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Early View

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Molecular Assessment of Mycobacterial Burden in the Treatment of Nontuberculous Mycobacterial (NTM) Disease

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Molecular Assessment of Mycobacterial Burden in the Treatment of Nontuberculous Mycobacterial (NTM) Disease

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Take Home Message

We describe the development of molecular assays capable of quantifying NTM DNA in sputum. The assays were applied to 410 samples obtained from a cohort including patients on NTM treatment. The assays provide a fast and accurate measure of molecular burden

Introduction

Nontuberculous mycobacterial (NTM) pulmonary infection is increasing in prevalence throughout the world [1-3] and associated with significant morbidity and mortality [4-6]. Mycobacteria are ubiquitous in the environment and their impact on individual patients is heterogenous. Determining when and if to treat patients can be difficult. This is compounded by the fact that treatment involves multiple antibiotics over many months with disappointing success rates. Efforts to tackle infections in NTM are hampered by a lack of reliable biomarkers for diagnosis, assessment of disease activity, and prognostication [7]. Consequently decisions are currently based on mycobacterial culture results. Mycobacteria however are slow growing and significantly can take up to 8 weeks to culture. Data-backed treatment decisions are therefore not timely and are based on out-of-date information. In addition, culture based results have significant issues with sensitivity owing to high false negative rates [8].

Culture independent tests based on direct DNA detection have the potential to significantly improve the current situation. Previous studies using DNA based approaches, to directly detect and identify NTM species without the need for culture from respiratory studies, have however provided disappointing results. The reported sensitivities are low with NTM detected in only 29–76% of culture positive samples [9-11]. In addition, these results continue to provide binary information with regards to the presence of mycobacteria. No techniques have been developed to accurately quantify NTM burden. To provide real time data for the latter would enable more accurate assessment of the impact of treatment on the clinical state and subsequent treatment management decisions.

This study therefore describes the development of a quantifiable molecular test for the six most commonly pathogenic NTM species in the UK and the longitudinal application of the assays targeting MAC and *M. abscessus* to a patient cohort over a period of 18 months.

Methods

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

Recruitment
A total of 38 patients with either CF or non-CF bronchiectasis were recruited to the study. Patients were assigned to one of three separate groups based on the following inclusion and exclusion criteria:

1. Patients due to start treatment for *Mycobacterium abscessus* complex or *Mycobacterium avium* complex (termed “abscessus treatment” and “avium treatment” respectively).
2. Patients who cultured *Mycobacterium abscessus* or *Mycobacterium avium* complex on more than one occasion but are treatment naïve (termed “abscessus control” and “avium control” respectively).
3. Patients with bronchiectasis (CF and non-CF) but who had never cultured mycobacteria (termed “control”).

Patients were reviewed on a monthly basis to monitor treatment compliance.

Sample Collection

Patients requiring treatment provided a sputum sample at screening, then on a weekly basis for the first month then monthly samples up to and including 12 months with final samples at 15 and 18 months. Abscessus control, avium control and control patients provided a sputum sample at screening, then on a weekly basis for the first month followed by monthly samples up to and including month three.

Initial screening sputum samples (for participants in Group 1 these samples were obtained before treatment commenced) were divided with a sterile scalpel blade into four. One aliquot was sent immediately for mycobacterial culture and one sample frozen immediately at -80^{o}C. The two other samples were kept at room temperature for 24 hours before being sent for culture and being frozen at -80^{o}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 (subsequent sputum samples were sent to the laboratory via first class postal delivery). All sputum samples were obtained spontaneously.

Upon receipt of subsequent samples these were again divided in to two aliquots with one aliquot being sent for mycobacterial culture and the other frozen at -80^{o}C.

Stored sputum samples were thawed prior to DNA extraction. Details of DNA extraction can be found in supplementary methods.

Assay Development

Due to it relatively large interspecies variability and its preservation between isolates of the same species the *hsp65* gene was selected as an assay target. Custom primers and probes (Table 1) for the detection and quantification of the 6 most commonly pathogenic mycobacteria were designed in house. The probes were tested in singleplex and multiplex. A series of pilot experiments were performed and it was determined that when used in singleplex the probes had a greater limit of detection. NTM quantification was subsequently performed in singleplex. For details of primer and probe design, including sensitivity, specificity and level of quantitation feasible, please see supplementary methods.

Table 1 – Custom designed primers and probes used for identification and quantification of six 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

Quantitative PCR (qPCR) Testing

DNA extracted samples were diluted in a 1:50 ratio with PCR grade water. For each qPCR reaction, 5µl of diluted sample was combined with the assay. For quantification standards 5µl aliquots of serially diluted plasmids were used. Each standard and extracted sample were tested in triplicate to ensure reliability of results.

Each sample was tested in singleplex against probes targeting *M. abscessus* complex, *M. avium and M. intracellulare (*the latter two forming the *M. avium* complex). Positive qPCR results required positivity in triplicate. Assay constituents as well as PCR cycling conditions are detailed in supplementary methods.

Results

Clinical Characteristics of the Cohort

A total of 410 sputum samples were obtained from the 38 patients (Table 2). Fifteen patients had recurrent isolates of MAC and 8 patients [53.3%] were started on treatment. Eighteen patients recurrently isolated *M. abscessus* and 14 of them [77.8%] were started on treatment. MAC treatment regimens were based on guideline-based therapy with rifampicin, ethambutol and either clarithromycin or azithromycin. The treatment of *M. abscessus* was also based on guidelines with intravenous meropenem, amikacin and tigecycline and oral clarithromycin or azithromycin for the initiation phase [12-14]. The continuation phase was more varied due to patient antibiotic intolerance as indicated in Table 2.

Eight of the 14 patients who commenced treatment for *M. abscessus* were culture negative (Figure 1) 12 months after initial culture conversion. These patients were therefore deemed to be cured by BTS and ATS criteria [12-14] . A total of 10 patients in the treatment group cultured more than one mycobacterial species illustrating the frequency of mixed isolates and the inherent difficulty faced when selecting treatment regimes. Four of the 8 patients who commenced treatment for MAC were culture negative (Figure 1) 12 months after initial culture conversion and hence deemed to be cured by BTS and ATS criteria [12-14] .

In the *abscessus* control and *avium* controls, all but one patient (Patient 44) cultured the expected NTM species at a time point in the study period (Figure 1). Individual NTM culture results, however, were inconsistent, with 28 of the 70 samples failing to culture NTM. All the patients recruited to the NTM culture negative group (control) remained negative for the duration of the study. No patients in the control groups were maintained on prophylactic macrolides.

Table 2 - Clinical characteristics of the study cohort. Shown in this table are the breakdown of patients by gender, age, smoking status, underlying disease, use of medication that potentially causes increased susceptibility to infection (immunomodulation was the use of monoclonal antibodies), pulmonary function results (before treatment initiation), BMI and antibiotics used to treat infection. *P*-values were obtained using Kruskal – Wallis rank sum test.

		Mycobacterium avium		***Mycobacterium abscessus***			***P* value**
		Treatment	Control	Treatment	Control	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	
Disease	Bronchiectasis (Bx)	7	4	2	1	2	0.01
	Cystic Fibrosis (CF)	1	3	12	3	3	
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
***M. avium* Treatment**	Rif, EMB, AZT	6					
	Rif, EMB, 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 regime - consists of 3 drugs where: Rif=Rifampicin, Eth=Ethambutol, AZT=Azithromycin, Clari=Clarithromycin.

M. abscessus treatment regime - 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.

Impact of Room Temperature Storage of Samples

For each patient screening sputum samples were taken prior to the commencement of treatment with subsequent samples being sent by patients to the laboratory via Royal Mail. To investigate the potential impact of the delay between sputum production and laboratory receipt and storage at -80^{o}C, the screening sputum sample was aliquoted into two (see methods, aliquots designated time point 1 and 2) and NTM copy number (defined as the number of *hsp65* gene copies identified by qPCR) compared between the two time points.

For the *M. abscessus* culture positive patients, rises in *M. abscessus* copy number between time points 1 and 2 were observed in 12 of the 18 patients but they were not statistically significant (Student's T test *P*- value = 0.53) (Figure 2a).

For *M. avium* complex culture positive patients, 8 of the 15 experienced a rise in MAC copy number between the two time points but again this was not statistically significant (Student's T test *P*- value = 0.32) (Figure 2b).

***M. abscessus* Treatment Group: Quantitative Assessment of Mycobacterial Burden**

One hundred and ninety-two samples were obtained for the *M. abscessus* treatment cohort (14 patients). Forty-six of the samples were culture positive for *M. abscessus.* In addition, 11 samples were culture positive for *M. avium*, 4 samples positive for *M. intracellulare*, 2 samples were positive for *M. xenopi* with 1 positive for *M. kansasii* (Figure 1).

For the *abscessus* treatment cohort, overall burden decreased significantly during antibiotic treatment (Figure 3a and Figure 4) (Friedman analysis of variance: F_r = 22.8, df 3, *P*- value = < 0.01) with significant differences between time points 1 and 3, time points 2 and 3 (*P*- values = 0.02) and time points 2 and 4 (*P*- value = 0.04) (Wilcoxon signed rank test). In contrast, for the *abscessus* control cohort the molecular load was more stable (Supplementary Figure 2) (Friedman analysis of variance: F_r = 2.2, df 3, *P*- value = 0.5).

There was an association between treatment adherence, negative culture and qPCR results (Figures 3c, b and a respectively). Four patients suffered intolerable treatment side effects (patients 2, 18, 23 and 35) and therefore had varying compliance. These four were the only patients who were culture and qPCR positive beyond time point 6 (one month of treatment). For three of the patients, *M. abscessus* burden appeared to be resolved by implementation of an alternate oral antibiotic regime. For the fourth patient (ID 23), *M. abscessus* copy number increased beyond and remained greater than pre-treatment levels at time point 6 and was only addressed by the reinstatement of an initiation therapy at time point 12.

***M. avium* Complex Treatment Group: Quantitative Assessment of Mycobacterial load.**

For the MAC treatment cohort (avium treatment 8 patients) a total of 117 samples were obtained. Of these, 24 were culture positive for *M. avium* and 12 for *M.intracellulare* with 3 samples being culture positive for *M. xenopi* (Figure 1).

For the majority of patients, treatment resulted in reduction of MAC burden (Figure 6). The effect demarcation was not as clear as that seen for the *abscessus* treatment cohort (Figure 4) most likely due to the absence of an intravenous induction phase in the MAC cohort. The decrease in MAC copy number during treatment did not reach significance (Figure 6) (Friedman analysis of variance: F_r = 3.75, df 3, *P*- value = 0.3).

No decrease in MAC copy number was observed amongst the MAC control (untreated) group (Supplementary Figure 3) (Friedman analysis of variance: F_r = 3, df 3, *P*- value = 0.4).

For three of the patients (1, 3 and 17) there were clear disparities between culture and molecular results with the latter showing positive despite the culture based analysis being negative throughout the clinical course (Figure 5b).

Some association with clinical course and patient compliance was also observed. Patients 34, 38 and 39 experienced problems at various stages of treatment resulting in the temporary cessation of treatment for a period of 2 months or more

(Figure 4). These points of treatment cessation were associated with peaks in MAC DNA copy number that in patient 39 were not replicated in culture analysis.

The Control group: Quantitative Assessment of Mycobacterial load

A total of 33 samples were available from the control cohort (N = 5 patients). No samples were culture positive for NTM species (Figure 1).

No positive results were obtained when the samples were screened using the qPCR assay targeting *M. abscessus*.

For the MAC qPCR assay, positive results were obtained for samples from three patients (Study IDs42, 45 and 46). These results were not reciprocated by culture analysis. No significant difference in MAC copy number between time points (Friedman analysis of variance: F_r = 5.69, df 3, *P*- value = 0.13) (Supplementary Figure 4).

Concordance Between Culture and Molecular Results

As currently diagnosis of NTM disease relies on culture of the pathological mycobacterial species [12,13] we next examined the concordance between the mycobacterial culture (the reference standard) results and qPCR assay results (Table 2 and supplementary results).

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 (Supplementary Figure 1a).

Owing to significant lack of concordance between culture-based results and the qPCR assays targeting *M. avium* and *M. intracellulare* the assays were combined to form a “MAC qPCR assay” whereby MAC copy number is equal to the sum of the *M. avium* and *M. intracellulare* qPCR assays. This was performed to mitigate for misidentification of culture based samples. It is an important consideration that culture samples were identified using a commercial test that is subject to misidentification of samples rather than whole genome sequencing. When combined, the assays showed 78% concordance with positive culture results with a test

sensitivity of 0.86; specificity = 0.62; positive predictive value = 0.35; negative predictive value = 0.95; resulting in an AUC of 0.741 (Supplementary Figure 1d).

Table 3 - Individual probe characteristics based on sputum samples culture-based results. Each probe result was compared to the corresponding sample culture result which was used as the reference standard test. The table details numbers of true positives, false positives, true negatives and false positives and the resulting sensitivity, specificity, positive predictive and negative predictive values for each probe.

	Probe			
	abscessus	**avium**	**intracellulare**	**MAC**
True positive	52	30	15	67
False positive	16	78	82	127
True negative	334	277	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
AUC	0.923	0.673	0.301	0.741

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

Discussion

The diagnosis of NTM pulmonary disease and the subsequent monitoring of treatment relies upon culture of the pathological bacteria. The shortfalls in this technique are demonstrated by the failure of samples originating from the same sputum to culture the same NTM species (patients 6 and 9, Figure 1). This study describes the design and application of a custom qPCR assay to a total of 410 samples from patients with treated and observed NTM pulmonary lung disease, demonstrating potential clinical utility for monitoring and treatment assessments in these conditions.

The molecular assays described are of potential clinical value as they enable the provision of rapid and real time biomarker data during the course of treatment, enabling clinicians to make treatment decisions with real time data. Target NTM DNA demonstrated high copy numbers prior to initiation of treatment with significant progressive reductions preceding culture conversion in several patients over the first time points. This was well demonstrated particularly in the *M. abscessus* treated cohort where a reduction in molecular load provided evidence of initial treatment

success. There was also some indication that DNA levels could be utilised to assess the need for or to establish the effectiveness of treatment changes. Several patients demonstrated increases in copy number accompanying interruption of treatments (Figure 3c). 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 following poor compliance. It may also be possible to utilise such data to determine the effectiveness of additional antibiotics to help patients with refractory disease. Presently treatment decisions are reliant on culture data that provide binary results and take many weeks to be obtained. A longitudinal improvement in NTM copy number would serve to reassure clinicians of treatment efficacy when culture is persistently positive (see patient 6) Similarly the delay incurred by culture techniques may hinder patient improvement and may allow progression of disease.

The treatment regimes of *M. avium* complex differ significantly between *M. abscessus* not just in terms of differing antibiotics but in that there is no initiation phase of treatment. Consequently, there is no initial rapid decline seen in *M. avium* complex copy number. Instead MAC DNA copy numbers remain high often for several months before reducing. The slower replication rates associated with MAC, in addition to the lack of an initiation phase could explain the difference in mycobacterial elimination.

In addition to providing rapid and quantitative data, there is also evidence to suggest that the qPCR assays are also more sensitive than standard culture alone. Although there was generally good concordance between molecular and culture data, there were occasions where negative culture results were accompanied by positive qPCR results. For samples taken from individuals undergoing treatment that exhibited this phenomenon it could be postulated that the assay was detecting DNA from dead bacteria. However the phenomenon was noted in samples not exposed to treatment. Samples from patient 6 at time points 1 and 2 (samples generated from the same divided sputum sample) demonstrated an incongruity between culture results (sample 1 was culture negative in contrast to sample 2 that cultured *M. abscessus*). Molecular analysis of these samples were both qPCR positive for *M. abscessus*

demonstrating the potential for false negatives in culture based techniques and suggesting that molecular techniques (once refined) will have a better sensitivity than culture alone. Improved sensitivity is important with regards to decisions pertaining to the completion of treatment and culture conversion definitions. For example, Patient 7 who became culture negative after 1 week of anti MAC treatment but was qPCR positive up until 10 months of treatment (time point 15) (Figure 5). In these circumstances it is entirely possible that the patient's medication will have been stopped prematurely, a factor that is believed to be linked to relapse of disease and accounts for the successful eradication of MAC lung disease in only 60 to 80% of patients, with 20 to 40% failing to respond to treatment, and a significant proportion of successfully treated patients experiencing disease recurrence [15-18].

A further area of disparity between the culture and molecular data relates to multiple species detection by PCR. This is in line with previous data [19,20] demonstrating mycobacteria appear in communities rather than the presence of individual isolates. Culture methods carry an inherent bias in favour of the reporting of fast growers whereas molecular methods, without the need for culture, would have less bias in this respect.

This study also looked at the impact of delays to NTM burden within sputum samples before their arrival at the laboratory for processing. This is extremely important presently with the huge increase in remote consultation and management, with many patients sending sputum samples via the post. To control for samples incurring a delay whilst they were sent to the laboratory, screening samples were divided with one undergoing a 24-hour delay in freezing. Molecular analysis of NTM copy number between these samples showed no significant changes indicating robustness of the assay with regard to sputum samples that cannot immediately be frozen. The impact of delayed freezing on sputum samples on culture-independent microbiological analyses has previously been investigated with a significant decrease in the abundance of anaerobes seen at 12 hours [21]. NTM are however aerobic bacteria with notoriously slow growth rates accounting for the differences in studies.

Mycobacterial culture results for the control group revealed all patients remained culture negative for the duration of the study. Three of the five patients however

showed qPCR positive results for the *Mycobacterium avium* complex (MAC). This is likely attributable to the high failure rate associated with traditional culture techniques that result in a high false negative rate of its own[22]. This study has demonstrated that culture techniques provide false negative results (see patient 6, samples 1 and 2) whilst other studies have shown histological evidence of NTM infection in asymptomatic patients with predisposing conditions [23].

There are some limitations of this study. For example, there were a few instances of false negatives in presence of a positive sputum culture suggesting some optimization of the molecular tests may be needed going forward. In addition, as recruitment was only via one referral centre, the study size is small. Nevertheless, there are significant strengths of the study, the development of an assay to enable rapid screening of sputum samples and within the study the high frequency sputum sample testing conducted that has provided insights and a much better temporal understanding of the mycobacterial response to antibiotics.

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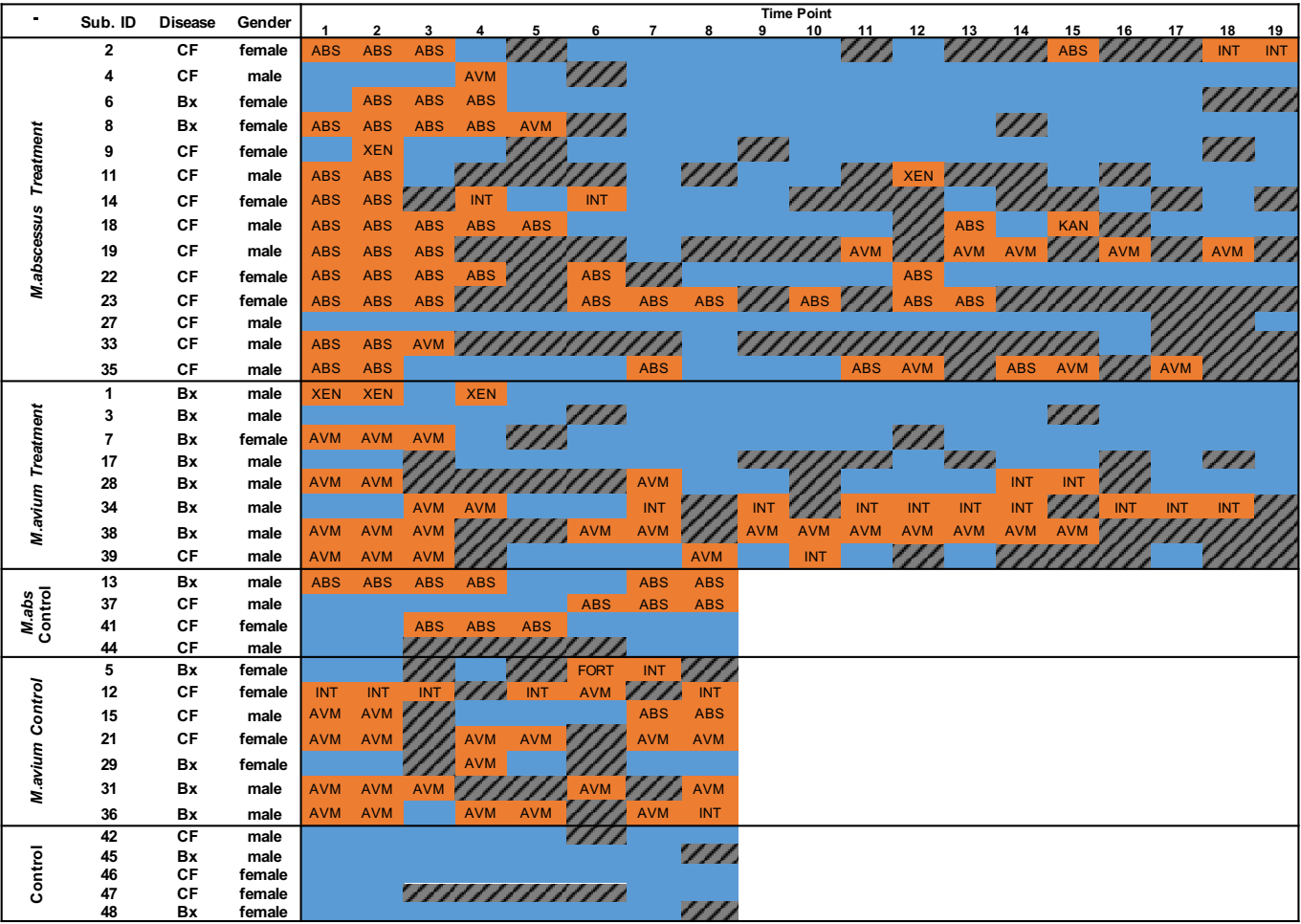


Figure 1 – Mycobacterial culture results by patient and time point. Blue square indicates a negative culture result whilst orange indicates positive. Name inside the orange box is NTM species (ABS = *M. abscessus,* AVM = *M. avium,* INT = *M. intracellulare,* XEN = *M. xenopi,* FORT = *M. fortuitum*). 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.

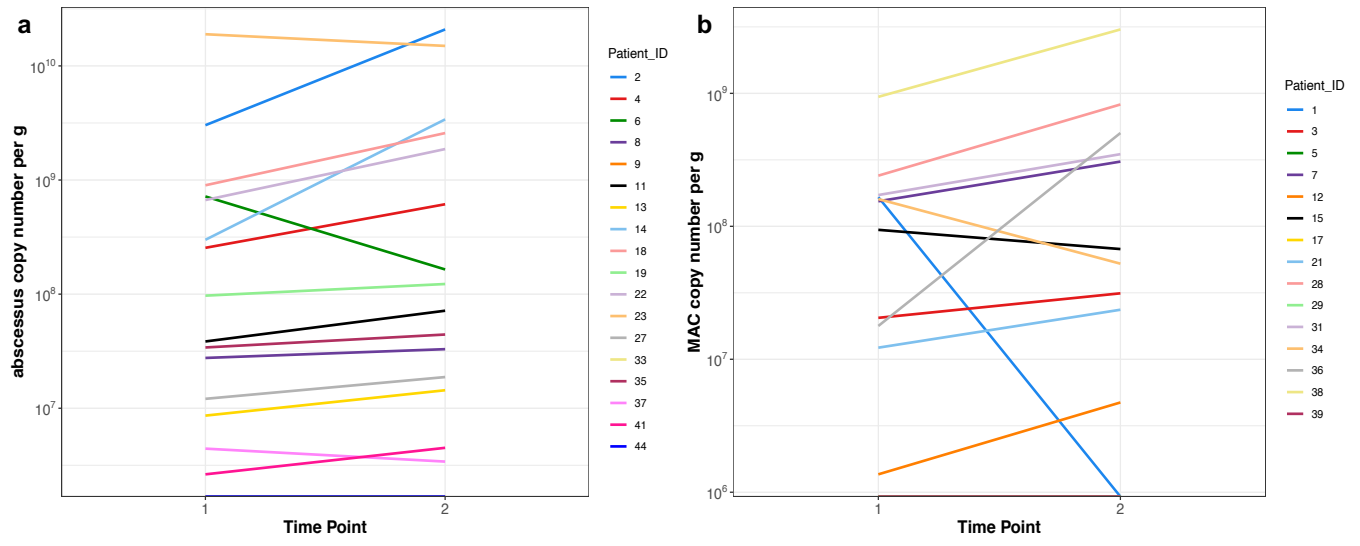
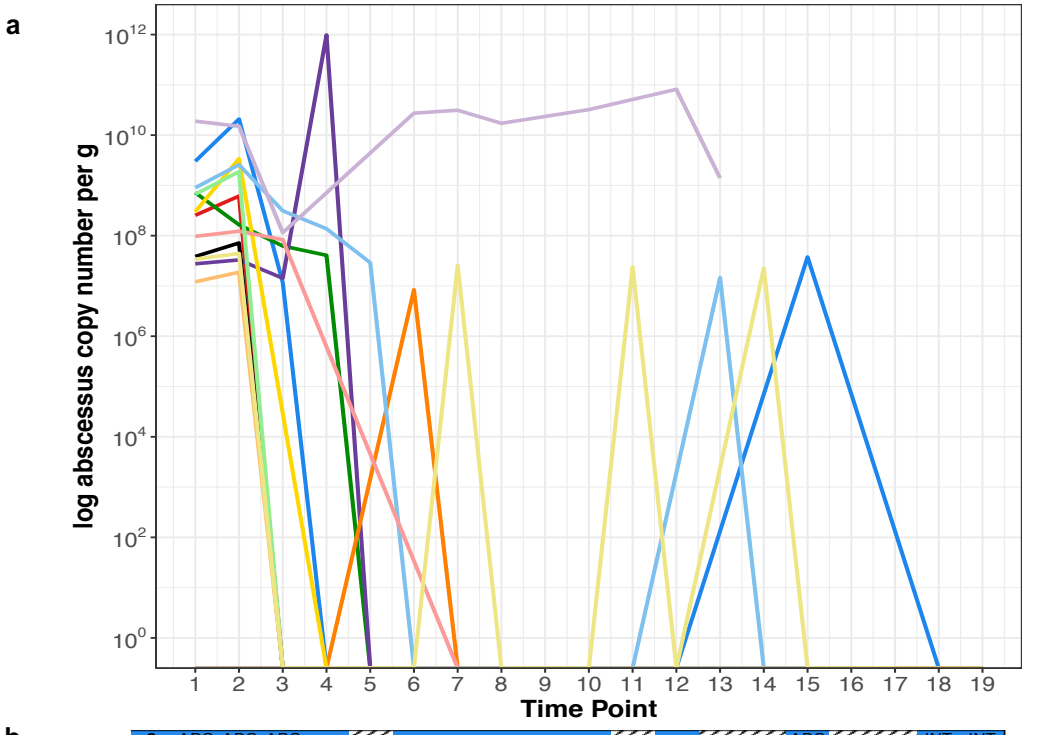
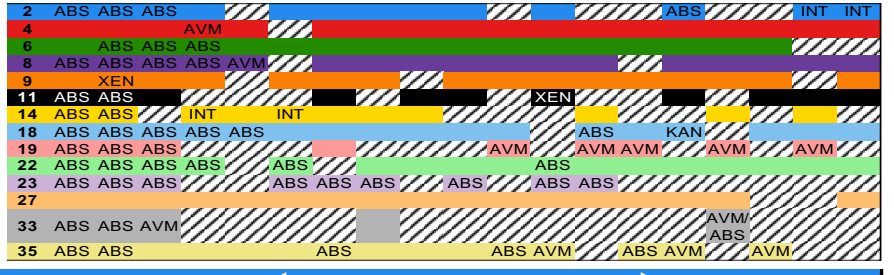


Figure 2 - Samples were derived from the same sputum sample but sample time point 2 incurred a 24 hours time delay before being processed.
a) – *M. abscessus* copy number between time points 1 and 2.
Mean copy number at time point 1 was 1.39 x 10^9 copies/g sputum (SD = ±4.44x10^9) and 2.49 x 10^9 copies/g sputum (SD = ±5.79x10^9) at time point 2. *P*- value = 0.53.
b) – MAC copy number between time points 1 and 2.
Mean copy number at time point 1 was 8.2x10^7 copies/g sputum (SD = ±2.39x10^8) and 2.32x10^8 copies/g sputum (SD = ±7.8x10^8) at time point 2. *P*- value = 0.32).





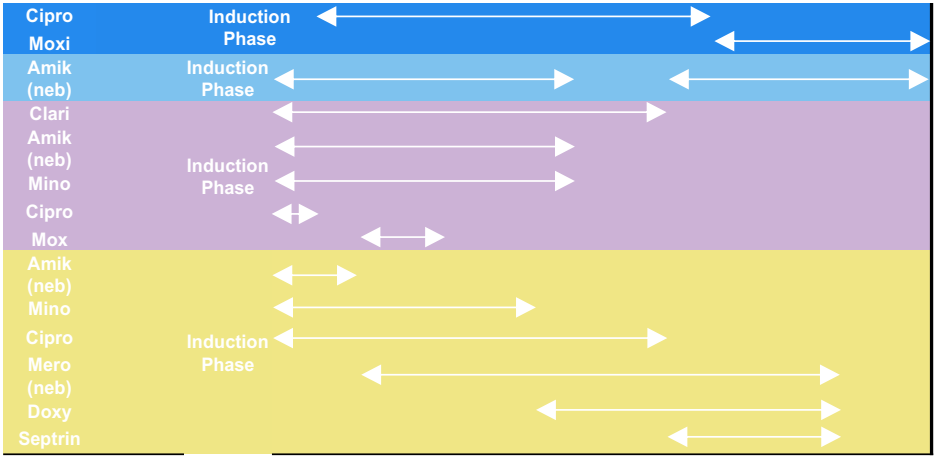


Figure 3 a) – Custom assay with *M. abscessus* probe used with samples from the *M. abscessus* treatment group. **b)** Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **c)** interrupted treatment regimens relating to each sample time point and patient. Interruptions in treatment correlated with increased NTM copy number. Only regimes that were interrupted are illustrated.

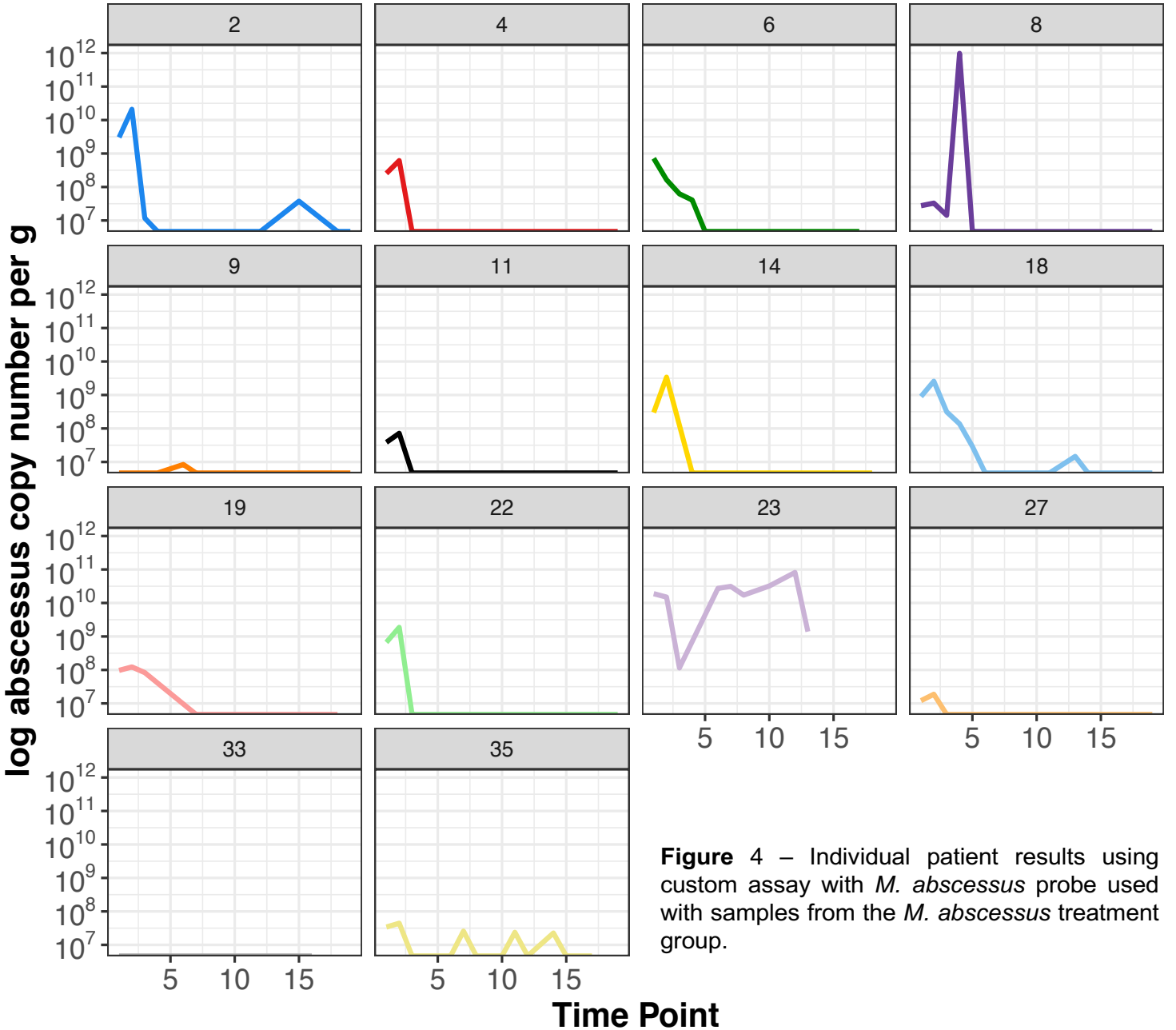
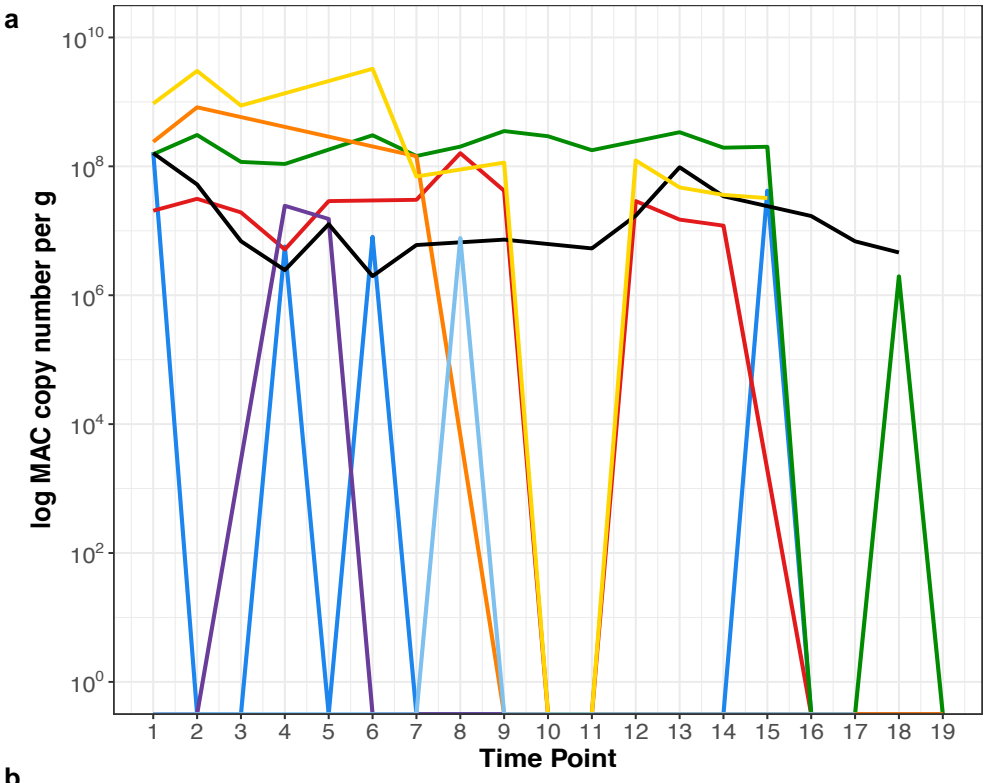


Figure 4 – Individual patient results using custom assay with *M. abscessus* probe used with samples from the *M. abscessus* treatment group.



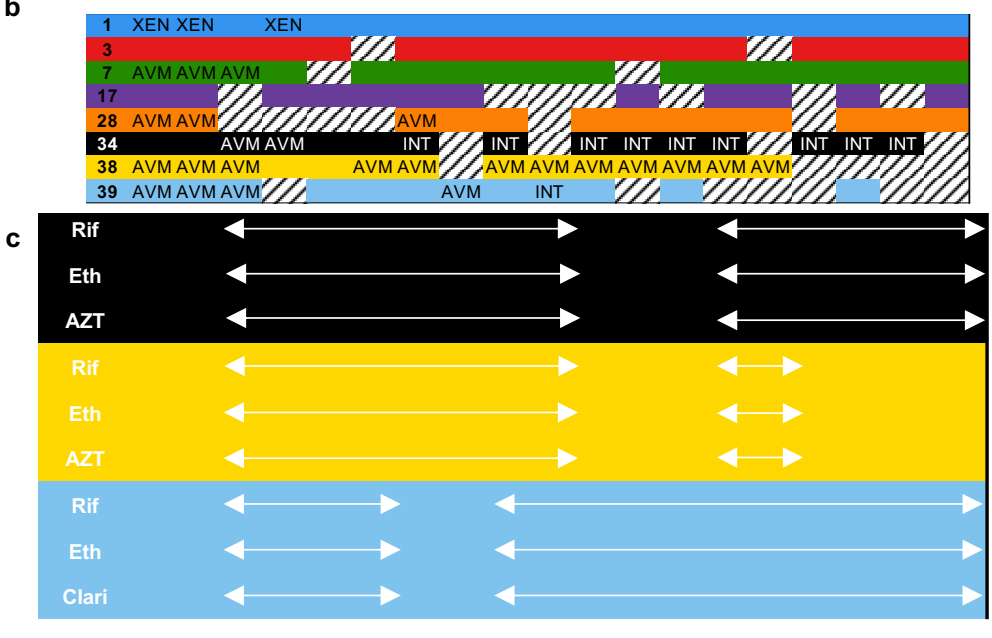


Figure 5 a) – Custom assay with *M. abscessus* probe used with samples from the *M. abscessus* treatment group. **b)** Patient culture results by time point are displayed below qPCR data. Hashed boxes indicate unavailable samples. **c)** interrupted treatment regimens relating to each sample time point and patient. Interruptions in treatment correlated with increased NTM copy number. Only regimes that were interrupted are illustrated.

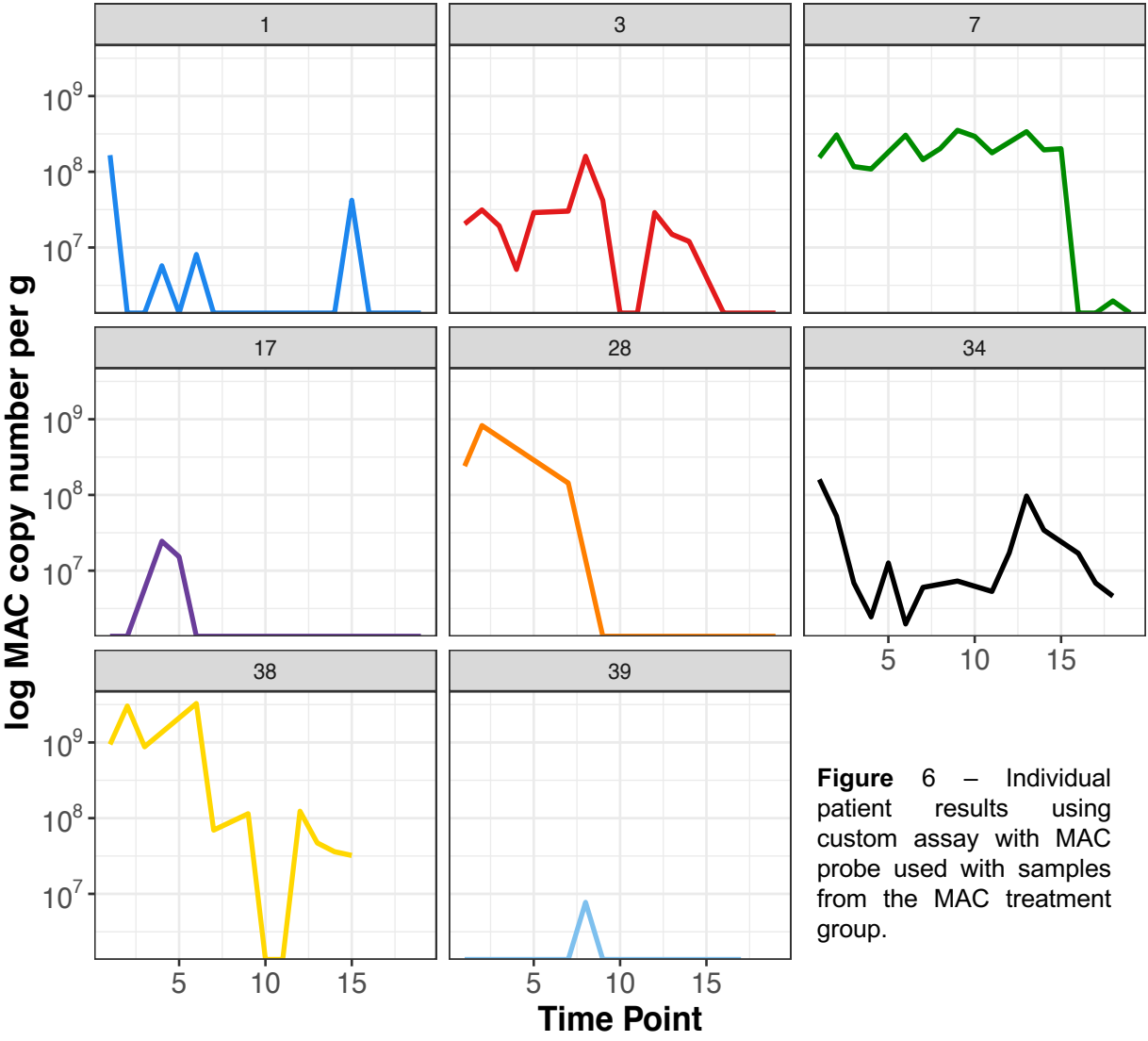


Figure 6 – Individual patient results using custom assay with MAC probe used with samples from the MAC treatment group.

Supplementary Methods

Patient Recruitment Inclusion and Exclusion Criteria

Inclusion criteria:

- Age 16 years or older (no upper age limit)
- Able to give informed consent
- Regular sputum production
- Patients recruited to treatment group should be treatment naïve prior to recruitment and have cultured the same NTM on more than one occasion
- Patients recruited to avium or abscessus control 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 as 'control' should have radiological evidence of bronchiectasis

Exclusion criteria:

- Change in baseline bronchiectasis or NTM therapy between 28 and 2 days before recruitment.

Mycobacterial Culture and DNA Extraction

Bacterial culture of sputum samples was performed by the Royal Brompton Clinical Microbiology Department with species identification using the GenoType Mycobacterium CM VER.2.0 (Hain Lifescience).

DNA was extracted from sputum samples for subsequent downstream analyses using a phenol:chloroform method. Briefly 300micrograms of sputum from each sample was placed into a Lysing Matrix E (LME) tube (MP Biomedicals, Solon, USA). Next five hundred µl of 10% w/v hexadecyl-trimethyl-ammonium bromide (CTAB) and 50µl of 0.1M aluminium ammonium sulphate solution was added followed by 500µl phenol:chloroform:isoamyl alcohol 25:24:1. The samples was then homogenized at 6000rpm for 1 minute using the MP bio FastPrep-24™ 5G bead beating instrument and then centrifuged at 16,000g for 5 minutes. The aqueous phase was transferred to a pre centrifuged 2mls phase-lock tube (Quantabio 5PRIME) which was then centrifuged at 16,000g for 10 minutes at 4^{o}C. Five hundred µl chloroform:isoamyl alcohol 5:1 was added and mixed gently followed by

centrifugation 4°C for 10 minutes. One µl linearized polyacrylamide (LPA) (Sigma-Aldrich, St. Louis, USA) was added to the aqueous phase plus 30% polyethylene glycol and the DNA allowed to precipitate overnight by storage at at 4°C.

The tubes were next centrifuged at 16,000g for 20 minutes at 4°C. The polyethylene glycol solution was aspirated and 500µl ice cold 70% ethanol added before centrifugation at 16,000g for 5 minutes at 4oC. The ethanol was removed by pipetting.

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 stored at -20°C until use.

Creation of *hsp65* Plasmids

Bacterial Strains

Lyophilised genomic DNA from NTM type strains was obtained from the Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkµlturen (DSMZ, Braunschweig, Germany)(Table 1) and reconstituted with molecular grade water. DNA was amplified using the Illustra™ Ready-To Go™ GenomiPhi™ HY DNA Amplification kit according to manufacturer's guidelines

Supplementary Table 1 – 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. *intracellµlare*	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
Haemophilus influenzae	4690
Staphylococcus aureus	20231
Streptococcus pneumoniae	20566

Amplification and Amplicon Cloning

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 were used to generate a 441 base pair amplicon of the *hsp65* gene. For each 25µl 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. PCR conditions were: 5 minutes at 95°C; 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. Amplicons of expected size were gel extracted and purified using the Monarch® DNA Gel Extraction Kit according to the manufacturers protocol and then cloned using the TOPO® TA Cloning® Kit for Sequencing according to the manufacturers protocol. DNA was extracted from positive clones using the PureLink® Quick Plasmid Miniprep and sequence of *hsp65* inserts confirmed by Sanger sequencing (Eurofins Genomics, Ebersberg, Germany).

Primer and Probe Design

hsp65 gene sequences for the 6 opportunistic mycobacteria described were downloaded from the National Centre for Biotechnology Information (NCBI) and aligned. Sequences were manually searched for discriminatory areas between species and primers (to generate a 140 base pair amplicon) and probes were designed. Primers and probes were sourced from Eurofins (Eurofins Genomics, Ebersberg, Germany) with 'high purity salt free' (HPSF) purification.

Establishing Annealing Temperatures

Theoretical melting temperatures for the designed primers were calculated and a temperature gradient PCR ranging from 52^{o}C to 70^{o}C was conducted before an annealing temperature of 66^{o}C was found to be optimal.

Primer Specificity

The custom primers were first interrogated using software to examine and explore biological data in relation to bacterial species (http://genome.usdc.edu). The software demonstrated PCR amplification limited to NTM species. Furthermore, primers were tested by performing PCR reactions with genomic DNA from various bacterial species and a positive control containing *M. abscessus* DNA. Amplification was then performed on an agarose gel that revealed amplification only in the positive control.

Probe Specificity

Assay specificity was established by testing each probe against a panel of plasmids containing both “target” and “off target” sequences. Probe specificity ranged from 92 – 100%.

Supplementary Table 2 – Specificity of each custom probe based on reactions against a panel of 15 different mycobacterial plasmids.

Probe Identity	Specificity
Abscessus complex	100%
Avium	92%
Intracellulare	100%
Kansasii	92%
Malmoense	92%
Xenopi	100%

Quantification of Mycobacterial DNA

The accurate quantification of mycobacteria was determined by the creation of a mock community into which serial dilutions of genomic NTM DNA were added. Mock community constituents and their end concentrations are shown in supplementary table 2.

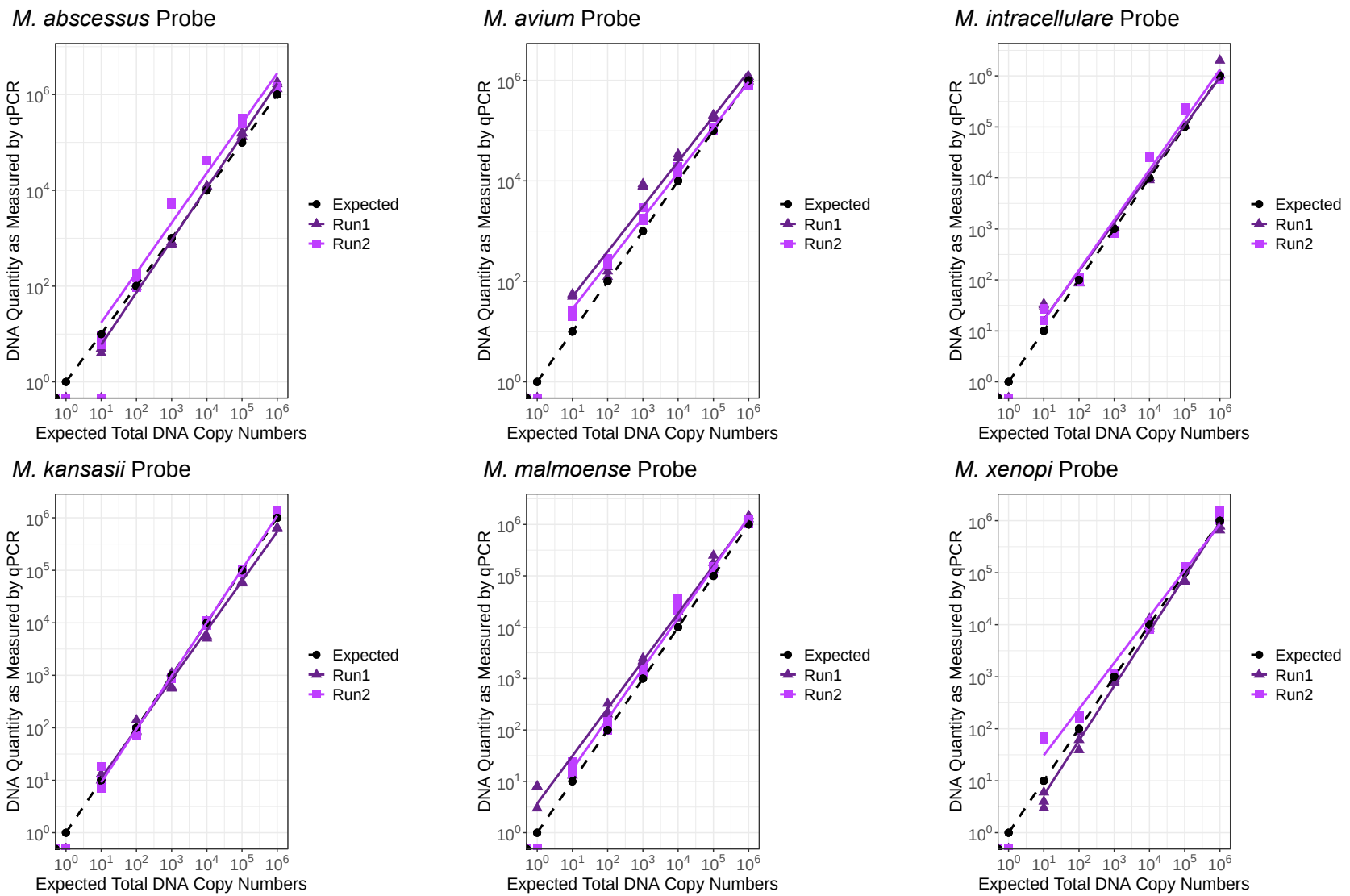
Supplementary Table 2 - Mock community constituents and their corresponding concentrations.

Species	DNA Concentration (copies/µl)
S. pneumoniae	2×10^5
S. aureus	2×10^5
H. influenzae	2×10^5
M. gordonae	2×10^5
H. sapiens	2×10^3

Serial dilutions of 1µl target NTM DNA (ranging from 1×10^6 to 1 copy/µl) were spiked into 4µl of the mock community. Measurement of target DNA was performed in triplicate.

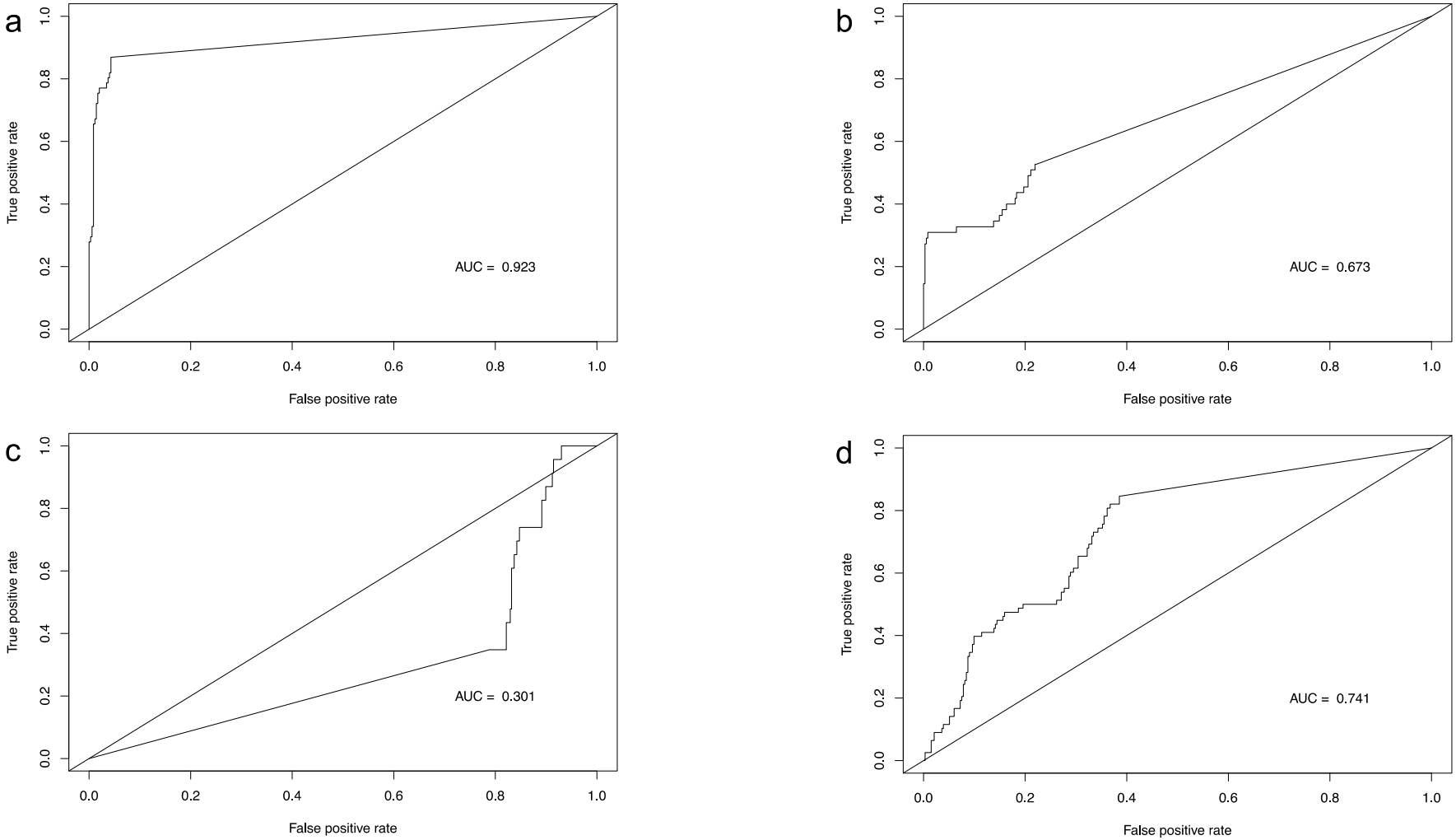
Results

To demonstrate each assay's reproducibility, testing was performed twice (supplementary figure 1). The distribution of all data sets was assessed for normality using the Shapiro-Wilk normality test which revealed a non-normal distribution. The Kruskal-Wallis one way analysis of variance showed no significant differences between measured and ideal values.

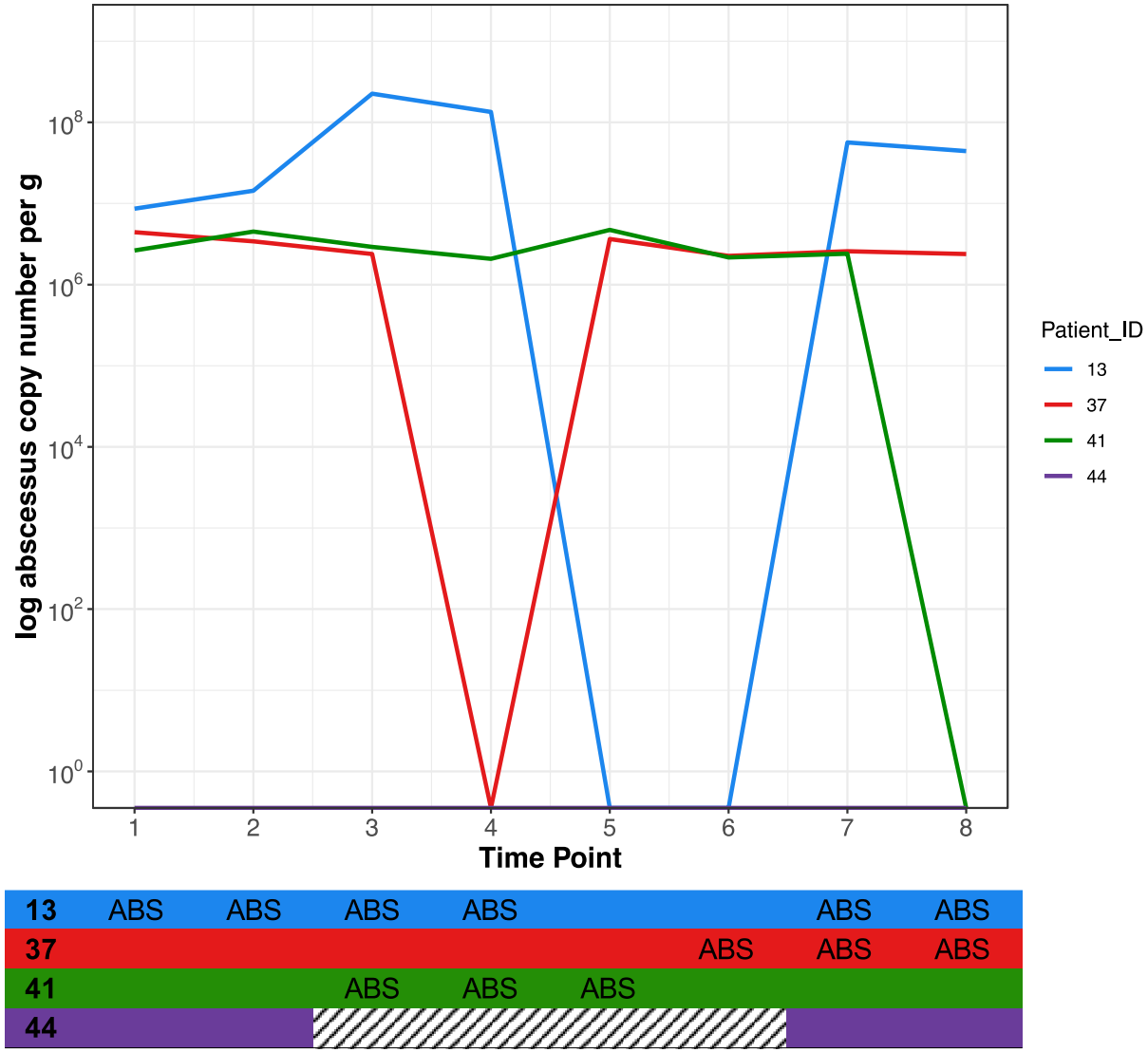


Supplementary Figure 1 - Figure illustrating the assay's ability to detect NTM species when present in a mock community. Measured DNA quantity within the assay is shown (Run 1). The experiment was repeated to show assay reproducibility (Run 2). The black dashed line illustrates the ideal values.

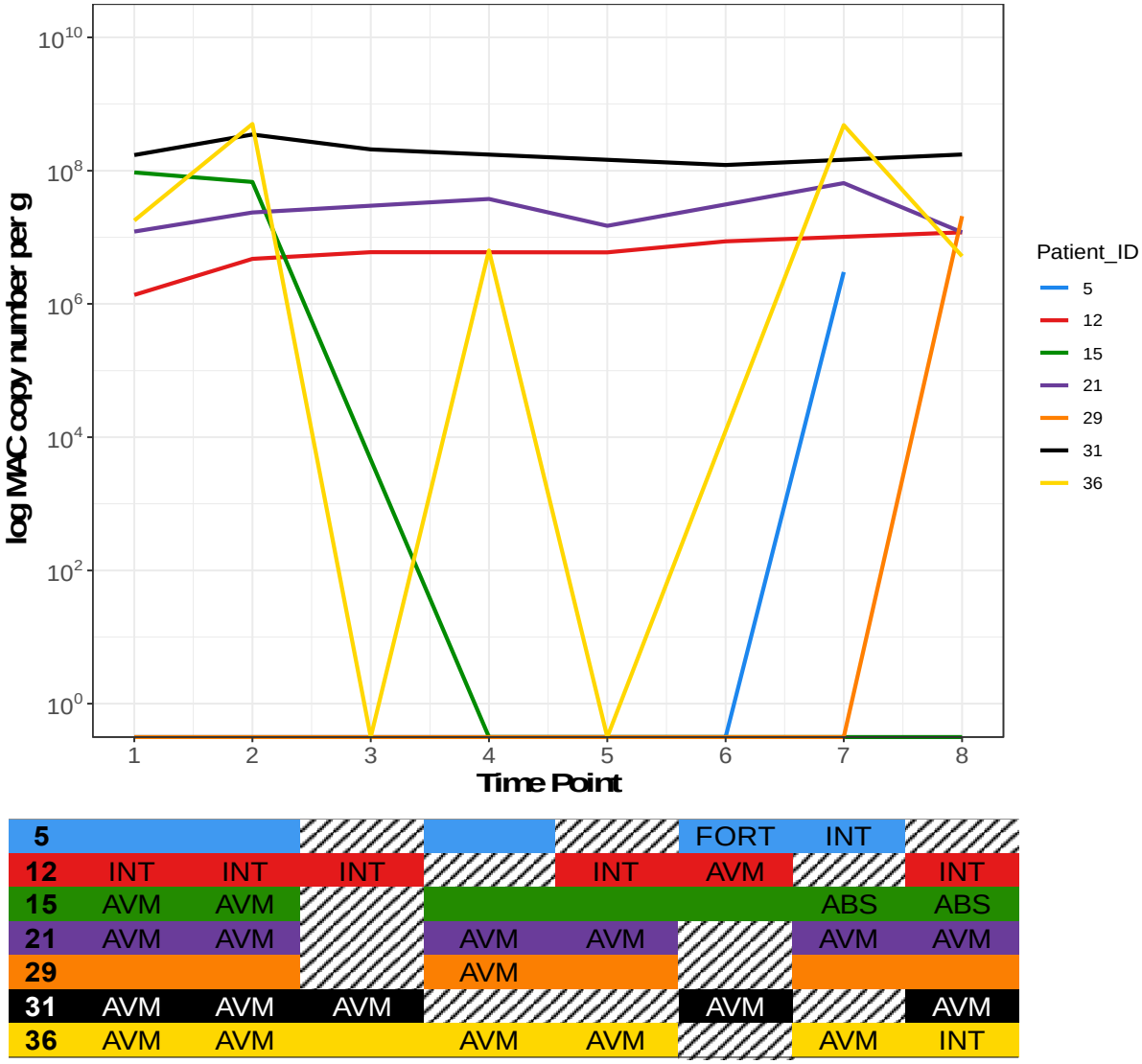
Supplementary Results



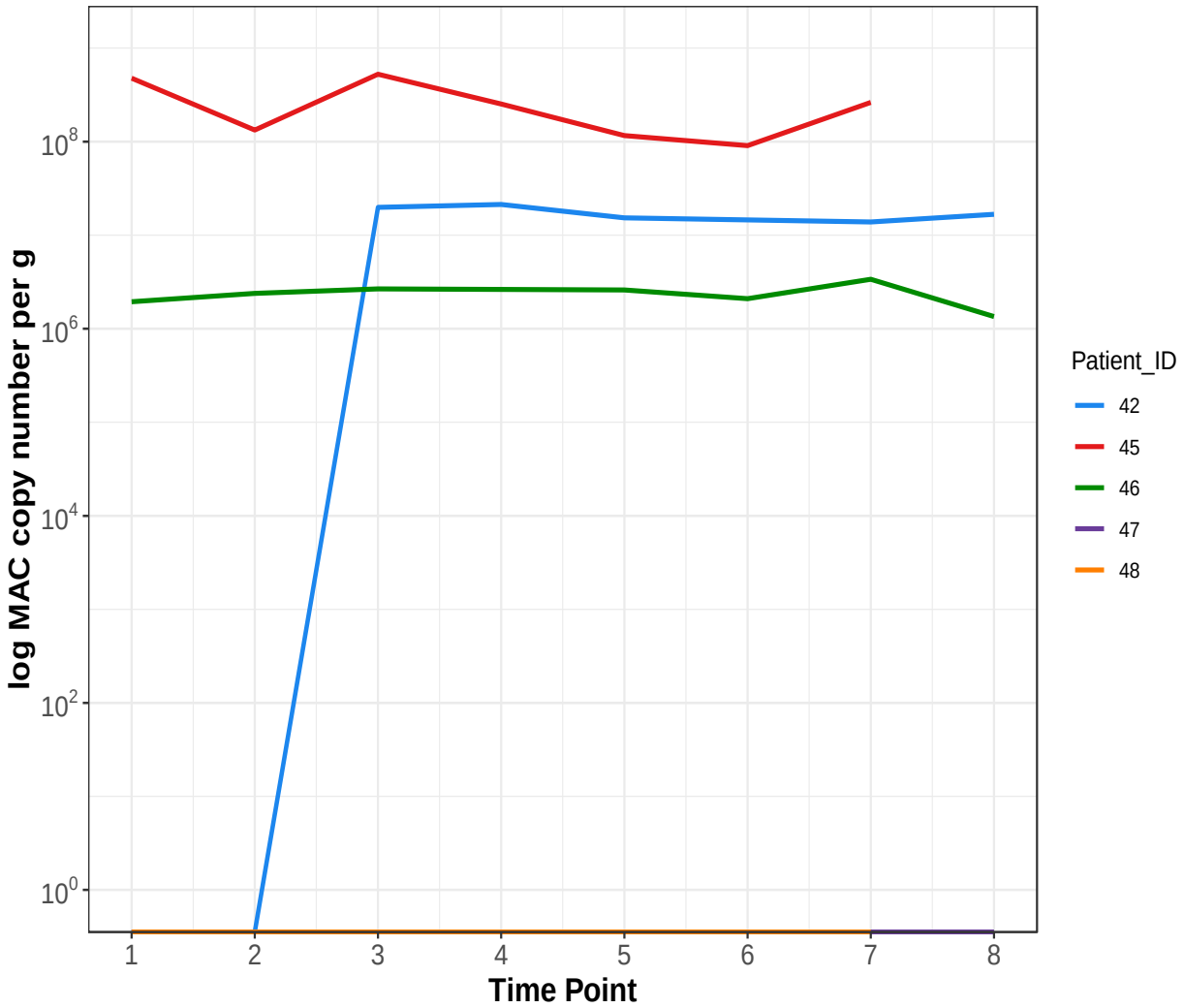
Supplementary Figure 1 – Receiver Operator Characteristic (ROC) Curves for a) the *M. abscesus* assay, AUC = 0.923 b) the *M. avium* assay, AUC = 0.673,c) for the *M. intracellulare* assay., AUC = 0.301 and d) the combined *M. avium* and *M. intracellulare* (MAC) assay AUC = 0.741. AUC: Area Under the Curve.



Supplementary Figure 2 – Custom assay with abscessus probe used with samples from the abscessus control (untreated) group.



Supplementary Figure 3 – Custom assay with MAC probe used with samples from the MAC control (untreated) group.



Supplementary Figure 4 - Custom assay with MAC probe used with samples from control (NTM culture negative) group. All samples were qPCR negative for *M. abscessus* and were culture negative throughout.