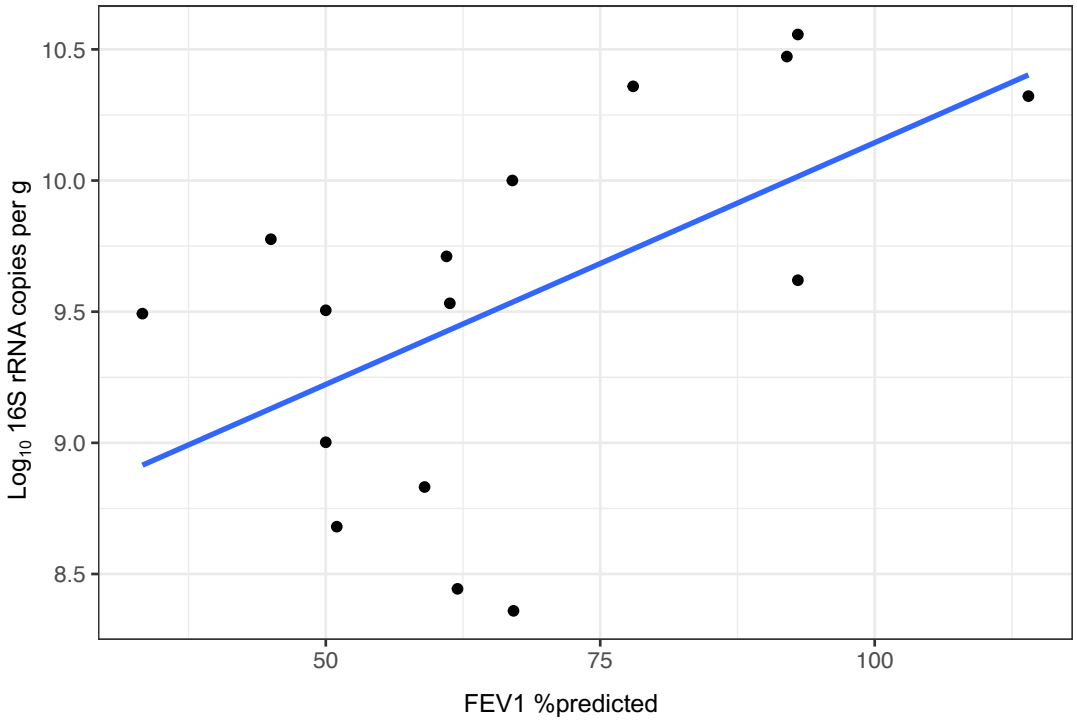
5.9 Exploring Clinical Outcomes in NTM

5.9.1 Relating Lung Function to Bacterial Burden

Results of lung function testing were recorded after recruitment but before the commencement of treatment. When all samples were considered together Log_{10} bacterial abundance was positively correlated with FEV1 %predicted and FVC %predicted (Figure 5.11) (Pearson's, *P*-values = 0.025 and 0.032 respectively).

When the *M. abscessus* treatment group was considered independently at time point 1, the association between bacterial abundance and pulmonary function testing was even more marked (FEV1 %predicted Pearson's *P*-value = 0.02, FVC %predicted Pearson's *P*-value = 0.004). In the *M. avium* treatment group, there was a significant positive correlation in FEV1 %predicted (Pearson's *P*-value = 0.02) however there was not a significant correlation in FVC %predicted (Pearson's *P*-value = 0.4).

a)



b)

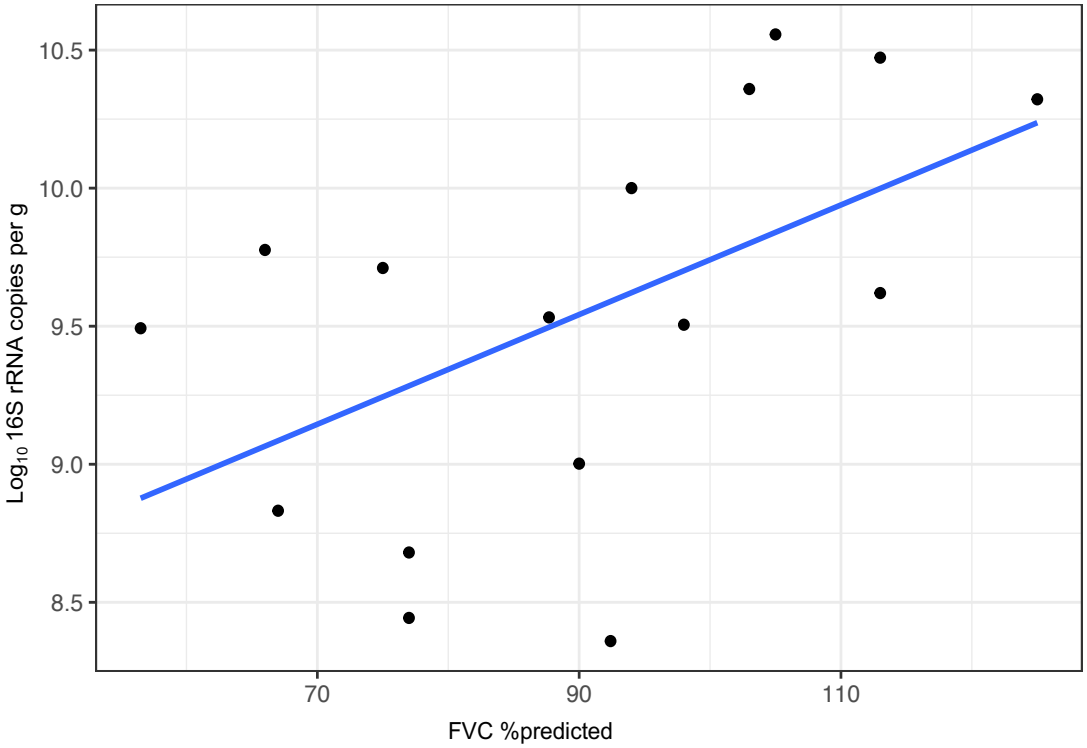


Figure 5.11 a) - correlation between bacterial abundance and FEV1 %predicted **b)** correlation between bacterial abundance and FVC %predicted. Patient FEV1 and FVC were calculated from lung function testing at time point 1. %predicted was calculated using population data for any person of similar age, sex, and body composition. X-axis = % predicted, Y-axis = Log_{10} 16S copies/g sputum, FEV1 = forced expiratory volume in 1 second, FVC = forced vital capacity.

5.9.2 Relating Lung Function to Alpha Diversity

All samples at time point 1 were considered together. There was a positive correlation between lung function and alpha diversity measures. The correlation between FEV1 %predicted and alpha diversity was significant although this was not reciprocated in FVC measures (Figure 5.12). Using Pearson's correlation for FEV1 %predicted: Simpson's diversity *P*-value = 0.02, Shannon's diversity *P*-value = 0.009, Observed *P*-value = 0.01, Pielou's evenness *P*-value = 0.02. Using Pearson's correlation for FVC %predicted Simpson's diversity *P*-value = 0.8, Shannon's diversity *P*-value = 0.6, Observed *P*-value = 0.4, Pielou's evenness *P*-value = 0.9.

Considering the *M. abscessus* and *M. avium treatment* groups independently, there was no significant correlation between alpha diversity metrics and pulmonary function. This is likely to be due to small patient numbers.

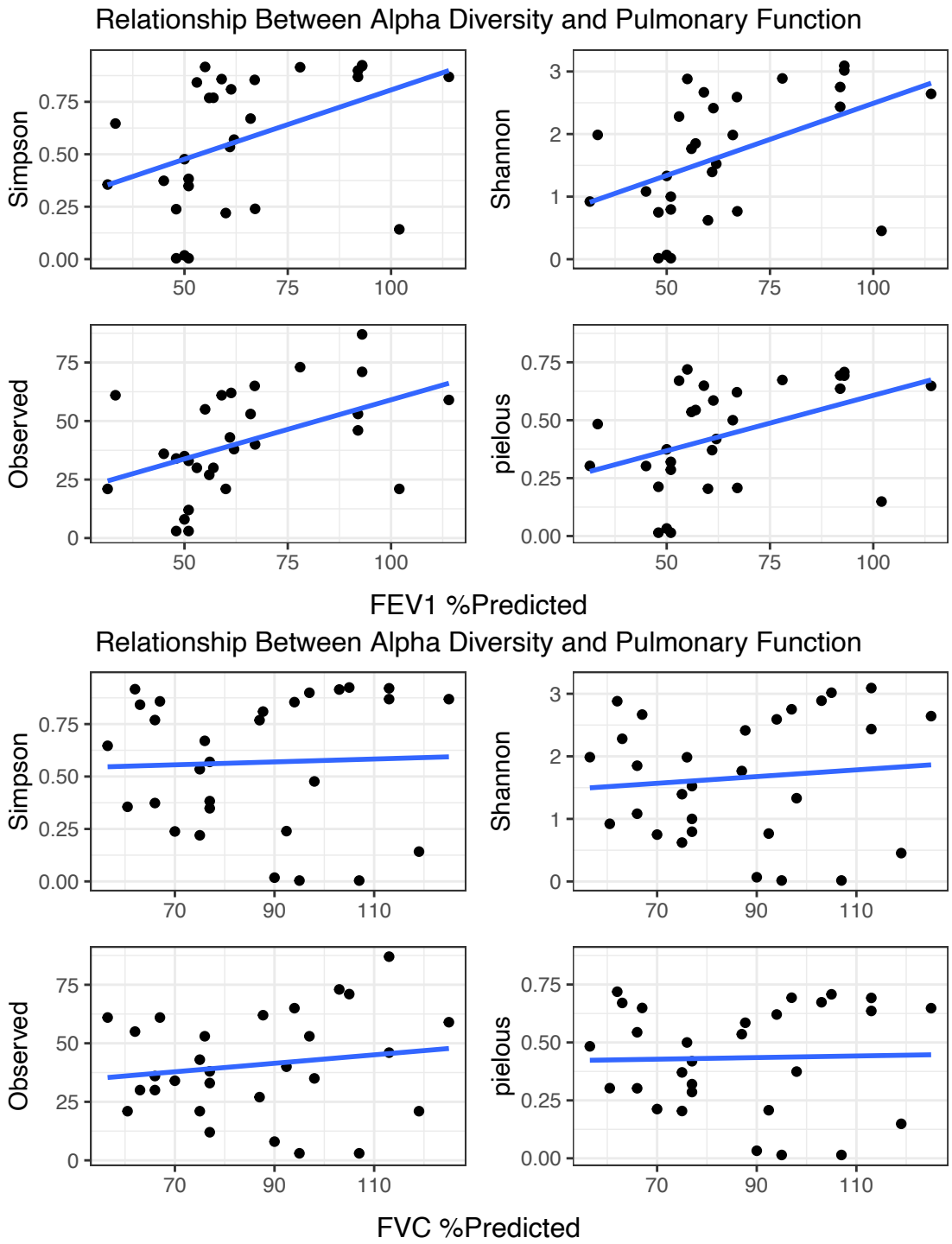


Figure 5.12 – Alpha diversity and lung function in all patients at time point 1. X-axis = % predicted, Y-axis = Log_{10} 16S copies/g sputum, FEV1 = forced expiratory volume in 1 second, FVC = forced vital capacity.

5.10 Longitudinal Assessment of Bacterial Groups

5.10.1 16S rRNA Quantitative PCR Copy Number

5.10.1.1 Assessment of Subjects Culturing *M. abscessus*

For patients on treatment for *M. abscessus*, shown in green in Figure 5.13, the mean 16S copy number at time point 1 was 1.07 x 10^{10} copies/g sputum (median = 5.5 x 10^{9}, standard deviation = 1.21 x 10^{10}). At time point 2, the mean 16S copy number was 1.27 x 10^{9} (median = 2.37 x 10^{8}, standard deviation = 2.22 x 10^{9}). At time point 3, the mean 16S copy number was 2.34 x 10^{9} (median = 1.2 x 10^{9}, standard deviation = 2.79 x 10^{9}). At time point 4, the mean 16S copy number was 2.13 x 10^{9} (median = 1.92 x 10^{9}, standard deviation = 1.7 x 10^{9}).

Statistical analysis by Kruskall – Wallis one-way analysis of variance showed a significant difference between overall time points (chi-squared = 9.3423, df = 3, *P*-value = 0.03). Bacterial abundance was significantly lower at time point 2 compared to time point 1 (*P*-value = 0.0014). Bacterial abundance steadily increased after time point 2 and reached significance after 3 months of treatment (*P*-value = 0.019).

For patients that cultured *M. abscessus,* but who were not on treatment (abscessus control group shown in red in Figure 5.13), there were no significant differences between time points. The mean 16S copy number at time point 1 was 4.74 x 10^{9} copies/g sputum (median = 5.14 x 10^{9}, standard deviation = 1.47 x 10^{9}). At time point 2, the mean 16S copy number was 9.35 x 10^{9} (median = 5.15 x 10^{9}, standard deviation = 8.69 x 10^{9}). At time point 3, the mean 16S copy number was 1.58x10^{10} (median = 6.58 x 10^{9}, standard deviation = 1.72 x 10^{10}). At time point 4, the mean 16S copy number was 9.18 x 10^{9} (median = 1.13 x 10^{10}, standard deviation = 6.86 x 10^{9}).

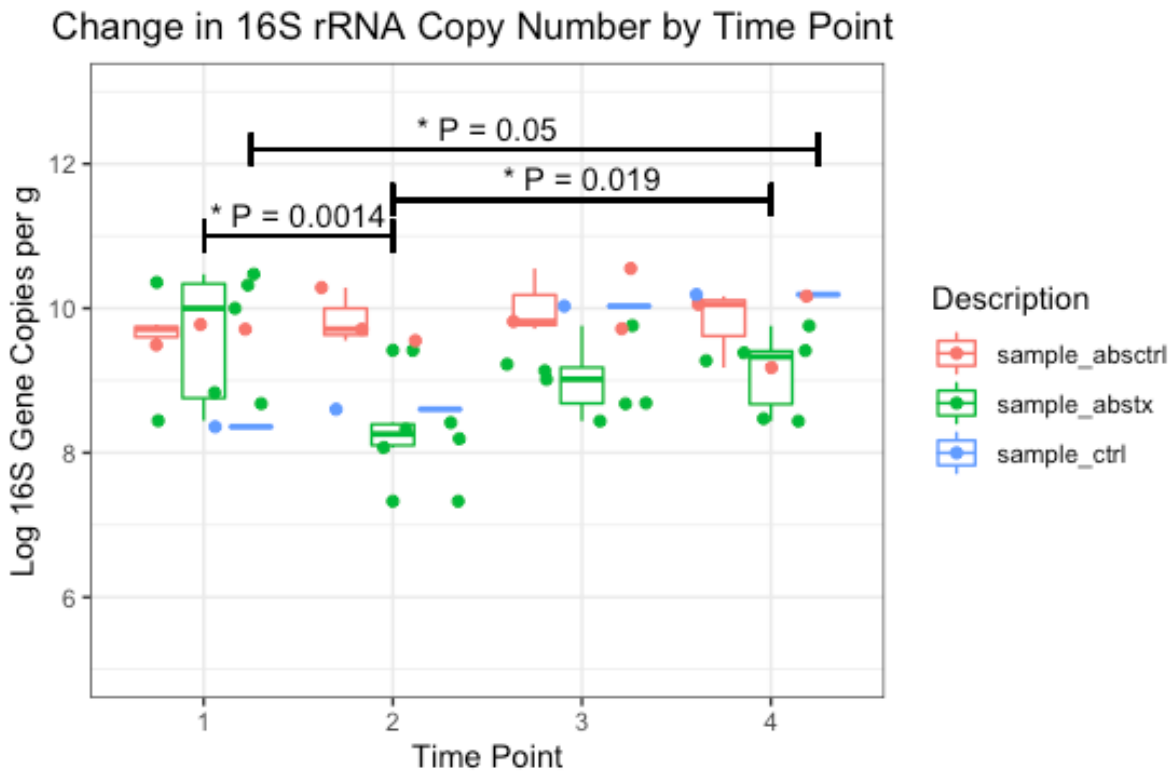


Figure 5.13 – Longitudinal comparison of 16S *rRNA* gene copy number (as determined by qPCR) for patients on treatment for *M. abscessus*. The boxplots in red show changes in 16S rRNA gene copy number for patients culturing, but not on treatment for *M. abscessus* over a 3-month period. The boxplots in green show changes in 16S rRNA gene copy number for patients on treatment for *M. abscessus* over a 3-month period. The boxplots in blue show changes in 16S rRNA gene copy number for patients who are NTM culture naive over a 3-month period. Time point 1 = baseline sample (pre-initiation of treatment), time point 2 = week 2 of observation or treatment, time point 3 = week 4 of observation or treatment, time point 4 = month 3 of treatment.

5.10.1.2 Assessment of Subjects Culturing *M. avium*

For patients on treatment for *M. avium*, shown in green in Figure 5.14, there were no significant differences between overall time points by Kruskall – Wallis one-way analysis of variance (chi-squared = 2.7949, df = 3, *P*-value = 0.32). The fall in bacterial abundance between time points 2 to 4 and 3 to 4 was however found to be significant by Dunn analysis (*P*-value = 0.05).

The mean 16S copy number at time point 1, pre-treatment initiation, was 3.59 x 10^9 copies/g sputum (median = 3.4 x 10^9, standard deviation = 5.1 x 10^8). At time point 2, 2 weeks of antibiotic treatment, the mean 16S copy number was 6.25 x 10^9 (median = 8.3 x 10^9, standard deviation = 4.92 x 10^9). At time point 3, 1 month of antibiotic treatment, the mean 16S copy number was 7.79 x 10^9 (median = 6.36 x 10^9, standard

deviation = 6.26 x 10^9). At time point 4, 3 months of antibiotic treatment, the mean 16S copy number was 1.79 x 10^9 (median = 1.32 x 10^9, standard deviation = 1.36 x 10^9).

For patients that cultured *M. avium,* but who were not on treatment (avium control group shown in red in Figure 5.14), there were no significant differences between overall time points by Kruskall – Wallis one-way analysis of variance chi-squared = 3, df = 3, *P*-value = 0.39)

The 16S copy numbers were at time point 1 3.6 x 10^{10}, time point 2 1.25 x 10^{10}, time point 3 2.56 x 10^{11} and at time point 4 1.88 x 10^9 copies/g sputum.

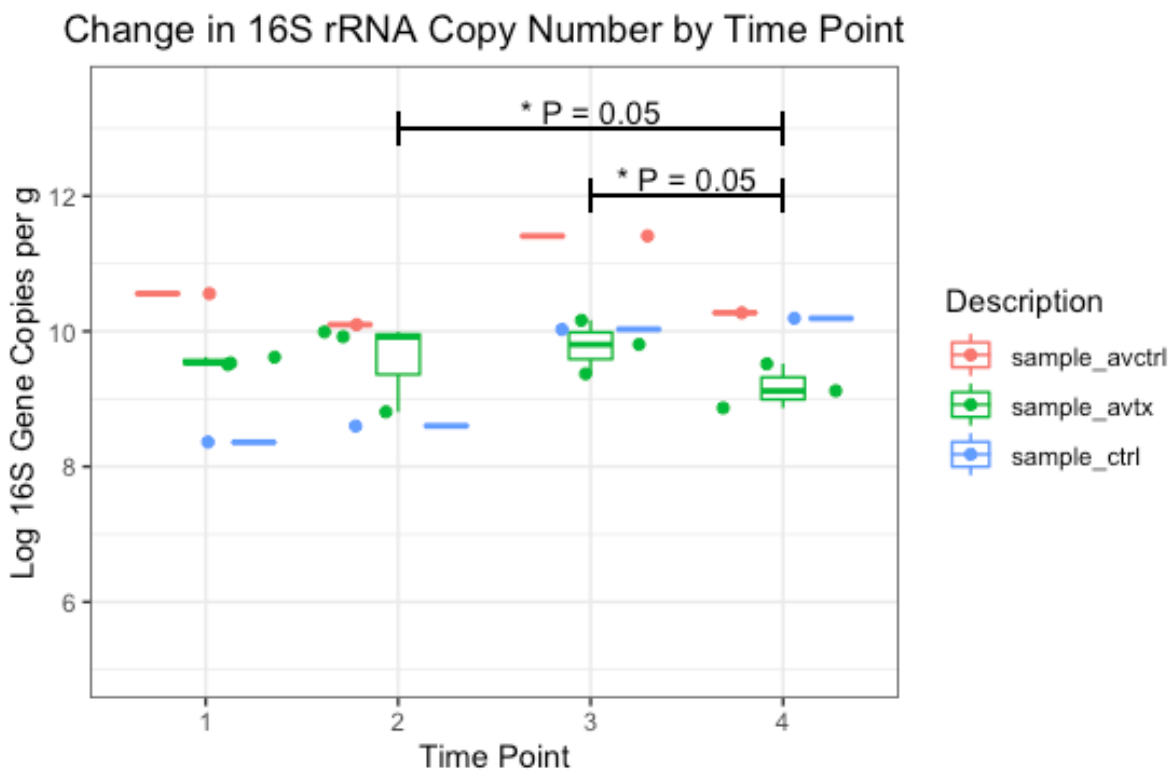


Figure 5.14 – Longitudinal comparison of 16S rRNA gene copy number (as determined by qPCR) for patients on treatment for *M. avium*. The boxplots in red show changes in 16S rRNA gene copy number for patients culturing, but not on treatment for *M. avium* over a 3-month period. The boxplots in green show changes in 16S rRNA gene copy number for patients on treatment for *M. avium* over a 3-month period. The boxplots in blue show changes in 16S rRNA gene copy number for patients who are NTM culture naïve over a 3-month period.

5.10.1.3 Assessment of non NTM control patients

For patients in the control group (shown in blue in Figures 5.13 and 5.14), the bacterial abundance at time point 1 was 2.29 x 10^8 copies/g sputum. The bacterial abundance at time point 2 was 4 x 10^8, at time point 3 1.07 x 10^{10} and at time point 4 1.55 x 10^{10}.

Statistical analysis by Kruskall – Wallis one-way analysis of variance showed no significant differences between overall time points (chi-squared = 3, df = 3, *P*-value = 0.39). Overall bacterial abundance increased with a significant difference between time point 1 and 4 (*P*-value = 0.05).

5.10.2 Differences in Alpha Diversity

5.10.2.1 Assessment of Subjects Culturing *M. abscessus*

The mean alpha diversity metrics at time point 1 for patients receiving treatment against *M. abscessus* were as follows: Simpson's diversity = 0.66, Shannon's diversity = 1.98, observed OTUs = 47.88, Pielou's evenness = 0.5.

The mean alpha diversity metrics for patients receiving treatment against *M. abscessus* decreased significantly between time points 1 and 2 (latter after 2 weeks of antibiotic therapy). At time point 2 the mean alpha diversity metrics were as follows: Simpson's diversity = 0.32 (difference from time point 1 to 2: Kendall's Tau = -0.44, *P*-value 0.049), Shannon's diversity = 0.85 (difference from time point 1 to 2: Kendall's Tau = -0.47, *P*-value 0.037), observed OTUs = 24 (difference from time point 1 to 2: Kendall's Tau = -0.5, *P*-value 0.028), Pielou's evenness = 0.26 (difference from time point 1 to 2: Kendall's Tau = -0.44, *P*-value = 0.049).

Between time point 2 and 3 (2 weeks versus 1 month of anti-microbial therapy), the mean alpha diversity metrics increased although this change was not significant. At time point 3 the mean alpha diversity metrics were as follows: Simpson's diversity = 0.55, Shannon's diversity = 1.38, observed OTUs = 24, Pielou's evenness = 0.41.

Between time point 3 and 4, 1 month and 3 months of anti-microbial therapy respectively, the mean alpha diversity metrics once again increased. This increase however was not significant. At time point 4 the mean alpha diversity metrics were:

Simpson's diversity = 0.56, Shannon's diversity = 1.39, observed OTUs = 24.86, Pielou's evenness = 0.42.

A significant difference in observed OTUs was noted between time points 1 and 2 (Kendall's Tau = -0.5, *P*-value = 0.028), 1 and 3 (Kendall's Tau = -0.43, *P*-value = 0.046), 1 and 4 (Kendall's Tau = -0.5, *P*-value = 0.027).

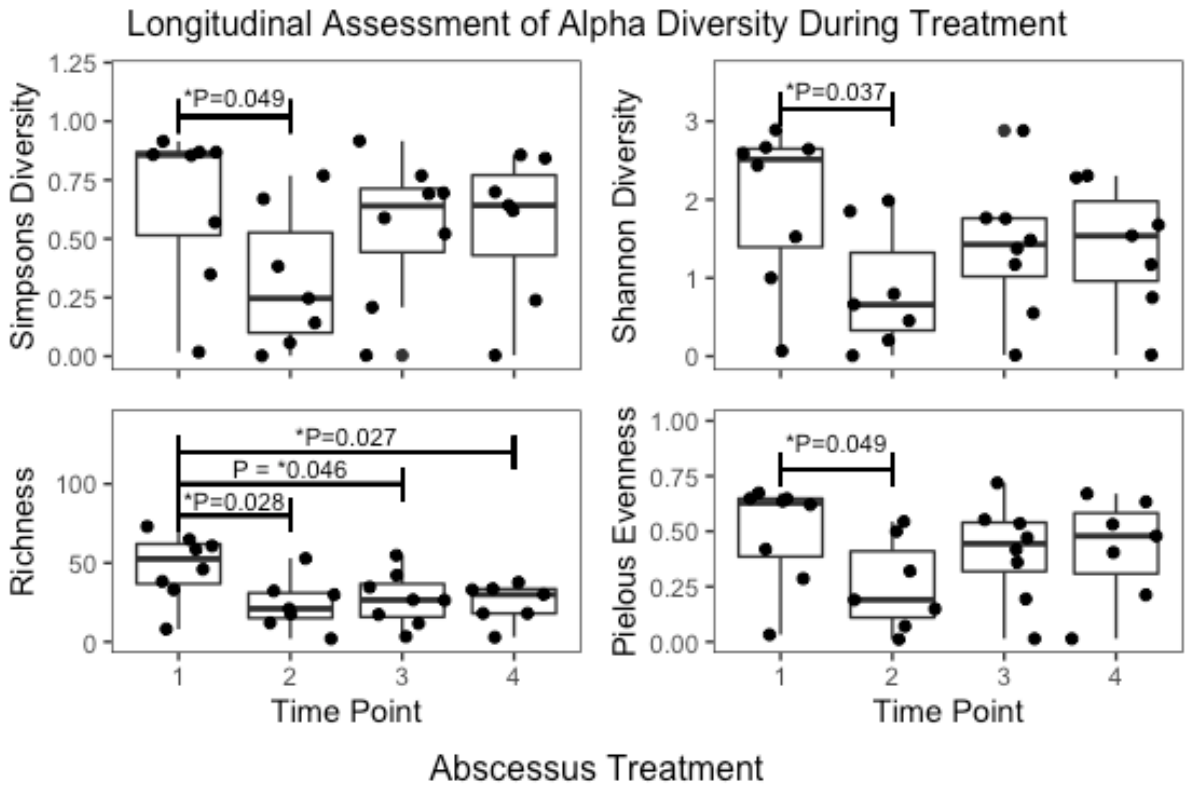


Figure 5.15 – Longitudinal assessment of alpha diversity for patients receiving treatment for *M. abscessus.* Time point 1 = pre-treatment, time point 2 = 2 weeks of treatment, time point 3 = 1 month of treatment, time point 4 = 3 months of treatment. The mean Simpson's diversity measure at time point 1 was 0.66 (range 0.02 – 0.91), Shannon diversity = 1.98 (range = 0.068 – 2.89), observed OTUs = 47.88 (range = 8 - 73), Pielou's evenness = 0.5 (range = 0.03 – 0.67). The mean Simpson's diversity at time point 2 was 0.32 (range = 0.002 – 0.77), Shannon diversity = 0.85 (range = 0.008 – 1.99), observed OTUs = 24 (range = 2 - 53), Pielou's evenness = 0.26 (range = 0.01 – 0.54). The mean Simpson's diversity at time point 3 was 0.55 (range = 0.004 – 0.92), Shannon diversity = 1.38 (range = 0.016 – 2.88), observed OTUs = 24 (3 - 55), Pielou's evenness = 0.41 (range = 0.01 – 0.72). The mean Simpson's diversity at time point 4 was 0.56 (range = 0.004 – 0.86), Shannon diversity = 1.39 (range = 0.02 – 2.3), observed OTUs = 24.86 (range = 3 - 38), Pielou's evenness = 0.42 (0.01 – 0.67).

In contrast, there were no significant differences seen in changes in alpha diversity between time points in the abscessus control group.

5.10.2.2 Assessment of Subjects Culturing *M. avium*

There were no significant differences in alpha diversity between time points in the *M. avium* treatment group (Figure 5.16).

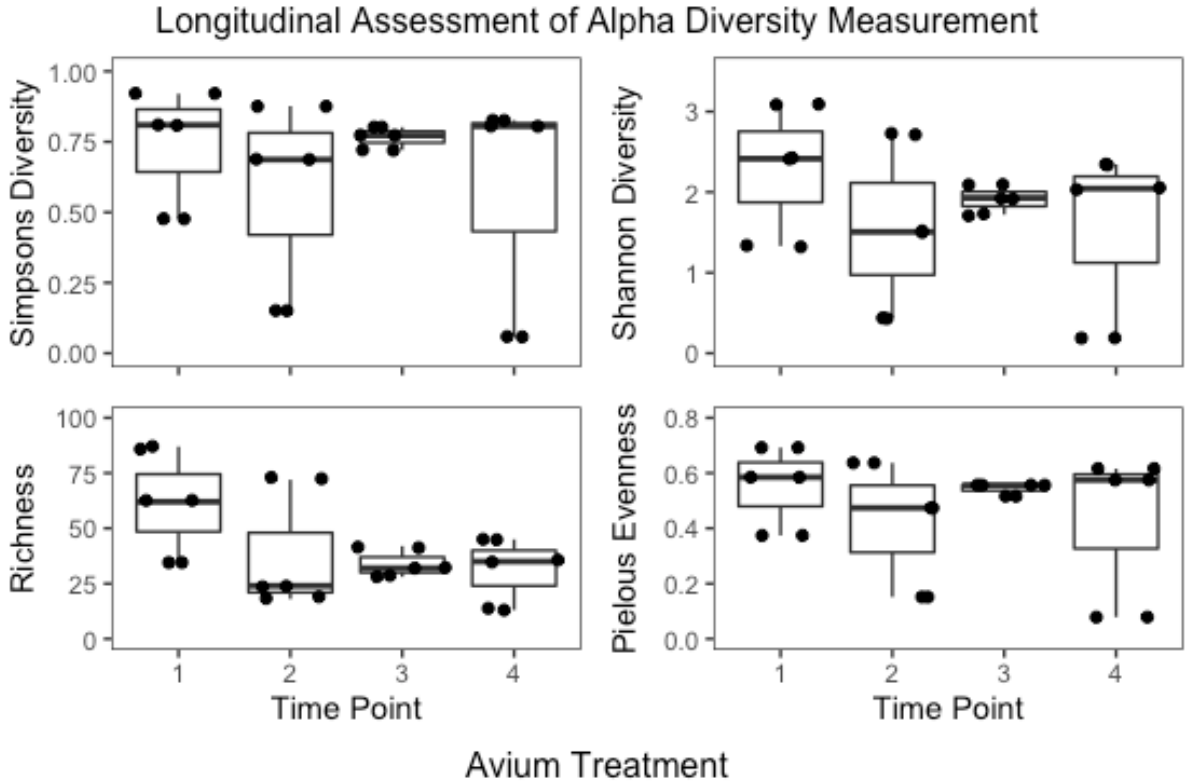


Figure 5.16 – Longitudinal assessment of alpha diversity for patients receiving treatment for *M. avium.* Time point 1 = pre-treatment, time point 2 = 2 weeks of treatment, time point 3 = 1 month of treatment, time point 4 = 3 months of treatment. The mean Simpson's diversity measure at time point 1 was 0.74 (range = 0.48 – 0.92), Shannon diversity = 2.28 (range = 1.33 – 3.09), observed OTUs = 61.33 (range = 35 – 87), Pielou's evenness = 0.55 (range = 0.37 -0.69). The mean Simpson's diversity at time point 2 was 0.57 (range = 0.15 – 0.88), Shannon diversity = 1.56 (range = 0.44 – 2.72), observed OTUs = 38 (range = 18 – 72), Pielou's evenness = 0.42 (range = 0.15 – 0.64). The mean Simpson's diversity at time point 3 was 0.76 (range = 0.72 – 0.8), Shannon diversity = 1.91 (range = 1.72 – 2.08), observed OTUs = 34 (range = 28 – 42), Pielou's evenness = 0.54 (range = 0.52 – 0.56). The mean Simpson's diversity at time point 4 was 0.56 (range = 0.059 – 0.83), Shannon diversity = 1.53 (range = 0.2 – 2.35), observed OTUs = 31 (range = 13 – 45), Pielou's evenness = 0.42 (range = 0.079 – 0.62).

Likewise, there were no significant differences in alpha diversity between time points in the *M. avium* control group (Figure 5.17).

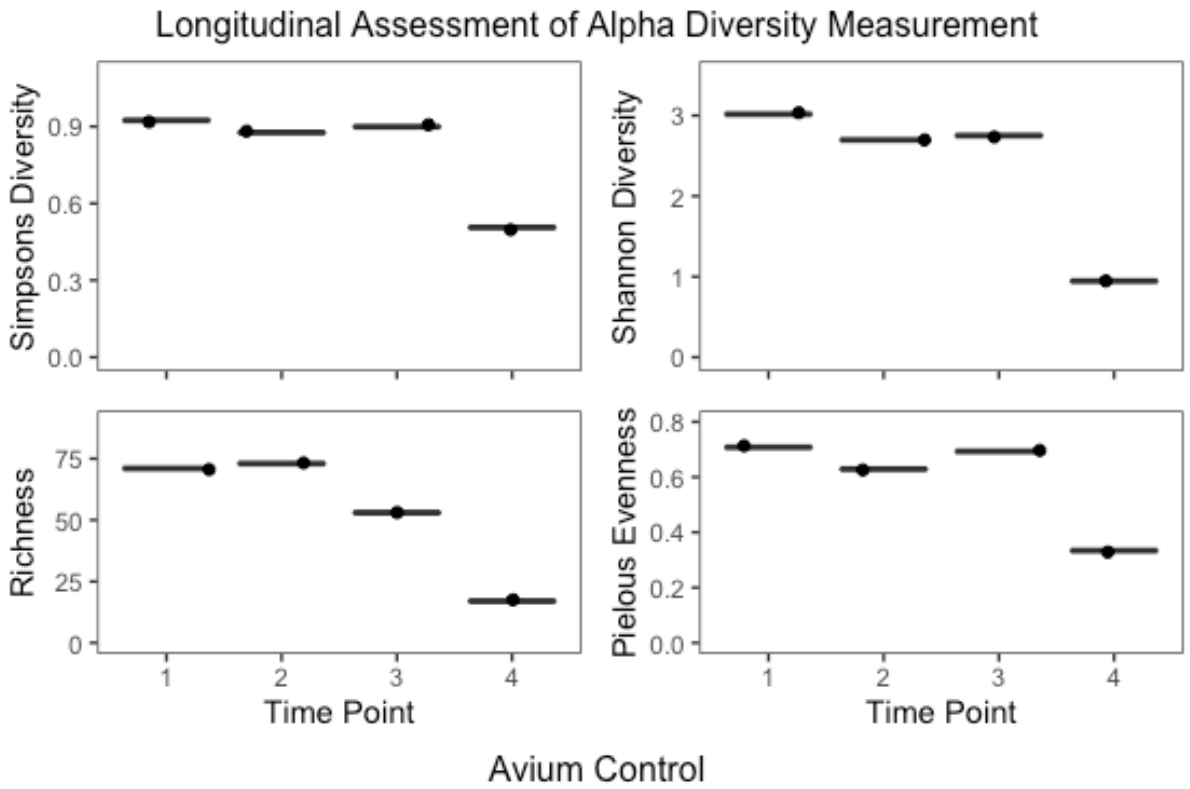


Figure 5.17 – Longitudinal assessment of alpha diversity for the patient culturing, but not on treatment for *M. avium.* Time point 1 = baseline, time point 2 = 2 weeks after baseline, time point 3 = 1 month after baseline, time point 4 = 3 months after baseline. The Simpson's diversity measure for the patient culturing but not on treatment for *M. avium* at time point 1 was 0.92, Shannon's diversity was 3.017, observed OTUs was 71, Pielou's evenness was 0.71. The Simpson's diversity measure at time point 2 was 0.88, Shannon's diversity was 2.7, observed OTUs was 73, Pielou's evenness was 0.63. The Simpson's diversity measure at time point 3 was 0.9, Shannon's diversity was 2.75, observed OTUs was 53, Pielou's evenness was 0.69. The Simpson's diversity measure at time point 4 was 0.51, Shannon's diversity was 0.95, observed OTUs was 17, Pielou's evenness was 0.33.

There were no significant differences in alpha diversity between time points in the control group (Figure 5.18).

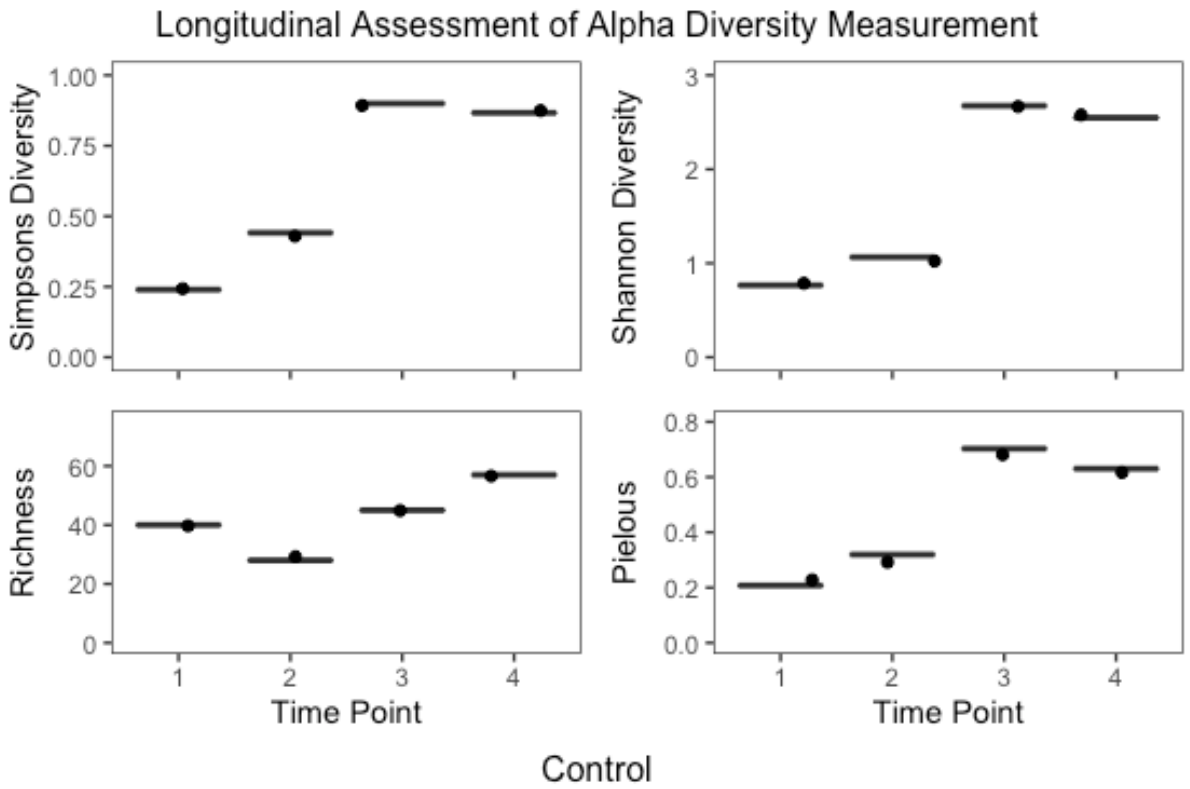


Figure 5.18 – Longitudinal assessment of alpha diversity for the NTM culture naive patient. Time point 1 = baseline, time point 2 = 2 weeks after baseline, time point 3 = 1 month after baseline, time point 4 = 3 months after baseline. The Simpson's diversity measure for the NTM culture naïve patient at time point 1 was 0.24, Shannon's diversity was 0.77, observed OTUs was 40, Pielou's evenness was 0.21. The Simpson's diversity measure at time point 2 was 0.44, Shannon's diversity was 1.06, observed OTUs was 28, Pielou's evenness was 0.32. The Simpson's diversity measure at time point 3 was 0.9, Shannon's diversity was 2.68, observed OTUs was 45, Pielou's evenness was 0.7. The Simpson's diversity measure at time point 4 was 0.87, Shannon's diversity was 2.55, observed OTUs was 57, Pielou's evenness was 0.63.

5.10.3 Differences in Beta Diversity

5.10.3.1 Assessment of Subjects Culturing *M. abscessus*

Beta diversity was assessed using adonis PERMOANOVA using Bray-Curtis distance matrix.

Overall there was no significant difference in beta diversity between time points for any of the three groups: *M. abscessus* treatment group (R^2 = 0.035, *P*-value = 0.41), *M. abscessus* control group (R^2 = 0.1 *P*-value = 0.32), control group (R^2 = 0.53, *P*-value = 0.33) (figure 5.19). For the latter group as only one sample was analysed the mean dissimilarity at each time point could not be determined.

The mean longitudinal Bray-Curtis dissimilarity for patients in the *M. abscessus* treatment group was: time point 1 = 0.82 (range = 0.35 – 1.0), time point 2 = 0.79 (range = 0.11 – 0.99), time point 3 = 0.88 (range = 0.64 – 1.0), time point 4 = 0.89 (range = 0.59 – 1.0).

The mean longitudinal Bray-Curtis dissimilarity for patients in the *M. abscessus* control group was: time point 1 = 0.26 (range = 0.24 – 0.32), time point 2 = 0.31 (range = 0.23 – 0.37), time point 3 = 0.34 (range = 0.18 – 0.48), time point 4 = 0.24 (range = 0.13 – 0.29).

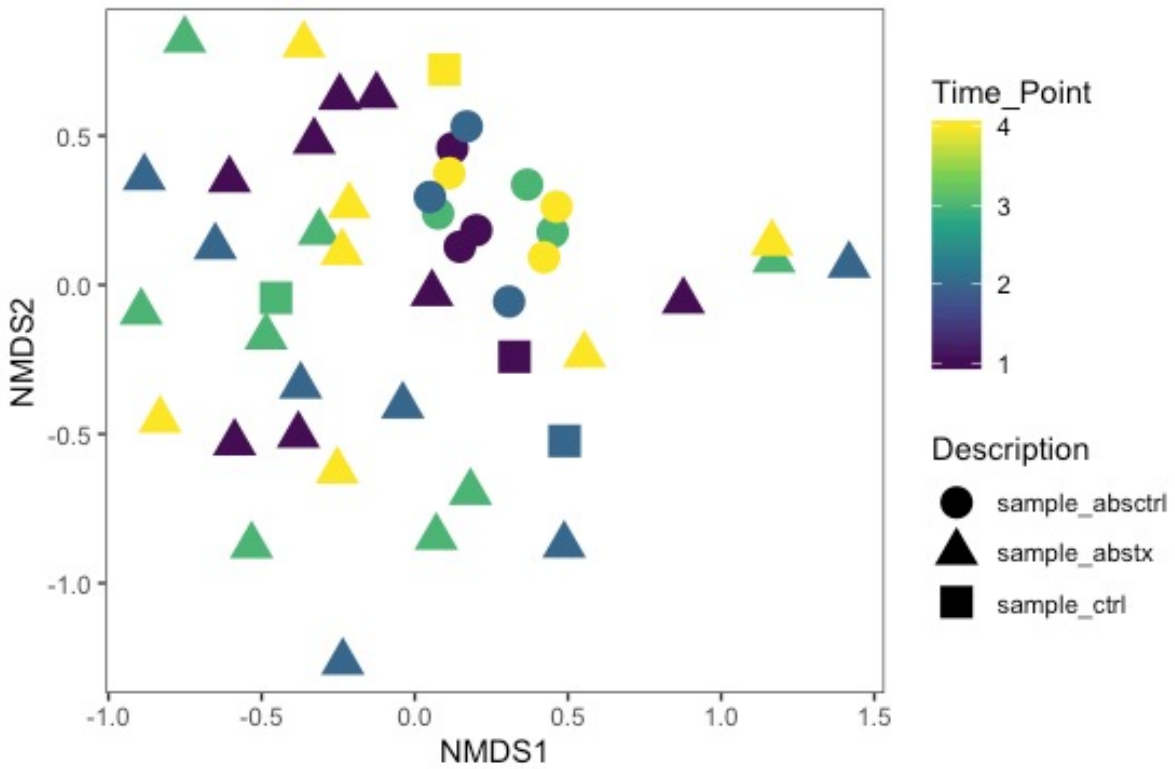


Figure 5.19 - Non-metric multidimensional scaling (NMDS) of samples taken longitudinally from patients: culturing *M. abscessus* but not on treatment (sample_absctrl), culturing *M. abscessus* and on treatment (sample_abstx) and the NTM culture naïve patient (sample_ctrl). There were no significant differences in beta diversity between time points in each group.

5.10.3.2 Assessment of Subjects Culturing *M. avium*

Overall there was no significant difference in beta diversity between time points in the *M. avium* treatment group (R^2 = 0.04, *P*-value = 0.81). The mean longitudinal Bray-Curtis dissimilarity for patients in the *M. avium* treatment group was: time point 1 = 0.7 (range = 0.6 – 0.801), time point 2 = 0.79 (range = 0.58 – 0.95), time point 3 = 0.44 (range = 0.23 – 0.57), time point 4 = 0.84 (range = 0.56 – 0.99).

There was no significant difference in beta diversity between time points in the *M. avium* control group (R^2 = 0.41, *P*-value = 0.33) or the control group (R^2 = 0.53, *P*-value = 0.33) (figure 5.20). As only one sample at each time point was analysed for each of these control groups, the mean dissimilarity at each time point could not be determined.

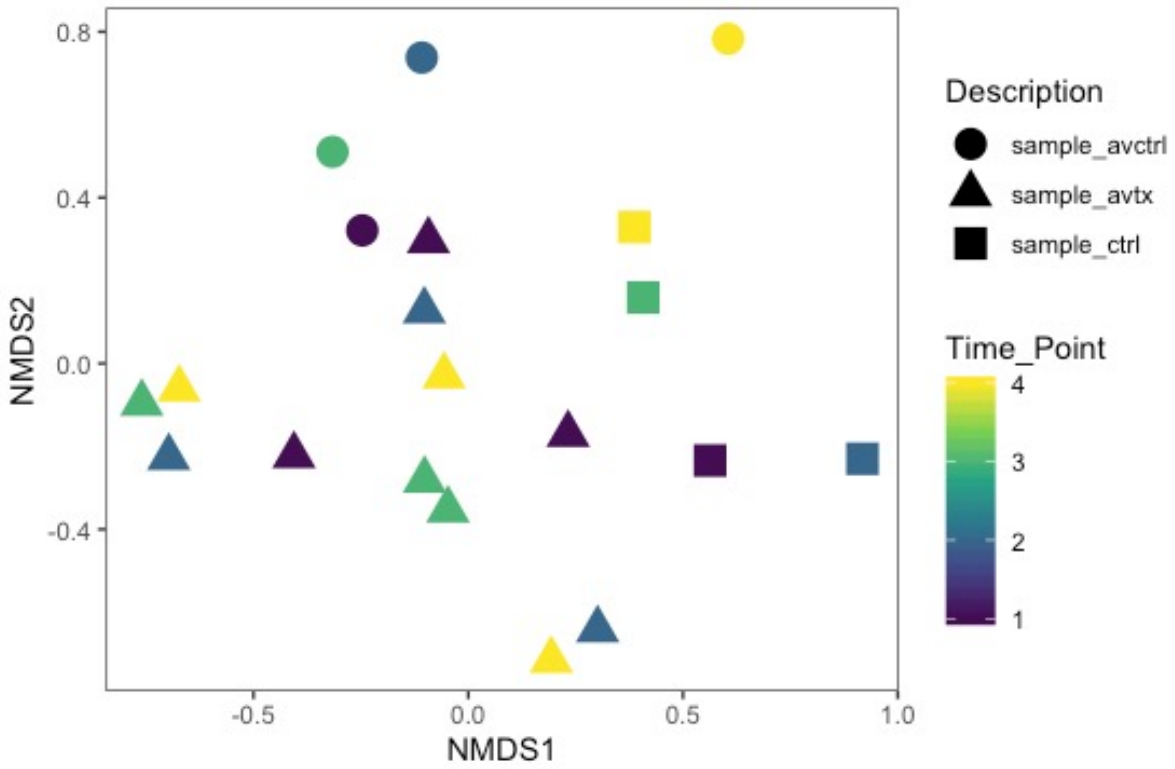


Figure 5.20 - Non-metric multidimensional scaling (NMDS) of samples taken longitudinally from patients: culturing *M. avium* but not on treatment (sample_avctrl), culturing *M. abscessus* and on treatment (sample_avtx) and the NTM culture naïve patient (sample_ctrl). There were no significant differences in beta diversity between time points in each group.

5.10.4 Differences in Specific Bacterial Abundances

As no differences in 16S rRNA gene copy count, alpha or beta diversity measures were observed in the non-treatment groups, changes in OTUs were only analysed in the treatment groups.

5.10.4.1 Assessment of Subjects on treatment for *M. abscessus*

At all 4 time points the *Pseudomonas* genus was amongst the most abundant genera constituting 25.3%, 28.4%, 18.8% and 23.9% of the relative abundances respectively.

Throughout the time points the total abundance of *Pseudomonas* changed very little (range = 1,500 – 2,023 reads). The second most common genus in the pre-treatment group was *Streptococcus* constituting 12.9% of the relative abundance made up of 1,035 reads. The total abundance of *Streptococcus* decreased after 2 weeks of treatment forming just 1.4% of the relative abundance (101 reads) before becoming the most abundant genera at time point 3 (30.3% of the relative abundance) and the second most abundant genera at time point 4 (22.4%).

The genus *Mycobacterium* was the sixth most abundant before the commencement of treatment comprising 315 reads and 3.9% of the relative abundance. After two weeks of treatment this fell to 106 reads (1.5% of the relative abundance). After one month of treatment no reads identified as *Mycobacteria* were identified and this remained the case at time point 4 (Table 5.11).

The most abundant constituent genera of each sample can be seen in Figure 5.21. Samples are arranged first by patient and then by time point.

Table 5.11 – The 15 most common genera seen in samples from patients on treatment for *M. abscessus* at different time points. Time point 1 = pre-initiation of anti-microbial therapy. Time point 2 = 2 weeks of treatment. Time point 3 = 1 month of treatment. Time point 4 = 3 months of treatment. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

	Abscessus Treatment Time Point 1			Abscessus Treatment Time Point 2			Abscessus Treatment Time Point 3			Abscessus Treatment Time Point 4		
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Pseudomonas	0.5	2023	25.3	0.6	1991	28.4	0.7	1500	18.8	0.5	1670	23.9
Streptococcus	0.9	1035	12.9	0.3	101	1.4	0.5	2421	30.3	0.5	1565	22.4
Staphylococcus	0.6	927	11.6	0.7	2592	37	1.2	879	11	0.4	41	0.6
Veillonella	0.7	478	6	0.2	12	0.2	0.2	50	0.6	0.5	367	5.2
Actinomyces	1.3	387	4.8	0.1	24	0.3	0.4	302	3.8	0.7	274	3.9
Mycobacterium	1	315	3.9	0.8	106	1.5	0	0	0	0	0	0
Granulicatella	1.5	312	3.9	0.2	8	0.1	0.5	85	1.1	0.3	41	0.6
Stomatobaculum	1.3	289	3.6	0.1	1	0	0	0	0	0	0	0
Stenotrophomonas	1	288	3.6	0.6	1115	15.9	0.9	66	0.8	0.4	881	12.6
Haemophilus	0.7	255	3.2	0.3	30	0.4	0.1	10	0.1	0.2	16	0.2
Neisseria	0.8	246	3.1	0.1	4	0.1	0.4	110	1.4	0.2	24	0.3
Prevotella	0.6	222	2.8	0.3	13	0.2	0.3	45	0.6	0.4	763	10.9
Rothia	1	155	1.9	0.5	329	4.7	0.7	674	8.4	1	291	4.2
Bifidobacterium	0.6	106	1.3	0.3	68	1	0.6	267	3.3	0.4	199	2.8
Enterococcus	1.6	90	1.1	0.8	22	0.3	1.2	1109	13.9	1	430	6.1

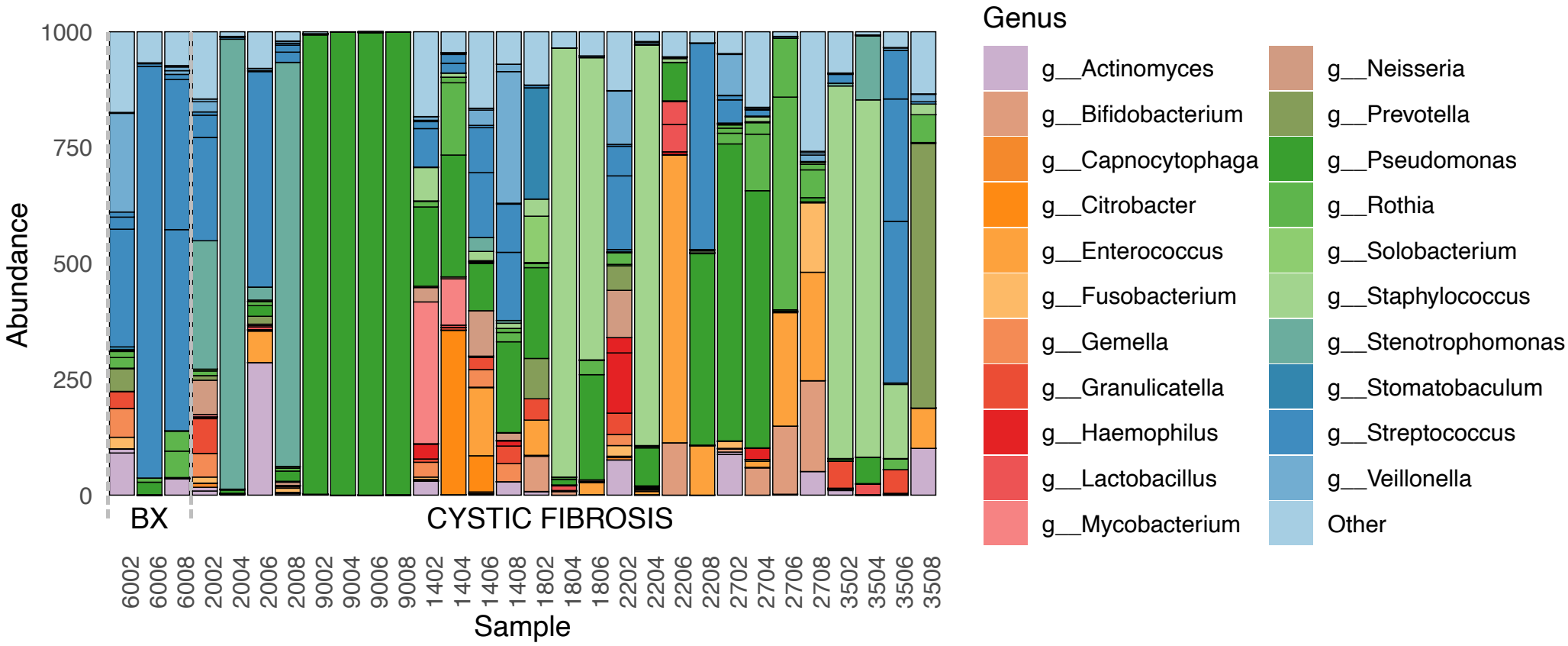


Figure 5.21 – Stacked bar chart showing most abundant genera by patients on treatment for *M. abscessus.* Samples are arranged by underlying disease (BX = bronchiectasis) then by time point.

Similarity percentage (simper) analysis was conducted by comparing samples from the *M. abscessus* treatment group at time points:

1. 1 (pre-initiation of treatment) and 2 (two weeks of treatment)
2. 1 (pre-initiation of treatment) and 3 (one month of treatment)
3. 1 (pre-initiation of treatment) and 4 (three months of treatment)

The OTUs that contributed the most to dissimilarity observed between samples from time points 1 and 2 were: *Staphylococcus_3526* (23.1% of total dissimilarity), *Pseudomonas_2114* (21.2%) and *Stenotrophomonas_2039* (10.8%). Differences between these three OTUs contributed to over 50% of the dissimilarity observed although no significant differences in OTU abundance were observed between the two time points. Of the ten OTUs contributing to over 75% of the dissimilarity between time points, a significant difference in OTU abundance was only observed for: *Veillonella_3368* (*P*-value = 0.03), *Streptococcus_487* (*P*-value = 0.02), *Actinomyces_778* (*P*-value = 0.001) and *Granulicatella_3662* (*P*-value = 0.02). All of these OTUs decreased significantly after the initiation of treatment.

Table 5.12 – Summary of simper analysis between samples from patients on treatment for *M. abscessus* at: **a)** time point 1 (tp1) and time point 2 (tp2) **b)** time point 1 (tp1) and time point 3 (tp3) **c)** time point 1(tp1) and time point 4 (tp4). Taxa = OTU identity. Contribution to Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity, all OTUs together add up to 100 (shown here are OTUs contributing to 75.8% - 81.5% of total dissimilarity); *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between bronchiectasis and cystic fibrosis.

a)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus tp1	Average Abundance Abscessus tp2	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Staphylococcus_3526	0.19	0.2	114.38	368.57	23.1	0.23	23.1	0.35
Pseudomonas_2114	0.18	0.17	250.88	282.14	21.2	0.44	44.3	0.35
Stenotrophomonas_2039	0.09	0.16	35.5	158.57	10.8	0.55	55.1	0.74
Streptococcus_574	0.03	0.04	62.5	5.86	3.7	0.59	58.8	0.35
Veillonella_3368	0.03	0.04	56	0.86	3.3	0.62	62.1	0.03
Streptococcus_487	0.03	0.04	57	3.29	3.4	0.65	65.5	0.02
Citrobacter_1503	0.03	0.06	0	50.86	3	0.69	68.5	0.12
Mycobacterium_949	0.02	0.05	38.88	14.29	3	0.71	71.5	0.62
Actinomyces_778	0.02	0.02	38.88	0.29	2.3	0.74	73.8	0
Granulicatella_3662	0.02	0.01	33.63	1.14	2	0.76	75.8	0.02

b)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus tp1	Average Abundance Abscessus tp3	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.16	0.18	250.88	182.88	18.5	0.18	18.5	0.92
Staphylococcus_3526	0.09	0.14	114.38	105.38	10.8	0.29	29.3	0.87
Streptococcus_574	0.06	0.07	62.5	109.75	7.4	0.45	44.8	0.71
Streptococcus_487	0.04	0.05	57	56.5	5.2	0.57	57.4	0.16
Rothia_1085	0.03	0.07	8.88	60.25	3.8	0.61	61.2	0.2
Veillonella_3368	0.03	0.04	56	4.88	3.3	0.65	64.5	0.05
Stenotrophomonas_2039	0.02	0.04	35.5	8.13	2.4	0.67	66.9	0.43
Mycobacterium_949	0.02	0.05	38.88	0	2.3	0.69	69.2	0.14
Actinomyces_778	0.02	0.02	38.88	0.25	2.3	0.71	71.5	0
Granulicatella_3662	0.02	0.01	33.63	10.38	1.8	0.81	81.5	0.17

c)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus tp1	Average Abundance Abscessus tp4	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.17	0.18	250.88	234.57	20.4	0.2	20.4	0.68
Stenotrophomonas_2039	0.08	0.15	35.5	125.86	9.1	0.29	29.5	0.74
Streptococcus_574	0.07	0.07	62.5	128	8.4	0.38	37.9	0.64
Staphylococcus_3526	0.06	0.13	114.38	5.71	7	0.45	44.9	0.81
Prevotella_2474	0.05	0.09	25.25	82	6	0.51	50.9	0.33
Veillonella_3368	0.04	0.05	56	46.57	4.8	0.56	55.7	0.68
Streptococcus_467	0.03	0.08	3.38	63.57	3.8	0.6	59.5	0.86
Streptococcus_487	0.03	0.04	57	24.86	3.9	0.63	63.4	0.1
Actinomyces_778	0.02	0.02	38.88	24.14	2.5	0.7	69.6	0.25
Mycobacterium_949	0.02	0.05	38.88	0	2.3	0.72	71.9	0.17
Granulicatella_3662	0.02	0.01	33.63	5.86	1.92	0.76	76	0.08

5.10.4.2 Assessment of Subjects on treatment for *M. avium*

Streptococcus was found to be the most abundant genus for three of the four time points. After three months of treatment, after *Pseudomonas* (32.6%) *Streptococcus* became the second most abundant genus (relative abundance of 27.8%). Overall, the total abundance of *Streptococcus* did not change considerably during the four time points (range = 833 – 1,331 reads). In contrast the total abundance of *Pseudomonas* increased conspicuously between time point 1 and 2 (396 to 1,032 reads) and remained high becoming the most abundant genus at time point 4. Interestingly the total abundance of *Granulicatella* mirrored that of *Pseudomonas* with the total abundance increasing steadily from 113to 384 reads after 3 months of treatment, becoming the third most abundant genera.

Reads assigned to the genus *Mycobacterium* were not identified at any time point in the *M. avium* treatment group.

Table 5.13 – The most common genera seen in samples from: patients on treatment for *M. avium* at different time points. Time point 1 = pre-initiation of anti-microbial therapy. Time point 2 = 2 weeks of treatment. Time point 3 = 1 month of treatment. Time point 4 = 3 months of treatment. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

	Avium Treatment Time Pont 1			Avium Treatment Time Pont 2			Avium Treatment Time Pont 3			Avium Treatment Time Pont 4		
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Streptococcus	0.49	1234	41.13	0.36	1055	35.17	0.45	1331	44.37	0.29	833	27.77
Pseudomonas	0.3	396	13.2	0.43	1032	34.4	0.3	672	22.4	0.13	978	32.6
Haemophilus	0.63	243	8.1	0.25	75	2.5	0.04	1	0.03	0.29	86	2.87
Neisseria	0.63	234	7.8	0.25	47	1.57	0.5	180	6	0.25	16	0.53
Veillonella	0.33	193	6.43	0.43	240	8	0.5	216	7.2	0.23	286	9.53
Granulicatella	0.54	113	3.77	0.46	155	5.17	0.46	213	7.1	0.69	384	12.8
Actinomyces	0.67	101	3.37	0.13	42	1.4	0.17	8	0.27	0.17	44	1.47
Gemella	1	93	3.1	0.43	114	3.8	0.71	163	5.43	0.43	48	1.6
Leptotrichia	0.67	62	2.07	0.25	6	0.2	0.08	1	0.03	0.25	11	0.37
Rothia	0.35	59	1.97	0.26	85	2.83	0.35	162	5.4	0.17	80	2.67
Capnocytophaga	0.67	45	1.5	0.22	11	0.37	0	0	0	0.22	29	0.97
Prevotella	0.31	41	1.37	0.19	38	1.27	0.08	22	0.73	0.12	66	2.2
Fusobacterium	0.5	40	1.33	0.25	6	0.2	0.25	2	0.07	0.5	46	1.53
Selenomonas	0.4	22	0.73	0.25	6	0.2	0.2	8	0.27	0.15	7	0.23
Parvimonas	1.5	20	0.67	0	0	0	0	0	0	0	0	0

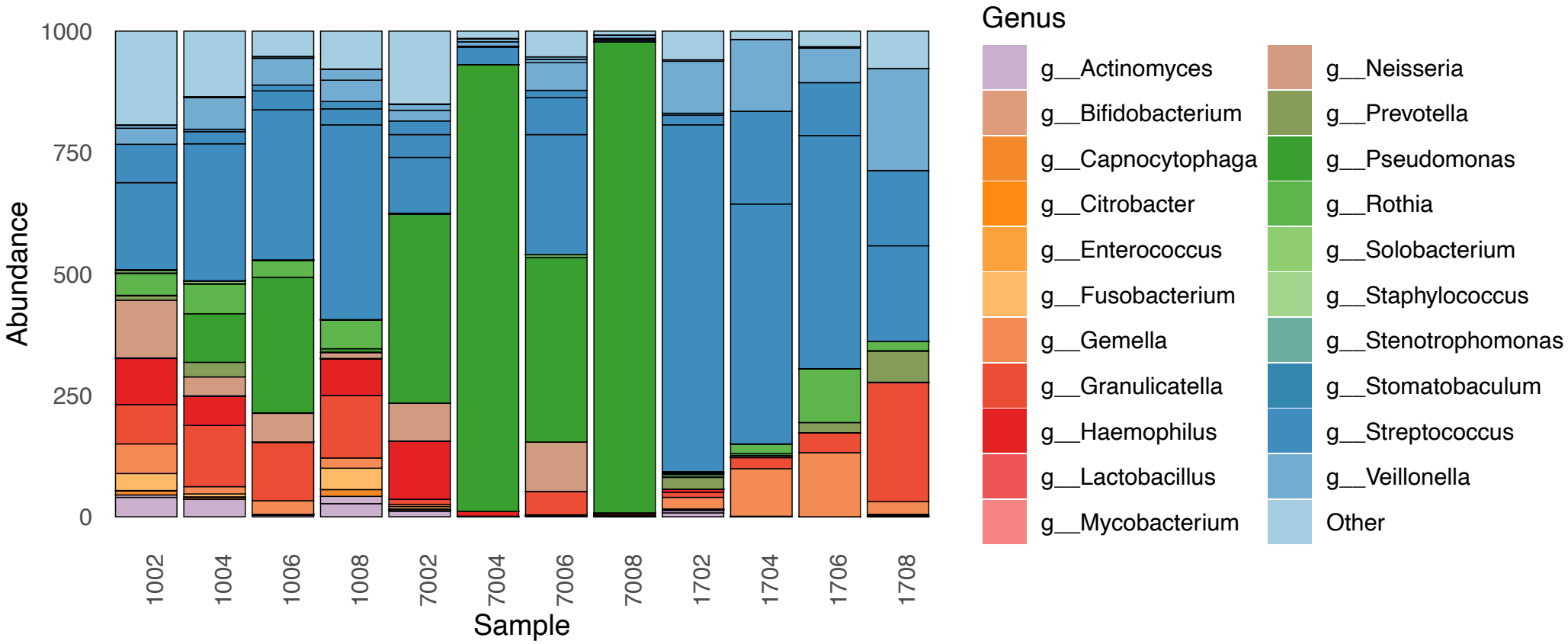


Figure 5.22 – Stacked bar chart showing most common genera by patients on treatment for *M. avium.* All samples in this group are derived from patients with cystic fibrosis.

The *Pseudomonas_2114* and *Streptococcus_574* OTUs contributed the most to the dissimilarities between samples from all time points. Between time point 1 and 2 *Pseudomonas_2114* contributed to 28.3% of the dissimilarity. The average sample abundance of *Pseudomonas_2114* increased from 130.3 at time point 1 to 341.3 reads at time point 2 although this change was not significant (*P*-value = 0.17). Between time points 1 and 3 the average abundance of *Streptococcus_574* and *Pseudomonas_2114* both increased (336 to 345.3 in the case of *Streptococcus_574* and 130.3 to 219.7 in the case of *Pseudomonas_2114*. Once again, these changes were not found to be significant. One OTU, the decrease in which was found to be significant, was *Actinomyces_778* (*P*-value = 0.05). This OTU contributed to 1.45% of the overall dissimilarity between time points 1 and 3. Between time points 1 and 4 there were no significant changes in OTUs and *Pseudomonas_2114* and *Streptococcus_574* contributed the most to dissimilarity between sample time points.

Table 5.14 – Summary of simper analysis between samples from patients on treatment for MAC at: **a)** time point 1 (tp1) and time point 2 (tp2) **b)** time point 1 (tp1) and time point 3 (tp3) **c)** time point 1(tp1) and time point 4 (tp4). Taxa = OTU identity. Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity, all OTUs together add up to 100 (shown here are OTUs contributing to 80.5% - 84.7% of total dissimilarity); *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between bronchiectasis and cystic fibrosis.

a)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Avium tp1	Average Abundance Avium tp2	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.18	0.18	130.33	341.33	28.3	0.28	28.3	0.17
Streptococcus_574	0.14	0.1	336	270.67	21.9	0.5	50.2	0.83
Streptococcus_487	0.04	0.03	42.33	72	5.5	0.56	55.7	0.83
Haemophilus_1826	0.03	0.02	71.67	23.33	5	0.61	60.7	0.37
Neisseria_1599	0.03	0.02	65.67	14	4.8	0.65	65.5	0.38
Veillonella_3368	0.03	0.02	54	74	4.6	0.7	70.1	0.83
Granulicatella_3662	0.03	0.02	34	50	4	0.74	74.1	0.83
Gemella_152	0.02	0.02	29.67	37.67	3.2	0.77	77.3	0.83
Rothia_1085	0.01	0.01	16	20.33	2	0.79	79.3	0.51
Actinomyces_778	0.01	0.01	16	12	1.4	0.81	80.7	0.27

b)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Avium tp1	Average Abundance Avium tp3	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Streptococcus_574	0.13	0.07	336	345.33	24	0.24	24	0.51
Pseudomonas_2114	0.1	0.09	130.33	219.67	19.1	0.43	43.1	0.82
Haemophilus_1826	0.04	0.03	71.67	0.33	19.1	0.5	49.8	0.25
Neisseria_1599	0.03	0.02	65.67	53.67	5	0.55	54.8	0.66
Granulicatella_3662	0.03	0.02	34	69.67	5.2	0.6	60	0.28
Gemella_152	0.03	0.02	29.67	53.33	4.5	0.64	64.5	0.83
Streptococcus_487	0.02	0.01	42.33	74.67	3.9	0.68	68.4	0.28
Rothia_1077	0.02	0.03	2	37	3.6	0.72	72	0.82
Veillonella_3368	0.02	0.01	54	61	3.5	0.76	75.5	0.51
Rothia_1085	0.01	0.01	16	13.67	1.8	0.77	77.3	0.83
Actinomyces_778	0.01	0.01	16	0.67	1.45	0.8	80.5	0.05

c)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Avium tp1	Average Abundance Avium tp4	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.18	0.2	130.33	326	27.2	0.27	27.2	0.49
Streptococcus_574	0.14	0.11	336	200.33	20.1	0.47	47.3	0.83
Granulicatella_3662	0.06	0.04	34	125.33	8.4	0.56	55.7	0.51
Veillonella_3368	0.04	0.03	54	87	5.8	0.62	61.5	0.83
Neisseria_1599	0.03	0.02	65.67	4.33	4.8	0.66	66.3	0.38
Haemophilus_1826	0.03	0.02	71.67	26.33	4.6	0.71	70.9	0.38
Streptococcus_487	0.03	0.02	42.33	63	4.4	0.75	75.3	0.83
Rothia_1085	0.01	0.01	16	19.67	1.8	0.77	77.1	0.51
Gemella_152	0.01	0.01	29.67	15.67	1.7	0.81	80.7	0.51
Actinomyces_778	0.01	0.01	16	9	1.28	0.85	84.7	0.27

5.10.5 Analysis of Patients with Recurrent Disease

Over the study period, all patients on treatment for *M. abscessus* became NTM culture negative at 3 months. Four of these patients however subsequently became culture positive after 3 months (Patients 2, 18, 22 and 35). These patients exhibited side effects to first line treatment agents and therefore omitted at least one agent for a period of up to two weeks. Another possibility however is that patients with resurgent disease exhibited a different microbiological make up that may have conferred some degree of treatment resistance and with this in mind, Patients 2, 18, 22 and 35 were considered independently of the *M. abscessus* treatment group (see Table 5.14).

Before the commencement of treatment, *Pseudomonas* was less abundant in these samples than in the whole *M. abscessus* treatment group(relative abundances of 6.3% vs 25.3% respectively) and remained relatively consistent during treatment (4.1%, 9.0%, 15.1% at other time points).

Table 5.15 - The most common genera seen in samples from patients with resurgent *M. abscessus* at different time points. Time point 1 = pre-initiation of anti-microbial therapy. Time point 2 = 2 weeks of treatment. Time point 3 = 1 month of treatment. Time point 4 = 3 months of treatment. Total abundance is the total number of OTUs in the group that belong to that genus. Relative abundance is the percentage contribution of each genera to the total ecological makeup of the group.

	Treatment Failure Time Pont 1			Treatment Failure Time Pont 2			Treatment Failure Time Pont 3			Treatment Failure Time Pont 4		
Genus	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**	**Mean Prevalence**	**Total Abundance**	**Relative Abundance**
Staphylococcus	0.4	853	26	0.5	2573	65.8	0.9	858	21.9	0.2	29	1
Streptococcus	0.3	557	17	0.1	28	0.7	0.3	1222	31.1	0.1	502	17.1
Stenotrophomonas	0.9	287	8.7	0.3	1109	28.4	0.6	36	0.9	0.1	875	29.7
Stomatobaculum	0.8	257	7.8	0	0	0	0	0	0	0	0	0
Granulicatella	1.1	266	8.1	0	2	0.1	0.2	54	1.4	0.1	1	0
Pseudomonas	0.2	206	6.3	0.1	159	4.1	0.3	353	9	0.1	443	15.1
Prevotella	0.3	168	5.1	0.1	3	0.1	0.2	42	1.1	0.1	583	19.8
Neisseria	0.4	213	6.5	0	0	0	0.2	8	0.2	0.1	7	0.2
Solobacterium	1	102	3.1	0	1	0	1	1	0	0	0	0
Bifidobacterium	0.3	97	3	0.1	9	0.2	0.1	116	3	0	0	0
Enterococcus	0.8	85	2.6	0.4	9	0.2	0.6	717	18.3	0.4	195	6.6
Actinomyces	0.5	123	3.7	0	2	0.1	0.2	295	7.5	0.2	115	3.9
Rothia	0.3	55	1.7	0.2	14	0.4	0.3	69	1.8	0.3	74	2.5
Lachnoanaerobaculum	0.4	8	0.2	0	2	0.1	0.1	154	3.9	0.4	118	4
Mycobacterium	0.4	6	0.2	0	0	0	0	0	0	0	0	0

Simper analysis was completed (Table 5.15) comparing OTUs in the treatment failure group (Patients 2, 18, 22 and 35) and those in the treatment success group (Patients 6, 9, 14, 27). *Pseudomonas-2114* OTU was significantly greater in the treatment success group than in the failure group at time point 1. No other OTUs were significantly different (*P*-value = 0.02). This difference remained significant at time point 2 (*P*-value = 0.03). *Pseudomonas-2114* OTU remained greater in the success group at time points 3 and 4 although this difference was not significant.

Table 5.16 – Summary of simper analysis between samples from patients treated unsuccessfully (Tx failure) and successfully (Tx success) for *M. abscessus* at: **a)** time point 1 (tp1) **b)** time point 2 (tp2) **c)** time point 3 (tp3) **d)** time point 4 (tp4). Taxa = OTU identity. Contribution to Average dissimilarity = OTU contribution to average between-group dissimilarity; average abundance = average abundance of each OTU per sample; % contribution to dissimilarity = degree to which each OTU contributes to total Bray Curtis dissimilarity; % cumulative contribution = degree to which each OTU in turn contributes to total Bray Curtis dissimilarity, all OTUs together add up to 100 (shown here are OTUs contributing to 80.5% - 84.7% of total dissimilarity); *P*-value = significance testing by Kruskal – Wallis rank sum test to establish if a difference in OTU abundance exists between treatment failure and treatment success groups.

a)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus Tx Failure tp1	Average Abundance Abscessus Tx Success tp1	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.21	0.19	50.75	451	26	0.26	26	0.02
Staphylococcus_3526	0.11	0.17	210.25	18.5	13	0.39	39	1
Streptococcus_574	0.05	0.04	95.5	29.5	5	0.44	44	0.77
Veillonella_3368	0.04	0.04	34.75	77.25	5	0.49	49	0.67
Mycobacterium_949	0.04	0.07	1.25	76.5	5	0.54	54	0.85

b)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus Tx Failure tp2	Average Abundance Abscessus Tx Success tp2	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Staphylococcus_3526	0.32	0.19	640.25	6.33	34	0.34	34	0.21
Pseudomonas_2114	0.28	0.16	39.5	605.67	29	0.63	63	0.03
Stenotrophomonas_2039	0.14	0.21	277.25	0.33	15	0.78	78	0.44
Citrobacter_1503	0.06	0.09	0	118.67	6	0.84	84	0.08
Rothia_1165	0.03	0.04	2	60.33	4	0.88	88	0.37

c)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus Tx Failure tp3	Average Abundance Abscessus Tx Success tp3	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.14	0.19	83.25	282.5	17	0.17	17	0.56
Streptococcus_467	0.12	0.19	26.75	223.25	13	0.3	30	0.76
Staphylococcus_3526	0.1	0.14	205.75	5	12	0.42	42	0.08
Enterococcus_90	0.1	0.1	179.25	98	12	0.54	54	0.56
Streptococcus_574	0.09	0.09	183	36.5	10	0.64	64	0.56

d)

Taxa	Contribution to Average Dissimilarity	Standard Deviation of Contribution	Average Abundance Abscessus Tx Failure tp4	Average Abundance Abscessus Tx Success tp4	% Contribution to Dissimilarity	Cumulative	% cumulative contribution	*P*-value
Pseudomonas_2114	0.1665417	0.1785	145.6667	301.25	19.3	0.1939	19.4	0.85
Streptococcus_467	0.117875	0.18788	26.75	223.25	10.7	0.3013	30.1	0.71
Staphylococcus_3526	0.102187	0.13561	205.75	5	11.8	0.4189	41.9	0.2
Enterococcus_90	0.102125	0.10493	179.25	98	11.7	0.536	53.6	0.27
Streptococcus_487	0.050062	0.069843	87.25	25.75	5.8	0.7668	76.7	1

5.11 Discussion

The healthy lung microbiome is a rich and diverse structure whose composition is altered by disease. It is unclear, however, the extent to which the altered microbiome itself can drive disease and therefore contributes directly to morbidity and mortality. It has been shown in cystic fibrosis patients that the airway microbiome evolves with increased age becoming less even and less diverse. These changes translate to a shift in airway microbiota with airways becoming dominated by Proteobacteria and characterised by a loss of pulmonary function [239-241]. The bacteria that come to dominate these airways are frequently implicated as infective agents associated with morbidity and declining pulmonary function that results in increased mortality [242, 243]. Non-Tuberculous Mycobacteria have been implicated as infective agents causing premature deterioration in pulmonary function [92, 244, 245] and hence their eradication is considered necessary when deemed as infective organisms.

Limited data is available however comparing the microbiome of patients culturing NTM and those that do not [158, 185] and no studies have looked at ecological differences between patients deemed as NTM colonised and those with NTM disease. Very few studies have focussed directly at the longitudinal effect antibiotic therapy has on the lung microbiome [246] and there have been no studies to date examining the effects prolonged antibiotic therapy has on the NTM affected lung. These deficiencies in knowledge are important to address in order to aid in the understanding of how NTM develop from being colonising agents into pathological entities and the effect this has on the airway microbiome. By understanding how the microbiome evolves with antibiotic therapy, we can see how dominant genera may be affected and replaced by other potentially troublesome bacteria.

The present study followed a total of 16 patients with background diseases of non-cystic fibrosis induced bronchiectasis (n=5) and cystic fibrosis (n=11) over the course of 3 months with patients providing samples at: baseline, 2 weeks, 1 month and 3 months.

Each of the 16 patients studied fell into one of five categories:

1. Treatment for *M. abscessus* (n=8).

2. Culture positive but not on treatment for *M. abscessus* (n=3).
3. Treatment for *M. avium* (n=3).
4. Culture positive but not on treatment for *M. avium* (n=1).
5. Mycobacterial culture naïve (n=1).

Consideration of disease status, time points and categories and the study findings are discussed below.

5.11.1 Differences Between Bronchiectasis and Cystic Fibrosis

To compare ecological differences between the microbiome in bronchiectasis and cystic fibrosis, samples at time point 1 (pre-initiation of treatment) were divided according to underlying disease and compared.

5.11.1.1 Differences in 16S rRNA Quantitative PCR Copy Number

The study found no significant difference in bacterial burden between patients with bronchiectasis and cystic fibrosis (*P*-value = 0.47). The median 16S copy number was lower in patients with bronchiectasis (bronchiectasis median= 3.4×10^9 copies/g sputum versus cystic fibrosis median = 5.14×10^9 copies/g sputum).

Within the bronchiectasis cohort, the 16S rRNA qPCR copy number was found to be higher than other studies examining treatment naïve patients with bronchiectasis [247]. The study by Cox *et al.* reported a median copy number of $2{\cdot}2 \times 10^8$ per ml of sputum at baseline (IQR $4{\cdot}8 \times 10^7$–$8{\cdot}6 \times 10^8$) with baseline defined as the start of a longitudinal observational study where patients did not have an infection. As indicated however, the bacterial burden in the Cox *et al.* study was measured as gene copy number per ml of sputum. In contrast, the present study measured by gram of sputum thereby making direct comparison difficult. These factors may therefore account for our observation of a higher bacterial burden in both groups.

Twomey *et al*. [248] examined and compared the microbiota abundant in sputum samples from adult cystic fibrosis patients presenting with exacerbation and those defined as having stable disease. The group found no significant difference in copy number between cohorts (approximate median copy number per ml = 1×10^9). The

finding of similar 16S rRNA qPCR copy number between stable disease and those with exacerbations mirrored the present study as did the median copy number although note should be made that calculations were once again performed per ml sputum making direct comparison between studies difficult.

5.11.1.2 Differences in Diversity

Before the initiation of treatment, patients with cystic fibrosis demonstrated lower levels of alpha diversity than patients with bronchiectasis although this difference was not statistically significant (Section 5.9.2). There was no significant difference in beta diversity between the groups (Section 5.9.3). Similarly, there were no significant differences in individual OTUs between the two groups.

At the time of writing there has been only one published study directly comparing the microbial diversity of lungs of patients with non CF bronchiectasis and cystic fibrosis [249]. The Maughan *et al*. study suffered from a relatively low proportion of reads in its sampling cohort although this was reflected in both the cohorts studied, cystic fibrosis and bronchiectasis, suggesting no bias. The study found no differences in either alpha or beta diversity metrics between the two groups. Like other studies analysing the microbiome in suppurative airways disease, Proteobacteria were found to dominate the airways with *Pseudomonas* being the most prevalent genus [247]. Other studies have noted existence of a small subset of patients which are dominated by the phylum Firmicutes e.g. *Veillonella* [233, 250, 251].

This present study supports the data found by Maughan *et al*. and suggests that there is no difference in diversity measures between patients with cystic fibrosis and those with bronchiectasis. It should be noted however that the present study incorporated other variables that concerned the presence or absence of NTM species as well as whether these species required treatment. Incorporating this number of variables together with relatively small patient numbers may impact and limit the level of interpretation possible.

5.11.2 Exploring Differences Between NTM Treatment and NTM Control Groups

There is an important distinction to be made between patients who culture NTM and require treatment versus those who do not. Distinguishing between these groups of patients is aided by guidelines produced by the American and British Thoracic societies and relies upon symptomatic or radiological evidence of disease in addition to microbiological confirmation [2, 95]. An extensive literature search as of November 2020 revealed no prior studies directly comparing the microbiome of patients deemed to require treatment for NTM and those who simply culture the bacteria. The present study is therefore the first to have examined this. It should be noted that sample numbers in this present study are limited (see Section 5.11.6) and therefore cautious interpretation is required.

5.11.2.1 Differences in 16S rRNA Quantitative PCR Copy Number

In this present study it was found that bacterial burden was higher in the *M. abscessus* treatment group compared to the *M. abscessus* colonised group and the NTM culture negative group (median pre-treatment 16S copy number = 5.5×10^9 copies/g sputum vs 5.14×10^9 vs 2.29×10^8 respectively). The difference in bacterial abundance between *M. abscessus* treatment group and NTM culture negative patients was found to be statistically significant by Kruskall-Wallis rank sum analysis (*P*-value=0.05).

In the *M. avium* analysis, bacterial burden was again found to be higher in patients culturing NTM. The 16S copy number in the *M. avium* control group at time point 1 was 3.6×10^{10} copies/g sputum compared to a median of 3.4×10^9 in the *M. avium* treatment group and 2.29×10^8 in the culture negative group. The difference between the *M. avium* control group and the culture negative group was found to be statistically significant (*P*-value=0.04).

Other studies that have looked at bacterial abundance in patients receiving NTM treatment and compared those subjects to controls (NTM culture naïve patients) found that bacterial burden was lower in the NTM treatment group [207] although the Cowman *et al.* study compared patients who had already been initiated on anti-microbial therapy and therefore is not directly comparable to the present study.

5.11.2.2 Differences in Diversity

Patients that cultured NTM (both *M. avium* and *M. abscessus*) were found to have a greater alpha diversity than those in the NTM culture negative group although these differences were not statistically significant. Within the *M. avium* subgroup, patients requiring treatment were found to have a lower alpha diversity than those who did not require treatment. The reverse was found in the *M. abscessus* group where patients who went on to receive treatment had a higher alpha diversity than those who did not.

No significant differences were found in beta diversity between the three groups: *M. abscessus* treatment, *M. abscessus* control and the NTM culture negative. The mean Bray-Curtis dissimilarity across all pairs of samples was lower for *M. abscessus* control patients (0.26) compared to the *M. abscessus* treatment group (0.82).

No significant differences were found in beta diversity between: *M. avium* treatment group, *M. avium* control group and the NTM culture negative group. The mean Bray-Curtis dissimilarity across all pairs of samples was 0.7 for the avium treatment group. These results are mirrored by other studies examining the microbiome in NTM positive and negative cases where no significant differences in diversity were noted [158, 207].

5.11.2.3 Differences in Specific Bacterial Abundances

The *M. abscessus* treatment, *M. abscessus* control and control groups were all dominated by Proteobacteria. These findings were in keeping with previous studies [231, 232]. Although being the most abundant genera, *Pseudomonas* was more abundant in the non–treatment group (average sample abundance = 250.9 reads for *M. abscessus* treatment, 676.3 for *M. abscessus* control, 872 for control groups). As a result, the *Pseudomonas* genera was found to contribute more to the relative abundance in the treatment naïve groups than to the treatment group (87.2% in the NTM naïve group, 68% in the *M. abscessus* control group and 25.3% in the treatment group). It is also of note that *Mycobacterium* genera was only seen in the treatment group and comprised 3.9% of the relative abundance between generas. No significant differences were found between specific OTUs between the *M. abscessus* treatment, *M. abscessus* control and control groups.

Amongst patients culturing *M. avium* the dominant phylum was Firmicutes. In contrast for patients culturing *M. abscessus, Streptococcus* and *Veillonella* were the most abundant genera. In the treatment group *Streptococcus* was the most abundant with *Pseudomonas* only contributing to 13.2% of the relative abundance. Once again, no significant differences were found between specific OTUs between the *M. abscessus* treatment, *M. abscessus* control and control groups.

Previously published literature has found that the genus *Stenotrophomonas* is significantly more abundant in cases of NTM disease [207]. This observation is not entirely surprising however when it is considered that Cowman *et al.* looked at patients already on treatment for NTM and compared the microbiome to treatment naïve patients, with *Stenotrophomonas* frequently observed after treatment.

5.11.3 Clinical Outcomes in NTM

The present study has shown that bacterial abundance is positively correlated with lung function as measured by FEV1 %predicted and FVC %predicted. These correlations reached statistical significance (Pearson's, *P*-value = 0.025 and 0.032 respectively). These findings were echoed in alpha diversity measures where a positive correlation between lung function and alpha diversity measures was noted.

These findings correlate with previous studies assessing the relationship between airway bacterial load and lung function that have repeatedly found lower bacterial load and diversity to be associated with more severe disease [252-257].

5.11.4 Longitudinal Assessment of Patients Culturing *M. abscessus*

The treatment of *M. abscessus* involves an initial intensive phase of intravenous and oral therapy consisting of at least four antibiotics over two weeks. This is followed by a continuation phase of nebulised and oral therapy consisting of at least three antibiotics. In this study each patient provided sputum at: baseline, after 2 weeks, after 1 month and after 3 months of treatment.

Patients receiving treatment for *M. abscessus* exhibited an initial fall in total bacterial DNA copy number after the initial 2 weeks of treatment from a mean abundance of

$1.07x10^{10}$ copies/g sputum to $1.27x10^{9}$ copies/g sputum. This change was found to be significant (*P*-value=0.0014). These changes were mirrored by changes in alpha diversity with all measures of alpha diversity decreasing significantly from time point 1 to 2. This means that with the initiation of antibiotic therapy, the total number of bacterial species making up the lung microbiome decreased mirroring a fall in the total bacterial abundance.

After week 2, both bacterial abundance and alpha diversity measures increased towards pre-treatment levels. After 3 months (time point 4) of anti-microbial therapy, the mean 16S copy number had increased to $2.13x10^{9}$. The difference between bacterial abundance at time points 2 and 4 was found to be statistically significant (*P*-value=0.019).

Analysis of specific generas, during treatment for *M. abscessus*, revealed a preservation of *Pseudomonas* throughout all time points. The total and relative abundances of *Pseudomonas* was maintained throughout treatment (relative abundance = 25.3%, 28.4%, 18.8% and 23.9% at each consecutive time point). The total and relative abundances of *Staphylococcus* fluctuated more throughout treatment. The relative abundance at time point 1 was 11.6%. This increased to 37% at time point 2 before decreasing to 11% and finally 0.6%. This suggests that during the initial intensive treatment regimen *Staphylococcus* was able to establish a resistant niche and this resulted in an increase in total abundance from 927 to 2,592 reads. Upon initiation of the continuation of the antibiotic regimen the total abundance fell to 879 and finally 41 reads. *Streptococcus* followed the opposite pattern to that of *Staphylococcus*, with the total abundance falling from 1,035 reads at time point 1 to 101 at time point 2 before re-establishing itself (2421 and 1565 reads at time points 3 and 4 respectively).

Changes in the average abundance of *Streptococcus_487* OTU were found to be significant between time point 1 and 2 (*P*-value=0.02). Changes in *Veillonella_3368* and *Actinomyces_778* reflected those of *Streptococcus* exhibiting a statistically significant drop in abundance between time points 1 and 2. These differences remained significant at time point 3. Changes in these genera accounted for over 60% of the cumulative bacterial abundance at time point 1. Of note is the genus

Mycobacteria. The total abundance of *Mycobacteria* at time point 1 was 315 reads. This decreased to 106 at time point 2 and then to 0 at time points 3 and 4.

5.11.5 Longitudinal Assessment of Patients Culturing *M. avium*

The treatment of the *Mycobacterium avium* complex involves the administration of three oral antibiotics: Rifampicin, Ethambutol and a macrolide such as Azithromycin or Clarithromycin, impact of treatment was therefore investigated. As for other groups within the study, patients provided sputum at: baseline, after 2 weeks, after 1 month and after 3 months.

In contrast to patients receiving treatment for *M. abscessus*, the study failed to find a significant difference in bacterial burden or alpha diversity amongst patients receiving treatment for *M. avium* between the first two time points.

There are two possibilities that may account for the differences between *M. abscessus* and *M. avium.* The first concerns the use of intravenous antibiotics in the initiation phase of *M. abscessus* treatment, something that is not emulated in the treatment of MAC. By using intravenous therapy, the pharmacokinetics of treatment differs from that of an oral regimen meaning that antibiotic plasma concentrations may be higher and therefore may have a more significant impact on the lung microbiome. Various studies have compared the mean areas under the concentration-time curves (AUC) of antibiotics when given orally and intravenously at the same dose finding that intravenous administration produces a higher AUC than oral [258, 259].

The second possibility is that the different antibiotics used in the treatments of *M. abscessus* and MAC have different pharmacodynamics and therefore appear in varying concentrations in plasma and have varying abilities to penetrate the lung. Van Ingen *et al.* showed that regimens for MAC lung disease yield low serum concentrations. The study included 481 patients and showed that peak serum concentrations (C_{max}) below target range were frequent for ethambutol (48% of patients); clarithromycin (56%); and azithromycin (35%). Concurrent administration of rifampicin (a potent inducer of the hepatic cytochrome P450 enzyme system) led to 68%, 23%, and 10% decreases in C_{max} of clarithromycin, azithromycin, and

moxifloxacin respectively. Serum drug levels associated with bactericidal activity were seldom met (57% of patients achieved levels for ethambutol, 42% for clarithromycin, 19% for amikacin, 18% for rifampicin, and 11% for moxifloxacin) [260]. It is likely therefore that low levels of these drugs reach the lungs and therefore a less dramatic effect on bacterial burden and alpha diversity is noted.

Analysis of specific genera and OTUs in the *M. avium* treatment group revealed a pre-treatment microbiome dominated by *Streptococcus* (rather than Proteobacteria as suggested by the literature) (41.13% of the relative abundance at time point 1). *Streptococcus* remained highly abundant during treatment (relative abundances of 35.2% at time point 2, 44.4% at time point 3 and 27.8% at time point 4.)

Interestingly the total abundance of *Pseudomonas* actually increased during treatment (396 reads at time point 1, 1,032 reads at time point 2, 672 reads at time point 3 and 978 reads at time point 4) becoming the most abundant genus at time point 4. Simper analysis revealed that these changes were not significant (*P*–value = 0.17) although this may be explained by the fact that only 3 samples at each time point were analysed. These changes suggest that *Pseudomonas* is resistant to antibiotics targeting MAC and that this bacterium may exploit the microbiological niche vacated by bacteria sensitive to these drug regimens.

Changes in specific OTUs revealed a significant change in only one (*Actinomyces_778*) between time points 1 and 3 (*P*-value=0.05).

5.11.6 Comparison with Existing Literature

At the time of writing very few studies observing longitudinal changes in the lung microbiome have been published. Those found through a literature search differed to the present study by recruiting patients with underlying structural lung disease but free of infection and then waiting an undefined period of time until exacerbation occurred [247, 261, 262]. At the time of writing, no other studies were found that looked specifically at NTM treatment and none were found that observed changes over a similarly long timeframe (3 months).

The present study observed that after the initiation of treatment bacterial abundance initially decreased significantly in the *M. abscessus* treatment group before increasing towards baseline thereafter. These changes were not mirrored in the *M. avium* treatment group although end bacterial abundance was significantly lower. These dynamic changes were mirrored by changes in alpha diversity measures with a change in species richness observed in both treatment subsets. There were however a low number of patients (n= 3) in this group and it is quite possible that with more patients a significant decrease in initial bacterial burden as well as diversity measures would have been observed. No significant changes were found in beta diversity in either patient group. These findings are supported by Cuthbertson *et al.* who found that whole community richness fell after the initiation of treatment before returning to baseline levels a month after exacerbation [263]. The same study found no significant changes in Bray-Curtis dissimilarity before, during and after treatment.

A number of studies have reported that community compositions do not significantly change during the course of treatment, and that changes in richness are accounted for by decreases in less abundant OTUs [263-266]. The present study demonstrated similar findings amongst certain genera but interestingly exposed the dynamic shift of others during treatment courses. Amongst patients on treatment for *M. abscessus,* the genus *Pseudomonas* remains persistent (relative abundance = 25.3% before initiation of treatment, 28.4% after 2 weeks of treatment, 18.8% after 4 weeks of treatment, 23.9% after 3 months of treatment) despite patients being treated with Meropenem and then with nebulised Amikacin (both antibiotics have anti-Pseudomonal properties) and differences in the OTU *Pseudomonas_2114* were found to be highly similar at different time points.

Other genera exhibited much more dynamic fluctuations over time. The relative abundance of the genera *Streptococcus* fell dramatically between time points 1 and 2 before increasing and becoming much more abundant after 1 and then 3 months of anti-microbial therapy (relative abundances = 12.9%, 1.4%, 30.3%, 22.4% respectively). Changes in the *Streptococcus_487* OTU were found to be significant between the time points. The antithesis of the *Streptococcus* genus seemed to be that of *Staphylococcus* whose relative abundance increased from 11.6% to 37% and then decreased to 11% and then 0.6%. It could be speculated that the dynamic changes in

microbiota reflect the changing nature of antibiotic treatment for *M. abscessus.* The use of early intensive treatment would seem to open a microbiological niche by the eradication of generas such as *Streptococcus* and *Actinomyces* allowing *Staphylococcus* abundance to increase. After the initial treatment is complete *Streptococcus* reassumes its role and becomes the most abundant genus at one month. Price *et al.* demonstrated a highly significant correlation between the results of *Pseudomonas* targeted quantitative polymerase chain reaction and sequencing data. Price *et al.* postulated that an increase in *Pseudomonas* abundance was due to the opening of a physical niche [264]. This change in *Pseudomonas* was not noted in the present study possibly because of the use of antibiotics with known antipseudomonal properties e.g. Meropenem and Amikacin.

Within the *M. avium* treatment group in the present study, the changes previously described by Price *et al*. were observed. The *Pseudomonas* genera increased from a relative abundance of 13.2% to 34.4% after only two weeks of treatment. This change was not deemed to be statistically significant but this is likely due to the small number of subjects in this group. The niche that was opened was likely created by the decrease in abundance of genera such as *Haemophilus* and *Actinomyces* (the latter exhibited a statistically significant drop between time points 1 and 3). *Pseudomonas* itself is a bacterium associated with considerable morbidity and premature deterioration in lung function and in so doing may contribute to mortality [242, 243, 264, 267, 268]. It could be hypothesised therefore that by initiating treatment directed at MAC, clinicians may inadvertently create a niche for *Pseudomonas* to become the dominant species. This may result in the same (or greater) degree of morbidity they were trying to avoid by treating MAC.

A further interesting finding lies within the group of patients where *M. abscesssus* became culture positive after 3 months (i.e. there was recurrence of disease) (Chapter 5, Section 5.10.5). *Pseudomonas* was less abundant in these samples before the onset of treatment than in the *M. abscessus* treatment group (relative abundances of 6.8% vs 25.3% respectively) and did not increase significantly during treatment. It is interesting to speculate that a higher abundance of *Pseudomonas* may help in the elimination of *M. abscessus* possibly by the production of antibacterial products such as phenazines. Phenazines are compounds produced by certain bacterial species that

have been implicated in the virulence and competitive fitness of producing organisms. Phenazine production by *Pseudomonas* has been found to contribute to the colonisation of lungs in patients with suppurative lung disease [269]. Indeed, the antibiotic Clofazimine (a type of phenazine) is used in the treatment of *M. abscessus* itself and thus a higher abundance of *Pseudomonas* (and therefore phenazine) may aid in *M. abscessus* elimination.

5.11.7 Study Limitations

This study utilised sputum samples for sequencing. These samples have the advantage of being easily produced but they are subject to oral contamination resulting in an alteration of the perceived microbiota. A further disadvantage is that sputum samples may originate from any part of either lung and may not reflect a site of infection. An approach that would allow for targeting of the infected lung and protect the sample from oral contamination is that of broncho-alveolar lavage. This is an invasive and costly procedure that can only be done in the hospital environment with appropriate support and therefore its use in repeated longitudinal sampling is limited.

A major factor in this study is the small number of patients studied. This study focused on a longitudinal microbiome analysis, each patient contributing 4 samples to reflect a 3 months observational period. Patients were also selected to reflect the entire study cohort (a total of five sub groups). As sequencing is limited to 96 samples (including controls) per run, only a small subset of patients was therefore studied. This has had an influence on significance values throughout the study and rendered potentially interesting findings non-significant.

5.12 Conclusion

In conclusion the work conducted in this chapter has shown:

- There is no significant difference between the microbiome in cystic fibrosis and (non-CF) bronchiectasis.
- There is an increased bacterial burden in NTM culture positive cases.
- Bacterial burden initially decreases after initiation of antibiotic therapy but begins to return to normal levels over several months of antibiotic therapy.

- This effect is mirrored by changes in alpha diversity (these effects were not seen in the *M. avium* treatment group possibly due to low sample numbers).
- A decrease in bacterial burden and alpha diversity is secondary to a loss of specific genera whilst others are resistant to antibiotic therapy and may increase, occupying the microbiological niche vacated by these bacteria.
- In the case of *M. avium* treatment, genera that become more abundant over time may include pathogenic bacteria such as *Pseudomonas* and therefore patient selection in the treatment of *M. avium* is of paramount importance.
- *Pseudomonas* is significantly more abundant amongst patients who undergo successful *M. abscessus* eradication raising the possibility that *Pseudomonas* may possess anti-NTM properties.

Chapter 6: Discussion

6.1 Difficulties in the Treatment of NTM

Non-Tuberculous Mycobacterial infection is an insidious and progressive disease that causes morbidity and mortality and can be a contraindication to lung transplantation (Chapter 1, Section 1.7). Successful eradication of the offending bacteria is hindered by antibiotic resistance, long treatment regimes, patient intolerance to medications and recurrence after treatment has ceased (Chapter 1, Section 1.6).

At present, isolation of NTM is dependent on culture-based techniques. These techniques can take up to 2 months and only gives binary information on the presence or absence of mycobacteria (Chapter 1, Section 1.5). Culture techniques also frequently fail to culture bacteria that molecular testing will identify leading to inaccurate information being presented to clinicians. There is an unmet need for the development of a technique that has a quick turnaround time, that offers increased reliability compared to culture and can give information on NTM DNA copy number thus providing clinicians assurances over treatment regimes.

Considering this, a new diagnostic technique for the identification and quantification of the six most commonly pathogenic NTM species was developed and applied to samples taken from patients with and without NTM isolated from sputum samples.

6.2 Developing a Molecular Diagnostic Test

Assays for the detection and quantification of the six most commonly pathogenic NTM species were developed by targeting the *hsp65* gene. Sequences were obtained from the National Centre for Biotechnology Information and aligned. A variable region was identified, and primers constructed around this region to generate an amplicon of 140 base pairs. Probes were designed to identify specific NTM species by targeting complementary regions unique to their corresponding species (see Chapter 3, Section 2).

In vitro specificity was determined by testing each probe in turn against a panel of plasmids, each containing an insert from a different mycobacterial species. Positive

probe reactions were recorded and specificity for each probe was determined using the expression: specificity = True Negatives / (True Negatives + False Positives). Probe specificity ranged from 91.6% to 100%. *In vitro* sensitivity was calculated using the expression: sensitivity = True Positives / (True Positives + False Negatives). *In vitro* probe sensitivity was calculated as 100% (see Chapter 3, Sections 3 and 4 respectively).

To assess the ability of each assay to quantify NTM copy number *in vitro,* a mock community designed to imitate bacterial communities found within the lung was constructed. Approximate quantities of target bacterial DNA were subsequently spiked into the community and each assay's quantification accuracy, as well as the limit of detection (the lowest theoretical quantity of DNA the assay can detect), was recorded.

Assays performed in singleplex (i.e. one probe used each time) demonstrated an excellent ability to detect and accurately quantify target genetic material. When genomic NTM DNA was quantified, statistical testing revealed no significant differences between expected DNA quantity and that measured by qPCR. In order to demonstrate reproducibility, a second independent run was performed. There were no significant differences between runs and the expected values (*P* values ranged from 0.78 to 0.99 using the Kruskal-Wallis rank test). The limit of detection was also noted for each run. Singleplex assays were able to consistently detect to a theoretical limit of 10 copies/µl with the lowest copy number accurately quantified as 3.235 copies (see Chapter 3, Section 5).

6.3 Study Cohort

A total of 38 patients were recruited to the study, 22 of whom required treatment for NTM, 11 patients consistently isolating but not on treatment for NTM and 5 patients from whom NTM had not been isolated (Chapter 2, Section 2.1.2). The only significant difference found amongst patient demographics concerned underlying lung disease where a greater number of patients on treatment for *M. abscessus* were found to have cystic fibrosis. This is likely to be the case as isolation of *M. abscessus* is more common in cystic fibrosis and may act as a barrier to lung transplantation necessitating attempts to eradicate the bacteria.

A cohort of the 38 patients were selected to investigate longitudinal changes in the bacterial lung microbiome (Chapter 4). This group consisted of a total of 16 patients, 11 that isolated *M. abscessus* (with 8 on treatment and 3 who were not), 4 that isolated MAC (3 of whom were on treatment) and 1 patient who had never isolated NTM.

One interesting finding amongst the study cohort concerned the relationship between bacterial abundance and alpha diversity to pulmonary function. Log_{10} bacterial abundance was positively correlated with FEV1 %predicted and FVC %predicted (Pearson's, *P*-values = 0.025 and 0.032 respectively). There was also a significantly positive correlation between FEV1 %predicted and alpha diversity measures (although this was not reciprocated in FVC) (Chapter 3, Section 3.3).

6.4 Longitudinal Assessment of NTM Burden

The assays for *M. abscessus, M. avium* and *M. intracellulare* were applied to 410 samples from 38 patients. Using culture dependent methods as the gold standard assay performances were determined.

The probe designed to identify *M. absecssus* demonstrated a high sensitivity (0.87) and specificity (0.95) when used on samples derived from sputum. The probes directed at *M. avium* and *M. intracellulare* (species that form MAC) did not exhibit such favourable results (*M. avium* probe sensitivity = 0.58, specificity = 0.78, *M. intracellulare* sensitivity = 0.65, specificity = 0.79). It was postulated that a confused nomenclature might have been used when reporting culture-based analysis and that this may have resulted in an inflated number of false negatives. This would have affected the sensitivity and negative predictive value of the assay. Consequently, the results for the *M. avium* and *M. intracellulare* probe were considered as one (MAC) resulting in a marked improved sensitivity of 0.86. As a result of combining both probes' false positive reads, the assay specificity decreased to 0.61 (see Chapter 4, Section 11).

It could also be posited that culture-based techniques suffer from a high number of false negatives and that the number of false positive reads demonstrated by these

molecular assays are not deficiencies in the assay but a shortfall in the gold standard test. This implication raises a number of concerns relating to current practice.

The first consideration is whether the correct NTM species has been selected for treatment. Chapter 4, Sections 4.7 and 4.9 considered the application of the assays to the *M. abscessus* control and treatment groups respectively. A large presence of MAC amongst samples that were culture negative for this species were found. If MAC is indeed present in these samples then it is possible that infection caused by several species is present or that the wrong mycobacterial species has been selected for treatment. These mixed NTM isolates were under appreciated by culture techniques but have been confirmed in previous studies [184, 253]. The present study would benefit from the application of the 3 other probes (for *M. kansasii, M.malmonse* and *M. xenopi*) to assess whether further species exist within samples and are under represented by culture-based techniques alone.

The second implication relates to length of treatment. Currently it is recommended that anti-NTM treatment continue for at least a year after the last positive culture result. If, as suspected, culture techniques suffer from a high number of false negatives this raises the question as to whether treatment regimens are being ceased prematurely. During the study, no treatment regimens were ceased before the corresponding qPCR assay had reached undetectable levels. The fact, however, that many samples were culture negative whilst being qPCR assay positive raises the possibility that treatment regimens may inadvertently be halted prematurely. The development of these qPCR assays has the potential to provide clinicians with accurate real time information than culture-based techniques ending premature cessation of treatment.

Regarding NTM quantification, currently no technology exists to compare measurements *in vivo.* Considering the assay directed against *M. abscessus* patients on treatment, a high NTM copy number at the pre-treatment time points was generally demonstrated followed by a rapid decrease (often to undetectable levels) during the initiation phase of anti-microbial therapy. The assay showed that patients who are compliant with medication maintain an undetectable bacterial load but an interruption of one or more antibiotics for a week or longer results in *M. abscessus* being detected by qPCR.

The assays directed against MAC showed a more gradual reduction in NTM burden, occurring over several months rather than weeks. Treatment regimens differ considerably between *M. abscessus* and MAC not only in the use of different antibiotics but also in that regimens directed at MAC do not have an intensive initiation phase. These differences are discussed extensively in Chapter 5, Section 5.11.5. It is therefore not surprising that reduction in MAC copy number occurs more slowly. The assay showed its superiority over culture-based techniques in the evaluation of Patient 7 who became culture negative after 1 week of anti-MAC treatment but was qPCR positive up until 10 months of treatment. In these circumstances, it is likely that the patient's medication will have been stopped prematurely and therefore may result in an early relapse of disease. A similar situation was noted in Patient 1 who was culture negative for MAC during the study period (but who had been culture positive prior to the commencement of treatment) but was intermittently qPCR positive up until month 10 of treatment.

The development of an accurate quantification tool has the potential to add detail to a Patient's clinical course. Currently clinical treatment suffers from a delayed reactive process whereby monitoring relies on sputum culture that can take weeks to present results leading to a delay in change of treatment. An added problem arises when clinicians are presented with recurrent positive culture results, yet the patient appears to be improving from a symptomatic point of view. In this situation a qPCR assay could reveal a slowly decreasing NTM burden and therefore reassure the clinician. Conversely the assays may reveal a significant and persistent disease that requires alteration to antimicrobial agents administered.

The qPCR assays developed in this thesis also have the potential to allow tailor made drug regimen based on real time quantitative results. Currently drug regimens are based on ATS recommendations in conjunction with *in vitro* drug susceptibility testing. Discrepancies between *in vitro* drug susceptibility and *in vivo* outcomes of drug treatment are however well established [95, 270, 271]. Real time quantification has the potential to allow clinicians to substitute antibiotics in and out of regimens based on whether bacterial copy numbers are reducing.

6.5 The Lung Microbiome and Non-Tuberculous Mycobacteria

There is relatively little literature utilising molecular methods to describe the bacterial microbiome and NTM. At the time of writing only two studies were found that utilised 16S rRNA gene sequencing to describe these relationships [158, 185]. No studies were found that looked at differences in bacterial ecology between patients deemed as NTM colonised and those with NTM disease. Very few studies have focussed directly at the longitudinal effect antibiotic therapy has on the lung microbiome [246] and no studies were found examining the effects prolonged antibiotic therapy has on the NTM affected lung.

The present study found no differences in bacterial burden or diversity between patients with cystic fibrosis and bronchiectasis (Chapter 5, Section 7). Bacterial burden was significantly higher in the *M. abscessus* treatment group compared to the NTM culture negative group. Bacterial abundance was again higher in patients with MAC than in the NTM negative group. There were no differences in diversity between treatment and control groups (Chapter 5, Section 8).

Patients on treatment for *M. abscessus* exhibited an initial decrease in total bacterial abundance between weeks 0 and 2 (time points 1 and 2) before increasing towards baseline over the course of the next 3 months. This observation was not seen with treatment regimens directed at MAC and may be explained by the lack of a treatment initiation phase or simply because only 3 patients were included in the MAC treatment group.

This study is unparalleled in its duration of microbial assessment and is one of only a few that has observed changes during anti-microbial therapy. The longitudinal evolution of the bacterial community amongst patients on treatment was explained partly by decreases in less abundant OTUs. Amongst the more common genera *Pseudomonas spp.* remained relatively unchanged in the *M. abscessus* treatment cohort despite the use of Carbapenems and nebulised Amikacin (both antibiotics have anti-pseudomonal properties). Amongst patients with MAC, the total abundance of *Pseudomonas spp.* actually increased during treatment with the genus becoming the most abundant after 3 months (time point 4) of treatment. This suggests that

Pseudomonas is resistant to antibiotics targeting MAC and that this bacterium may exploit the microbiological niche vacated by bacteria sensitive to these drug regimens. Other abundant genera that experienced dynamic shifts included *Staphylococcus* and *Streptococcus*. These two genera appeared to behave asynchronously with *Streptococcus* experiencing an initial significant reduction in abundance before becoming more abundant after a month (time point 3) of antibiotic therapy.

Owing to the relatively low abundance of *Pseudomonas* amongst patients who experienced a relapse of *M. abscessus* beyond 3 months, it could be postulated that *Pseudomonas* aids the elimination of NTM. Theoretical support for this notion concerns *Pseudomonas'* production of phenazines, a compound with anti-microbial properties [269].

The overall conclusions from the work detailed in this thesis are:

- There is no significant difference between the microbiome in bronchiectasis and cystic fibrosis.
- There is no significant difference between the microbiome in patients requiring treatment for NTM and those who do not.
- Patients receiving treatment experience an initial decrease in bacterial burden over the first weeks of treatment followed by a gradual increase towards baseline over the next weeks to months. This change is mirrored in measures of alpha diversity.
- Changes in abundance and diversity are accounted for by decreases in specific bacteria whilst the abundance of other bacteria increases, occupying the microbial niche created. These bacteria (for example *Pseudomonas* spp.) are often associated with morbidity.
- Commencement of anti-NTM medication results in microbiome evolution. Some patients developed a microbiome dominated by bacteria that can cause much morbidity. Prior to the commencement of anti-microbials directed at NTM, it should be established that treatment would be of benefit.

- A molecular assay has been developed that has shown high *in vitro* sensitivity and specificity for the detection and accurate quantification of the 6 most commonly pathogenic NTM species.
- The assays were successfully utilised against DNA extracted from human sputum samples.
- In the treatment of *M. abscessus* copy number drastically reduced, often to undetectable levels, during the initiation stage of treatment. There was a good concordance between culture-based technique results and the qPCR assay.
- In the treatment of MAC, copy number reduced more gradually before becoming undetectable over the course of several months. Culture-based methods often showed negative results when the molecular assay remained positive. This may have led to premature cessation of treatment and may account for the high relapse rates noted in NTM disease.
- There was a remarkable association between NTM copy number and the cessation of one or more antibiotics (i.e. when one antibiotic was stopped because of patient intolerance, NTM copy number increased, often despite being unrecordable prior to this). Owing to the time taken for culture-based methods to be reported as positive, it is likely that clinicians receive this information several weeks after NTM copy number has actually increased. The qPCR assays developed in this thesis function in real time and results can be obtained in approximately 48 hours allowing clinicians to act on problematic results sooner than currently possible.

6.6 Future Work

These findings have originated from a single cohort treated at the same hospital and therefore the findings require independent validation. This would ideally be multi-centre, both to recruit the required number of subjects and to provide an even mix of patients and management methods. This is especially pertinent in the case of the qPCR assays developed in this body of work and that have been used exclusively in this study cohort.

The longitudinal bacterial microbiome study targeting the 16S rRNA gene also requires independent validation. Although studies observing longitudinal changes in

the lung microbiome have been previously published, the studies differed to the present study in that they recruited patients with underlying structural lung disease but free of infection and then waited an undefined period of time until symptomatic exacerbation occurred [247, 261, 262]. At the time of the present study, no studies have been conducted looking specifically at NTM treatment and none that have sought to observe changes over a similarly long timeframe (3 months). Consequently, independent verification is required to assess the dynamic changes of the lung microbiome over a similarly long period. The present study suffered from an overall lack of patients in the MAC treatment cohort where changes were not statistically significant despite mirroring those seen in the *M. abscessus* group. Increasing the number of participants in this cohort would likely remedy this.

The overall accuracy of each custom qPCR assay was potentially hindered by the DNA extraction methodology. The benefit of the phenol-chloroform method described in Chapter 2, Section 2.2 is that it preserves much bacterial (as well as human) DNA allowing for accurate 16S rRNA gene sequencing. This resulted in samples that were highly concentrated in genetic material that in turn gave rise to PCR inhibition. To overcome this inhibition all samples were diluted to the same degree prior to qPCR with the custom assays and this blanket dilutional approach may have resulted in over or under diluted samples. An additional osmotic lysis stage performed during DNA extraction may have the benefit of removing unwanted (and potentially PCR inhibiting) DNA allowing more accurate quantification. Samples that undergo qPCR with these assays in the future should be tested in multiple dilutions to avoid over dilution of NTM genetic material.

The changing nature of the mycobacterial microbiome can also be investigated in the future by using the same samples and sequencing the *hsp65* gene. This has been performed previously [253] but only in the context of comparing single samples from different patients who are either treatment naïve or at different stages of anti-NTM treatment. Using the same samples utilised in the 16S rRNA study will provide interesting information on the dynamic shifts that occur amongst NTM species as well as within the bacterial microbiome as a whole.

Metagenomic sequencing does suffer from shortcomings that may be overcome by newer technologies. Gene sequencing cannot distinguish between live and dead microbes, a shortcoming that is especially pertinent to this thesis [272]. Also, gene sequences produced during the PCR process are relatively short. Consequently, distinction between genetically related species is challenging [273]. Finally, 16S rRNA and *hsp65* sequencing analyses are limited to the detection of bacteria. Viruses and fungi do not carry these genes. Shotgun metagenomic sequencing (the process of breaking DNA up randomly into numerous small segments and then using overlapping reads to reassemble genetic sequences) can capture DNA-based viruses and eukaryotic DNA, including fungi, in addition to bacteria [273] and allows a deeper taxonomical analysis of the microbiome than 16S rRNA sequencing providing better discriminatory power between microbes [274, 275]. Owing to the high abundance of human DNA in samples obtained from the respiratory tract, shotgun metagenomic sequencing can become overwhelmed with eukaryotic sequences. Metatranscriptomics incorporates depletion methods that involves the physical removal of highly abundant genetic material from the samples, as they often constitute upward of 90% of all data if not removed and do not contribute towards most downstream analyses

Many of the shortcomings of metagenomic testing may be addressed by metatranscriptomic sequencing. This technique examines RNA-level genetic content and therefore allows the inclusion of RNA-based respiratory viruses in lung microbiota analysis [276, 277]. As RNA is the result of DNA transcription, metatranscriptomics focusses on living microbiota and is therefore a more accurate assessment of real time microbiome changes than DNA sequencing. By assessing gene expression of the microbiota, it can enable a better assessment of microbial functional activity and the interaction with the host as well as how expressed functions may impact disease progression and severity [278-280]. Metatranscriptomics also incorporates depletion methods that involve the physical removal of highly abundant genetic material (such as those derived from eukaryotic cells) from the samples with the effect of increasing the recovery of expressed genes. Future studies addressing the lung microbiome will likely utilise these technologies to refine our understanding of the dynamic nature of the lung microbiome and its interaction with the host.

Although the field of metatranscriptomics has shown great promise in obtaining insight into the active members, genes, and pathways within a microbiome, this relatively new field of research is hampered by a lack of adequate reference genomes [281]. This means that in any metatranscriptomic study there is often a fraction of reads that cannot be mapped back to a reference dataset and remain taxonomically uncharacterised.

The research described in this thesis has provided new insights into the evolving community structure of both mycobacteria and other taxa within the lungs of subjects on treatment for NTM infection. The study is unparalleled in terms of the duration of anti-microbial treatment and is unique in that it follows a cohort of patients with NTM disease before and during their treatment regime. A new and exciting real time molecular assay for the detection and quantification of the six most pathogenic mycobacteria has been successfully developed and tested on a cohort of patients. Whilst this technique requires optimisation and validation against an independent cohort, these findings may lead to a promising new tool for the diagnosis and monitoring of NTM disease that, in the longer term, may aid the management of patients with this challenging disease.

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Appendix

Table A1 – Sample mapping used for 16S rRNA sequencing. Sample ID = Sample name, Sample Well = sample location on 96 well plate, I7 index ID = identity of reverse primer index, I5 index ID = identity of forward primer index.

Sample_ID	Sample_Well	I7_Index_ID	I5_index_ID	Sample_ID	Sample_Well	I7_Index_ID	I5_index_ID
1002	a1	N701	S502	2202	e1	N701	S507
1004	a2	N702	S502	2204	e2	N702	S507
1006	a3	N703	S502	2206	e3	N703	S507
1008	a4	N704	S502	2208	e4	N704	S507
1017	a5	N705	S502	2217	e5	N705	S507
2002	a6	N706	S502	2702	e6	N706	S507
2004	a7	N707	S502	2704	e7	N707	S507
2006	a8	N710	S502	2706	e8	N710	S507
2008	a9	N711	S502	2708	e9	N711	S507
2018	a10	N712	S502	2719	e10	N712	S507
6002	a11	N714	S502	3402	e11	N714	S507
6004	a12	N715	S502	3404	e12	N715	S507
6006	b1	N701	S503	3406	f1	N701	S508
6008	b2	N702	S503	3409	f2	N702	S508
6017	b3	N703	S503	3417	f3	N703	S508
7002	b4	N704	S503	3502	f4	N704	S508
7004	b5	N705	S503	3504	f5	N705	S508
7006	b6	N706	S503	3506	f6	N706	S508
7008	b7	N707	S503	3508	f7	N707	S508
7017	b8	N710	S503	3517	f8	N710	S508
9002	b9	N711	S503	3702	f9	N711	S508
9004	b10	N712	S503	3704	f10	N712	S508
9006	b11	N714	S503	3706	f11	N714	S508
9008	b12	N715	S503	3708	f12	N715	S508
9017	c1	N701	S505	3802	g1	N701	S510
1302	c2	N702	S505	3804	g2	N702	S510
1304	c3	N703	S505	3806	g3	N703	S510
1306	c4	N704	S505	3809	g4	N704	S510
1308	c5	N705	S505	3815	g5	N705	S510
1402	c6	N706	S505	3902	g6	N706	S510
1404	c7	N707	S505	3905	g7	N707	S510
1406	c8	N710	S505	3906	g8	N710	S510
1408	c9	N711	S505	3908	g9	N711	S510
1418	c10	N712	S505	3917	g10	N712	S510
1502	c11	N714	S505	4102	g11	N714	S510
1504	c12	N715	S505	4104	g12	N715	S510
1506	d1	N701	S506	4106	h1	N701	S511
1508	d2	N702	S506	4108	h2	N702	S511
1702	d3	N703	S506	4602	h3	N703	S511
1704	d4	N704	S506	4605	h4	N704	S511
1706	d5	N705	S506	4606	h5	N705	S511
1708	d6	N706	S506	4608	h6	N706	S511
1717	d7	N707	S506	4802	h7	N707	S511
1802	d8	N710	S506	4804	h8	N710	S511
1804	d9	N711	S506	4806	h9	N711	S511
1806	d10	N712	S506	4807	h10	N712	S511
1808	d11	N714	S506	MockCommunity 1	h11	N714	S511
1817	d12	N715	S506	NegativeControl 1	h12	N715	S511

Table A2 – Sequences for each forward and reverse primer used in 16SrRNA sequencing.

16S_1_F_miseq	Adaptor	Index	Pad	Link	Primer	Total
16S 564 FS502	AATGATACGGCGACCACCGAGATCTACAC	CTCTCTAT	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS503	AATGATACGGCGACCACCGAGATCTACAC	TATCCTCT	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS505	AATGATACGGCGACCACCGAGATCTACAC	GTAAGGAG	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS506	AATGATACGGCGACCACCGAGATCTACAC	ACTGCATA	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACACTGCATATCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS507	AATGATACGGCGACCACCGAGATCTACAC	AAGGAGTA	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS508	AATGATACGGCGACCACCGAGATCTACAC	CTAAGCCT	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS510	AATGATACGGCGACCACCGAGATCTACAC	CGTCTAAT	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S 564 FS511	AATGATACGGCGACCACCGAGATCTACAC	TCTCTCCG	TCCGGTCGCG	GCC	AYTGGGYDTAAAGNG	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCCGGTCGCGGCCAYTGGGYDTAAAGNG
16S_2_R_miseq						
16S 785 RN701	CAAGCAGAAGACGGCATACGAGAT	TCGCCTTA	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATTCGCCTTAAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN702	CAAGCAGAAGACGGCATACGAGAT	CTAGTACG	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATCTAGTACGAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN703	CAAGCAGAAGACGGCATACGAGAT	TTCTGCCT	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATTTCTGCCTAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN704	CAAGCAGAAGACGGCATACGAGAT	GCTCAGGA	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATGCTCAGGAAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN705	CAAGCAGAAGACGGCATACGAGAT	AGGAGTCC	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATAGGAGTCCAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN706	CAAGCAGAAGACGGCATACGAGAT	CATGCCTA	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATCATGCCTAAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN707	CAAGCAGAAGACGGCATACGAGAT	GTAGAGAG	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATGTAGAGAGAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN710	CAAGCAGAAGACGGCATACGAGAT	CAGCCTCG	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATCAGCCTCGAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN711	CAAGCAGAAGACGGCATACGAGAT	TGCCTCTT	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATTGCCTCTTAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN712	CAAGCAGAAGACGGCATACGAGAT	TCCTCTAC	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATTCCTCTACAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN714	CAAGCAGAAGACGGCATACGAGAT	TCATGAGC	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATTCATGAGCAGGCGGCCGCGTTACNVGGGTATCTAATCC
16S 785 RN715	CAAGCAGAAGACGGCATACGAGAT	CCTGAGAT	AGGCGGCCG	CGT	TACNVGGGTATCTAATCC	CAAGCAGAAGACGGCATACGAGATCCTGAGATAGGCGGCCGCGTTACNVGGGTATCTAATCC

Table A3 – Performance of the assay containing *M. abscessus* complex probe used against mock community containing the *M. abscessus* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			11.80		
		1,000,000,000	1,000,000,000	0	11.68	11.67	0.14
		1,000,000,000			11.53		
		100,000,000			14.87		
		100,000,000	100,000,000	0	14.92	14.89	0.02
		100,000,000			14.88		
		10,000,000			18.24		
		10,000,000	10,000,000	0	18.22	18.21	0.04
		10,000,000			18.16		
		1,000,000			21.32		
		1,000,000	1,000,000	0	21.21	21.25	0.06
		1,000,000			21.23		
		100,000			22.95		
		100,000	100,000	0	22.87	22.89	0.06
		100,000			22.84		
		10,000			26.07		
		10,000	10,000	0	26.00	26.07	0.08
		10,000			26.15		
		1,000			29.55		
		1,000	1,000	0	29.59	29.54	0.06
		1,000			29.48		
ABSCESSUS PLASMID		943,559.50			20.72		
	1000000	1,252,172.88	1,147,197.38	176,383.82	20.37	20.49	0.20
		1,245,859.75			20.37		
		90,587.84			23.72		
	100000	97,645.21	97,552.72	6,919.10	23.36	23.47	0.22
		104,425.12			23.33		
		13,952.51			25.99		
	10000	16,982.03	13,425.52	3,847.18	25.75	26.08	0.38
		9,342.01			26.49		
		1,534.75			28.75		
	1000	1,300.59	1,539.06	240.65	28.96	28.76	0.20
		1,781.83			28.57		
		259.65			30.98		
	100	246.14	214.62	66.64	31.04	31.26	0.44
		138.07			31.77		
		20.85			34.13		
	10	18.09	20.69	2.53	34.31	34.15	0.16
		23.13			34.00		
		4.84			35.96		
	1	4.60	4.67	0.14	35.90	35.92	0.03
		4.59			35.90		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A4 – Performance of the assay containing *M. avium* probe used against mock community containing the *M. avium* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			10.38		
		1,000,000,000	1,000,000,000	0	9.94	11.55	0.14
		1,000,000,000			10.01		
		100,000,000			13.29		
		100,000,000	100,000,000	0	13.28	14.65	0.02
		100,000,000			13.29		
		10,000,000			16.48		
		10,000,000	10,000,000	0	16.42	18.10	0.04
		10,000,000			16.27		
		1,000,000			18.69		
		1,000,000	1,000,000	0	18.76	23.54	0.06
		1,000,000			18.84		
		100,000			21.07		
		100,000	100,000	0	20.93	25.98	0.06
		100,000			21.13		
		10,000			24.13		
		10,000	10,000	0	24.15	30.85	0.08
		10,000			24.15		
		1,000			29.56		
		1,000	1,000	0	28.52	34.77	0.06
		1,000			29.48		
AVIUM PLASMID		889,155.50			19.17		
	1000000	800,095.31	842,158.23	44,734.66	19.19	19.16	0.03
		837,223.88			19.13		
		75,961.18			22.26		
	100000	71,434.92	73,529.49	2,281.89	22.25	22.24	0.03
		73,192.36			22.20		
		9,907.83			24.93		
	10000	9,359.04	11,144.52	2,631.63	24.83	24.69	0.34
		14,166.70			24.30		
		650.60			28.17		
	1000	658.56	606.20	83.89	28.19	28.29	0.19
		509.44			28.52		
		47.82			31.52		
	100	58.75	54.91	6.15	32.71	32.32	0.70
		58.17			32.75		
		-	-	-	-		
	10	-	-	-	-	-	-
		-	-	-	-		
		-	-	-	-		
	1	-	-	-	-	-	-
		-	-	-	-		
		-	-	-	-		
	0	-	-	-	-	-	-
		-	-	-	-		

Table A5 – Performance of the assay containing *M. intracellulare* probe used against mock community containing the *M. intracellulare* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			11.86		
		1,000,000,000	1,000,000,000	0	11.75	11.82	0.06
		1,000,000,000			11.85		
		100,000,000			16.10		
		100,000,000	100,000,000	0	16.28	16.30	0.21
		100,000,000			16.53		
		10,000,000			19.83		
		10,000,000	10,000,000	0	19.71	19.68	0.17
		10,000,000			19.49		
		1,000,000			22.19		
		1,000,000	1,000,000	0	21.65	21.91	0.27
		1,000,000			21.89		
		100,000			25.04		
		100,000	100,000	0	24.56	24.66	0.34
		100,000			24.39		
		10,000			28.19		
		10,000	10,000	0	28.10	28.09	0.10
		10,000			27.99		
		1,000			31.88		
		1,000	1,000	0	31.60	31.70	0.16
		1,000			31.60		
INTRACELLULARE PLASMID		1,586,860.00			21.39		
	1000000	2,272,590.50	1,796,483.17	413,300.06	20.90	21.35	0.43
		1,529,999.00			21.75		
		317,066.66			23.60		
	100000	188,382.41	247,916.21	64,878.88	24.31	23.96	0.36
		238,299.58			23.99		
		11,500.16			28.13		
	10000	13,495.86	13,978.26	2,751.20	27.91	27.88	0.27
		16,938.76			27.60		
		813.03			31.76		
	1000	1,043.34	973.90	139.75	31.42	31.52	0.06
		1,065.32			31.39		
		88.87			34.78		
	100	91.83	105.11	25.61	34.74	34.58	0.32
		134.63			34.22		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A6 – Performance of the assay containing *M. kansasii* probe used against mock community containing the *M. kansasii* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			8.80		
		1,000,000,000	1,000,000,000	0	7.84	8.15	0.56
		1,000,000,000			7.82		
		100,000,000			12.06		
		100,000,000	100,000,000	0	12.08	12.11	0.06
		100,000,000			12.18		
		10,000,000			15.34		
		10,000,000	10,000,000	0	15.34	15.26	0.13
		10,000,000			15.11		
		1,000,000			18.41		
		1,000,000	1,000,000	0	18.36	18.43	0.09
		1,000,000			18.53		
		100,000			22.05		
		100,000	100,000	0	21.93	21.93	0.13
		100,000			21.80		
		10,000			29.55		
		10,000	10,000	0	30.10	29.99	0.40
		10,000			30.33		
		1,000			35.31		
		1,000	1,000	0	38.61	36.58	1.78
		1,000			35.82		
KANSASII PLASMID		1,420,155.38			18.19		
	1000000	1,479,569.75	1,421,652.50	57,183.39	18.23	18.26	0.09
		1,365,232.38			18.35		
		118,341.28			22.14		
	100000	122,657.74	116,190.81	7,768.70	22.09	22.17	0.11
		107,573.40			22.29		
		10,640.98			25.88		
	10000	11,377.59	10,672.17	690.36	25.77	25.87	0.10
		9,997.93			25.97		
		1,046.75			29.47		
	1000	1,023.86	971.52	111.08	29.50	29.59	0.18
		843.94			29.80		
		107.93			32.99		
	100	128.70	116.19	11.02	32.72	32.88	0.14
		111.93			32.93		
		17.62			36.88		
	10	2.87	12.61	8.44	37.77	37.05	0.65
		17.34			36.50		
		0			-		
	1	0	-	-	-	-	-
		0			-		
		0			-		
	0	0	-	-	-	-	-
		0			-		

Table A7 – Performance of the assay containing *M. malmoense* probe used against mock community containing the *M. malmoense* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			8.68		
		1,000,000,000	1,000,000,000	0	7.64	8.31	0.58
		1,000,000,000			8.60		
		100,000,000			11.74		
		100,000,000	100,000,000	0	11.82	11.81	0.06
		100,000,000			11.87		
		10,000,000			15.32		
		10,000,000	10,000,000	0	15.23	15.24	0.07
		10,000,000			15.18		
		1,000,000			18.73		
		1,000,000	1,000,000	0	19.01	18.94	0.19
		1,000,000			19.08		
		100,000			20.87		
		100,000	100,000	0	20.97	20.94	0.06
		100,000			20.97		
		10,000			24.32		
		10,000	10,000	0	24.64	24.52	0.17
		10,000			24.60		
		1,000			27.97		
		1,000	1,000	0	28.05	27.98	0.07
		1,000			27.92		
MALMOENSE PLASMID		992,283.63			19.24		
	1000000	976,266.13	927,287.40	99,029.58	18.80	18.99	0.23
		813,312.44			18.93		
		88,088.53			23.24		
	100000	92,908.59	89,800.44	2,696.36	23.02	23.42	0.51
		88,404.20			24.00		
		12,220.06			26.79		
	10000	12,526.21	12,433.14	185.02	26.61	26.67	0.11
		12,553.16			26.60		
		1,020.13			33.34		
	1000	1,342.95	1,519.83	607.75	33.35	33.35	0.02
		2,196.40			33.37		
		204.44			36.51		
	100	204.36	203.17	2.13	37.15	36.71	0.39
		200.71			36.46		
		10.93			36.65		
	10	5.29	6.93	3.48	35.23	35.77	0.76
		4.58			35.44		
		1.02			37.54		
	1	2.60	1.30	1.18	36.23	37.70	1.55
		0.28			39.33		
		0.00			-		
	0	0.00	-	-	-	-	-
		0.00			-		

Table A8 – Performance of the assay containing *M. xenopi* probe used against mock community containing the *M. xenopi* plasmid.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			7.55		
		1,000,000,000	1,000,000,000	0	7.54	7.84	0.52
		1,000,000,000			8.44		
		100,000,000			11.66		
		100,000,000	100,000,000	0	11.60	11.69	0.11
		100,000,000			11.82		
		10,000,000			15.15		
		10,000,000	10,000,000	0	15.06	15.07	0.07
		10,000,000			15.01		
		1,000,000			18.33		
		1,000,000	1,000,000	0	18.50	18.45	0.11
		1,000,000			18.54		
		100,000			22.00		
		100,000	100,000	0	21.33	21.73	0.35
		100,000			21.85		
		10,000			24.62		
		10,000	10,000	0	25.60	25.37	0.67
		10,000			25.88		
		1,000			28.53		
		1,000	1,000	0	28.56	28.52	0.05
		1,000			28.46		
XENOPI PLASMID		818,649.00			19.36		
	1000000	851,633.44	849,321.14	29,583.84	19.57	19.52	0.14
		877,680.97			19.63		
		93,235.43			23.45		
	100000	94,244.91	92,523.34	2,167.20	23.41	23.49	0.10
		90,089.69			23.60		
		9,404.59			27.36		
	10000	9,619.62	9,672.76	298.31	27.24	27.42	0.21
		9,994.07			27.64		
		809.70			31.00		
	1000	858.55	835.17	24.49	30.69	31.10	0.48
		837.27			31.63		
		88.09			33.91		
	100	80.60	86.00	4.72	33.42	33.74	0.27
		89.31			33.88		
		16.71			35.24		
	10	11.25	17.90	7.32	35.42	35.26	0.15
		25.75			35.12		
		3.84			37.76		
	1	2.91	3.31	0.48	37.06	37.55	0.43
		3.18			37.84		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A9 – Performance of the assay containing *M. abscessus* probe used against mock community containing *M. abscessus* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			12.41		
		1,000,000,000	1,000,000,000	0	12.01	12.17	0.21
		1,000,000,000			12.11		
		100,000,000			15.40		
		100,000,000	100,000,000	0	15.45	15.43	0.03
		100,000,000			15.44		
		10,000,000			18.85		
		10,000,000	10,000,000	0	18.87	18.83	0.05
		10,000,000			18.78		
		1,000,000			20.68		
		1,000,000	1,000,000	0	20.75	20.76	0.08
		1,000,000			20.84		
		100,000			24.01		
		100,000	100,000	0	23.96	23.98	0.03
		100,000			23.97		
		10,000			27.99		
		10,000	10,000	0	27.98	27.97	0.04
		10,000			27.92		
		1,000			30.96		
		1,000	1,000	0	31.02	31.00	0.04
		1,000			31.03		
ABSCESSUS GENOMIC RUN 1		1306465.00			17.99		
	1000000.00	1658755.00	1594635.00	262060.80	17.67	17.74	0.23
		1818685.00			17.55		
		134772.13			21.05		
	100000.00	154743.88	150056.96	13562.99	20.86	20.91	0.12
		160654.88			20.81		
		12104.81			24.29		
	10000.00	12342.86	12038.83	341.82	24.26	24.30	0.04
		11668.83			24.34		
		833.97			27.89		
	1000.00	768.33	772.69	59.22	28.00	27.99	0.10
		715.78			28.09		
		95.98			33.89		
	100.00	96.85	101.11	8.14	33.88	33.82	0.11
		110.50			33.70		
		0.00			-		
	10.00	4.62	3.00	2.60	37.97	38.01	0.05
		4.37			38.05		
		-			39.42		
	1.00	-	-	-	-	-	-
		-			-		
		-			-		
	0.00	-	-	-	-	-	-
		-			-		
ABSCESSGENOMIC RUN 2		1010229.00			18.33		
	1000000.00	1420614.00	1294594.00	246797.12	17.68	17.88	0.39
		1452939.00			17.63		
		319143.50			20.53		
	100000.00	244822.75	285784.50	37739.17	21.04	20.76	0.26
		293387.25			20.69		
		43557.19			23.95		
	10000.00	42947.66	42639.84	1103.92	24.37	24.25	0.27
		41414.69			24.44		
		5609.72			28.27		
	1000.00	5671.63	5450.79	330.35	28.24	28.32	0.12
		5071.01			28.46		
		181.24			34.83		
	100.00	92.24	140.62	45.01	36.13	35.39	0.67
		148.38			35.22		
	10.00	9.13	7.71	2.01	36.68	36.81	0.18
		6.29			36.94		
	1.00	-	-	-	-	-	-
		-			-		
		-			-		
	0.00	-	-	-	-	-	-
		-			-		

Table A10 – Performance of the assay containing *M. avium* probe used against mock community containing the *M. avium* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			11.88		
		1,000,000,000	1,000,000,000	0	11.42	11.55	0.29
		1,000,000,000			11.36		
		100,000,000			14.70		
		100,000,000	100,000,000	0	14.63	14.65	0.04
		100,000,000			14.62		
		10,000,000			18.11		
		10,000,000	10,000,000	0	18.14	18.10	0.05
		10,000,000			18.04		
		1,000,000			23.35		
		1,000,000	1,000,000	0	23.62	23.54	0.17
		1,000,000			23.65		
		100,000			26.08		
		100,000	100,000	0	26.18	25.98	0.26
		100,000			25.69		
		10,000			30.76		
		10,000	10,000	0	30.37	30.85	0.54
		10,000			31.44		
		1,000			34.69		
		1,000	1,000	0	34.85	34.75	0.09
		1,000			34.69		
AVIUM GENOMIC RUN 1		1,061,078.75			22.67		
	1000000	962,585.06	1,071,496.35	114,476.16	22.83	22.66	0.18
		1,190,825.25			22.47		
		197,441.41			25.53		
	100000	205,328.84	192,053.93	16,636.31	25.46	25.58	0.15
		173,391.53			25.75		
		28,326.11			28.83		
	10000	29,448.41	30,576.02	2,978.35	28.77	28.71	0.16
		33,953.53			28.52		
		8,711.29			30.84		
	1000	7,803.93	8,181.39	472.50	31.02	30.95	0.10
		8,028.95			30.98		
		124.27			38.07		
	100	193.03	158.81	34.38	-	37.69	0.53
		159.13			37.32		
		53.24			-		
	10	56.13	53.15	3.02	38.45	38.45	#DIV/0!
		50.09			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		
AVIUMGENOMIC RUN 2		820,033.38			21.78		
	1000000	815,157.56	831,069.23	23,464.23	21.79	21.76	0.05
		858,016.75			21.70		
		112,978.39			25.10		
	100000	112,185.33	109,115.19	6,017.52	25.12	25.16	0.09
		102,181.86			25.27		
		14,761.74			28.52		
	10000	19,664.72	18,996.71	3,943.63	28.04	28.12	0.36
		18,563.67			27.81		
		1,634.42			29.42		
	1000	2,935.69	7,789.66	926.44	29.56	29.60	0.20
		1,798.88			29.82		
		289.48			31.65		
	100	250.56	2,487.02	182.93	31.40	31.51	0.12
		221.01			31.49		
		22.56			33.57		
	10	25.61	22.88	2.58	33.33	33.48	0.13
		20.48			33.53		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A11 – Performance of the assay containing *M. intracellulare* probe used against mock community containing the *M. intracellulare* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			10.78		
		1,000,000,000	1,000,000,000	0	10.97	10.82	0.14
		1,000,000,000			10.70		
		100,000,000			13.14		
		100,000,000	100,000,000	0	13.40	13.32	0.16
		100,000,000			13.42		
		10,000,000			16.04		
		10,000,000	10,000,000	0	16.07	16.00	0.09
		10,000,000			15.90		
		1,000,000			18.32		
		1,000,000	1,000,000	0	18.41	18.39	0.06
		1,000,000			18.43		
		100,000			28.17		
		100,000	100,000	0	28.22	28.16	0.07
		100,000			28.09		
		10,000			30.66		
		10,000	10,000	0	31.11	30.86	0.23
		10,000			30.82		
		1,000			34.61		
		1,000	1,000	0	34.94	34.74	0.18
		1,000			34.66		
INTRACELLULARE GENOMIC RUN 1		1,025,867.53			18.77		
	1000000	1,045,173.05	1,360,102.91	562,276.45	18.42	18.44	0.31
		2,009,268.14			18.14		
		107,738.46			22.25		
	100000	107,581.94	106,795.78	1,499.26	22.08	21.98	0.33
		105,066.95			21.62		
		10,189.15			24.70		
	10000	10,114.18	9,795.56	617.92	24.71	24.77	0.12
		9,083.37			24.91		
		1,048.13			28.08		
	1000	1,048.21	1,052.75	7.93	28.16	28.19	0.12
		1,061.90			28.32		
		88.24			30.68		
	100	86.42	88.67	2.49	30.68	30.65	0.06
		91.35			30.57		
		31.56			33.21		
	10	29.04	31.67	2.59	33.13	33.16	0.04
		34.21			33.14		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		
INTRACELLULAREGENOMIC RUN 2		843,772.38			22.30		
	1000000	909,792.56	878,590.04	33,158.23	21.93	22.07	0.20
		882,205.19			21.99		
		210,573.97			24.63		
	100000	203,487.81	216,361.44	16,544.81	24.69	24.58	0.14
		235,022.53			24.43		
		26,691.13			24.49		
	10000	25,169.98	25,976.63	764.75	24.43	25.60	1.98
		26,068.77			27.89		
		912.67			30.94		
	1000	948.57	887.03	77.60	30.93	31.05	0.21
		799.85			31.29		
		93.39			38.88		
	100	116.11	100.56	13.48	37.93	38.57	0.55
		92.18			38.91		
		26.85			37.41		
	10	16.11	23.80	6.71	38.65	37.82	0.72
		28.44			37.40		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A12 – Performance of the assay containing *M. kansasii* probe used against mock community containing the *M. kansasii* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			10.78		
		1,000,000,000	1,000,000,000	0	10.97	10.82	0.14
		1,000,000,000			10.70		
		100,000,000			13.14		
		100,000,000	100,000,000	0	13.40	13.32	0.16
		100,000,000			13.42		
		10,000,000			16.04		
		10,000,000	10,000,000	0	16.07	16.00	0.09
		10,000,000			15.90		
		1,000,000			18.32		
		1,000,000	1,000,000	0	18.41	18.39	0.06
		1,000,000			18.43		
		100,000			28.17		
		100,000	100,000	0	28.22	28.16	0.07
		100,000			28.09		
		10,000			30.66		
		10,000	10,000	0	31.11	30.86	0.23
		10,000			30.82		
		1,000			34.61		
		1,000	1,000	0	34.94	34.74	0.18
		1,000			34.66		
KANSASII GENOMIC RUN 1		646,002.03			19.47		
	1000000	640,181.25	633,103.05	17,543.83	19.48	19.32	0.27
		613,125.88			19.01		
		59,918.09			22.37		
	100000	57,586.74	58,105.93	1,616.36	23.04	22.82	0.40
		56,812.97			23.07		
		10,592.91			24.87		
	10000	5,567.11	7,064.78	3,067.04	25.80	25.78	0.90
		5,034.31			26.67		
		603.67			29.01		
	1000	563.00	756.81	301.16	29.11	28.44	1.07
		1,103.77			27.20		
		84.24			31.85		
	100	140.06	103.64	31.57	31.12	31.59	0.41
		86.62			31.81		
		10.16			34.91		
	10	12.85	11.86	1.47	34.57	34.69	0.19
		12.55			34.60		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		
KANSASIIGENOMIC RUN 2		1,096,252.63			21.14		
	1000000	1,398,987.00	1,397,452.04	300,434.88	20.84	20.86	0.27
		1,697,116.50			20.60		
		102,721.63			24.06		
	100000	89,904.43	96,929.61	6,496.97	24.23	24.14	0.08
		98,162.78			24.12		
		9,521.83			27.00		
	10000	11,004.63	9,792.53	1,101.98	26.82	26.97	0.14
		8,851.12			27.09		
		865.72			29.97		
	1000	920.53	880.71	34.83	29.89	29.95	0.05
		855.89			29.98		
		73.28			33.02		
	100	92.05	85.03	10.24	32.74	32.84	0.15
		89.76			32.77		
		7.18			34.99		
	10	18.07	11.71	5.67	34.94	34.85	0.19
		9.89			34.63		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A13 – Performance of the assay containing *M. malmoense* probe used against mock community containing the *M. malmoense* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			9.02		
		1,000,000,000	1,000,000,000	0	8.86	8.94	0.08
		1,000,000,000			8.94		
		100,000,000			12.29		
		100,000,000	100,000,000	0	12.23	12.26	0.03
		100,000,000			12.27		
		10,000,000			15.62		
		10,000,000	10,000,000	0	15.65	15.63	0.02
		10,000,000			15.63		
		1,000,000			19.17		
		1,000,000	1,000,000	0	18.87	19.03	0.15
		1,000,000			19.04		
		100,000			22.45		
		100,000	100,000	0	22.66	22.66	0.21
		100,000			22.87		
		10,000			26.21		
		10,000	10,000	0	26.16	26.29	0.19
		10,000			26.50		
		1,000			29.41		
		1,000	1,000	0	28.95	29.25	0.26
		1,000			29.39		
MALMOENSE GENOMIC RUN 1		1,470,506.50			18.58		
	1000000	1,439,481.63	1,396,898.17	101,813.73	18.61	18.66	0.11
		1,280,706.38			18.78		
		248,413.33			21.23		
	100000	180,413.52	185,794.69	60,108.97	21.70	21.71	0.49
		128,557.23			22.21		
		20,040.59			24.97		
	10000	15,298.36	16,740.53	2,865.49	25.38	25.26	0.25
		14,882.64			25.42		
		2,116.25			28.32		
	1000	2,210.19	2,271.59	193.49	28.26	28.22	0.13
		2,488.33			28.08		
		222.37			31.68		
	100	327.39	250.86	67.00	31.10	31.53	0.38
		202.81			31.81		
		23.52			35.02		
	10	13.37	20.44	6.14	35.86	35.28	0.50
		24.45			34.96		
		7.59			36.71		
	1	8.46	6.48	2.72	36.54	37.05	0.75
		3.38			37.91		
		-			-		
	0	-	-	-	-	-	-
		-			-		
MALMOENSE GENOMIC RUN 2		1,033,501.75			18.93		
	1000000	1,245,434.50	1,185,070.92	132,165.49	18.82	18.86	0.07
		1,276,276.50			18.81		
		149,983.06			22.07		
	100000	110,903.09	123,985.21	22,514.96	22.22	22.17	0.09
		111,069.47			22.22		
		21,864.78			25.62		
	10000	29,819.73	29,136.63	6,955.49	25.39	25.42	0.20
		35,725.36			25.24		
		1,429.45			27.68		
	1000	1,318.60	1,433.01	116.22	28.28	28.42	0.82
		1,550.97			29.30		
		144.72			31.78		
	100	98.57	127.34	25.09	31.84	31.86	0.10
		138.72			31.98		
		17.58			35.98		
	10	23.72	18.56	4.75	35.96	35.97	0.01
		14.38			35.98		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A14 – Performance of the assay containing *M. xenopi* probe used against mock community containing the *M. xenopi* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			7.47		
		1,000,000,000	1,000,000,000	0	7.26	7.34	0.11
		1,000,000,000			7.30		
		100,000,000			11.00		
		100,000,000	100,000,000	0	11.02	11.02	0.01
		100,000,000			11.02		
		10,000,000			14.01		
		10,000,000	10,000,000	0	14.11	14.05	0.05
		10,000,000			14.04		
		1,000,000			17.47		
		1,000,000	1,000,000	0	17.53	17.59	0.17
		1,000,000			17.78		
		100,000			20.87		
		100,000	100,000	0	20.89	20.85	0.04
		100,000			20.81		
		10,000			24.31		
		10,000	10,000	0	24.26	24.30	0.04
		10,000			24.33		
		1,000			26.89		
		1,000	1,000	0	28.05	27.67	0.68
		1,000			28.07		
XENOPI GENOMIC RUN 1		782,932.00			17.91		
	1000000	784,069.44	741,506.54	72,738.27	17.90	17.99	0.15
		657,518.19			18.16		
		108,574.37			20.80		
	100000	67,902.10	82,430.49	22,688.06	21.48	21.23	0.38
		70,815.01			21.42		
		13,202.88			23.88		
	10000	9,393.01	10,332.97	2,534.23	24.38	24.27	0.34
		8,403.01			24.54		
		902.29			27.81		
	1000	825.21	833.06	65.67	27.94	27.93	0.12
		771.66			28.04		
		87.47			24.46		
	100	61.02	62.51	24.25	28.38	27.40	2.60
		39.04			29.38		
		5.98			31.98		
	10	4.01	4.41	1.41	32.26	32.28	0.31
		3.24			32.60		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		
XENOPI GENOMIC RUN 2		1,596,791.50			16.84		
	1000000	1,451,446.50	1,359,859.00	293,641.51	16.88	16.74	0.21
		1,031,339.00			16.50		
		115,424.44			20.44		
	100000	118,554.19	122,279.02	9,294.72	20.27	20.28	0.15
		132,858.44			20.14		
		8,228.34			23.05		
	10000	10,415.20	9,886.94	1,467.60	23.81	23.48	0.39
		11,017.29			23.58		
		1,158.79			27.72		
	1000	1,135.29	1,091.69	96.59	27.20	27.64	0.40
		980.98			27.98		
		158.33			28.01		
	100	182.11	168.81	12.14	28.65	28.42	0.36
		165.97			28.60		
		61.57			31.50		
	10	60.73	64.44	5.72	31.42	31.47	0.04
		71.02			31.49		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A15 – Performance of the assay containing the *M. abscessus, M. avium* and *M. intracellulare* probe used against mock community containing the *M. abscessus* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			11.22		
		1,000,000,000	1,000,000,000	0	11.11	11.14	0.08
		1,000,000,000			11.08		
		100,000,000			14.28		
		100,000,000	100,000,000	0	14.30	14.23	0.10
		100,000,000			14.12		
		10,000,000			17.54		
		10,000,000	10,000,000	0	17.51	17.50	0.04
		10,000,000			17.46		
		1,000,000			19.62		
		1,000,000	1,000,000	0	19.85	19.80	0.16
		1,000,000			19.92		
		100,000			22.21		
		100,000	100,000	0	22.17	22.21	0.04
		100,000			22.26		
		10,000			25.48		
		10,000	10,000	0	25.57	25.59	0.12
		10,000			25.72		
		1,000			28.67		
		1,000	1,000	0	28.84	28.79	0.11
		1,000			28.87		
ABSCESSUS GENOMIC MULTIPLEX		779,109.74			28.82		
	1000000	630,791.00	723,866.02	81,074.15	20.47	23.17	4.89
		761,697.31			20.23		
		54,798.52			23.52		
	100000	58,545.83	52,907.06	6,785.20	23.43	23.57	0.17
		45,376.82			23.75		
		1,932.26			27.69		
	10000	1,930.62	1,876.98	94.33	27.69	27.73	0.06
		1,768.06			27.80		
		96.20			22.81		
	1000	23.36	46.46	43.11	33.19	29.80	6.05
		19.82			33.40		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A16 – Performance of the assay containing the *M. abscessus, M. avium* and *M. intracellulare* probe used against mock community containing the *M. avium* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			10.89		
		1,000,000,000	1,000,000,000	0	10.79	10.82	0.07
		1,000,000,000			10.77		
		100,000,000			14.13		
		100,000,000	100,000,000	0	14.19	14.18	0.05
		100,000,000			14.22		
		10,000,000			17.57		
		10,000,000	10,000,000	0	17.59	17.56	0.05
		10,000,000			17.50		
		1,000,000			19.98		
		1,000,000	1,000,000	0	19.98	19.99	0.01
		1,000,000			20.00		
		100,000			22.67		
		100,000	100,000	0	22.50	22.55	0.10
		100,000			22.48		
		10,000			25.97		
		10,000	10,000	0	26.13	26.04	0.08
		10,000			26.02		
		1,000			29.23		
		1,000	1,000	0	29.03	29.09	0.12
		1,000			29.02		
AVIUM GENOMIC MULTIPLEX		130,907.63			21.52		
	1000000	150,959.33	138,070.81	11,184.91	21.35	21.46	0.10
		132,345.47			21.51		
		15,013.94			24.13		
	100000	12,824.68	13,915.52	1,094.65	24.32	24.22	0.10
		13,907.94			24.22		
		719.72			27.78		
	10000	644.73	709.12	59.80	27.91	27.80	0.10
		762.91			27.71		
		108.61			30.06		
	1000	106.82	102.47	9.12	30.08	30.13	0.11
		92.00			30.26		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A17 – Performance of the assay containing the *M. abscessus, M. avium* and *M. intracellulare* probe used against mock community containing the *M. intracellulare* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			10.89		
		1,000,000,000	1,000,000,000	0	10.79	10.82	0.07
		1,000,000,000			10.77		
		100,000,000			14.13		
		100,000,000	100,000,000	0	14.19	14.18	0.05
		100,000,000			14.22		
		10,000,000			17.57		
		10,000,000	10,000,000	0	17.59	17.56	0.05
		10,000,000			17.50		
		1,000,000			19.98		
		1,000,000	1,000,000	0	19.98	19.99	0.01
		1,000,000			20.00		
		100,000			22.67		
		100,000	100,000	0	22.50	22.55	0.10
		100,000			22.48		
		10,000			25.97		
		10,000	10,000	0	26.13	26.04	0.08
		10,000			26.02		
		1,000			29.23		
		1,000	1,000	0	29.03	29.09	0.12
		1,000			29.02		
INTRACELLULARE GENOMIC MULTIPLEX		429,877.50			21.13		
	1000000	643,374.50	567,700.21	119,549.41	20.60	20.79	0.29
		629,848.63			20.63		
		64,106.71			23.59		
	100000	57,119.26	55,472.28	9,564.86	23.74	23.79	0.23
		45,190.87			24.05		
		3,736.55			27.28		
	10000	3,761.10	3,850.76	176.99	27.27	27.24	0.06
		4,054.63			27.17		
		421.30			30.10		
	1000	319.47	385.35	57.13	30.46	30.23	0.20
		415.28			30.12		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A18 – Performance of the assay containing the *M. kansasii, M. malmoense* and *M. xenopi* probe used against mock community containing the *M. kansasii* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			8.97		
		1,000,000,000	1,000,000,000	0	8.87	8.90	0.06
		1,000,000,000			8.87		
		100,000,000			11.47		
		100,000,000	100,000,000	0	11.54	11.54	0.08
		100,000,000			11.62		
		10,000,000			14.64		
		10,000,000	10,000,000	0	14.54	14.57	0.06
		10,000,000			14.54		
		1,000,000			17.93		
		1,000,000	1,000,000	0	18.01	18.01	0.08
		1,000,000			18.09		
		100,000			21.17		
		100,000	100,000	0	21.12	21.14	0.03
		100,000			21.14		
		10,000			24.32		
		10,000	10,000	0	24.30	24.37	0.10
		10,000			24.48		
		1,000			27.78		
		1,000	1,000	0	27.72	27.79	0.07
		1,000			27.86		
KANSASII GENOMIC MULTIPLEX		249,347.94			19.96		
	1000000	307,168.34	295,072.03	41,035.60	19.67	19.74	0.20
		328,699.81			19.58		
		34,149.63			22.70		
	100000	31,259.23	32,366.39	1,559.30	22.82	22.78	0.07
		31,690.31			22.81		
		3,373.71			25.89		
	10000	2,562.96	2,794.94	504.51	26.27	26.17	0.24
		2,448.15			26.33		
		291.65			29.27		
	1000	255.18	273.90	18.25	29.45	29.36	0.09
		274.88			29.35		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A19 – Performance of the assay containing the *M. kansasii, M. malmoense* and *M. xenopi* probe used against mock community containing the *M. malmoense* genomic DNA.

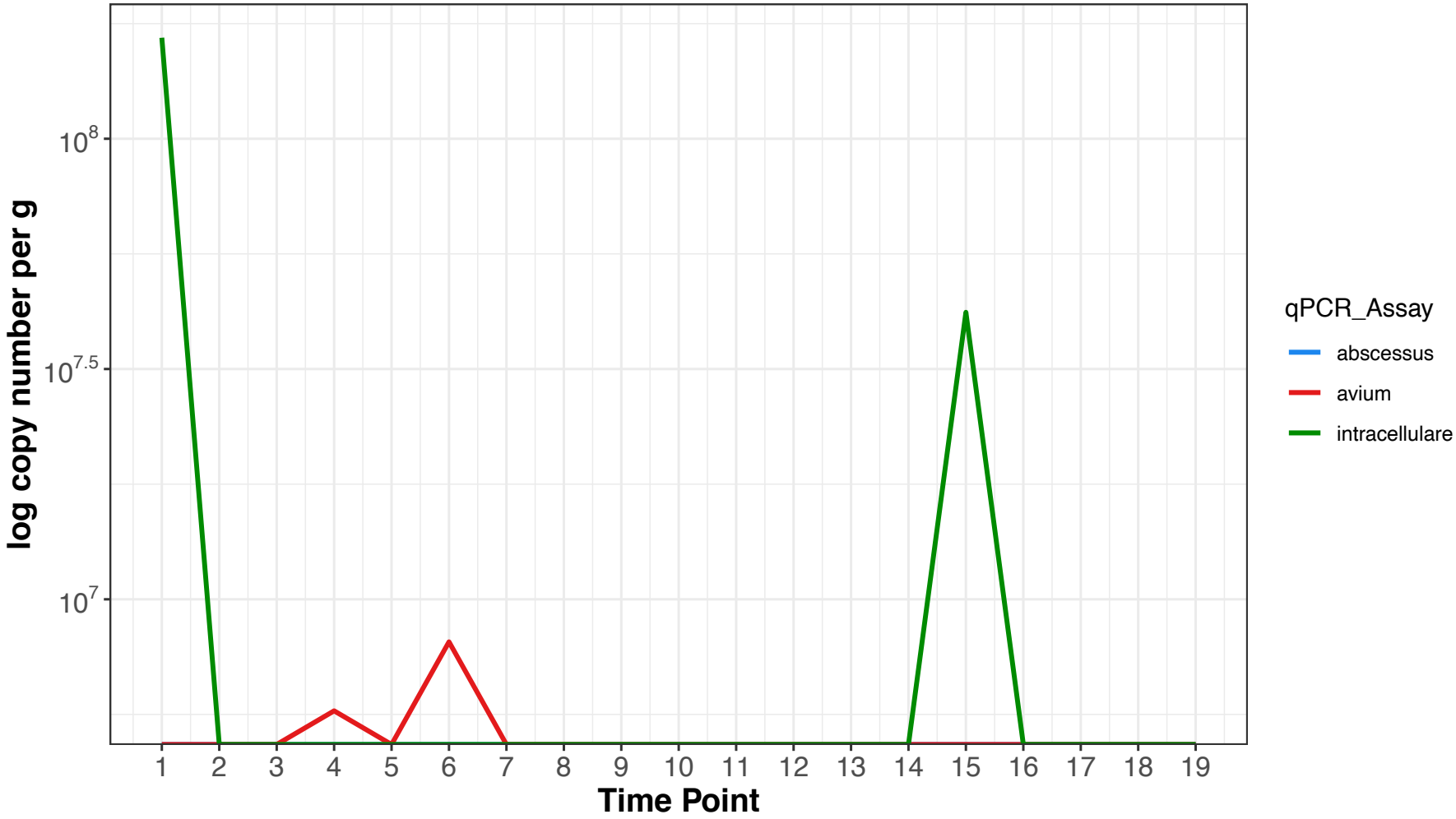
	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			8.80		
		1,000,000,000	1,000,000,000	0	8.57	8.66	0.12
		1,000,000,000			8.62		
		100,000,000			10.48		
		100,000,000	100,000,000	0	10.69	10.60	0.11
		100,000,000			10.64		
		10,000,000			13.15		
		10,000,000	10,000,000	0	13.28	13.24	0.08
		10,000,000			13.30		
		1,000,000			16.38		
		1,000,000	1,000,000	0	16.15	16.31	0.14
		1,000,000			16.40		
		100,000			18.94		
		100,000	100,000	0	19.04	19.11	0.21
		100,000			19.34		
		10,000			22.15		
		10,000	10,000	0	22.15	22.19	0.07
		10,000			22.27		
		1,000			25.38		
		1,000	1,000	0	25.41	25.39	0.02
		1,000			25.38		
MALMOENSE GENOMIC MULTIPLEX		226,077.27			18.33		
	1000000	229,097.95	222,613.05	8,747.57	18.31	18.35	0.05
		212,663.92			18.40		
		16,071.94			21.58		
	100000	12,980.79	13,562.56	2,274.98	21.84	21.80	0.20
		11,634.96			21.97		
		806.96			25.25		
	10000	934.66	901.62	83.21	25.07	25.12	0.12
		963.23			25.03		
		85.61			28.01		
	1000	90.79	85.07	6.00	27.94	28.02	0.09
		78.82			28.11		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

Table A20 – Performance of the assay containing the *M. kansasii, M. malmoense* and *M. xenopi* probe used against mock community containing the *M. xenopi* genomic DNA.

	Intended Quantity (total copy number)	Quantity	Quantity Mean	Quantity SD	CT	Ct Mean	Ct SD
STANDARDS		1,000,000,000			12.57		
		1,000,000,000	1,000,000,000	0	11.49	11.81	0.66
		1,000,000,000			11.36		
		100,000,000			15.14		
		100,000,000	100,000,000	0	15.28	15.10	0.21
		100,000,000			14.87		
		10,000,000			18.48		
		10,000,000	10,000,000	0	18.83	18.70	0.19
		10,000,000			18.79		
		1,000,000			22.64		
		1,000,000	1,000,000	0	22.61	22.69	0.11
		1,000,000			22.82		
		100,000			25.97		
		100,000	100,000	0	25.82	25.82	0.16
		100,000			25.66		
		10,000			29.13		
		10,000	10,000	0	28.86	29.06	0.18
		10,000			29.19		
		1,000			32.96		
		1,000	1,000	0	32.90	32.93	0.03
		1,000			32.92		
XENOPI GENOMIC MULTIPLEX		133,592.45			25.37		
	1000000	95,753.43	101,806.25	29,233.60	25.88	25.83	0.43
		76,072.87			26.23		
		9,173.93			29.46		
	100000	11,901.64	10,509.98	1,364.71	29.06	29.26	0.20
		10,454.35			29.26		
		1,496.05			32.23		
	10000	1,260.58	1,503.61	246.90	32.49	32.23	0.25
		1,754.20			31.99		
		347.63			34.46		
	1000	157.23	207.05	123.45	35.67	35.42	0.86
		116.31			36.13		
		-			-		
	100	-	-	-	-	-	-
		-			-		
		-			-		
	10	-	-	-	-	-	-
		-			-		
		-			-		
	1	-	-	-	-	-	-
		-			-		
		-			-		
	0	-	-	-	-	-	-
		-			-		

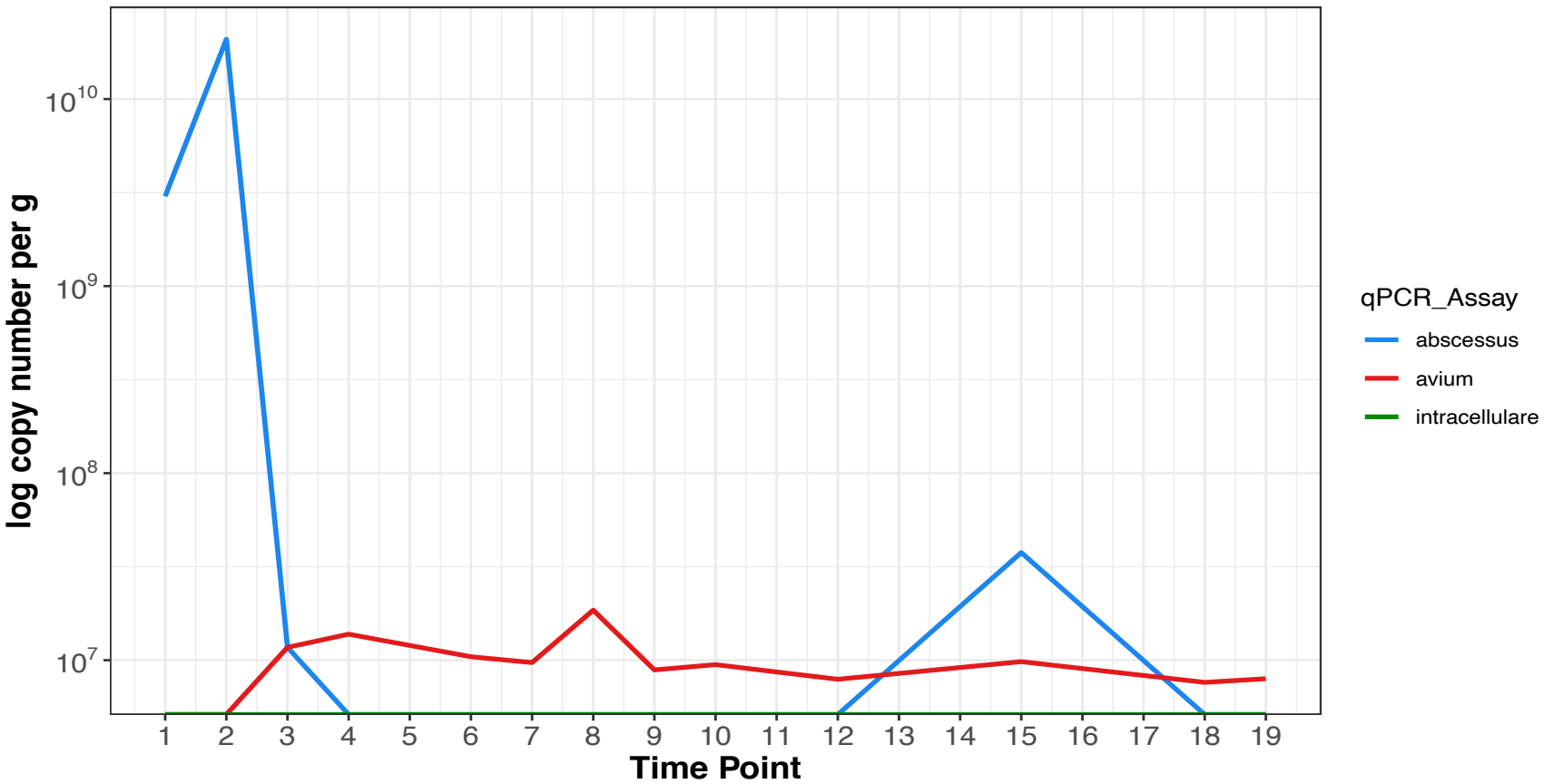
Table A21 - 16S Mock community composition.

Full Name	Strain Number
Actinomyces odontolyticus	DSM-19120
Bifidobacterium dentium	DSM-20436
Burkholderia cepacia	DSM-7288
Chlamydophila pneumoniae	DSM-19748
Corynebacterium pseudodiphtheriticum	DSM-44287
Enterococcus faecalis	DSM-20478
Escherichia coli	DSM-30083
Fusobacterium nucelatum subsp. nucleatum	DSM-15643
Granuclicatella adiacens	DSM-9848
Haemophilus influenzae	DSM-4690
Haemophilus parainfluenzae	DSM-8978
Leptotrichia buccalis	DSM-1135
Moraxella catarrhalis	DSM-9143
Mycobacterium bovis	DSM-43990
Mycobacterium psychrotolerans	DSM-44697
Mycoplasma penumoniae	DSM-22911
Neisseria flavescens	DSM-17633
Neisseria meningitidis	DSM-10036
Nocardia farcinica	DSM-43665
Pasteurella multocida subsp. multocida	DSM-16031
Pseudomonas fluorescens	DSM-50090
Rothia mucilaginosa	DSM-20746
Sallmonella enterica subsp. enterica	DSM-17058
Staphylococcus aureus subsp. aureus	DSM-20231
Streptococcus agalactiae	DSM-2134
Streptococcus mitis	DSM-12643
Streptococcus pneumoniae	DSM-20566
Streptococcus pyogenes	DSM-20565
Streptococcus pseudopneumoniae	DSM-18670
Streptococcus constellatus subsp. constellatus	DSM-20575
Streptococcus infantis	DSM-12492
Streptococcus parasanguinus	DSM-6778
Streptococcus sanguinis	DSM-20567
Treponema denticola	DSM-14222
Veillonella dispar	DSM-20735
Vibrio natriegens	DSM-759



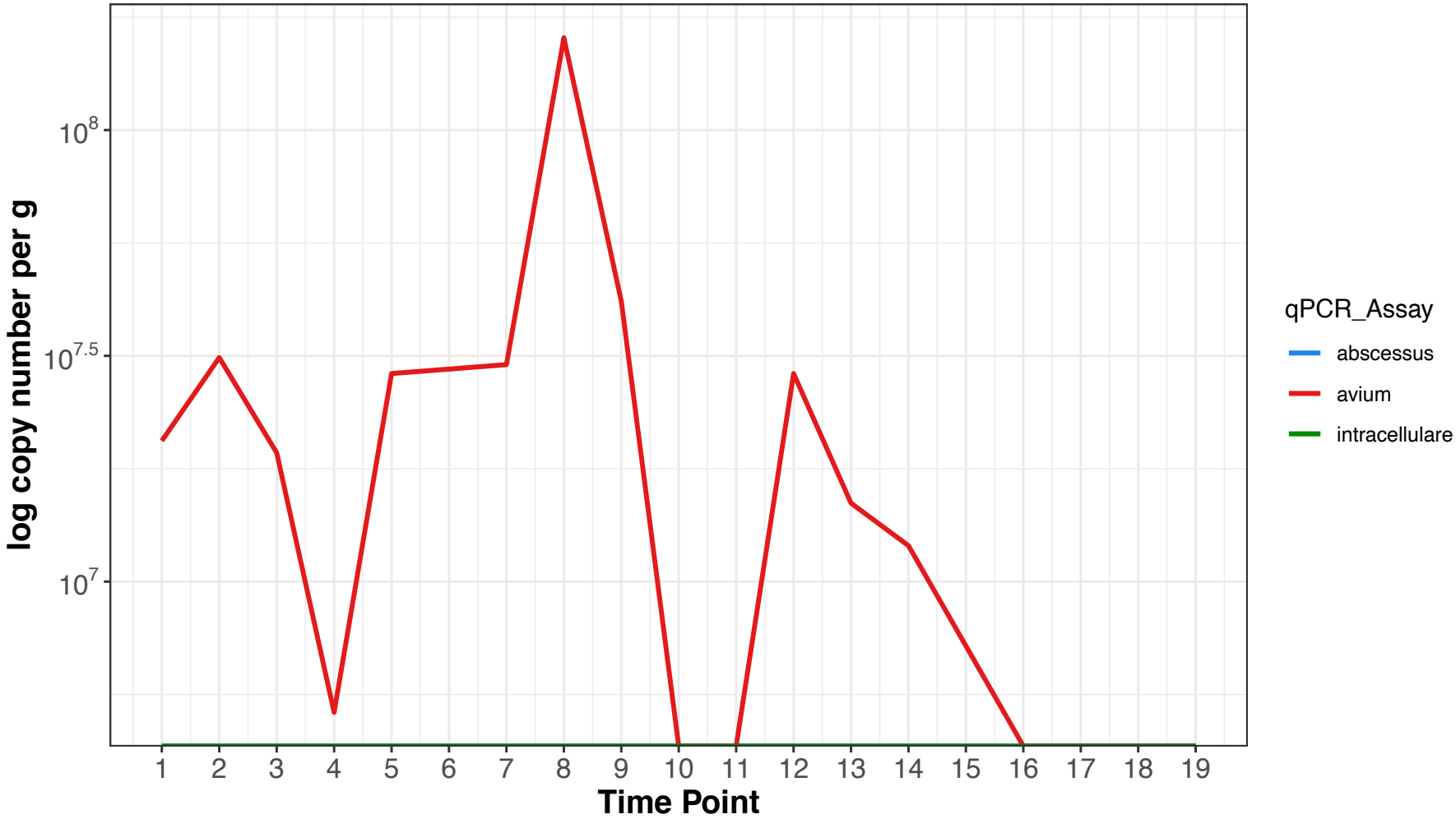
CULTURE RESULT	XEN	XEN		XEN															
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A1 – Longitudinal NTM copy numbers for Patient 1 with custom qPCR assays (Chapter 4). Patient 1 underwent treatment for *M. avium* with Rifampicin, Ethambutol and Azithromycin; was 59 years of age; BMI of 20.3; FEV1 of 13% predicted; FVC of 48% predicted. NTM culture results and treatment at each time point are shown in the table.



CULTURE RESULT	ABS	ABS	ABS												ABS			INT	INT
Amikacin (IV) / Meropenem / Tigecycline			Y	Y	Y														
Aithromycin / Amikacin (neb) / Doxy / Ciprofloxacin						Y	Y	Y	Y	Y	Y	Y	Y	Y					
Aithromycin / Amikacin (neb) / Doxy Moxifloxacin															Y	Y	Y	Y	Y

Figure A2 – Longitudinal NTM copy numbers for Patient 2 with custom qPCR assays (Chapter 4). Patient 2 underwent treatment for *M. abscessus* with Amikacin, Meropenem and Tigecycline as the initiation phase and Azithromycin, Amikacin (nebulised), Doxycycline and Moxifloxacin as the continuation phase; was 23 years of age; BMI of 22.9; FEV1 of 67% predicted; FVC of 94% predicted. NTM culture results as well as treatment at each time point are shown in the table.



CULTURE RESULT																			
Rifampicin/ Ethambutol/ Clarithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A3 – Longitudinal NTM copy number for Patient 3 with custom qPCR assays (Chapter 4). Patient 3 underwent treatment for *M. avium* with Rifampicin, Ethambutol and Clarithromycin; was 59 years of age; BMI of 20.2; FEV1 of 109% predicted; FVC of 110% predicted. NTM culture results as well as treatment at each time point are shown in the table.

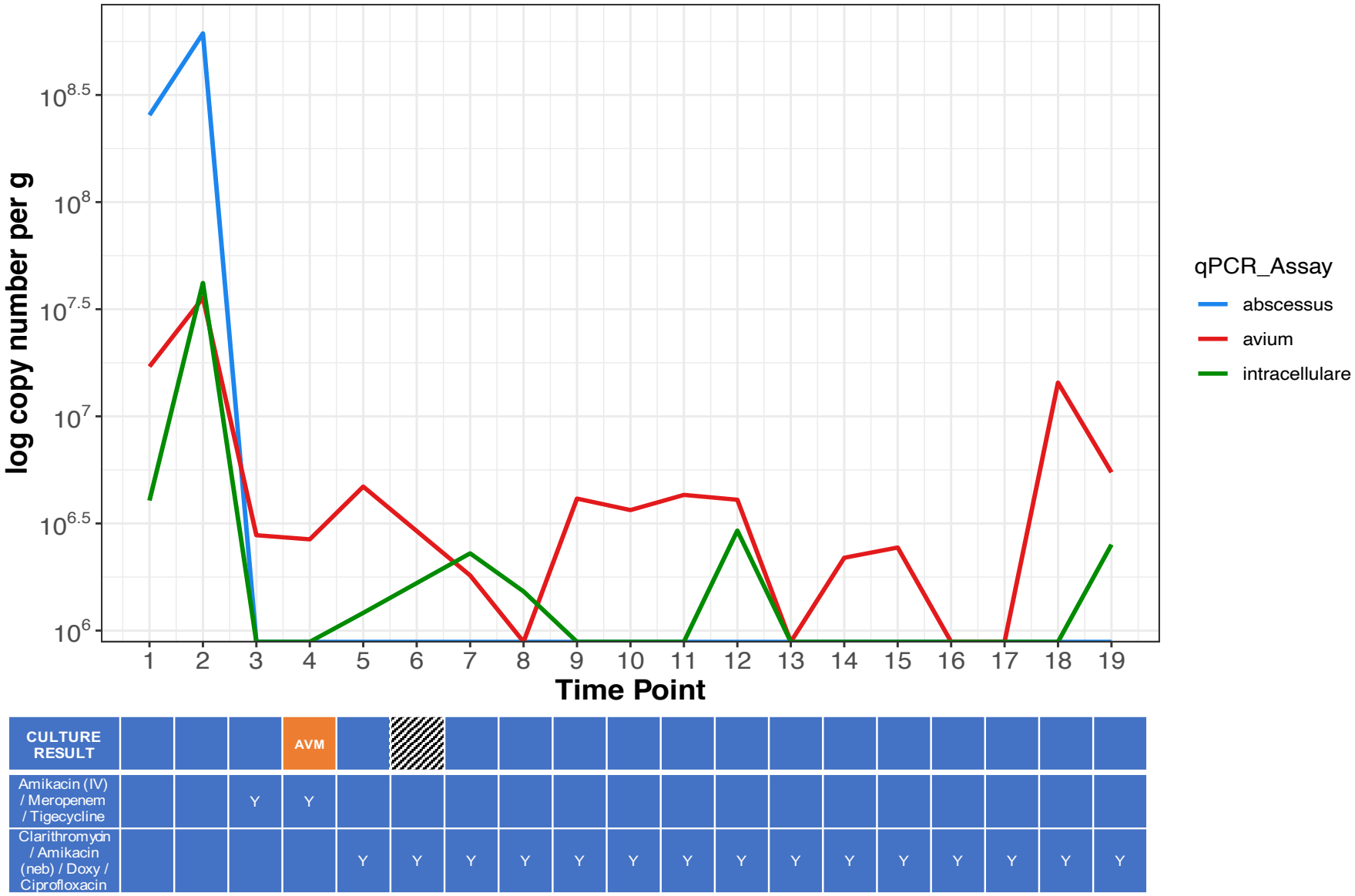
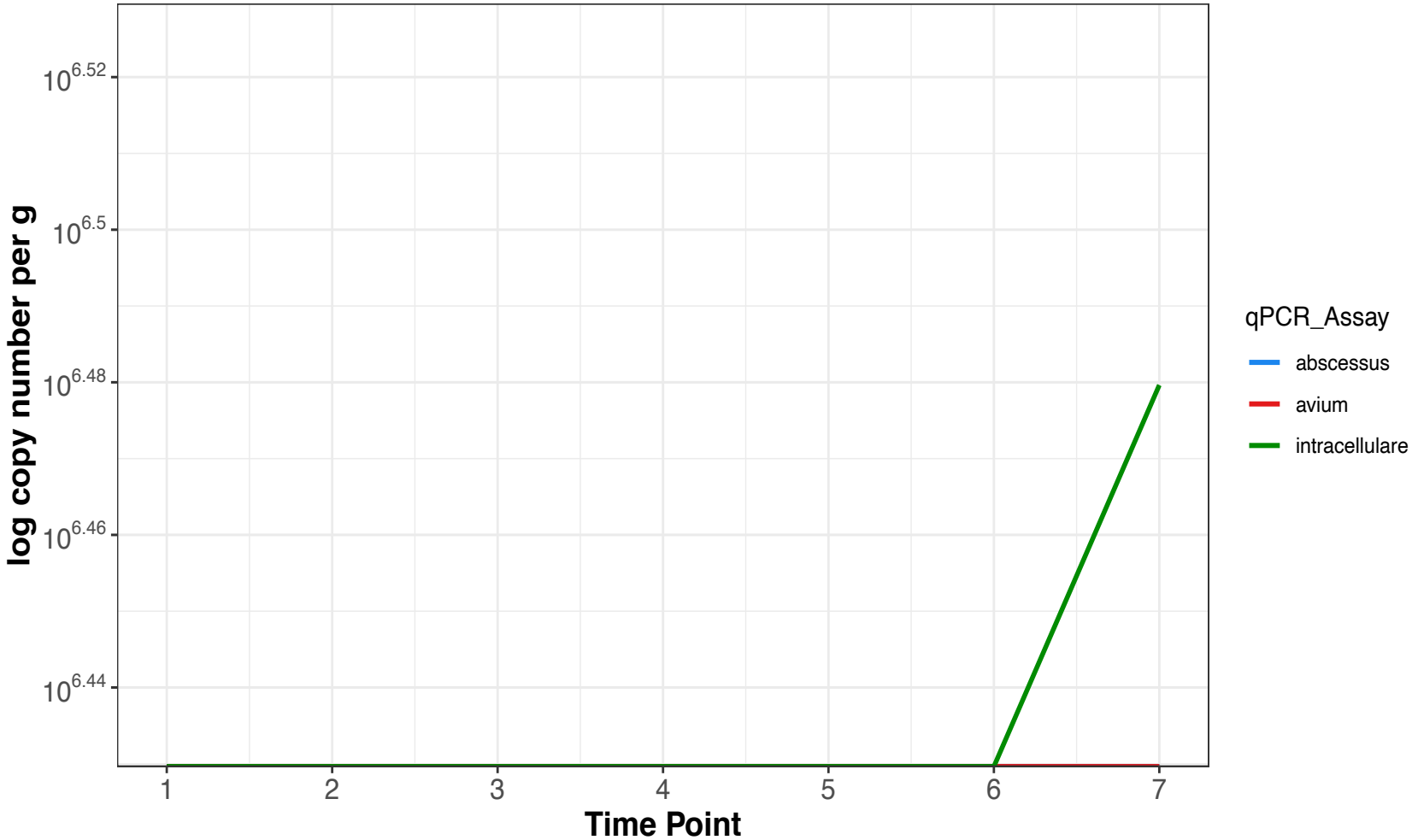
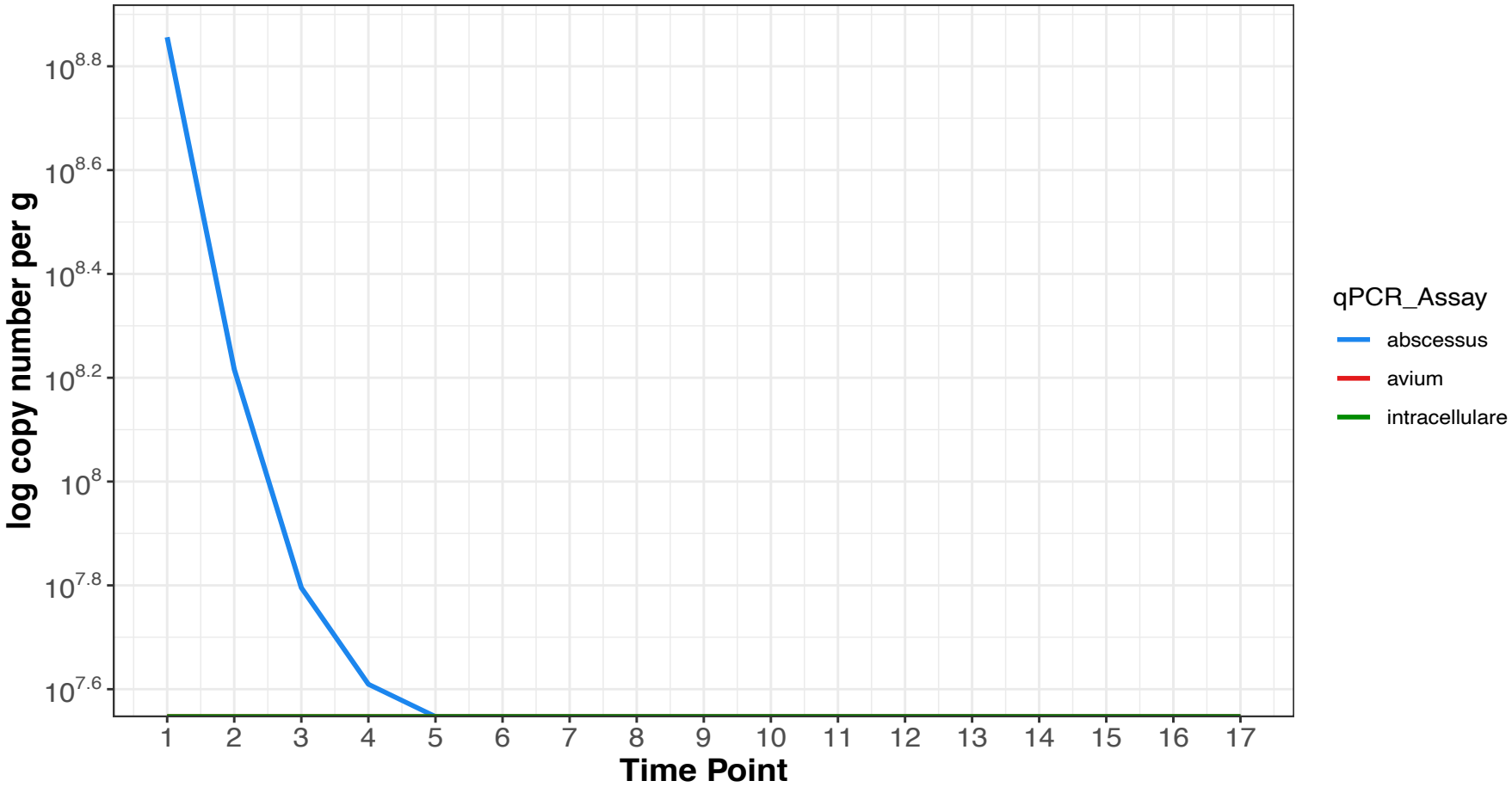


Figure A4 – Longitudinal NTM copy number for patient 4 with custom qPCR assays (Chapter 4). Patient 4 underwent treatment for *M. abscessus* with Amikacin, Meropenem and Tigecycline as the initiation phase and Clarithromycin, Amikacin (nebulised), Doxycycline and Ciprofloxacin as the continuation phase; was 17 years of age; BMI of 21.6; FEV1 of 99% predicted; FVC of 97% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



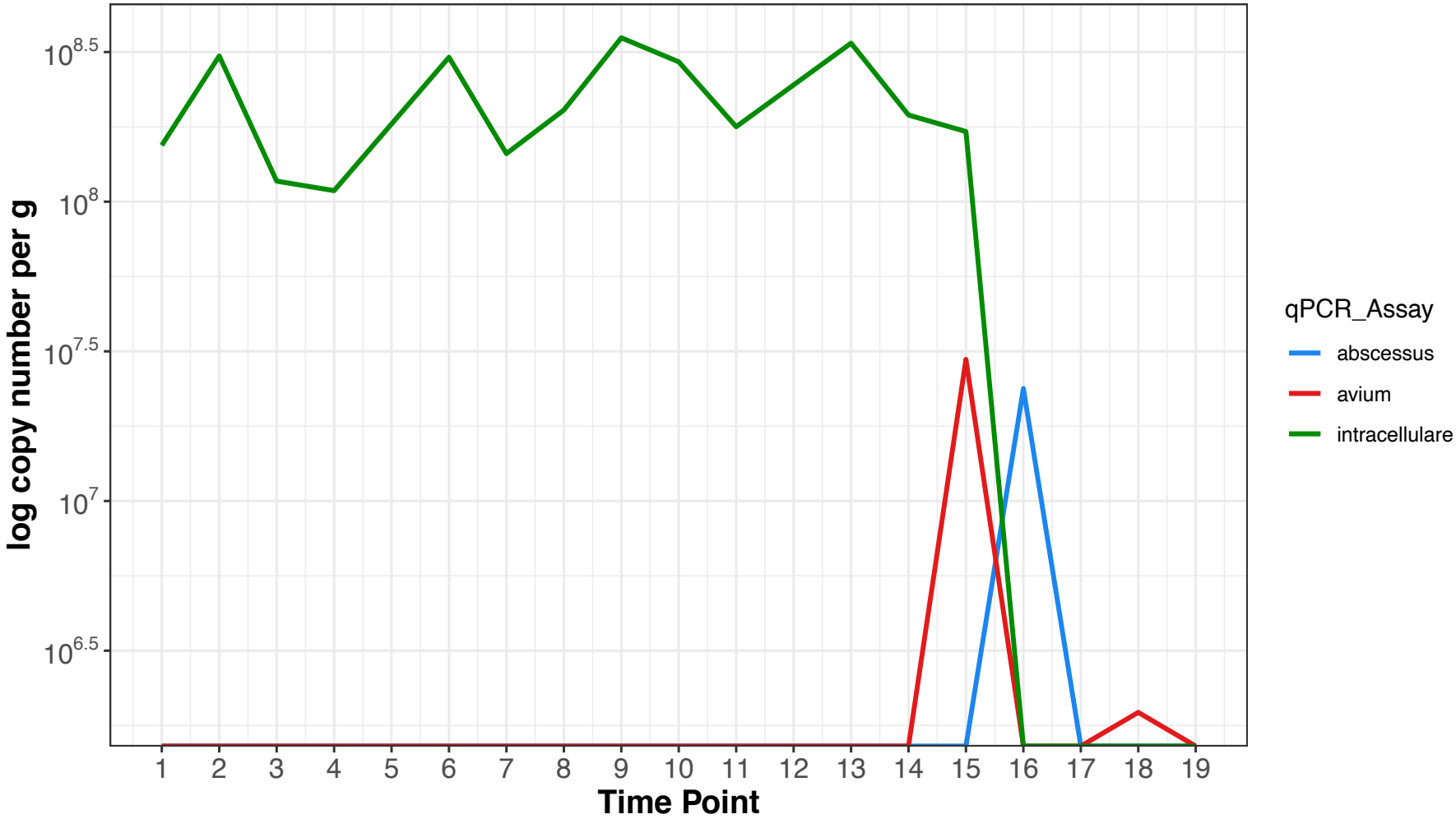
CULTURE RESULT						FORT	INTRA	

Figure A5 – Longitudinal NTM copy number for Patient 5 with custom qPCR assays (Chapter 4). Patient 5 isolated MAC but was not on treatment for the study period; was 63 years of age; BMI of 22.4; FEV1 of 87% predicted; FVC of 109% predicted. NTM culture results at each time point are shown in the table.



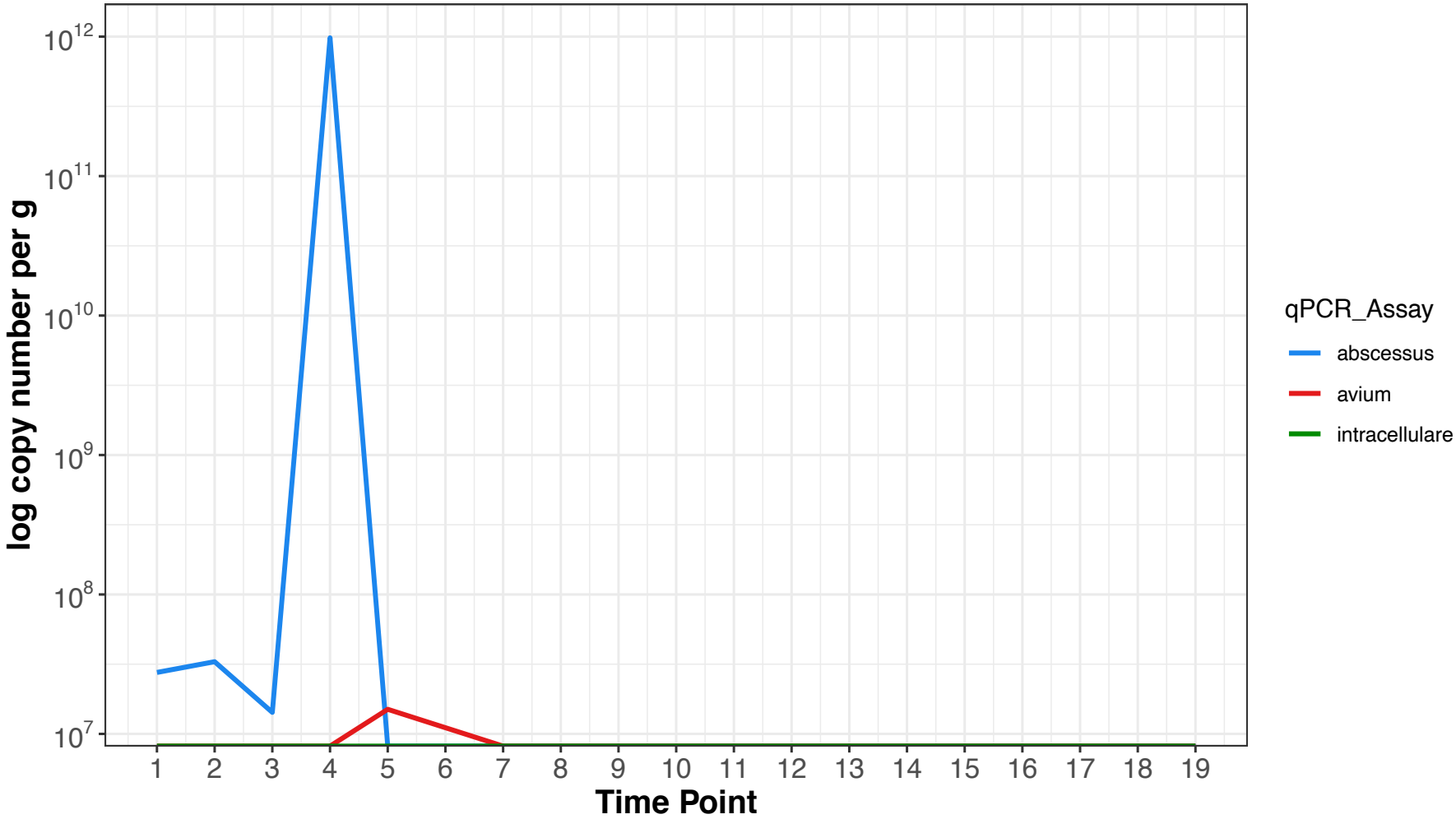
CULTURE RESULT		ABS	ABS	ABS													
Amikacin (IV) / Meropenem / Clarithromycin			Y	Y													
Clarithromycin / Amikacin (neb) / Minocycline / Clofazamine					Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A6 – Longitudinal NTM copy number for Patient 6 with custom qPCR assays (Chapter 4). Patient 6 underwent treatment for *M. abscessus* with Amikacin, Meropenem and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Minocycline and Clofazamine as the continuation phase; was 43 years of age; BMI of 23.3; a FEV1 of 114% predicted; FVC of 125% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT	AVM	AVM	AVM																
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A7 – Longitudinal NTM copy number for Patient 7 with custom qPCR assays (Chapter 4). Patient 7 underwent treatment for *M. avium* with Rifampicin, Ethambutol and Azithromycin; was 47 years of age; BMI of 23; a FEV1 of 101.3% predicted; FVC of 127.7% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT	ABS	ABS	ABS	ABS	AVM														
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A8 – Longitudinal NTM copy number for Patient 8 with custom qPCR assays (Chapter 4). Patient 8 underwent treatment for *M. avium* with Rifampicin, Ethambutol and Azithromycin; was 62 years of age; BMI of 23.2; a FEV1 of 101.3% predicted; FVC of 127.7% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

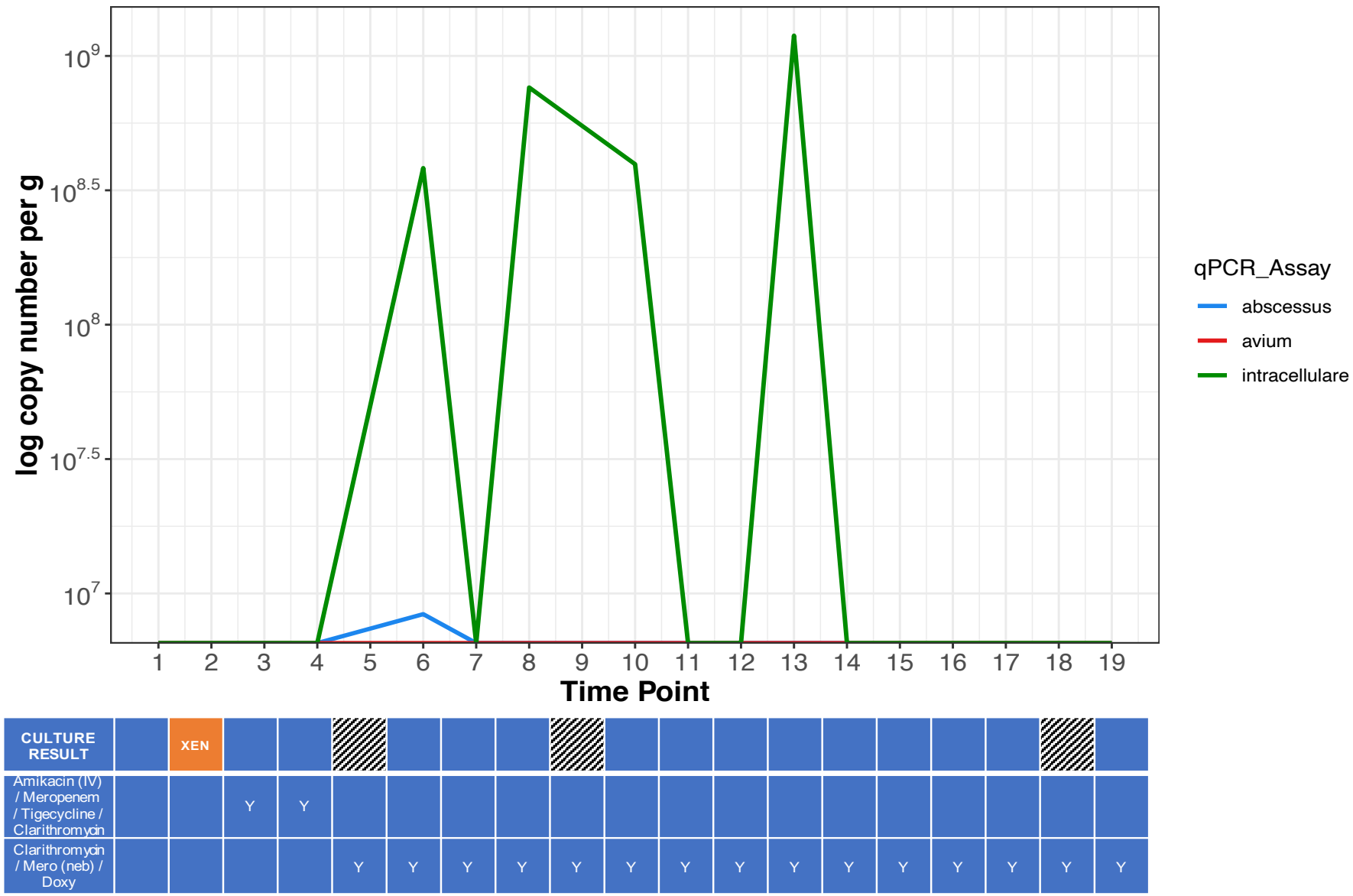


Figure A9 - Longitudinal NTM copy number for Patient 9 with custom qPCR assays (Chapter 4). Patient 9 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Meropenem (nebulised) and Doxycycline as the continuation phase; was 46 years of age; BMI of 20.0; a FEV1 of 50% predicted; FVC of 90% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

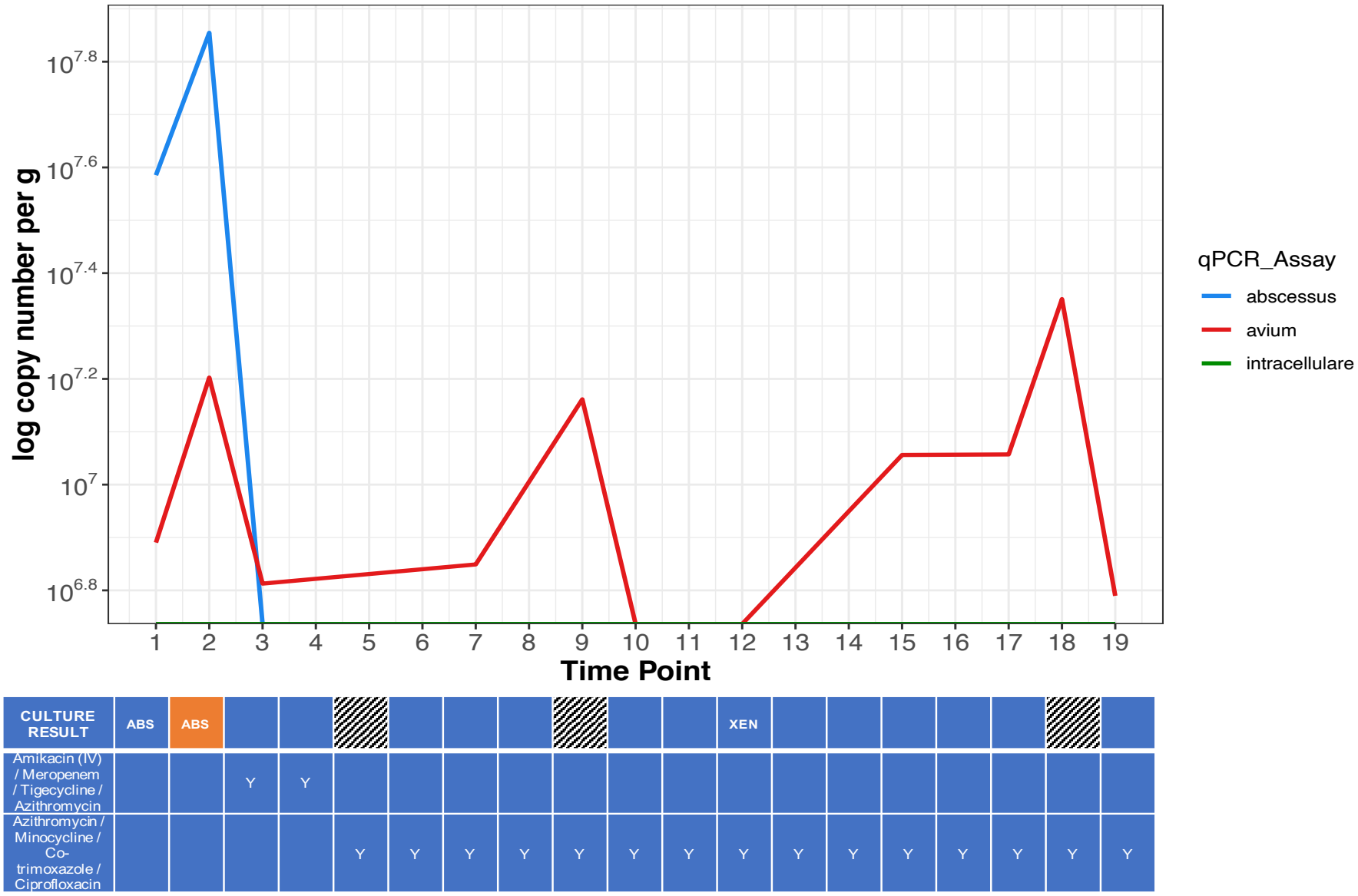
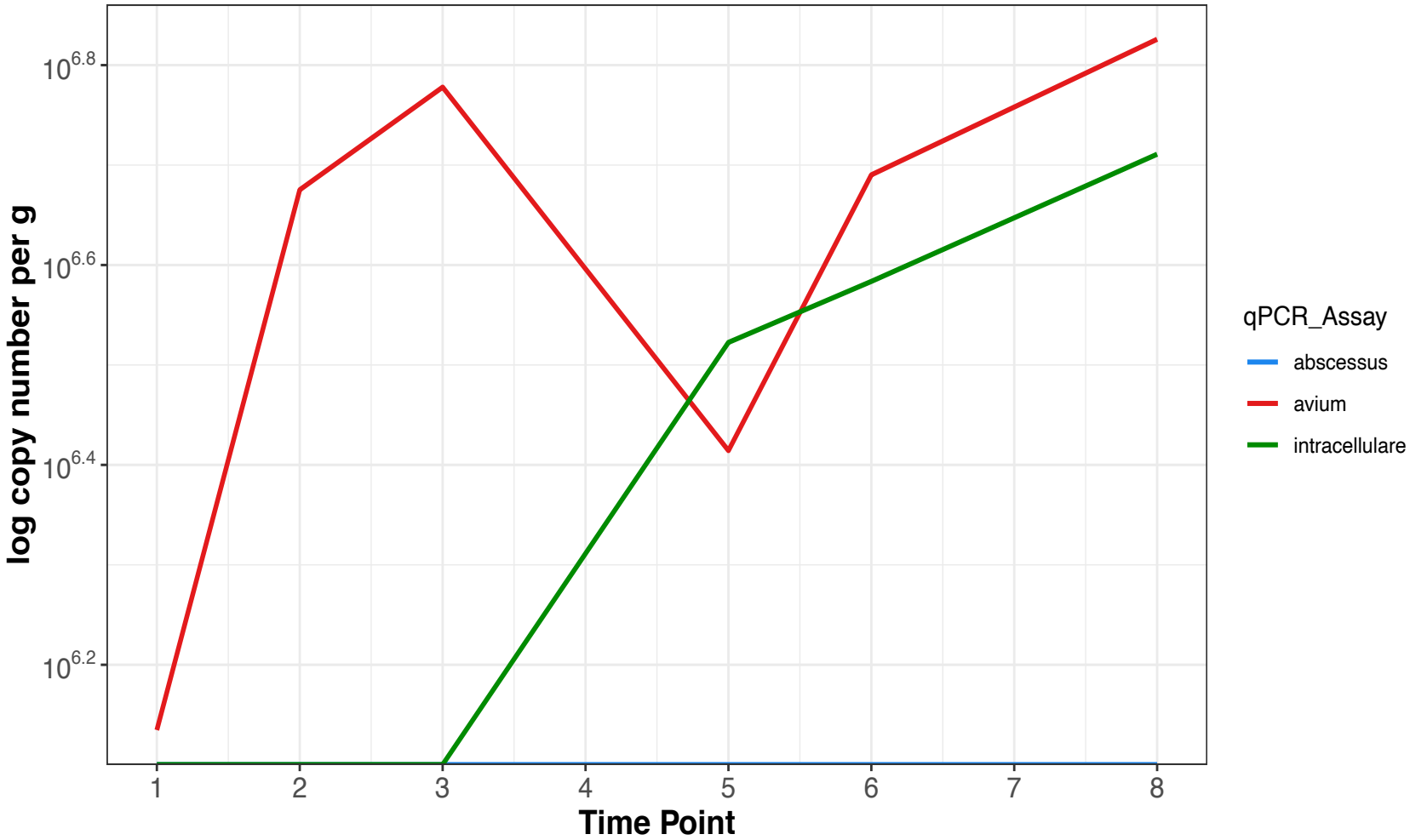
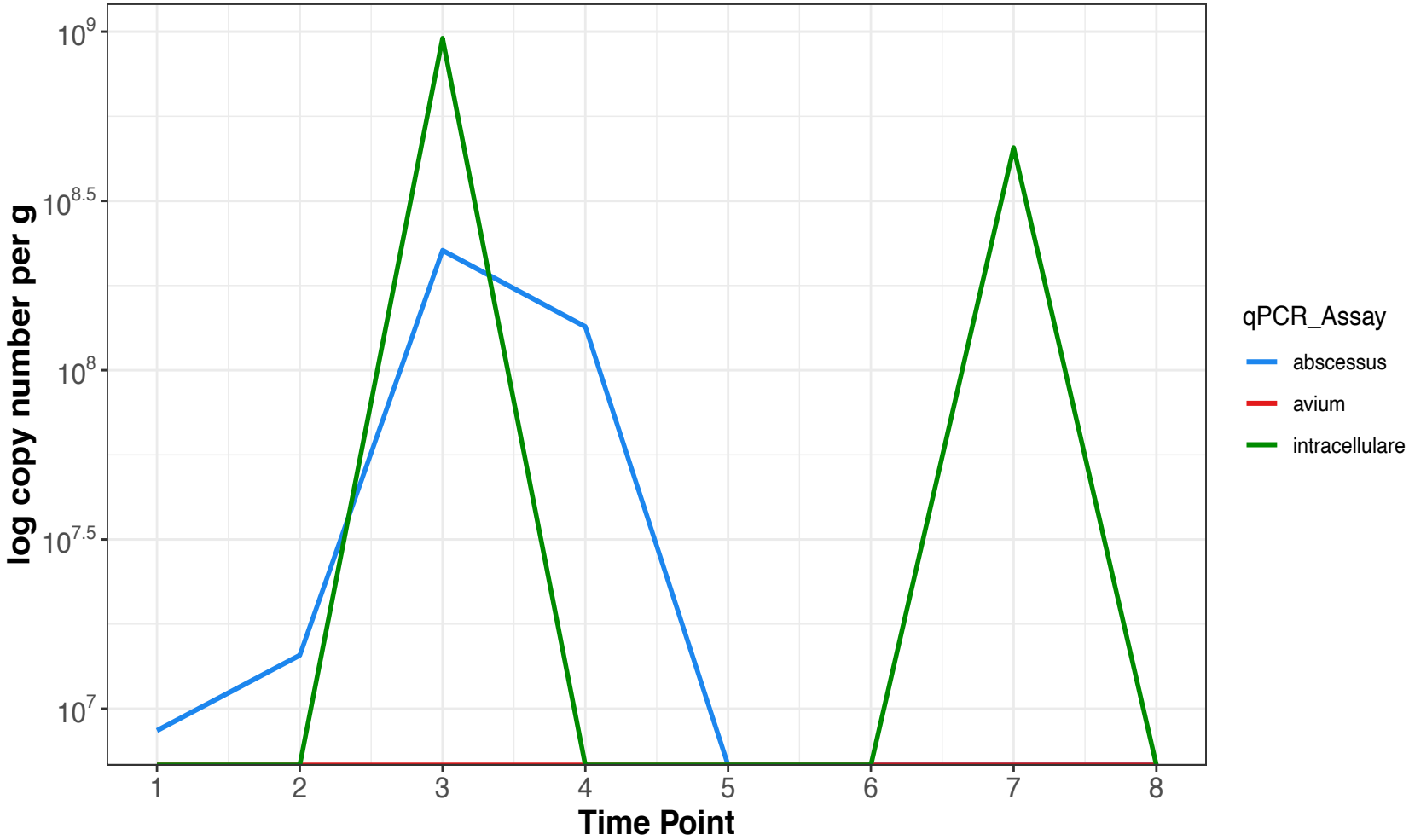


Figure A10 – Longitudinal NTM copy number for Patient 11 with custom qPCR assays (Chapter 4). Patient 11 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Azithromycin as the initiation phase and Azithromycin, Minocycline, Co-trimoxazole and Ciprofloxacin as the continuation phase; was 26 years of age; BMI of 18.6; a FEV1 of 17% predicted; FVC of 45% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



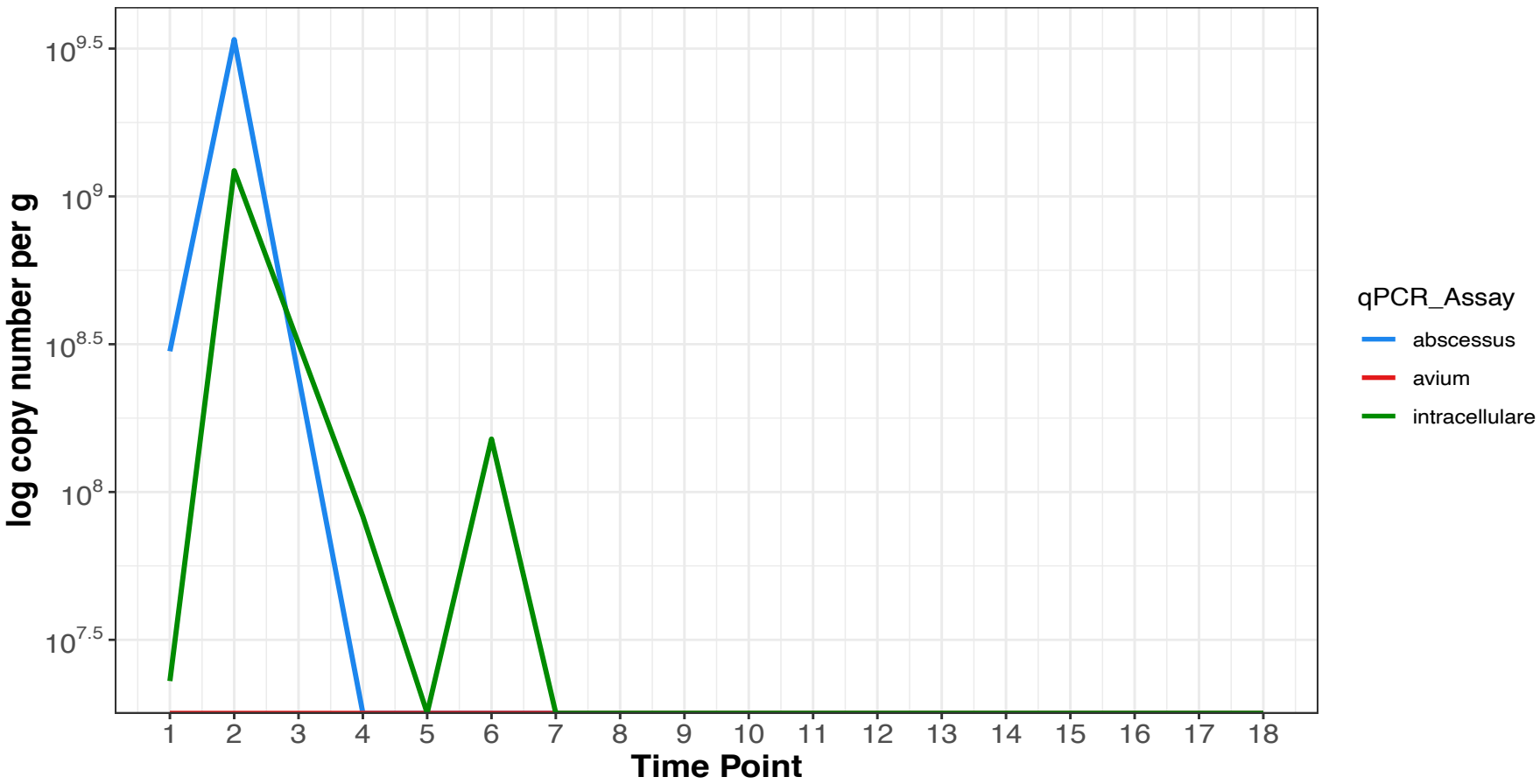
CULTURE RESULT	INT	INT	INT		INT	AVM		INT

Figure A11 – Longitudinal NTM copy number for Patient 12 with custom qPCR assays (Chapter 4). Patient 12 isolated MAC but was not on treatment for the study period; was 29 years of age; BMI of 19.1; a FEV1 of 54% predicted; FVC of 55% predicted. NTM copy numbers were ascertained using the custom qPCR assays described in Chapter 4. NTM culture results at each time point are shown in the table.



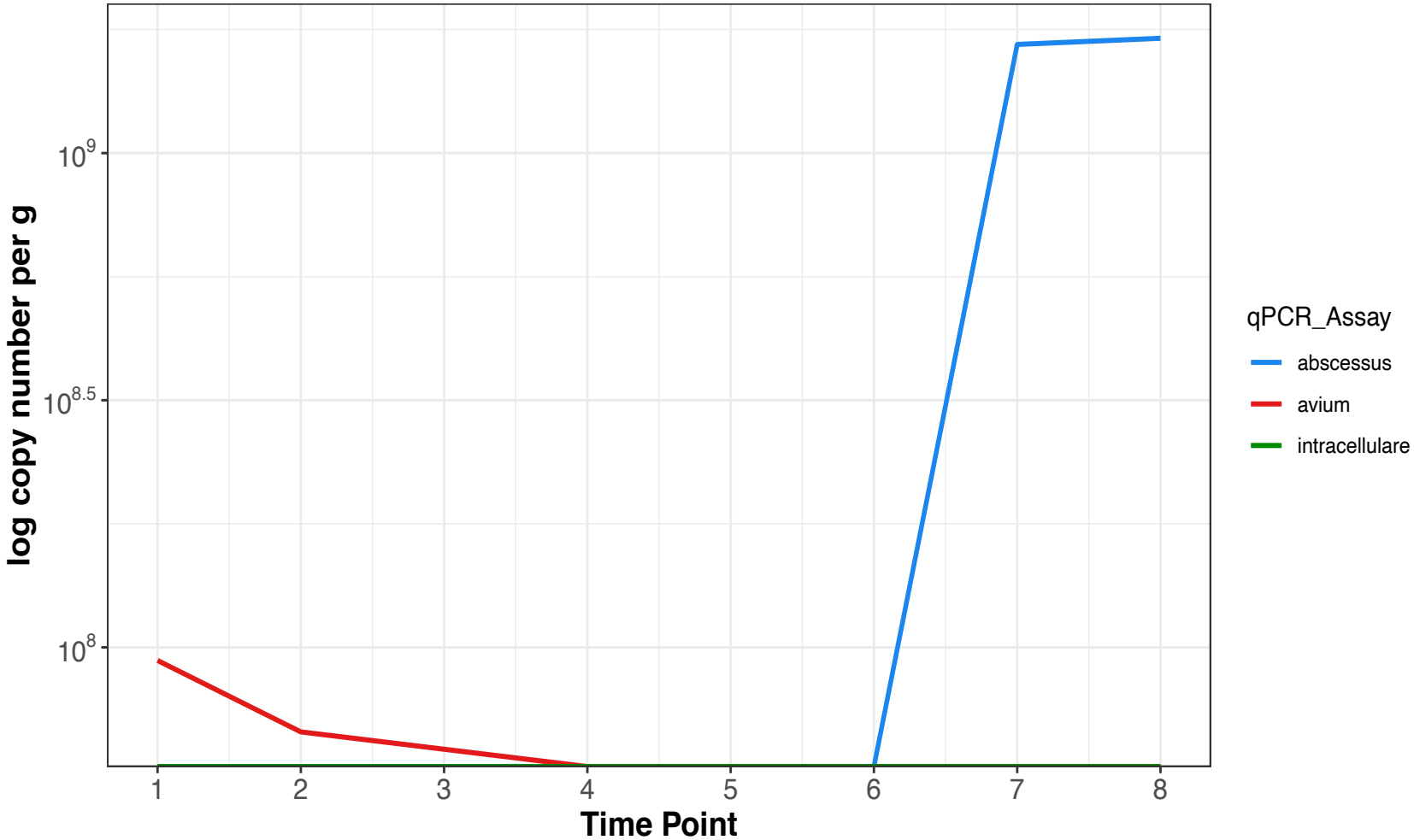
CULTURE RESULT	ABS	ABS	ABS	ABS			ABS	ABS

Figure A12 – Longitudinal NTM copy number for Patient 12 with custom qPCR assays (Chapter 4). Patient 13 isolated *M. abscessus* but was not on treatment for the study period. Patient 13 was 72 years of age, BMI of 22.4; FEV1 of 33.3% predicted; FVC of 56.5% predicted. NTM culture results at each time point are shown in the table.



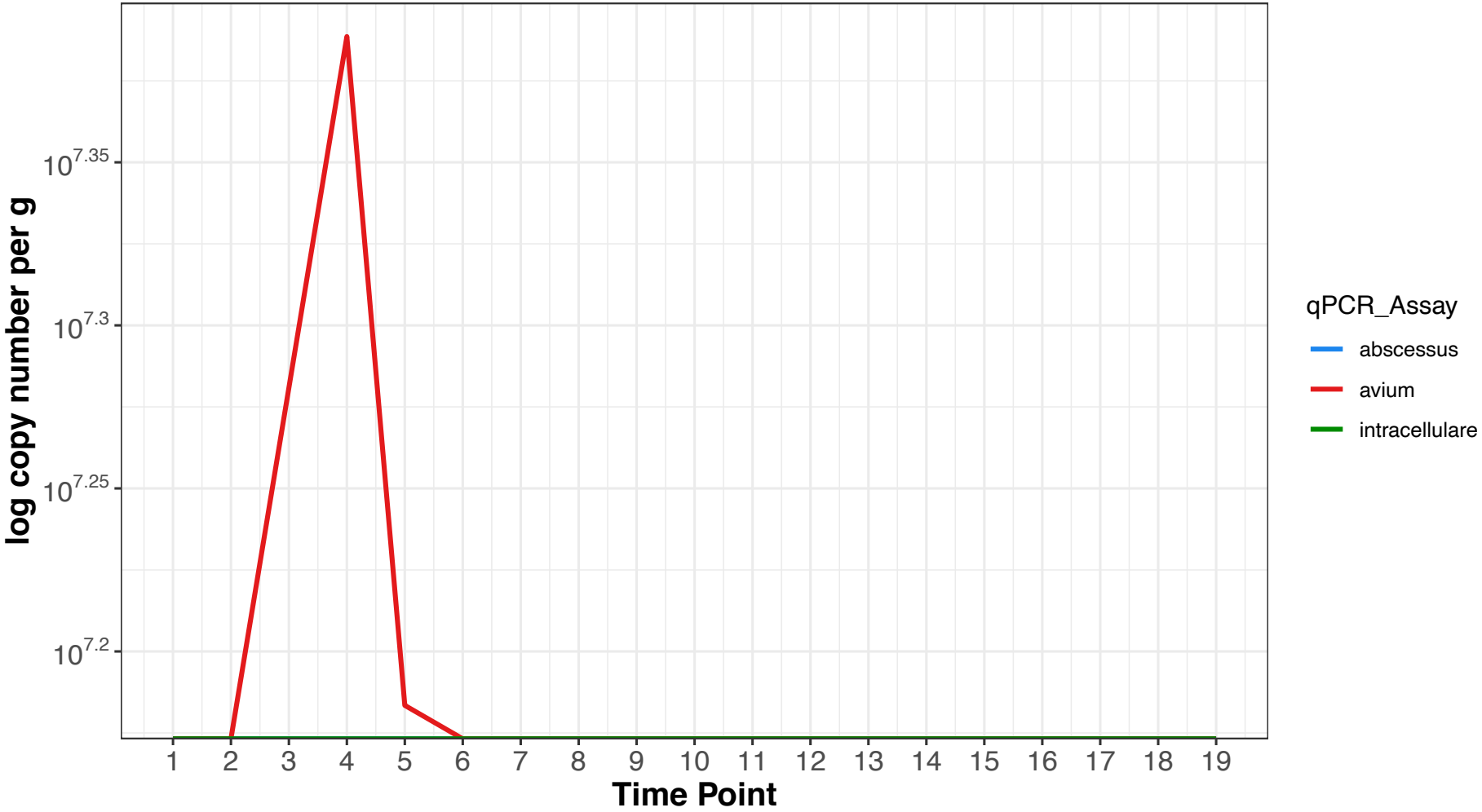
CULTURE RESULT	ABS	ABS		INT		INT												
Amikacin (IV) / Meropenem / Tigecycline / Clarithromycin			Y	Y														
Clarithromycin / Amikacin (neb) / Doxycycline / Moxyloxacin					Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A13 - Longitudinal NTM copy number for Patient 14 with custom qPCR assays (Chapter 4). Patient 14 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Doxycycline and Moxifloxacin as the continuation phase. Patient 14 was 19 years of age; BMI of 20.1, a FEV1 of 89% predicted; FVC of 97% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



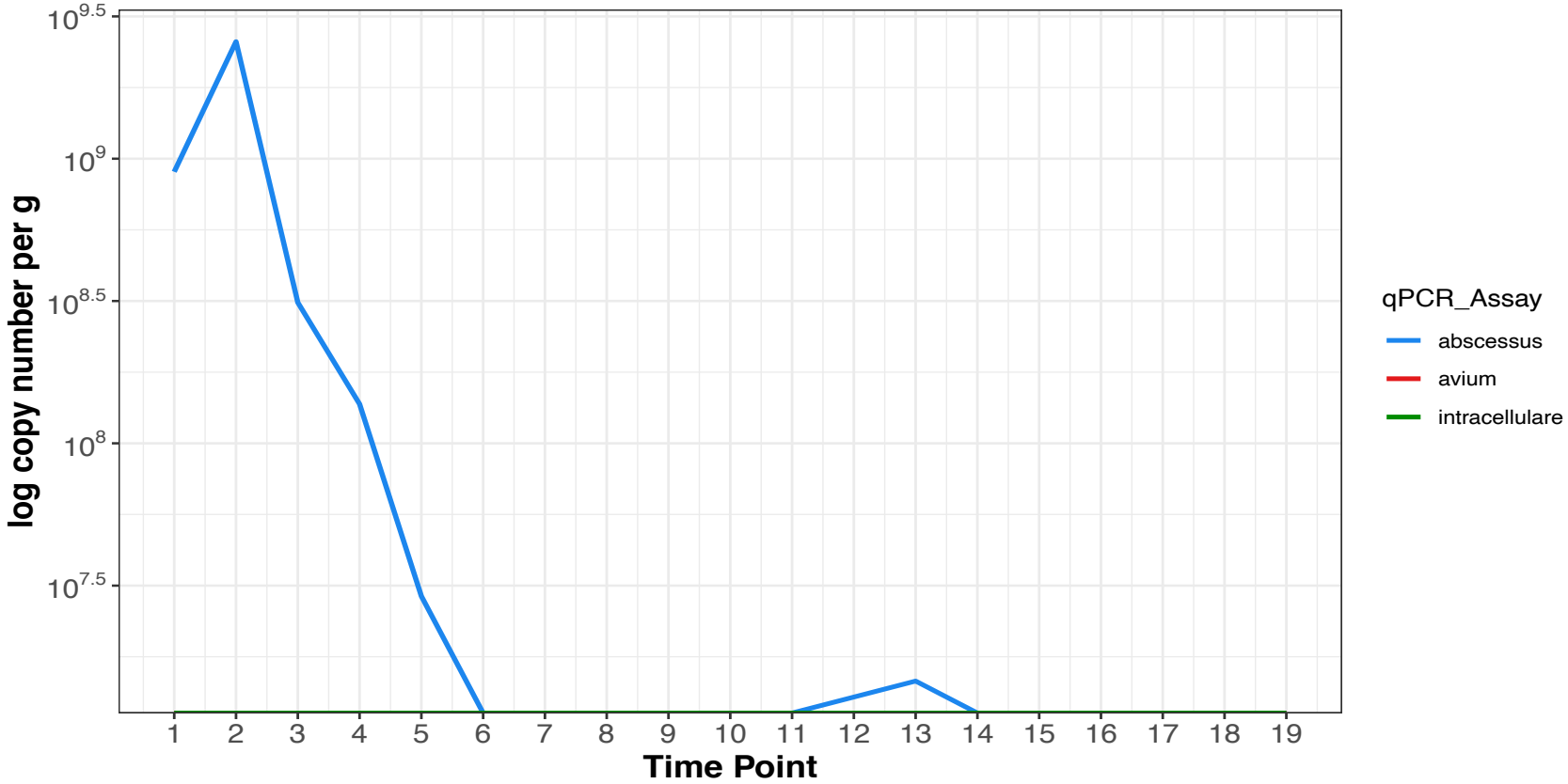
CULTURE RESULT	AVM	AVM					ABS	ABS

Figure A14 – Longitudinal NTM copy number for Patient 15 with custom qPCR assays (Chapter 4). Patient 15 isolated MAC but was not on treatment for the study period; was 18 years of age; BMI of 19; FEV1 of 72% predicted; FVC of 88% predicted. NTM culture results at each time point are shown in the table.



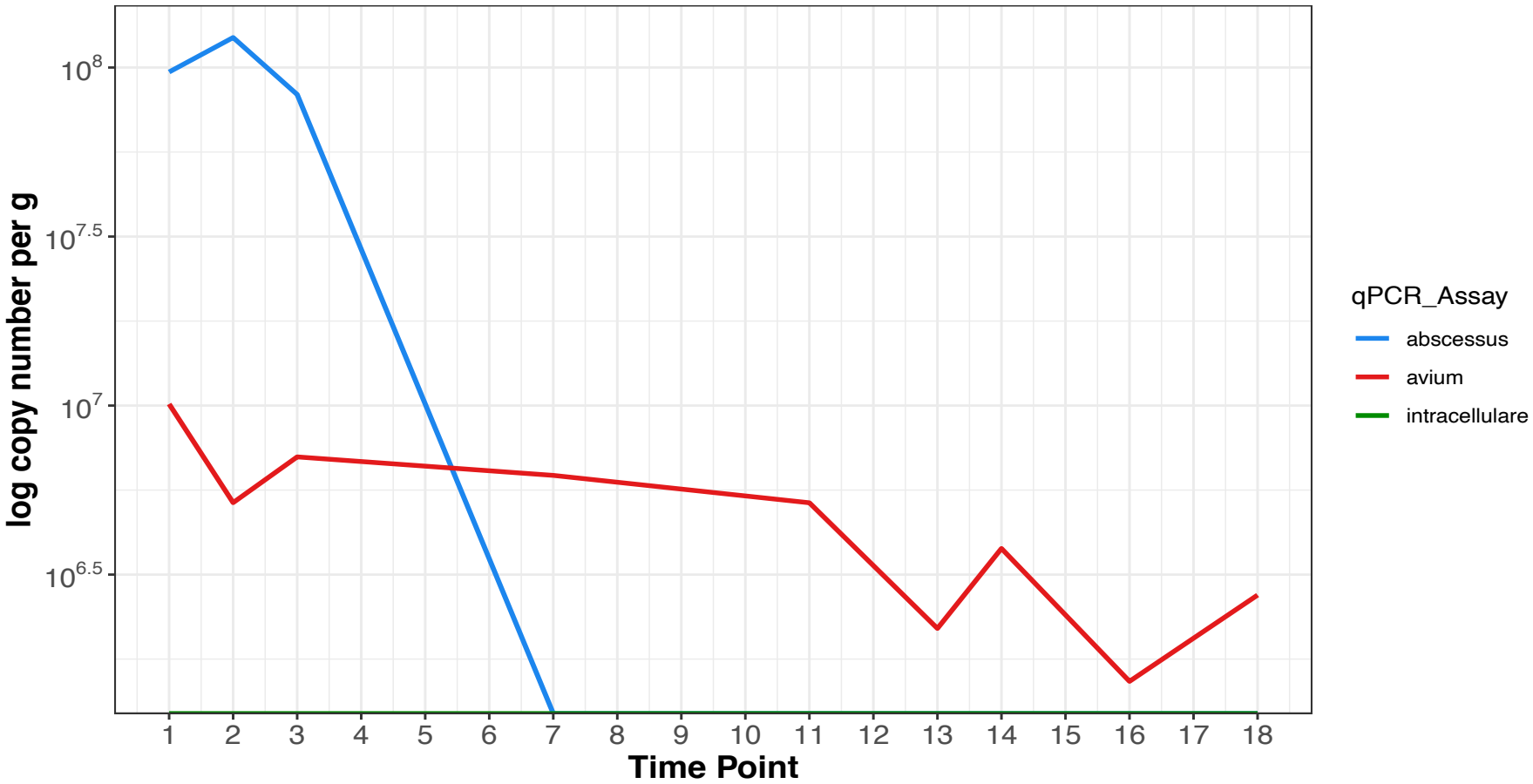
CULTURE RESULT																		
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A15 – Longitudinal NTM copy number for Patient 17 with custom qPCR assays (Chapter 4). Patient 17 underwent treatment for MAC with Rifampicin, Ethambutol and Azithromycin. Patient 17 was 61 years of age; BMI of 21.6; FEV1 of 50% predicted; FVC of 98% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT	ABS	ABS	ABS	ABS	ABS								ABS		KAN				
Amikacin (IV) / Meropenem / Tigecycline / Clarithromycin			Y	Y															
Clarithromycin / Amikacin (neb) / Minocycline / Co-trimoxazde					Y	Y	Y	Y	Y	Y	Y			Y	Y	Y	Y	Y	Y
Clarithromycin / Minocycline / Co-trimoxazde												Y	Y						

Figure A16 – Longitudinal NTM copy number for Patient 18 with custom qPCR assays (Chapter 4). Patient 18 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Minocycline and Co-trimoxazole as the continuation phase. At time points 12 and 13 nebulised Amikacin was held but reintroduced later. Patient 18 was 28 years of age; BMI of 21.4; FEV1 of 92% predicted; FVC of 113% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT	ABS	ABS	ABS								AVM		AVM	AVM		AVM		AVM
Amikacin (IV) / Tigecycline / Clarithromycin			Y	Y														
Clarithromycin / Amikacin (neb) / Minocycline / Ciprofloxacin					Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A17 – Longitudinal NTM copy number for Patient 19 with custom qPCR assays (Chapter 4). Patient 19 underwent treatment for *M. abscessus* with Amikacin, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Minocycline and Ciprofloxacin as the continuation phase. Patient 19 was 23 years of age, BMI of 16.5; FEV1 of 66% predicted; FVC of 75% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

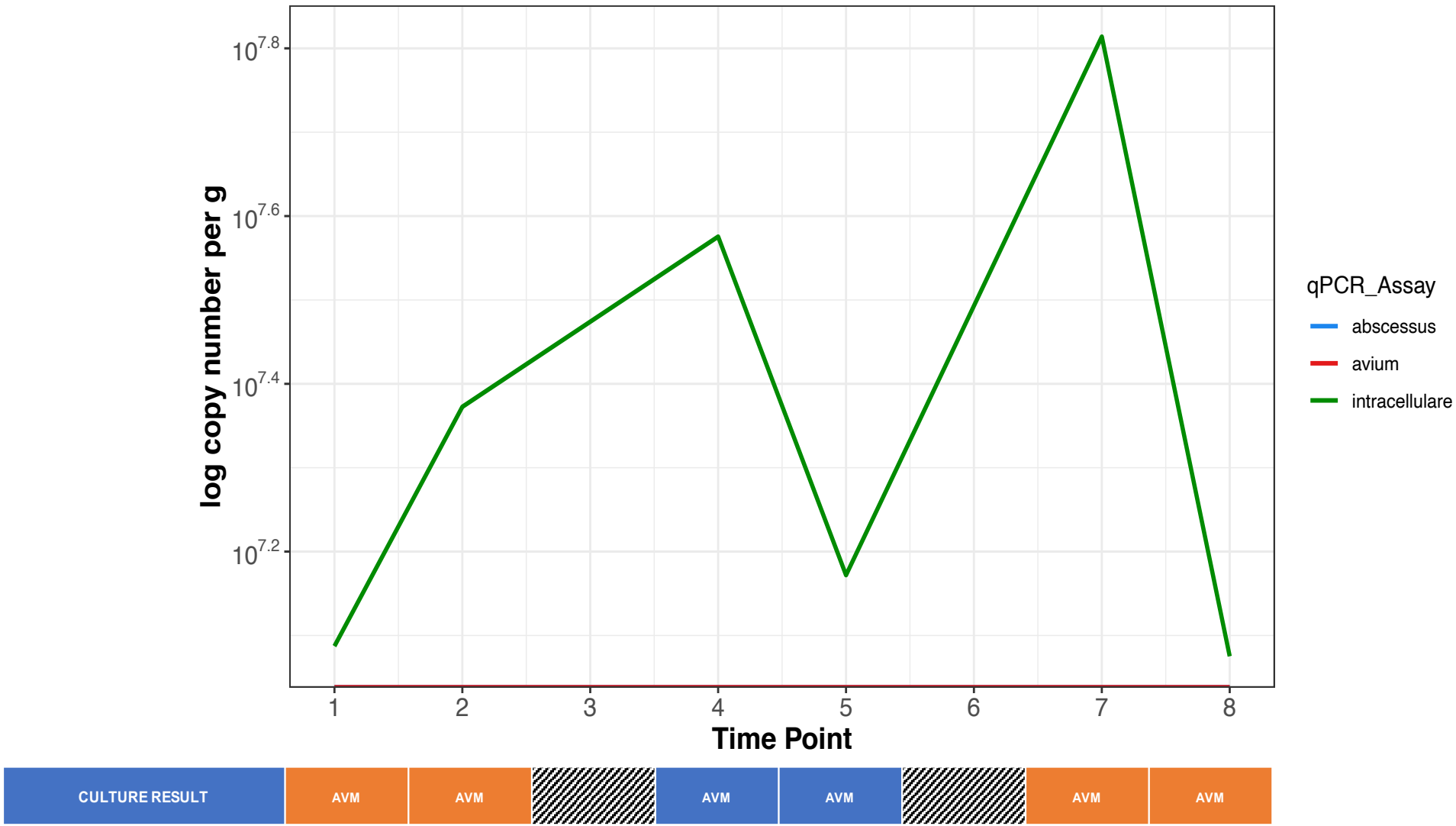
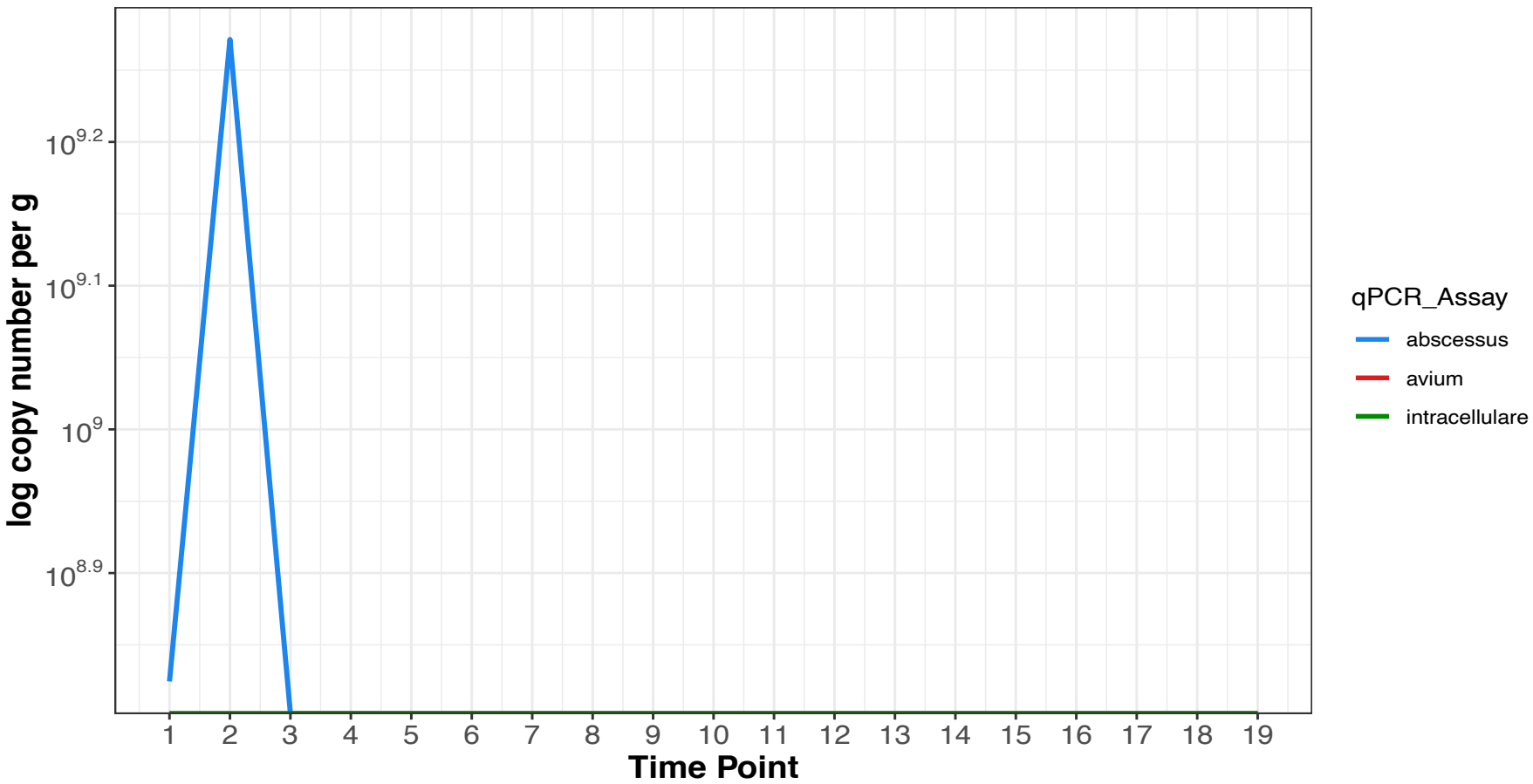


Figure A18 – Longitudinal NTM copy number for Patient 21 with custom qPCR assays (Chapter 4). Patient 21 isolated MAC but was not on treatment for the study period. Patient 21 was 25 years of age; BMI of 21; FEV1 of 75% predicted; FVC of 93% predictedNTM culture results at each time point are shown in the table.



CULTURE RESULT	ABS	ABS	ABS	ABS		ABS						ABS							
Amikacin (IV) / Meropenem / Tigecycline / Clarithromycin			Y	Y															
Clarithromycin / Amikacin (neb) / Minocycline / Ciprofloxacin					Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A19 – Longitudinal NTM copy number for Patient 22 with custom qPCR assays (Chapter 4). Patient 22 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Minocycline and Ciprofloxacin as the continuation phase. Patient 22 was 30 years of age; BMI of 21; FEV1 of 78% predicted; FVC of 103% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

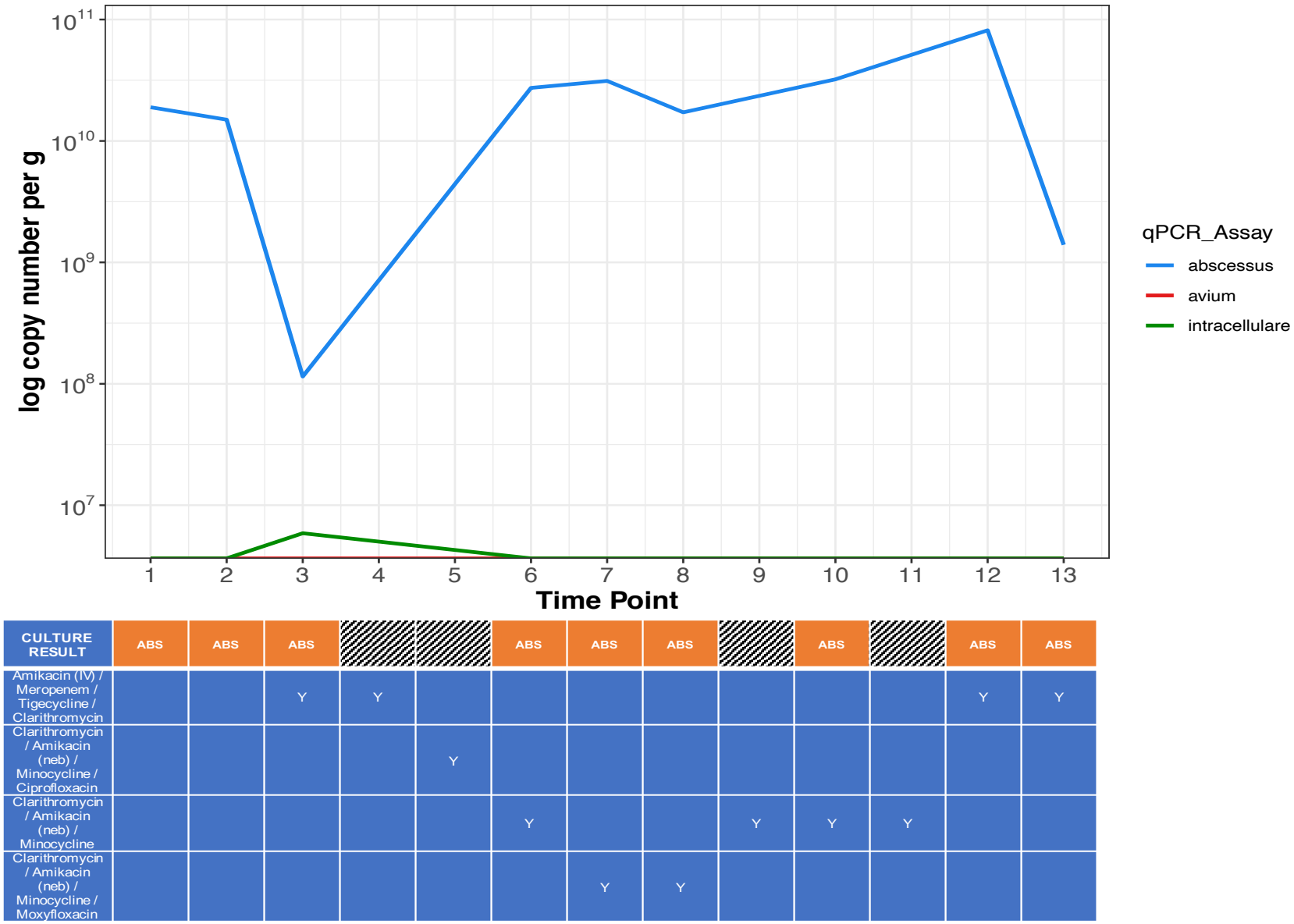
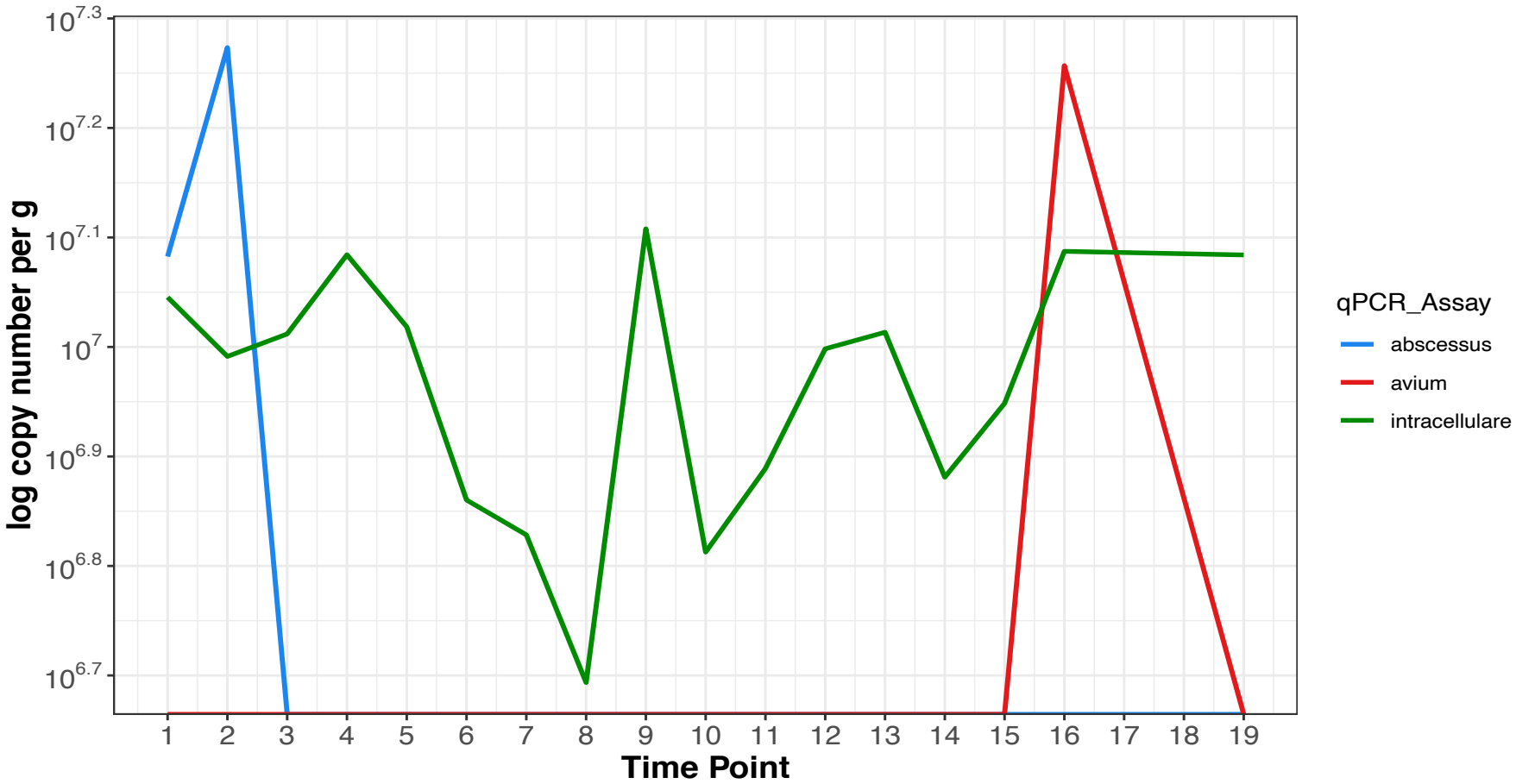
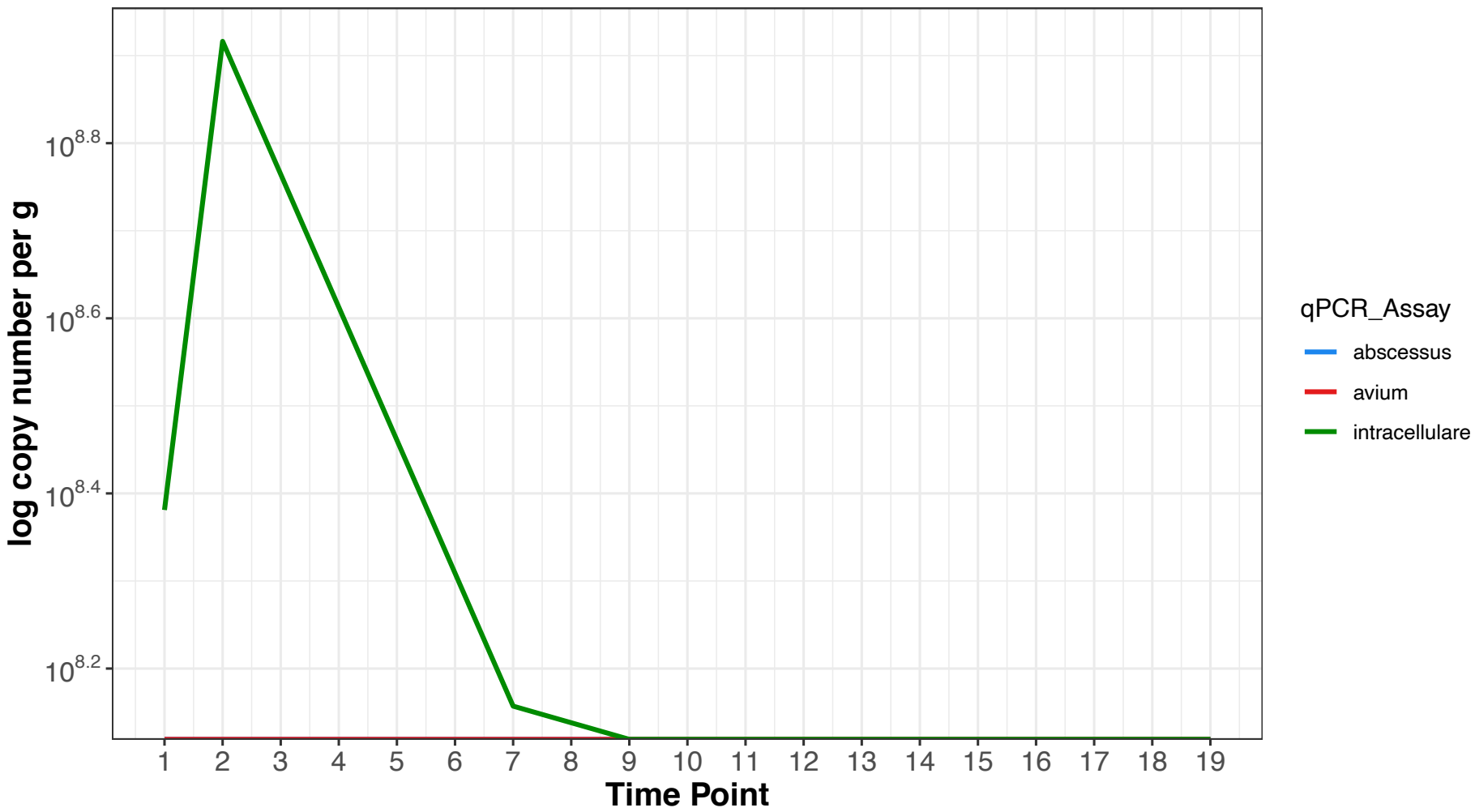


Figure A20 - Longitudinal NTM copy number for Patient 23 who underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin (initiation phase) and Clarithromycin, Amikacin (nebulised), Minocycline and Ciprofloxacin (continuation phase). Ciprofloxacin was dropped at time point 6 before Moxifloxacin was introduced. Moxifloxacin was dropped between time points 9 -11. At time points 12 and 13 patient was re-admitted for intravenous treatment. Patient 23 was 17 years of age; BMI of 19.1; FEV1 of 65% predicted; FVC of 83% predicted.



CULTURE RESULT																			
Amikacin (IV) / Meropenem / Tigecycline / Clarithromycin			Y	Y															
Clarithromycin / Amikacin (neb) / Minocycline / Ciprofloxacin					Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A21 – Longitudinal NTM copy number for patient 27 with custom qPCR assays (Chapter 4). Patient 27 underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Clarithromycin as the initiation phase and Clarithromycin, Amikacin (nebulised), Minocycline and Ciprofloxacin as the continuation phase. Patient 27 was 37 years of age; BMI of 23.9, a FEV1 of 92% predicted; FVC of 107% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT	AVM	AVM					AVM							INT	INT				
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

Figure A22 – Longitudinal NTM copy number for Patient 28 with custom qPCR assays (Chapter 4). Patient 28 underwent treatment for MAC with Rifampicin, Ethambutol and Azithromycin. Patient 28 was 53 years of age; BMI of 26; FEV1 of 35% predicted; FVC of 50% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

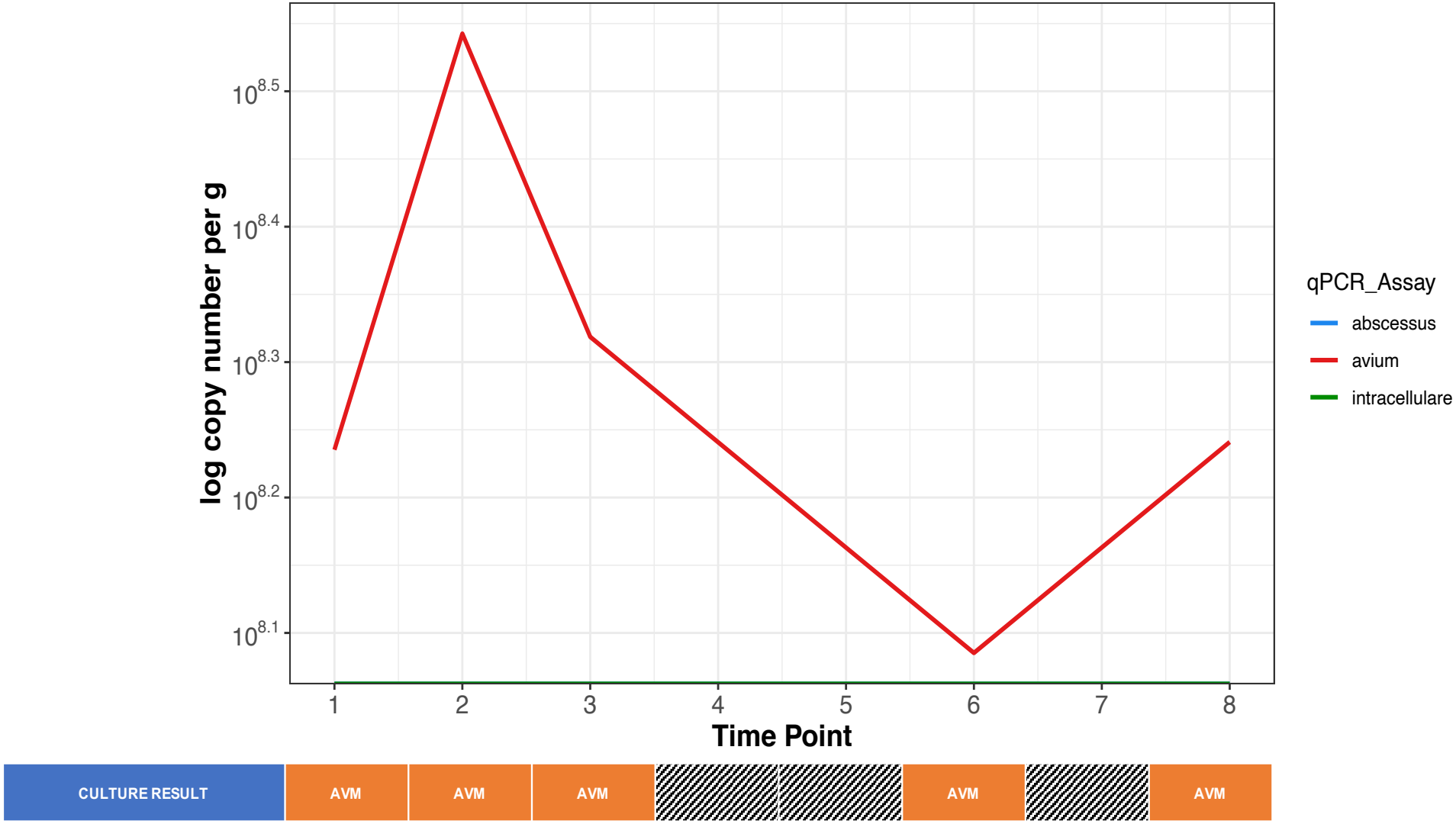
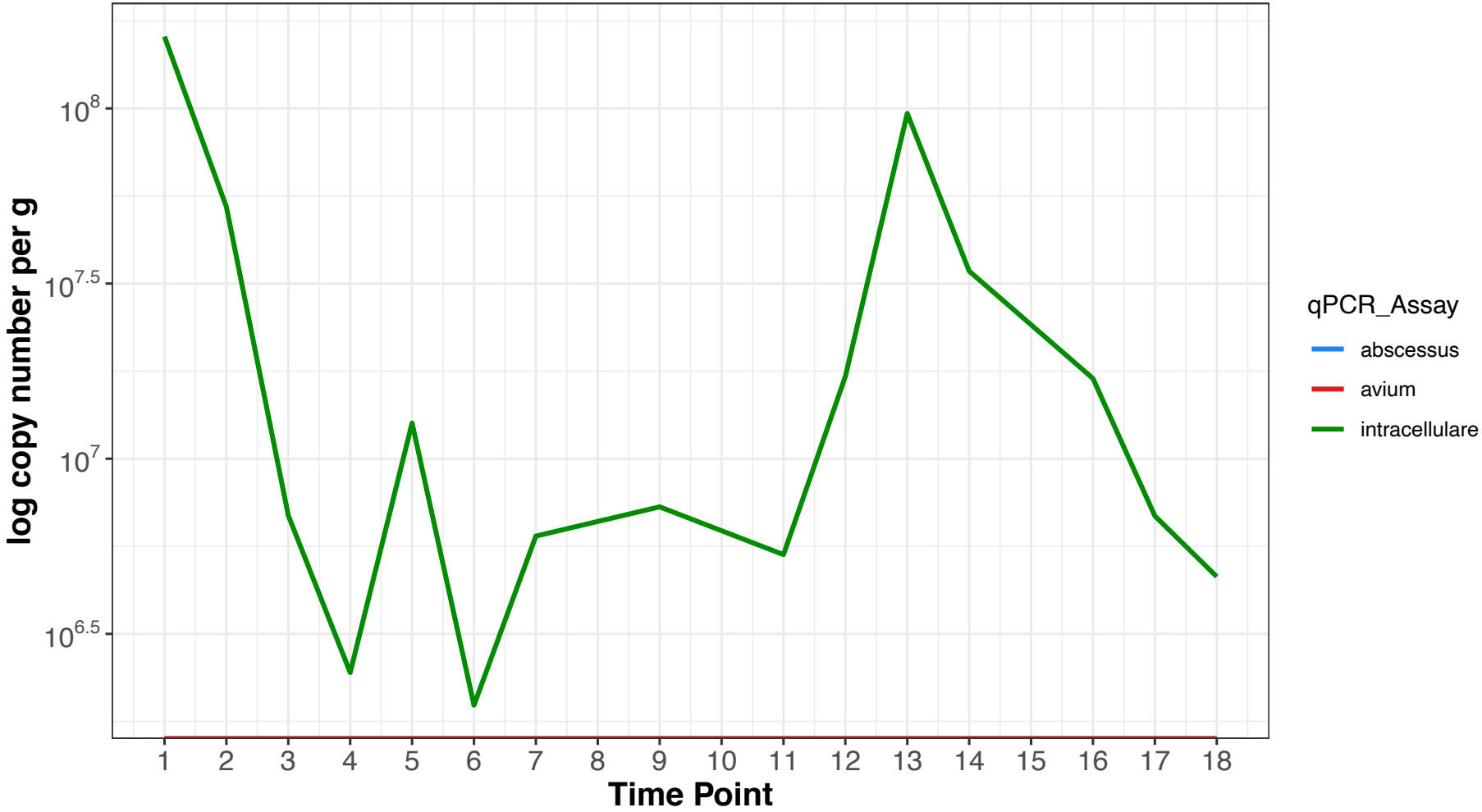
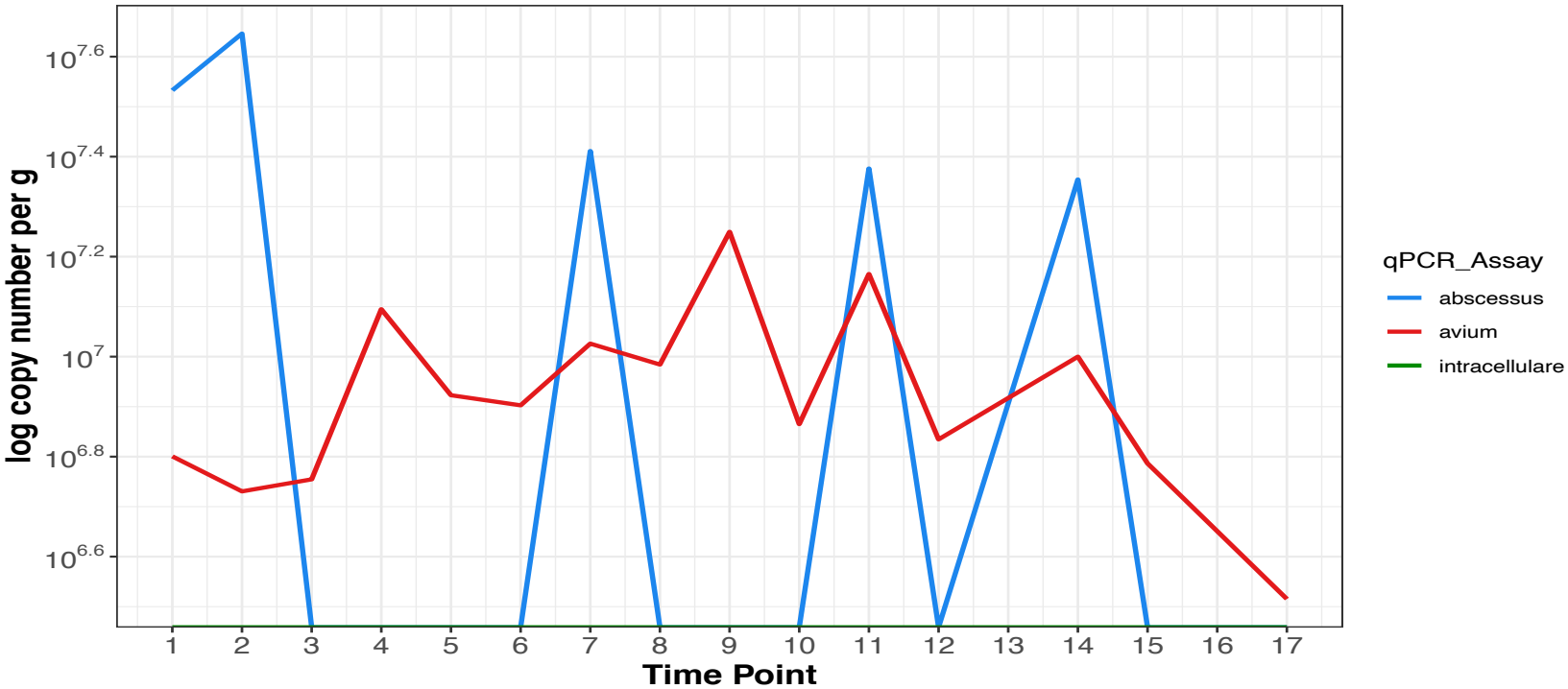


Figure A23 – Longitudinal NTM copy number for Patient 31 with custom qPCR assays (Chapter 4). Patient 31 isolated MAC but was not on treatment for the study period. Patient 31 was 79 years of age; BMI of 21.8; FEV1 of 80% predicted and a FVC of 100% predicted. NTM culture results at each time point are shown in the table.



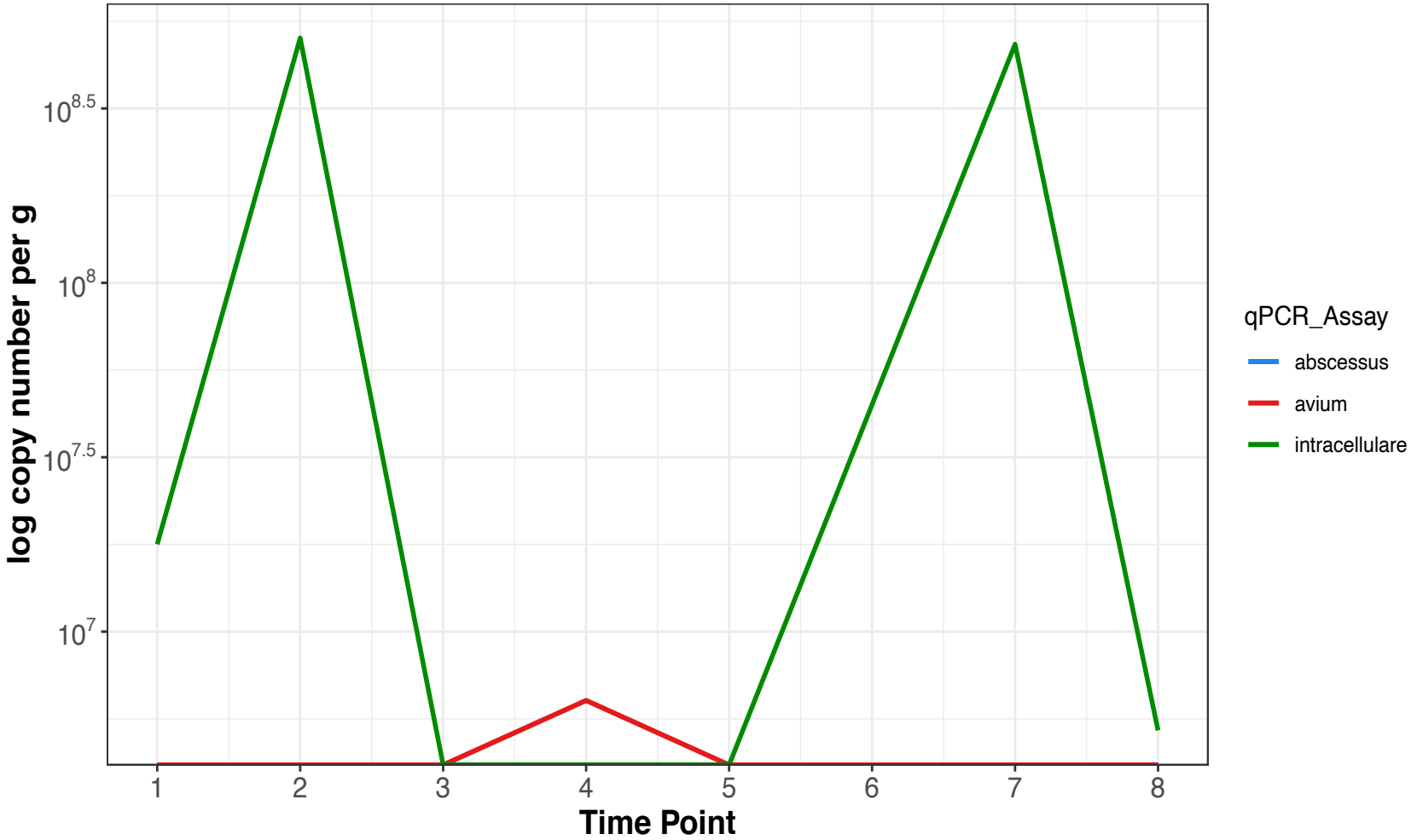
CULTURE RESULT			AVM	AVM			INT		INT		INT	INT	INT	INT		INT	INT	INT
Rifampicin/ Ethambutol/ Azithromycin			Y	Y	Y	Y	Y	Y	Y	Y				Y	Y	Y	Y	Y

Figure A24 – Longitudinal NTM copy number for Patient 34 with custom qPCR assays (Chapter 4). Patient 34 underwent treatment for MAC with Rifampicin, Ethambutol and Azithromycin. At time points 11 to 14 medication was held owing to side effects. Patient 34 was 65 years of age; BMI of 19.4; FEV1 of 89% predicted; FVC of 90% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



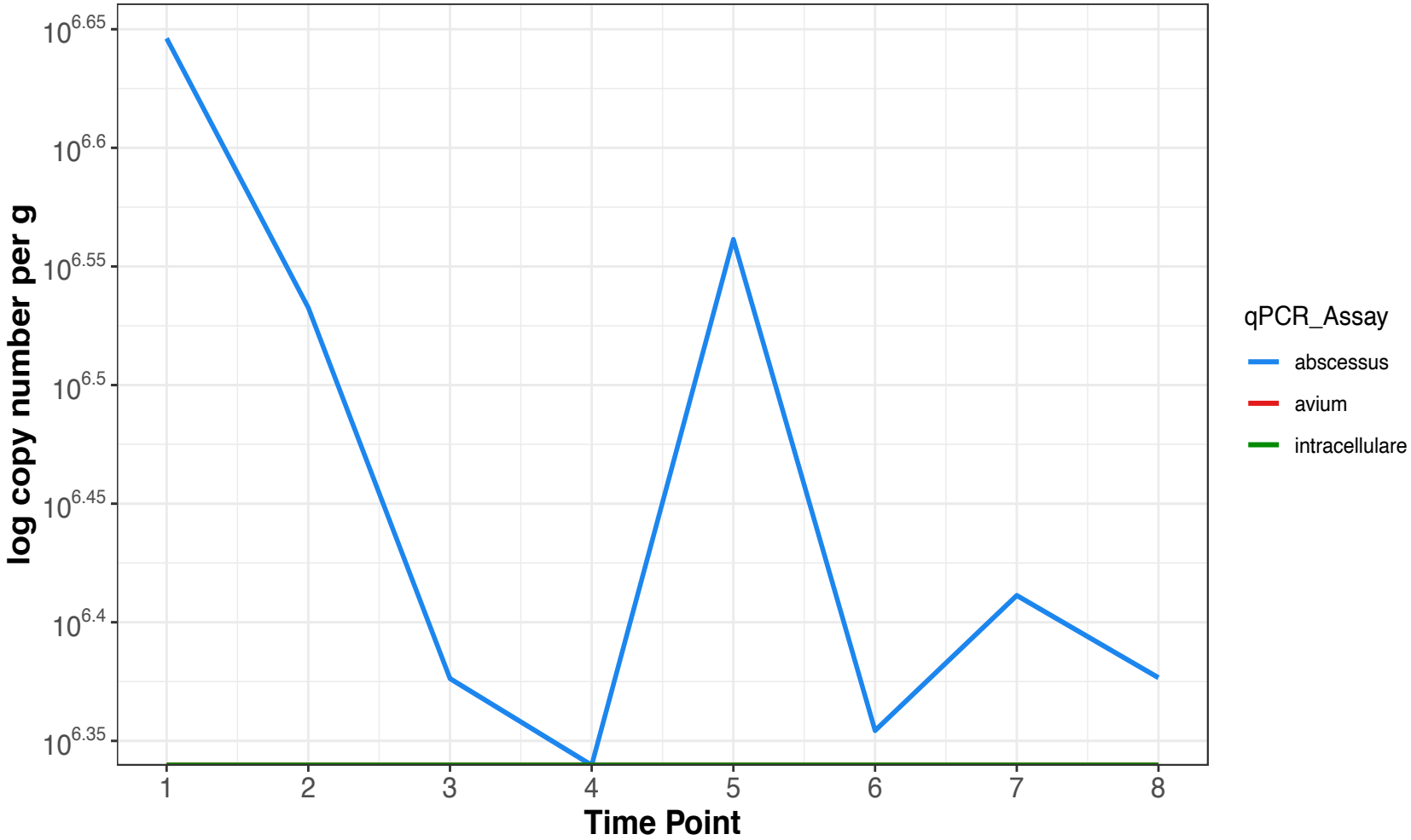
CULTURE RESULT	ABS	ABS					ABS				ABS	AVM		ABS	AVM		AVM
Amikacin (IV) / Meropenem / Tigecycline / Azithromycin			Y	Y													
Azithromycin / Amikacin (neb) / Minocycline / Ciprofloxacin					Y	Y											
Azithromycin / Meropenem (neb) / Minocycline / Ciprofloxacin							Y	Y	Y	Y							
Azithromycin / Meropenem (neb) / Doxycycline / Ciprofloxacin											Y	Y	Y				
Azithromycin / Meropenem (neb) / Co-trimoxazole / Doxycycline														Y	Y	Y	Y

Figure A25 – Longitudinal NTM copy number for Patient 35 who underwent treatment for *M. abscessus* with Amikacin, Meropenem, Tigecycline and Azithromycin (initiation phase) and Azithromycin, Amikacin (nebulised), Minocycline and Ciprofloxacin (continuation phase). Nebulised Amikacin was dropped at time point 7 and replaced by nebulised Meropenem. At time point 11 Minocycline was substituted for Doxycycline. At time point 14 Ciprofloxacin was dropped for Co-trimoxazole. Patient was 31 years of age; BMI of 18.2; FEV1 81% predicted; FVC 107% predicted.



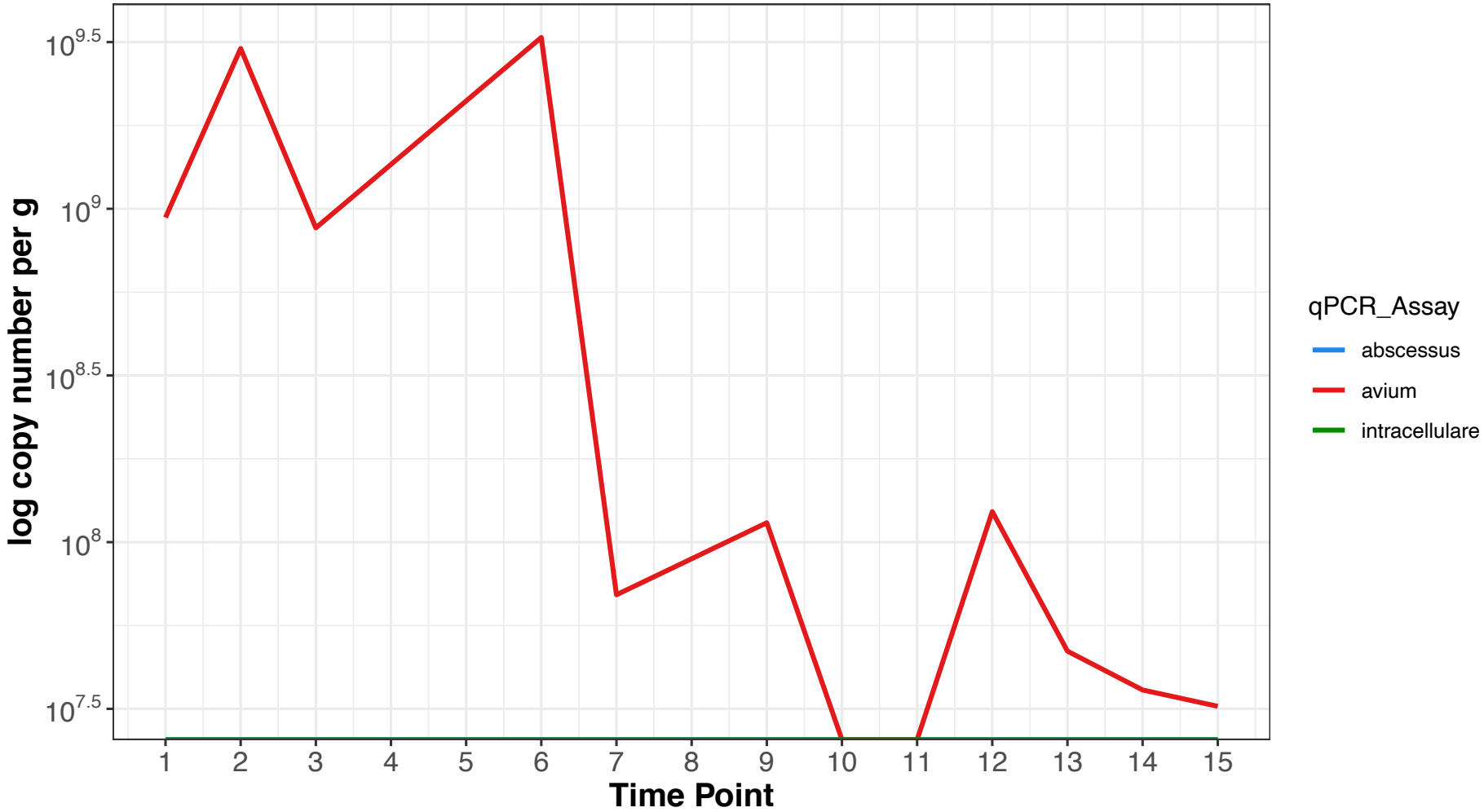
CULTURE RESULT	AVM	AVM		AVM	AVM		AVM	INT

Figure A26 – Longitudinal NTM copy number for Patient 36 with custom qPCR assays (Chapter 4). Patient 36 isolated MAC but was not on treatment for the study period. Patient 36 was 58 years of age, had a BMI of 21.8, a FEV1 of 53.5% predicted and a FVC of 100% predicted. NTM culture results at each time point are shown in the table.



CULTURE RESULT						ABS	ABS	ABS

Figure A27 – Longitudinal NTM copy number for Patient 37 with custom qPCR assays (Chapter 4). Patient 37 isolated *M. abscessus* but was not on treatment for the study period. Patient 37 was 36 years of age, had a BMI of 26.8, a FEV1 of 61% predicted and a FVC of 75% predicted. NTM culture results at each time point are shown in the table.



CULTURE RESULT	AVM	AVM	AVM			AVM	AVM		AVM	AVM	AVM	AVM	AVM	AVM	AVM
Rifampicin/ Ethambutol/ Azithromycin				Y	Y	Y	Y	Y	Y	Y				Y	Y

Figure A28 – Longitudinal NTM copy number for Patient 38 with custom qPCR assays (Chapter 4). Patient 38 underwent treatment for MAC with Rifampicin, Ethambutol and Azithromycin. Patient 38 was 70 years of age; BMI of 20.5; FEV1 of 72.6% predicted; FVC of 81.5% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.

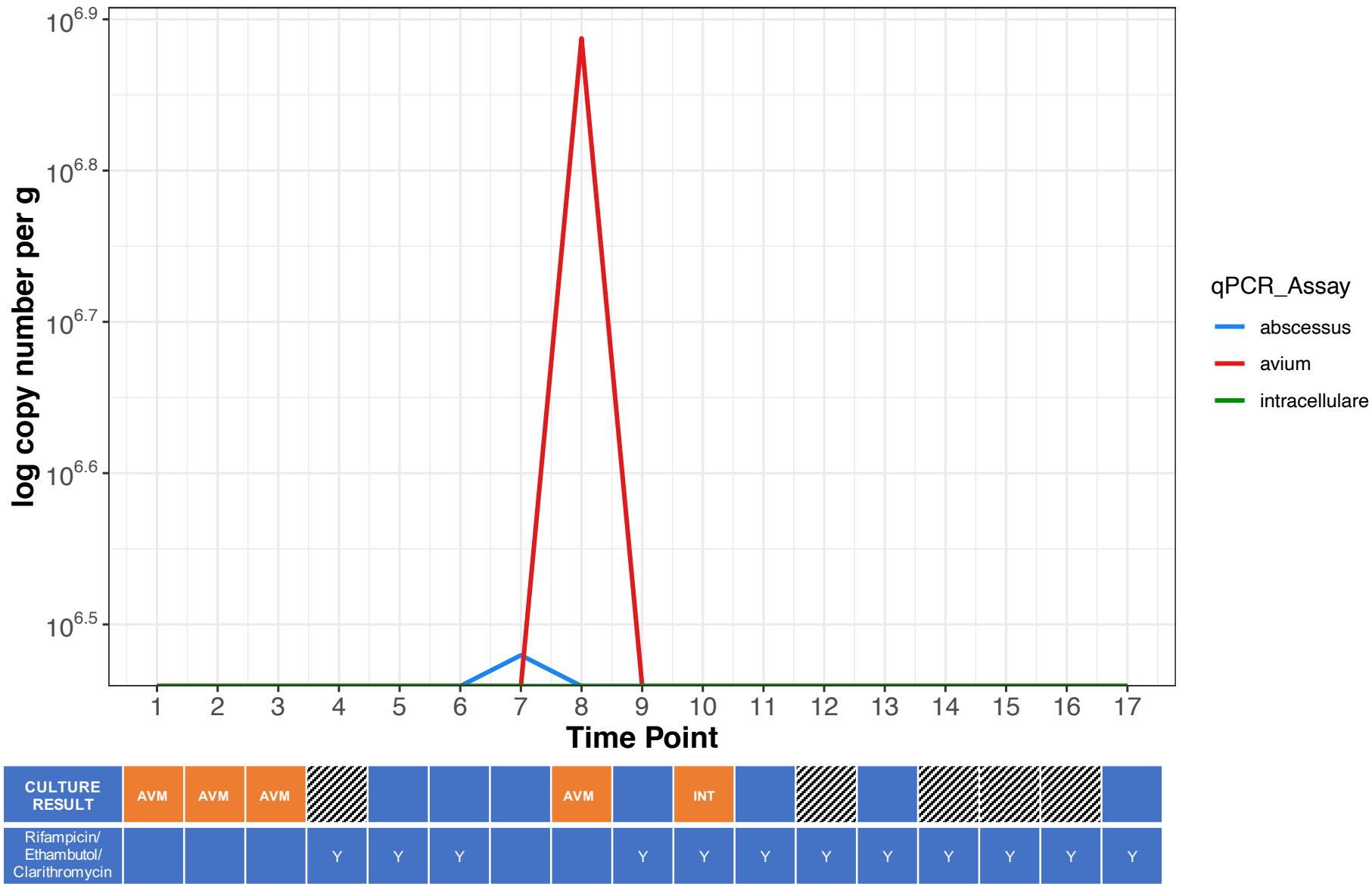
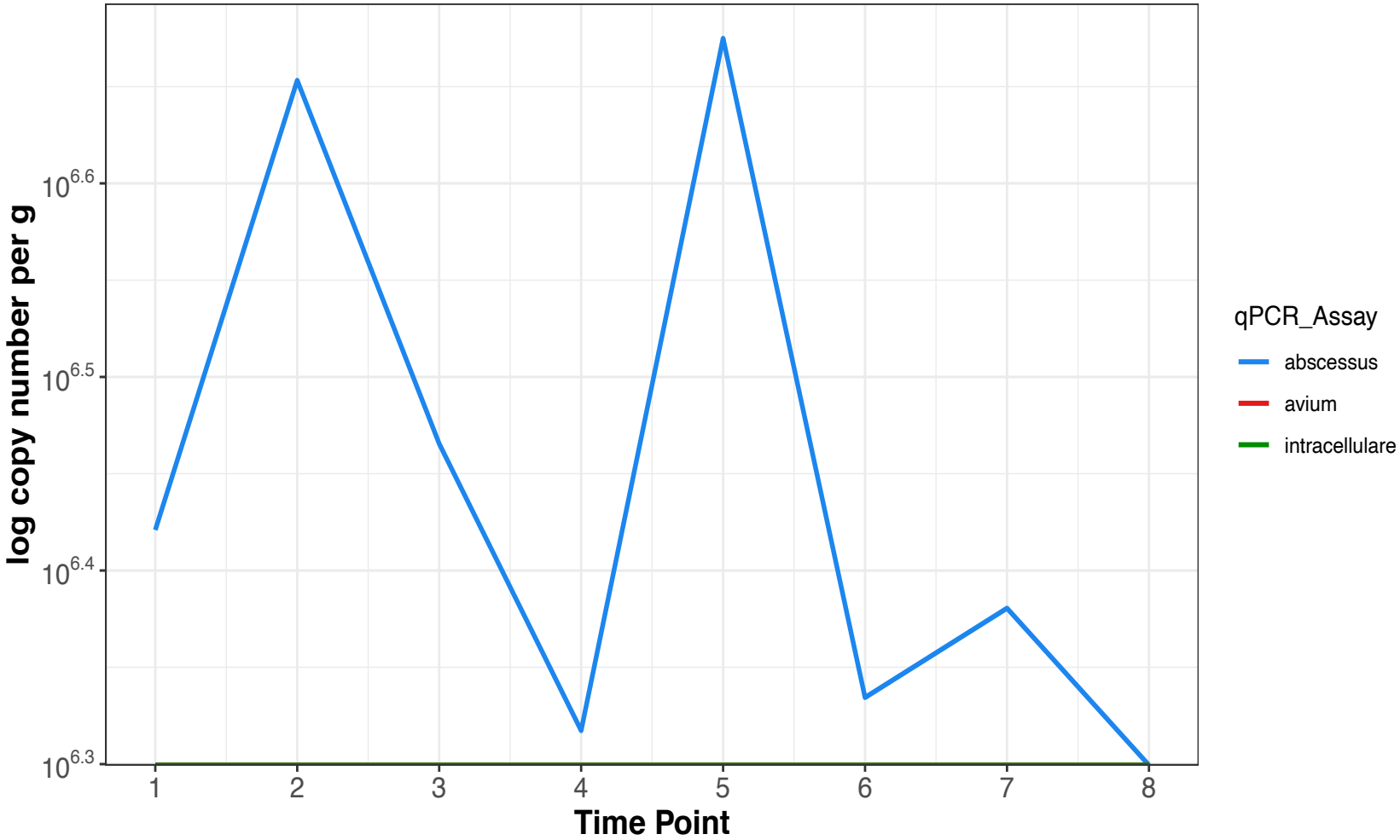


Figure A29 – Longitudinal NTM copy number for Patient 39 with custom qPCR assays (Chapter 4). Patient 39 underwent treatment for MAC with Rifampicin, Ethambutol and Clarithromyin. These medications were held between months 7 and 8 but reintroduced without issue. Patient 39 was 46 years of age; BMI of 20.6; FEV1 of 39% predicted; FVC of 67% predicted. NTM culture results as well as treatment regimens at each time point are shown in the table.



CULTURE RESULT			ABS	ABS	ABS			

Figure A30 – Longitudinal NTM copy number for Patient 41 with custom qPCR assays (Chapter 4). Patient 41 isolated *M. abscessus* but was not on treatment for the study period. Patient 41 was 32 years of age, had a BMI of 17.8, a FEV1 of 45% predicted and a FVC of 66% predicted. NTM culture results at each time point are shown in the table.

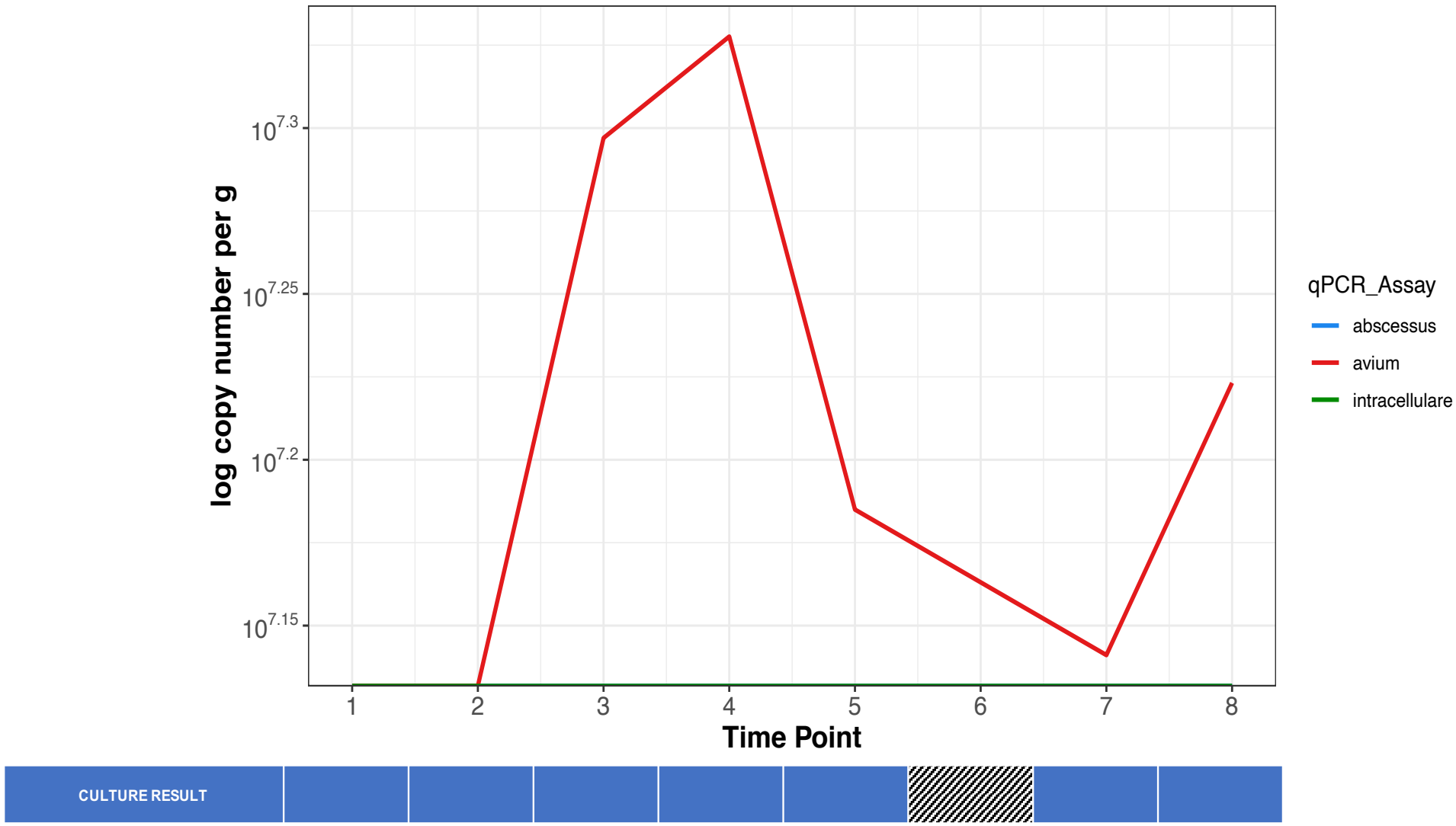


Figure A31 – Longitudinal NTM copy number for Patient 42 with custom qPCR assays (Chapter 4). Patient 42 had never isolated NTM species by culture methods. Patient 42 was 36 years of age, had a BMI of 21.8, a FEV1 of 27.9% predicted and a FVC of 59.7% predicted. NTM culture results at each time point are shown in the table.

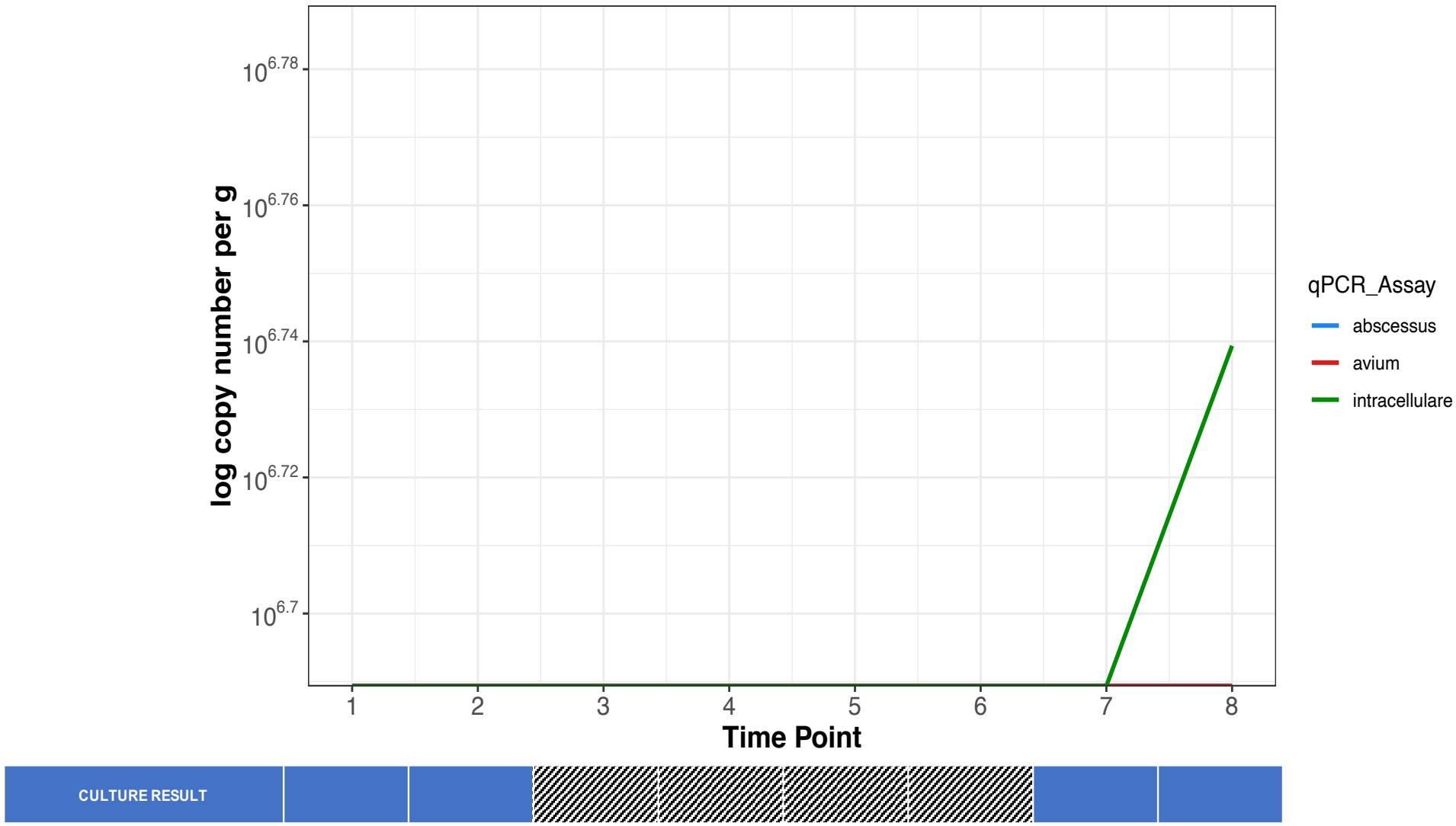


Figure A32 – Longitudinal NTM copy number for Patient 44 with custom qPCR assays (Chapter 4. Patient 44 had never isolated NTM species by culture methods. Patient 44 was 51 years of age, had a BMI of 23.2, a FEV1 of 63% predicted and a FVC of 81% predicted. NTM culture results at each time point are shown in the table.

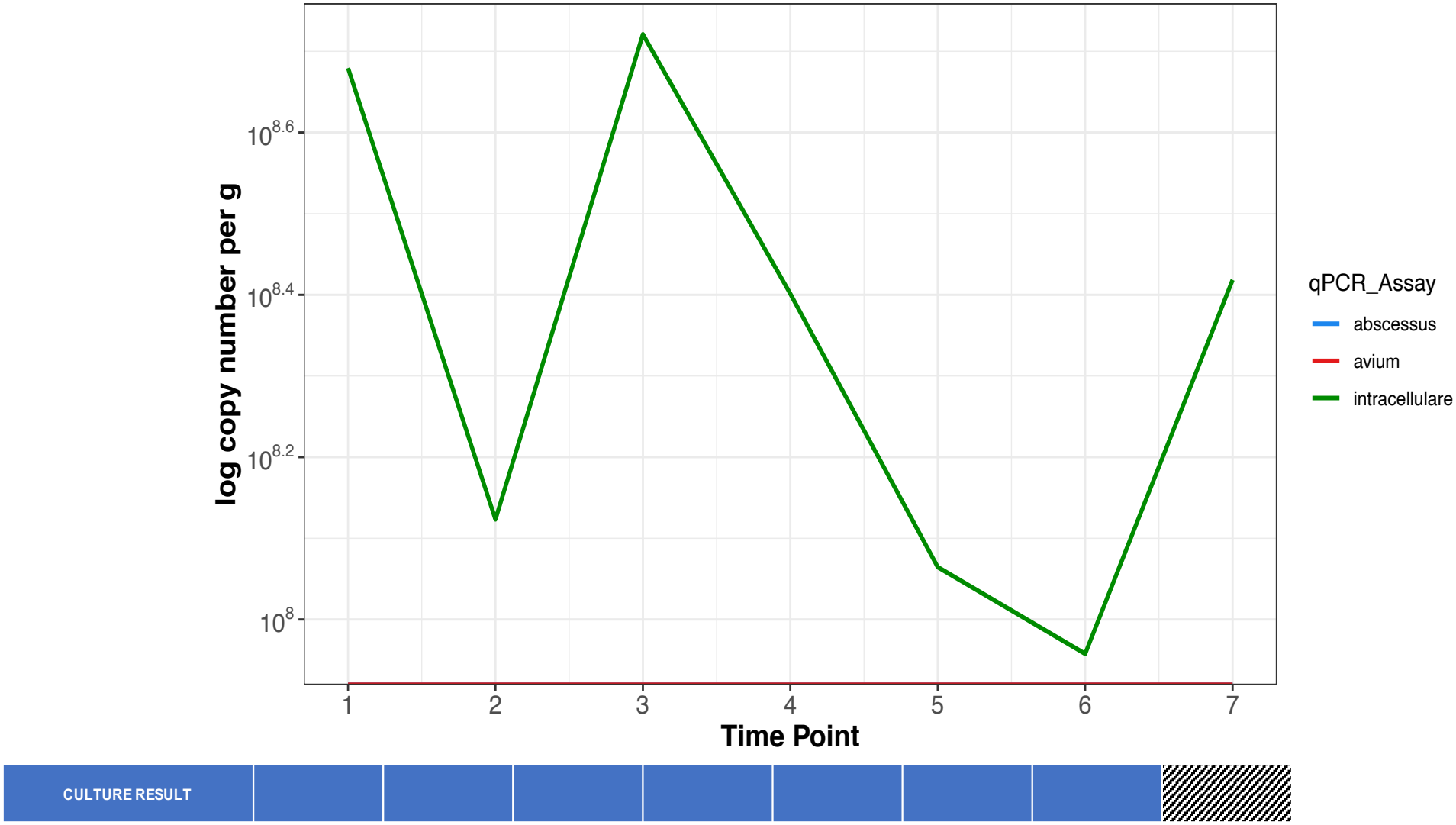
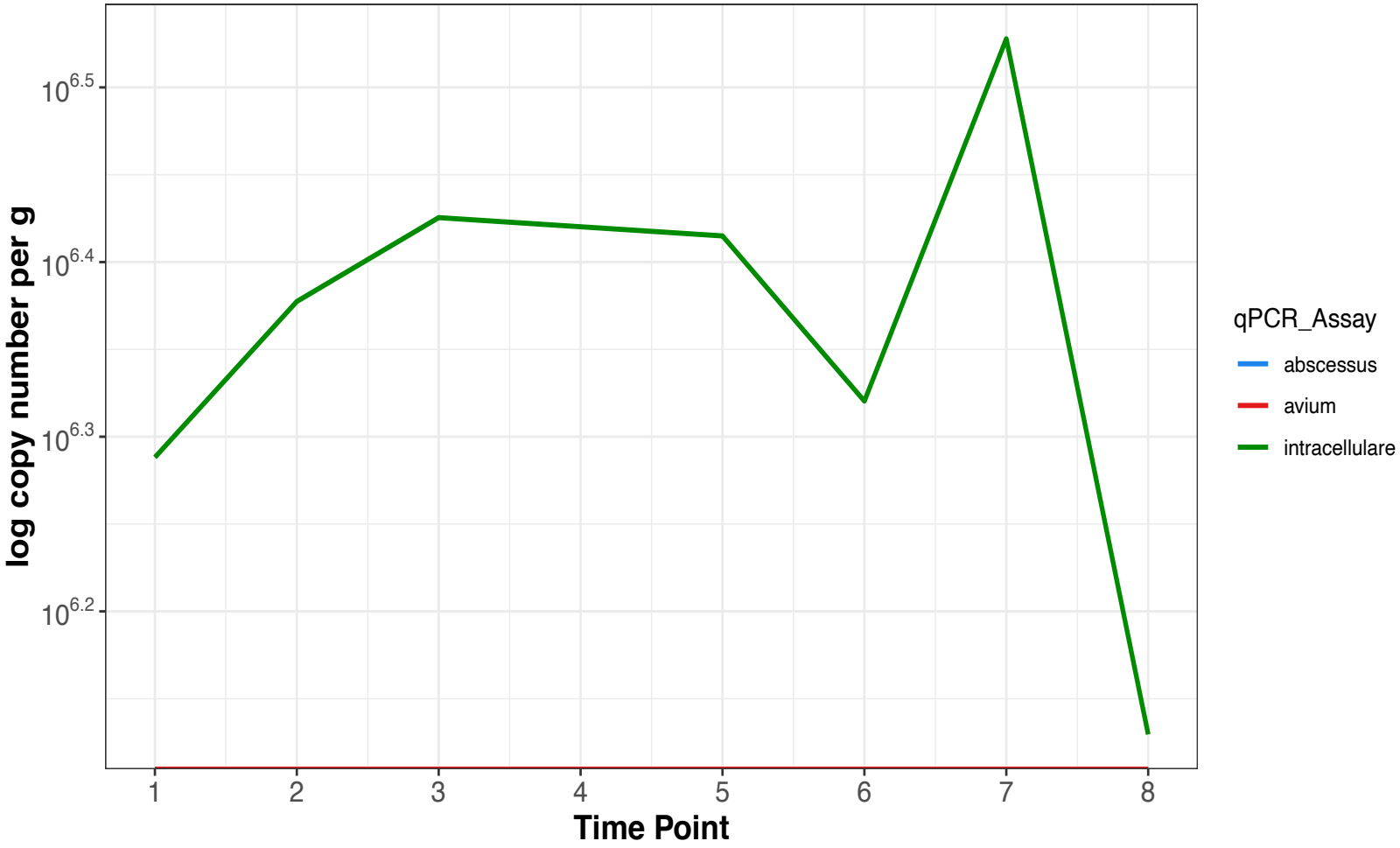


Figure A33 – Longitudinal NTM copy number for Patient 45 with custom qPCR assays (Chapter 4). Patient 45 had never isolated NTM species by culture methods. Patient 45 was 75 years of age. Unfortunately, no data on BMI or lung function testing was available for the patient. NTM culture results at each time point are shown in the table



CULTURE RESULT								

Figure A34 – Longitudinal NTM copy number for Patient 46 with custom qPCR assays (Chapter 4). Patient 46 had never isolated NTM species by culture methods. Patient 46 was 30 years of age, had a BMI of 21.2, a FEV1 of 67.1% predicted and a FVC of 92.4% predicted. NTM culture results at each time point are shown in the table

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Byung Woo Jhun, Seong Mi Moon, Kyeongman Jeon, O Jung Kwon, Heejin Yoo, Keumhee C. Carriere, Hee Jae Huh, Nam Yong Lee, Sung Jae Shin, Charles L. Daley, Won-Jung Koh
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Dear Huw

Non-tuberculous mycobacterial pulmonary disease
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European Respiratory Journal 2019 54: 1900250; DOI: 10.1183/13993003.00250-2019
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Dear Huw

The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study
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European Respiratory Journal 2013 42: 1604-1613; DOI: 10.1183/09031936.00149212
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