

***Pseudomonas aeruginosa* in the era of modulator therapies for Cystic Fibrosis**

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Declaration of Originality

I declare that the work in this manuscript has been carried out by myself unless where stated. Data collection and analysis of prescribed anti-pseudomonal inhaled therapies (chapter 3) was performed with assistance from Dr Dominic Fenn, Royal Brompton Hospital (Honorary), London. Bootstrapping analysis (chapter 3) was performed by Dr Mark Rosenthal, Royal Brompton Hospital, London. MALDI-TOF analysis of *Pseudomonas aeruginosa* cultured in the huff tube lab model was performed in conjunction with a MSc project under supervision of Dr Gerald Larrouy-Maumus, Imperial College, London. Training and testing of dogs to detect *Pseudomonas aeruginosa* cultured in the huff tube model, and subsequent data analysis, was performed in conjunction with the dog handlers, staff and statisticians of the Medical Detection Dogs charity, Milton Keynes.

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Abstract

Introduction

CFTR modulators (CFTRm) improve CFTR function, improving lung function and pulmonary exacerbation rate. Impact on sputum expectoration and *Pseudomonas aeruginosa* (Pa) infection remains unclear. Primary hypotheses explored were: (i) CFTRm reduce Pa culture frequency, density and shift virulence/persistence behaviours, (ii) alternative, non-invasive infection surveillance methods can be developed for those no longer expectorating.

Methods

A retrospective cohort study of people with Leeds defined chronic Pa infection pre-CFTRm was performed, examining sputum sample provision, Pa isolation frequency/abundance pre-/post-CFTRm. Virulence/persistence behaviours in Pa clinical isolates, induced *in vivo* pre-/post-CFTRm and *in vitro* in experiments mimicking lung environmental changes from CFTRm were examined. A lab model for a novel, non-sputum based, airway sampling method called 'huff tubes' was tested with PCR, mass spectrometry and sniffer dogs.

Results

Use of CFTRm led to reduction in number of surveillance samples provided; a majority no longer met Standards of Care. Adjusting for number of samples provided per year, there was reduction in Pa positivity rate and abundance in those with chronic Pa infection. Restoration of CF lung microenvironment oxygen state may lead to changes in virulence (increased pyoverdine production) and persistence phenotype (decreased mucoid alginate). PCR showed promise as an alternative Pa detection method in the huff tube model; sniffer dogs exceeded previous limits of detection of Pa. However, clinical pilot of huff tubes failed to capture sufficient bacteria for testing.

Conclusion

Reduction in surveillance sample provision poses a risk of missing lower airway infection and challenges current accepted definitions of chronic airway infection. Novel ways to sample the airway are urgently needed; alternatives to standard culture are available to detect Pa. Changes to the CF lung microenvironment post-CFTRm and the effect on Pa phenotype warrants further exploration as changes may be exploited to better treat those with persisting infection post-CFTRm.

Abbreviations

ALI	Air-liquid interface
AQs	2-alkyl-4-quinolones
ASL	Airway surface liquid
ASM	Artificial sputum media
AST	Antimicrobial sensitivity testing
Ax	<i>Achromobacter xylosoxidans</i>
BAL	Bronchoalveolar lavage
Bcc	<i>Burkholderia cepacia complex</i>
BMI	Body Mass Index
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic Fibrosis
CFQ-R	Cystic Fibrosis quality of life – respiratory domain questionnaire
CFTR	Cystic Fibrosis Transmembrane Regulator
CFTRm	Cystic Fibrosis Transmembrane Regulator modulator
CT	Computed tomography imaging
dH ₂ O	Deionised water
eDNA	Extracellular DNA
ELX	Elexacaftor
ENaC	Epithelial Sodium Channel
eNose	Electronic nose
EPR	Electronic patient record
EPS	Exopolysaccharide
ETI	Elexacaftor/Tezacaftor/Ivacaftor
F	<i>F508del</i>

HBE	Human bronchial epithelial (cells)
HCCA	Cyano-4-hydroxycinnamic acid
Hi	<i>Haemophilus influenzae</i>
HRA	Health Research Authority
IL-1 β	Interleukin 1 β
IL-8	Interleukin 8
IVA	Ivacaftor
LAMP	Loop-mediated isothermal amplification
LCI	Lung clearance index
LUM	Lumacaftor
MAIT	Mucosal associated invariant T cells
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight
MBW	Multiple breath washout
MDDs	Medical Detection Dogs
<i>MF</i>	Minimal function variant
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
NE	Neutrophil elastase
NTM	Non-tuberculous mycobacterium
OD ₆₀₀	Optical density measured at 600nm wavelength
Pa	<i>Pseudomonas aeruginosa</i>
PBS	Phosphate buffer solution
PCL	Periciliary layer
PCR	Polymerase chain reaction
PERT	Pancreatic enzyme replacement therapy

PEx	Pulmonary exacerbation
ppFEV ₁	Percent predicted forced expiratory volume in one second
pppy	Per person per year
pwCF	People with Cystic Fibrosis
qPCR	Quantitative polymerase chain reaction
QS	Quorum sensing
RBH	The Royal Brompton Hospital
REC	Research and Ethic committee
<i>RF</i>	Residual function variant
RFI	Relative fluorescent intensity
Sa	<i>Staphylococcus aureus</i>
Sma	<i>Serratia marcescens</i>
Smt	<i>Stenotrophomonas maltophilia</i>
SOP	Standard operating procedure
SRC-PAPA	Strategic Research Centre for Personalised approach to <i>Pseudomonas aeruginosa</i>
TEZ	Tezacaftor
TFTC	Too few to count
TMTC	Too many to count
VOCs	Volatile organic compounds

Contents

Chapter 1 – Introduction

1.1. Cystic Fibrosis	17
1.2. <i>CFTR</i> variants	18
1.3. CF pathophysiology	20
1.4. Airway infection in CF lung disease	24
1.5. <i>Pseudomonas aeruginosa</i>	25
1.6. Virulence and persistence adaptations of Pa	26
1.7. Conventional treatments and monitoring in CF lung disease	28
1.8. <i>CFTR</i> modulator therapies	30
1.9. The impact of <i>CFTRm</i> on the CF airway microenvironment and Pa infection.....	32
1.10. Existing and evolving challenges of Pa detection	34
1.11. Alternative Pa detection methods: Polymerase Chain Reaction and Mass Spectrometry	36
1.12. Alternative Pa detection methods: Sniffing out the scent of Pa	38
1.13. The Strategic Research Centre sample repository	39
1.14. Hypotheses	40
1.15. Aims and Objectives	40

Chapter 2 – General methods and methodology development

2.1. General methods	42
2.1.1. The SRC-PAPA repository and isolate storage.....	42
2.1.2. Broth and agar culture media	43
2.1.3. Revival of stored isolates.....	44
2.1.4. Growth measurement by serial dilution and colony counting	44
2.1.5. Growth measurement by Optical Density.....	46
2.1.6. Measurement of Relative Fluorescent Intensity	48
2.1.7. Measurement of Minimum Inhibitory Concentration	48
2.2. Methodology development.....	52

2.2.1. Congo red and mucoidy scoring.....	52
2.2.2. Developing a lab model for huff tubes.....	54
2.2.3. Polymerase chain reaction	71
2.3. Ethics approval	72
2.4. Statistical analysis.....	73

Chapter 3 – Results: Changes in airway sampling and *Pseudomonas aeruginosa* isolation after the introduction of Elexacaftor/Tezacaftor/Ivacaftor

3.1. Introduction	74
3.2. Methods.....	76
3.3. Results.....	78
3.4. Discussion	92

Chapter 4 – Results: Phenotypic impact of CFTRm on *Pseudomonas aeruginosa* isolates from people with Cystic Fibrosis

4.1. Introduction	96
4.2. Materials and methods.....	99
4.2.1. Phenotypic assays for Pa isolates from pwCF before and after CFTRm initiation	99
4.2.2. Modelling pre- and post- CFTRm treatment changes to the CF airway and their impact on <i>Pseudomonas aeruginosa</i> phenotype.....	102
4.3. Results - Phenotypic assays for Pa isolates in the SRC-PAPA repository before and after their CFTRm initiation	108
4.3.1. Demographics.....	108
4.3.2. Growth measurement by OD ₆₀₀	112
4.3.3. Growth measured by colony count.....	114
4.3.4. Relative fluorescent intensity.....	120
4.3.5. Mucoïd phenotype	125
4.4. Results - Modelling pre- and post- highly effective modulator treatment CF airway changes and their impact on <i>Pseudomonas aeruginosa</i> phenotype.	129
4.4.1. Stability of pH during passage.....	129
4.4.2. Growth and growth rate	131

4.4.3. Pyoverdine production.....	142
4.4.4. Mucoidy.....	144
4.4.5. Antimicrobial sensitivity testing.....	146
4.5. Discussion	148

Chapter 5 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: PCR, LAMP and MALDI-TOF

5.1. Introduction.....	153
5.2. Materials and methods.....	156
5.2.1. Polymerase chain reaction	156
5.2.2. Loop-mediated isothermal amplification.....	157
5.2.3. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry	159
5.3. Results.....	160
5.3.1. PCR	160
5.3.2. LAMP	170
5.3.3. Sensitivity of PAL1 and 2 PCR primers in the huff tube lab model when used with LB ...	178
5.3.4. MALDI-TOF Mass Spectrometry	180
5.4. Discussion	181

Chapter 6 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: sniffer dogs and a clinical pilot of huff tubes

6.1. Introduction.....	187
6.2. Methods – training and testing the huff tube lab model with sniffer dogs	189
6.3. Results – training and testing the huff tube lab model with sniffer dogs	194
6.4. Methods – Clinical pilot of huff tubes with sniffer dogs.....	196
6.5. Results – Clinical pilot of huff tubes tested with sniffer dogs	202
6.6. Discussion	211

Chapter 7 – Discussion

7.1. Summary of findings.....	215
7.2. Post-approval studies for ETI.....	218

7.3. Impact of ETI on sputum production and Pa infection.....	220
7.4. Impact of ETI on CF lung microenvironment and CF pathogenesis.....	221
7.5. Impact of ETI on Pa virulence and persistence.....	224
7.6. Advances and ongoing challenges in Pa detection.....	226
7.7. Strengths and limitations.....	229
7.8. Future directions	231

References.....	234
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Appendices

Appendix 1: SRC-PAPA repository collection and storage SOP	255
Appendix 2: SRC-PAPA Revival of frozen stored repository samples SOP	258
Appendix 3: Miles and Misra Method of Colony Counting	262
Appendix 4: Resazurin assay.....	264
Appendix 5: Huff tube lab model SOP	265
Appendix 6: LAMP colorimetric indicator protocol – New England Biolabs	267
Appendix 7: Medical Detection Dogs – SOP for transport, storage, handling and disposal of samples.....	269

Figures

Chapter 2 – General methods and methodology development

Figure 2.1: Comparison of OD ₆₀₀ and CFU/ml measurements of clinical Pa isolates	46
Figure 2.2: Subjective scoring system for mucoidy on congo red agar	53
Figure 2.3: Preliminary workflow of huff-tube lab model development	57
Figure 2.4: Starting concentration of inoculum onto gauze and post-incubation OD	59
Figure 2.5: Example of resazurin assay colour changes	60
Figure 2.6: Resazurin viability assay to detect the minimum starting concentration to produce viable growth on gauze	61
Figure 2.7: Growth curves demonstrating late growth of Pa isolates at very low starting concentrations.....	63
Figure 2.8: Final workflow of huff-tube lab model.....	65
Figure 2.9: Sensitivity of the huff tube lab model	66
Figure 2.10: Growth curves of Bcc isolates in Pa selective broth.....	68
Figure 2.11: Unexpected growth of a single Sa isolate in Pa selective broth.....	68
Figure 2.12: The huff tube lab model used with proposed alternative Pa detection methods	70

Chapter 3 – Results: Changes in airway sampling and *Pseudomonas aeruginosa* isolation after the introduction of Elexacaftor/Tezacaftor/Ivacaftor

Figure 3.1: Diagram of included study population and subgroups	78
Figure 3.2: Change in paired airway samples (number and type) per person per year	80
Figure 3.3: Sub-analysis of number of sputum samples provided pppy by ppFEV ₁	81
Figure 3.4: Proportion of people meeting CF Standards of Care pre- and post-ETI.....	82
Figure 3.5: Change in paired Leeds defined chronic infection status over time	83
Figure 3.6: Changes to bacterial growth from paired airway samples pppy.....	84
Figure 3.7: Sensitivity analysis of bacterial growth from paired sputum samples pppy	85

Figure 3.8: Sub-analysis of Pa isolation frequency by sex, genotype, pre-ETI baseline ppFEV ₁ and change in ppFEV ₁	86
Figure 3.9: Changes in mucoid Pa phenotype from paired airway samples pppy	87
Figure 3.10: Sensitivity analysis of mucoid Pa phenotype from paired sputum samples pppy	88
Figure 3.11: Sub-analysis of mucoid Pa isolation frequency by sex, genotype, pre-ETI baseline ppFEV ₁ and change in ppFEV ₁	89
Figure 3.12: Change in median Pa abundance in Pa positive paired airway samples pppy	90

Chapter 4 – Results: Phenotypic impact of CFTRm on *Pseudomonas aeruginosa* isolates from people with Cystic Fibrosis

Figure 4.1: Workflow of serial passage through a range of pH and oxygen state environments for 11 Pa isolates and phenotype assays	104
Figure 4.2: Pa isolate timeline relative to CFTRm start date	110
Figure 4.3: Absolute OD ₆₀₀ for each patient's isolates over time pre- and post-CFTRm	112
Figure 4.4: OD ₆₀₀ for each patient's isolates relative to baseline over time pre- and post-CFTRm	113
Figure 4.5: Pooled OD ₆₀₀ for all isolates pre- compared to all isolates post-CFTRm	114
Figure 4.6: Absolute CFU/ml for each patient's isolate over time pre- and post-CFTRm	115
Figure 4.7: CFU/ml for each patient's isolate relative to baseline over time pre- and post-CFTRm	116
Figure 4.8: Pooled CFU/ml for all isolates pre- compared to all isolates post-CFTRm	117
Figure 4.9: Correlation of OD ₆₀₀ and CFU/ml for each isolate	120
Figure 4.10: Correlation of RFI and CFU/ml for each isolate	122
Figure 4.11: Absolute CFU adjusted RFI for each patient's isolate over time pre- and post-CFTRm	123
Figure 4.12: CFU adjust RFI for each patient's isolate relative to baseline over time pre- and post-CFTRm	124
Figure 4.13: Pooled CFU adjusted RFI for all isolates pre- compared to all isolates post-CFTRm ..	125
Figure 4.14: Subjective mucoidy score for each patient's isolate relative to baseline over time pre- and post-CFTRm	127

Figure 4.15: Changes in broth pH after 24 hours incubation at different oxygen states.....	130
Figure 4.16: Absolute OD ₆₀₀ for all isolates at each pH and oxygen state.....	132
Figure 4.17: Relative OD ₆₀₀ for all isolates at each pH and oxygen state	133
Figure 4.18: Pooled OD ₆₀₀ for all isolates at each condition, relative to results for normoxic pH 7.4	134
Figure 4.19: Absolute CFU/ml for all isolates at each pH and oxygen state.....	135
Figure 4.20: Relative CFU/ml for all isolates at each pH and oxygen state	136
Figure 4.21: Pooled CFU/ml for all isolates at each condition, relative to results for normoxic pH 7.4	137
Figure 4.22: Correlation between CFU/ml and OD ₆₀₀ values at different conditions.....	138
Figure 4.23: Growth curve plots of serial OD ₆₀₀ measurements used to calculate doubling times	141
Figure 4.24: Comparison of doublings times at each environmental condition	141
Figure 4.25: Absolute CFU adjusted RFI for all isolates at each pH and oxygen state	142
Figure 4.26: Relative CFU adjusted RFI for all isolates at each pH and oxygen state.....	143
Figure 4.27: Pooled CFU adjusted RFI for all isolates at each condition, relative to results for normoxic pH 7.4	144
Figure 4.28: Muroid scores for all biological repeats for isolates at each environmental condition	145
Figure 4.29: MIC to tobramycin for isolates at each environmental condition	147
Figure 4.30: Pooled median MIC at each environmental condition.....	148

Chapter 5 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: PCR, LAMP and MALDI-TOF

Figure 5.1: First attempt to detect Pa directly from bacteria cultured in ceftrimide broth.....	161
Figure 5.2: Comparison of PCR performed on samples directly from ceftrimide broth against those resuspended in PBS.	163
Figure 5.3: Comparison of different <i>Taq</i> polymerase brands when performing PCR with samples taken directly from ceftrimide broth.....	164

Figure 5.4: Concentrating bacterial samples in a multi-well plate for PCR to attempt to improve sensitivity when sampling directly from directly from cultured cetrimide broth	166
Figure 5.5: Exploring failures to reproduce results when attempting PCR by concentrating samples prior to analysis	168
Figure 5.6: Final attempt to concentrate bacterial samples for PCR to attempt to improve sensitivity when sampling directly from cultured cetrimide broth.....	169
Figure 5.7: Comparing use of LAMP colorimetric indicators to detect Pa in samples taken directly from cultured cetrimide broth and samples resuspended in PBS.....	173
Figure 5.8: Using PCR to detect Pa in the huff tube lab model when LB is used as culture media instead of cetrimide.....	176
Figure 5.9: Using LAMP to detect Pa in the huff tube lab model when LB is used as culture media instead of cetrimide.....	178
Figure 5.10: Examining specificity of PCR primers in the huff tube lab model	179
Figure 5.11: Limits of detection of Pa in the huff tube lab model using MALDI-TOF.....	180

Chapter 6 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: sniffer dogs and a clinical pilot of huff tubes

Figure 6.1: The dogs trained and tested in the Pa detection project.....	190
Figure 6.2: Samples identified by sniffer dogs as Pa from the huff tube model	195
Figure 6.3: Instructions for home huff tube sampling for pilot study	200
Figure 6.4: Sample return slip for clinical pilot of huff tubes	201
Figure 6.5: Confirmatory PCR results of huff samples collected in LB in clinical pilot	210

Tables

Chapter 2 – General methods and methodology development

Table 2.1: Median MIC for examined isolates and expected testing ranges	50
Table 2.2: Specificity - summary of non-Pa strain survival in the huff tube lab model.....	69

Chapter 3 – Results: Changes in airway sampling and *Pseudomonas aeruginosa* isolation after the introduction of Elexacaftor/Tezacaftor/Ivacaftor

Table 3.1: Baseline demographics	79
--	----

Chapter 4 – Results: Phenotypic impact of CFTRm on *Pseudomonas aeruginosa* isolates from people with Cystic Fibrosis

Table 4.1: Demographics of people providing isolates used in phenotype assays	109
Table 4.2: Date of Pa isolate provision and clinical reporting of mucoid status by RBH lab.....	111
Table 4.3: Comparison of biological repeats of OD ₆₀₀ and CFU/ml for each isolate	118
Table 4.4: Comparison of biological repeats of RFI for each isolate	121
Table 4.5: Comparison of technical and biological repeats of subjective mucoidy score.....	129

Chapter 5 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: PCR, LAMP and MALDI-TOF

Table 5.1: Pilot PCR thermal cycler settings	157
Table 5.2: First attempt at using LAMP with colorimetric indicators to detect presence of Pa DNA in the huff tube model.....	171

Chapter 6 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: sniffer dogs and a clinical pilot of huff tubes

Table 6.1: Clinic comparison of cetrimide and LB broth, sample types and timing of incubation of samples to evaluate failure of the first clinical pilot of huff tubes.....	206
Table 6.2: Results of clinical pilot of huff tubes using LB broth media	209

Chapter 1 – Introduction

1.1. Cystic Fibrosis

Cystic Fibrosis (CF) is an autosomal inherited condition affecting approximately 70-100,000 people worldwide [1]. Disease causing variants are carried by one in 25 people, it is described in all ethnicities globally and is the most common autosomal recessive disease amongst Caucasian populations [2]. CF is a multi-system disorder characterised by the production of excessively viscous secretions throughout the body. Its effects are seen before birth, with congenital bilateral absence of the vas deferens (CBAVD) in affected males, pancreatic insufficiency and meconium ileus, and is progressive throughout life with destructive bronchiectasis, poor nutrition and growth, gut and liver disease and CF-related diabetes. Advances in CF care have dramatically changed outcomes; the respiratory and gut complications of a disease that caused death in childhood have been targeted and led to improvement in survival. In 2020, at the time of starting this MD(Res) project, the median predicted survival in the UK was 51.6 years, an improvement of over 10 years since 2010 (41.4 years), according to UK CF Patient Registry data [3].

The association of cystic and fibrotic destruction of the pancreas along with progressive bronchiectasis in a single disease entity was made by Dr Dorothy Andersen in the 1930s [4]. Work by Dr Paul di Saint Agnese, during a heat wave in the 1950s, identified that people with CF (pwCF) have elevated concentration of chloride in their sweat, and are highly susceptible to dehydration in hot weather [5]. This led to the development of the 'sweat test' as the diagnostic test for CF, with normal sweat chloride concentration being $<30\text{mmol/L}$ and abnormal being $>60\text{mmol/L}$. In 1983, it was discovered that chloride impermeability led to sweat test abnormalities and suggested that pathophysiology of CF related to a defect in chloride channel conductance. Rapid progress in the field of genetics in the 1980s led first to the localisation of the defective gene causing CF to the long arm of chromosome 7 and later its exact position and sequence was found [6]. The gene was called

the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene, consisting of 250,000 base pairs and codes for a protein consisting of 1480 amino acids, known as CFTR protein [7–9]. CFTR is a transmembrane ion channel responsible for flow of chloride and bicarbonate anions and is found at the apical surface of epithelial cells [10].

1.2. *CFTR* variants

Over 2000 *CFTR* variants leading to reduced quantity or impaired function of CFTR protein have been identified since the discovery of the gene [2,11]. *F508del* is the commonest variant, accounting for 70-85% of variants worldwide. Six classes of *CFTR* variant are commonly recognised, with classification based on how protein synthesis is affected [12]:

- Class I variants, such as *G542X* and *W1282X*, are commonly called nonsense variants and are usually caused by premature termination codons leading to truncation of *CFTR* mRNA and protein, causing it to be non-functional.
- Class II variants, such as *F508del*, lead to protein misfolding and reduced delivery, as a result, to the cell surface.
- Class III (gating) variants, such as *G551D*, show protein delivery to cell surface but the channel does not open correctly meaning impaired chloride conductance.
- Class IV variants, such as *R117H*, also deliver protein correctly to the cell surface, the channel can open, but chloride conductance through the channel is significantly reduced.
- Class V variants, such as *3849+10kb C>T*, result in reduced protein transcription and overall, less CFTR quantity at the cell surface.
- Class VI variants, such as *c.120del123*, lead to reduced CFTR protein stability and increased CFTR turnover, so that cell surface CFTR quantity is not maintained.

Though in reality variants may exhibit defects from more than one class, for example, the commonest *CFTR* variant, *F508del*, causes defective protein trafficking to the cell surface (predominantly class II), but also demonstrates defective protein channel gating and shortened cell-surface half-life (features of class III and VI). There is a recognised relationship between degrees of *CFTR* function and disease severity, and a tight correlation between the level of sweat chloride and spectrum of *CFTR* related comorbidities, ranging from healthy, to isolated CBAVD, then pancreatic sufficient CF and to pancreatic insufficient CF [13]. This largely relates to the combination of *CFTR* genes a person with CF has. Class I, II and III variants are typically termed 'minimal function' (*MF*), except for *F508del* which is prevalent enough to usually be abbreviated as *F* [14]. Those carrying two copies of *F508del* (*F/F*), one copy of *F508del* and another minimal function variants (*F/MF*), or two minimal function variants (*MF/MF*), have very little residual *CFTR* function and expect a more severe CF phenotype with pancreatic insufficiency. Class IV, V or VI variants are termed residual function variants (*RF*), and those carrying at least one of these (*F/RF* or *MF/RF* or *RF/RF*) will likely have a milder disease course, usually with pancreatic sufficiency.

Whilst class I and II variants are associated with worse pulmonary function, CF lung disease correlates less well with variant class alone, and the wide variability in CF lung disease is also affected by environmental encounters and modifier genes [15,16]. Environmental encounters, such as infecting organisms in the airway (discussed below), nutritional status, treatment use and concordance, smoking and socioeconomic status have all been shown to play a role in CF phenotype [17]. Similarly, modifier genes are genes other than the *CFTR* variant that affect disease expression. Mechanisms of action could be directly on the degree of expression of the *CFTR* gene or impacting the downstream effects of *CFTR* dysfunction by improving or worsening susceptibility to infection and subsequent inflammatory responses and mucociliary clearance. A number of modifier genes have been investigated and are well-reviewed elsewhere [15,17].

1.3. CF pathophysiology

Understanding of CF pathophysiology has developed over decades, but many aspects are still being investigated. Knowledge of CFTR function, and in turn the consequences of its dysfunction have been critical in understanding CF pathophysiology.

CFTR function

CFTR is an ion channel responsible for flow of chloride and bicarbonate anions and in turn the salt and water balance across epithelial surfaces including lung, liver, intestine, pancreas and sinuses [10]. In the lung, the apical surface of airway epithelia is ciliated and covered in fluid, known as the airway surface layer (ASL) [18]. The ASL is formed of two components, the periciliary layer (PCL), lying adjacent to the apical surface and coating the cilia, and mucous layer on top of this. The mucous layer is escalated out of the airway by cilia beat action, in a process known as mucociliary clearance. This transports bacteria and organic and cellular debris away from the lung in the healthy airway.

Hydration of this ASL is maintained by the actions of ion channels that cross the apical and basolateral surfaces of epithelial cells. In the resting state, active absorption of sodium into the cell, by the Epithelial Sodium Channel (ENaC), leads to passive absorption of chloride across the cell surface. Water follows sodium and chloride into the cell via aquaporin channels.

CFTR functions as an inhibitor of ENaC. When increased hydration of the ASL is required, ENaC is inhibited, active sodium absorption into the cell is reduced, and cyclic-AMP dependent activation of CFTR causes the channel to open and secrete intracellular chloride. The presence of sodium and chloride in the ASL draws intracellular water across, in turn hydrating it. A balance of CFTR and ENaC control is always occurring, in a process termed 'tonic inhibition' [19].

CFTR dysfunction and low volume ASL

In CF, defective production or dysfunction of CFTR leads to uninhibited action of ENaC and reduced ability to secrete chloride into the airway. This leads to dehydration of the ASL and impaired mucociliary clearance, in a process termed as 'the low volume hypothesis'. Widely accepted as being an important part of CF pathogenesis, the dehydration of airway mucous in turn leads to mucus obstruction in the smaller airways. This predisposes to bacterial infection with opportunistic pathogens; infection becomes chronic and neutrophilic inflammation occurs. A cycle of infection and inflammation leads to bronchiectasis over time [18]. This progressive lung disease process will eventually cause respiratory failure; the principal cause of premature death in pwCF [20]. In addition to chronic infection and inflammation, episodes of acute infective exacerbation, known as pulmonary exacerbation (PEx) occur as part of the respiratory disease course. A unifying agreed definition of PEx does not exist, but in guidelines on the management of PEx, it is accepted that clinical features of PEx include increased cough, increased sputum production, chest pain, shortness of breath, weight loss, and drop in lung function [21]. PEx require prompt treatment, and recurrent or severe PEx may lead to more rapid respiratory decline.

However, this model maybe over simplified, and resolution of airway hydration alone does not prevent CF lung disease [2]. The pH, relative oxygen state, presence of extracellular DNA (eDNA), mucin structure, biochemical composition, neutrophilic inflammation and neutrophil elastase (NE) activity in mucus and ASL make up a distinct CF airway microenvironment. They all likely contribute independently to CF pathogenesis and the bacteria that infect the airway.

CFTR dysfunction and ASL pH

CFTR also conducts bicarbonate; CFTR dysfunction reduces pH of airway surface liquid in animal models and measurement of exhaled breath condensate pH in pwCF is below neutral pH [22–29]. In experiments using CF animals in their infancy, before significant chronic infection or inflammation

takes place in the airway, acidified ASL has been shown to independently affect function of antimicrobial peptides and impair bacterial killing [27,28]. In a study comparing newborn CF and wild type pigs, bacteria were placed directly onto the airway, *in vivo*, and *in vitro* within primary epithelial cultures, with bacterial survival examined using immunofluorescent staining [27]. Bacteria were killed rapidly when in contact wild type ASL, but lack of CFTR in CF pig ASL showed reduced bacterial killing. The speed of killing suggested chemical, rather than immunological or inflammatory, properties of ASL to be responsible. The ASL in CF pigs was found to be more acidic and restoring ASL pH toward neutral in CF pigs restored ASL bacterial killing ability.

Airway surface pH may also be secondarily reduced in the presence of inflammation and infection. It has also been shown that CFTR dependent bicarbonate secretion is needed for normal mucus secretion from mucus glands in a CF mouse model and in turn, impaired bicarbonate secretion with CFTR dysfunction, can impair mucus function, adhesion and detachment, further increasing mucus viscosity [30,31].

Low oxygen and high eDNA microenvironments

Within thick mucus plugs on the epithelial surface of the CF airway there is a rapid gradient of oxygen concentration [32]. This relative hypoxic state is thought to provide favourable conditions for CF pathogens to evade innate immune clearance and build antibiotic tolerance. Relative hypoxia can also lead to neutrophilic inflammation even in the absence of infecting bacteria [33]. High eDNA content in the CF mucus leads to persistence of chronic infecting bacteria, forming the principal component of bacterial biofilms [34]. Presence of high eDNA sputum content is also associated with greater decline in lung function and more frequent hospitalisation rates [35]. Mucins are large glycoprotein molecules responsible for the viscous and elastic properties of normal mucus. In CF, dehydration of ASL causes mucin strands to become entangled, and reactive oxygen species from

inflammation cause mucin cross links, impairing mucus function and ability to clear pathogen from the airway [36].

The role of inflammation in CF pathophysiology

Airway inflammation occurs from an early age in the CF airway, and exploring its role in CF pathogenesis in detail is beyond the scope of this thesis, but it is well reviewed elsewhere [37]. From early life, airway inflammation is present with persistent neutrophil recruitment and activity within the CF airway. Other inflammatory markers seen in bronchoalveolar lavage (BAL) fluids include NE and interleukin 8 (IL-8) [38]. Despite increased neutrophil activity, CF neutrophils also appear unable to adequately clear CF airway pathogens. Debate exists as to whether infection, and failure to control infection, is the sole driver of inflammation or if inherent defect in neutrophils themselves occurs due to CFTR dysfunction, but there is accumulating evidence that the latter may be important [39,40].

NE is released following neutrophil degranulation and is a damaging protease enzyme, degrading proteins that act as controllers of normal inflammatory response relating to cell death, phagocytosis of bacterial pathogens and protection against further protease action [37]. Macrophage dysfunction is exhibited in CF as well, with reduced ability to switch between pro- and anti-inflammatory states and can be forced toward a more pro-inflammatory state by relative hypoxic environments [41]. T-lymphocytes are also expressed differentially in the CF lung, with upregulation of Th17 which perpetuate abnormal neutrophil responses. The results of this difference in the CF airway is a pro-inflammatory state that is ineffective in clearing bacterial infection [37]. Targeting airway inflammation has long been desired as a therapeutic target but no clearly successful anti-inflammatory therapies have been found.

1.4. Airway infection in CF lung disease

Failure to clear microorganisms in the CF lung, through loss of mucociliary clearance, leads to chronic airway infection and contributes to the driving of a persistent pro-inflammatory state [10]. In early childhood, infection with organisms such as *Staphylococcus aureus* (Sa) and *Haemophilus influenzae* (Hi) is common [42]. These are common infections in childhood airways anyway, but rates of detection are higher in those infants and young children with CF. Generally adopted definitions of all infection chronicity in CF come from 'Leeds' criteria that were designed originally to define infection status of Pa [43]. An individual is classified as having chronic infection if >50% of samples per year grow Pa, non-chronic (intermittent) in those growing Pa in >1 sample but <50% of total samples, and negative if not growing on any samples in a year.

At the time of designing this research project in 2020, the UK CF registry reported that 24% of children aged 0-3 years intermittently grew Sa, and 7% were chronically infected. 28% of the same group had at least one growth of Hi [3]. Intermittent Sa prevalence stayed stable up to 16 years (23-30%), then reduced in adulthood with a mean of 18% in those over 16 years. Chronic Sa peaked at 19% at age 16-23, reducing to an average of 16% for the remaining years. Prevalence of Hi decreased throughout childhood.

As CF lung disease progresses, *Pseudomonas aeruginosa* (Pa) becomes the predominant organism and is clinically the most significant [42,44]. In the UK, in 2020, intermittent Pa was detected in 18% of children aged 0-3, and this increased to 20% of 12-15 year olds [3]. Mean prevalence at over 16 years was 18%. Chronic Pa infection increased with age, staying relatively low in early childhood, below 4% at age 8-11, increasing to 10% at 12.15 years. It increased further in adulthood, peaking at 43% at age 36-39 years, before decreasing. Mean prevalence above 16 years was 32%.

1.5. *Pseudomonas aeruginosa*

Pa is a Gram-negative bacterium with a range of virulence systems including toxin secretion and biofilm formation, allowing it to readily overcome impaired CF host defence systems [44]. It is considered to be the most clinically relevant because it is so prevalent and it is associated with accelerated decline of percentage predicted forced expiratory volume in one second (ppFEV₁) and mortality [45–48]. Reviewing US CF registry data in 1998 of children aged 1-5 years in 1990, those who were Pa culture positive in 1990 had a 2.6 (95%CI 1.6-4.1) times higher risk of death, lower ppFEV₁ and weight centiles and increased risk of hospitalisation for PEx [46]. Similarly, mortality data reviewed between 1995 and 2005 for a single large CF centre showed that the proportion infected with Pa was higher in the group that died [45]. In an Australian observational cohort of 56 children born 1990-1992, who underwent annual BAL, 43% had Pa positive cultures at some point before age 7 [48]. Of the 4 that died before 7 years, all had Pa isolates that exhibited mucoid chronic infection phenotype. Pa infection was associated with worse quality of life scores, greater variability in ppFEV₁, increased hospital time and higher rates of nebulised mucolytic use, although at age 7 there was no statistical difference in best ppFEV₁. A further US study showed that time to first positive Pa culture was predictive of deteriorating chest radiograph (by standardised scoring system) and more rapid decline in FEV₁/FVC ratios [47].

Early recognition of Pa, through microbiological culture of airway samples, is essential to improve the chances of successful eradication with antibiotics [49]. When infection becomes chronic it is manifest by phenotypic and behavioural changes in Pa isolates that downregulate acute complex virulence systems and enhance airway persistence, such as mucoidy and biofilm formation [50]. Mucoid Pa is often considered the hallmark of chronic Pa infection and is characterised by overproduction of exopolysaccharide (EPS) alginate which affords Pa protection from dehydration and other stressors [51]. Those with established chronic mucoid Pa show an even steeper decline in

ppFEV₁ [52]. Management has traditionally then switched to chronic suppression as attempts at eradication are mostly futile.

1.6. Virulence and persistence adaptations of Pa

Pa is a highly adapted bacterium with an arsenal of virulence and persistence mechanisms that help establish and maintain infection in the CF lung [44].

Acute virulence

Bacterial motility is possible because of specialised surface proteins; rotating flagella provide swimming movement through fluid media and swarming movement of bacterial clusters across a surface, while pili allow crawling (twitching) of bacterium across a surface. Flagella and pili play an important role in cell surface adhesion and coverage while flagella also act as a recognition site for host immune response [53,54]. Knock-out mice models show reduced mortality and pneumonia when flagella or pili are not present [55].

Acute virulence is enhanced by toxin production including elastase, protease and hydrogen cyanide that effect host cell cytotoxicity or protein degradation [56]. Presence of high quantities of hydrogen cyanide in the microenvironment may lead to inhibition of ciliary function, further reducing mucociliary clearance of infecting bacteria [57]. Pyoverdine is a siderophore involved in acquisition of iron for Pa metabolism and virulence signalling pathways that control several of the acute toxins that Pa secretes [58]. Inhibition of pyoverdine production can limit the acute virulence of Pa infection in lab models [59]. Secretion of toxins can occur by diffusion or pumping, into the local environment or directly into host cells, by specially adapted surface protein secretion systems [60]. Production of acute virulence factors is coordinated by quorum sensing (QS), a cell density

dependent regulatory system that controls the expression of multiple genes related to toxin production, secretion systems and motility [56,61].

Environmental selective pressures in the CF lung

The CF lung microenvironment can enhance acute virulence response with studies showing that relative hypoxia increased antibiotic resistance by altering the composition of multi-drug efflux pumps [62]. However, whilst impairments in the host defence of CF airways allow Pa to take advantage, the CF host environment remains a uniquely hostile place for bacteria to thrive. Airway dehydration, variable oxygen state within mucus plugs, low pH, oxidative and nitrosative stresses, host adaptive immune responses, sub-lethal concentrations of antibiotics and competition from other organisms lead to selective pressures on Pa [50,63]. Despite this, Pa frequently manages to establish chronic infection.

Persistence adaptations to environmental pressures

The phenotype of chronic Pa is different to that of acute Pa, evolving under these selective pressures that lead to changes in gene transcription and in turn, virulence and persistence factor expression. Mucoide EPS alginate production is one such persistence factor, with secretion of EPS that provides a protective barrier to environmental stressors. [51]. Mucoide phenotype has been shown to develop in response to low oxygen environmental conditions [64].

Mucoide EPS alginate can also be a component of Pa biofilms, along with protein and eDNA and conversion from the planktonic state being triggered by increased levels of intracellular cyclic-di-GMP [65]. Pa biofilms allow the slowing of metabolic activity and doubling times and increased tolerance to antibiotics [66]. They trigger an intense antibody response and increased recruitment and activity of alveolar macrophages around the biofilm [67,68]. This inflammatory response leads

directly to alveolar damage and loss of total alveolar volume contributing to a person's lung function [69]. Macrophages are not found inside the biofilm; their inability to penetrate mucoid alginate EPS and the production of rhamnolipids by Pa within biofilms, confers resistance to phagocytosis [66,70]. Pa has been shown to produce more biofilm in more acidic pH environments [71].

When switching to a chronic state, Pa generally loses twitching and swimming motility, with loss of pili and flagella, making them less readily phagocytosed as phagocytic immune cell receptors recognise flagellar components and there is loss of flagellin mediated toll like receptor signalling [72]. QS is downregulated through mutational inactivation of the transcriptional regulator, *lasR*, leading to reduced expression of virulence toxins and secretion systems [73,74]. Pyoverdine production is reduced in low oxygen state and alternative mechanisms for iron acquisition are sought [75]. Finally, chronic Pa shows metabolic flexibility to utilise alternative nutritional sources such as amino acids and adapts to relative anaerobiosis in low oxygen environments [63]. In turn chronic Pa isolates confer reduced mortality in mouse models [76].

1.7. Conventional treatments and monitoring in CF lung disease

Until recently, treatments for CF were only able to target reversing the consequences of CF pathophysiology [2]. Airway hydration is targeted by the use of hypertonic saline nebulisers, to combat airway dehydration and draw water into the ASL from the epithelial surface. Mucous obstruction is targeted with use of recombinant human DNase (RhDNase) nebulised treatment that digests the eDNA component of CF mucus, making it less viscous. Mucociliary clearance is augmented by performing airway clearance physiotherapy to support mucus expectoration, and families are taught airway clearance techniques from the time of diagnosis. Supporting good lung health also comes from maintaining growth by managing the non-pulmonary consequences such as

pancreatic insufficiency with use of pancreatic enzyme replacement therapy (PERT) and diagnosing and treating CF-related diabetes.

Preventing airway infection in CF is challenging. Therefore, identifying airway infection promptly and attempting to eradicate known CF pathogens is also a mainstay of conventional treatment. CF Standards of Care recommend regular surveillance sampling of the lower airway at least four times per year, as discussed below [77]. Typical CF pathogens, like *Sa* and *Pa*, are treated by agreed protocol antibiotics in an attempt to eradicate infection. In *Pa* infection, use of oral and IV antibiotics in eradication is supported by the use of inhaled treatments as well, like tobramycin, colistin and aztreonam. When *Pa* infections persist, despite attempts to eradicate, chronic infection can be suppressed with long term use of inhaled antibiotics, to try to reduce persisting bacterial load. Additional antibiotics may be needed with episodes of PEx, and in severe PEx, admission to hospital and prolonged course of IV antibiotics for 10 to 14 days may be recommended [77].

In addition to airway microbiology surveillance, monitoring disease progression through other parameters over time is required. Lung function by measurement of ppFEV₁ is tracked at clinical reviews; an acute drop in ppFEV₁ may represent new infection and signify the need for more invasive microbiological investigation and consideration of treatment empirically with antibiotics if no organism is found. Chronic decline in ppFEV₁ is also important to recognise as some airway infections can present insidiously, or changes to treatment concordance need to be considered. In childhood however, advances in standard CF care over the past 20 years has meant that ppFEV₁ is often preserved well above 80%, and multiple breath washout (MBW) can provide calculation of lung clearance index (LCI) as more sensitive measure of lung ventilation inhomogeneity [78]. Both ppFEV₁ and LCI are common outcome measures in trials as well. Imaging of the chest is also used to monitor disease progression. Computed tomography (CT) imaging can provide information regarding structural lung disease like bronchial wall thickening and bronchiectasis, as well mucous obstruction and features specific to certain types of infection, including fungal and non-tuberculosis

mycobacterial (NTM) disease [79]. The utility of regular CT imaging has been debated and is not adopted in all CF centres or as part of Standards of care.

1.8. CFTR modulator therapies

Prior to 2020, the significant gains in median predicted survival in the UK are largely attributable to use of these outlined conventional therapies, in combination with comprehensive multi-disciplinary clinical care and employment of internationally agreed Standards of Care [77].

Whilst such treatments that target disease consequence have had significant impact on life expectancy and morbidity, therapies targeting the cause of CF remained elusive for a further 20 years despite the discovery of the *CFTR* gene in 1989. Utilising a high throughput screening programme of potential CFTR drug targets, small molecule therapies have been developed more recently which aim either to **potentiate** ion conductance by CFTR or to **correct** defective synthesis and transport more CFTR protein to the cell surface [80]. These therapies have been termed as CFTR modulators (CFTRm). Significant advances in the use and understanding of CFTRm in CF have occurred since this MD(Res) project was conceived, in September 2020, and then undertaken. The background of CFTRm is therefore framed in the context of relevant literature available at the time of starting. Research conducted in the wider field since the start of this project is presented in context of the results within the discussion of each results chapter (chapters 3-6) and in the summary discussion chapter (chapter 7).

In 2011, Ramsey *et al* reported the results of a placebo-controlled trial of Ivacaftor (IVA), a CFTR potentiator, which improves CFTR chloride conductance in people with the *G551D* variant (a class III gating variant) [81]. The results showed a significant improvement in ppFEV₁, sweat chloride concentration, frequency of PEx, weight gain and respiratory domain CF quality of life (CFQ-R) scores over the course of the 48-week study. Most unexpectedly, restoration of pancreatic exocrine

function, was observed in trials in those under 5 years [82–84]. The impact demonstrated in US registry data showed similar efficacy and safety to the trials, and went further to show benefits of a significantly lower risk of death (0.6% vs 1.6%, $p=0.0110$), transplantation (0.2% vs 1.1%, $p=0.0017$), hospitalisation (27.5% vs 43.1%, $p<0.0001$) and PEx (27.8% vs 43.3%, $p<0.0001$) compared to non-IVA-treated patients [85]. These trends were reflected in the UK registry patients too [86]. In both registries, there was lower prevalence of CF-related complications and higher mean ppFEV₁. However, isolated gating variants contribute to only 5% of those causing CF and so this CFTRm was eligible for a small minority of pwCF.

The commonest variant, *F508del*, constitutes approximately 70% of all *CFTR* variants. People who are homozygous *F508del* (*F/F*) or heterozygous *F508del* in combination with other minimal function defects (*F/MF*) are responsible for 85-90% of CF cases [1,3]. Targeting this variant has been a goal for research and led initially to the development of dual therapies that use a ‘corrector’ drug to improve delivery of CFTR to the cell surface, in combination with IVA as a potentiator. The first dual therapy was Lumacaftor (LUM)/IVA; this showed relatively modest improvement in ppFEV₁ of 2.4-4 percentage points and in *F/F* patients only [87]. However, despite this, LUM/IVA still demonstrated 30-32% reduction in PEx and improved Body Mass Index (BMI), and slowing of lung function decline compared to those not eligible. Tezacaftor (TEZ)/IVA was the second iteration of dual therapy to come from the drug development programmes, its efficacy in trials being very similar to LUM/IVA but appeared to have a more tolerable side effect profile [88].

The relatively modest apparent correction of *F508del*-CFTR by dual therapies led to the consideration of adding a second corrector molecule, to act at a different protein misfolding site, to enhance the effect. Four small molecule candidates were investigated in combination with TEZ/IVA in phase I and II trials in two groups of patients, *F/F* and *F/MF*. VX-445, VX-659, VX-440 and VX-152 all had adequate safety profiles, but VX-659 and VX-445 demonstrated ppFEV₁ increases of 13.3 and 13.8% respectively, leading to phase III double blind placebo controlled trials in pwCF >12 years, with

both combinations [89]. VX-659, in combination with TEZ/IVA showed improvements in ppFEV₁ of 13.3% in *F/MF*, confirming findings from phase I/II trials, and showed a 9.7% improvement in *F/F* pwCF who were already using TEZ/IVA [90].

However, results for VX-445, now called Elexacaftor (ELX), in combination with TEZ/IVA (ELX/TEZ/IVA, referred to as ETI from here on) were superior. In the *F/F* cohort, when compared to controls using TEZ/IVA, a mean increase of 10% ppFEV₁ was seen at week 4, sweat chloride reduced by -45.1mmol/L and CFQ-R scores improved [91]. A similar effect was seen in the *F/MF* group when compared to placebo: 13.8% increase in ppFEV₁ at week 4, and 14.3% increase at week 24 [92]. PEx rate reduced by 63%, sweat chloride reduction of -41.8mmol/L and improved CFQ-R score. The two cohorts tolerated the drug similarly and well. The impact was still apparent in trials in younger pwCF. A phase IIIb trial of efficacy and safety of ETI in *F/MF* heterozygous 6-11 year olds showed 9.5% increase in ppFEV₁ at 24 weeks, despite a higher baseline ppFEV₁. Lung clearance index (LCI) reduced by a mean of -2.29, and similar reduction of sweat Cl⁻, and improved CFQ-R were observed [93]. This means CFTRm therapy, defined principally as either IVA monotherapy or ETI depending on eligible genotype, is now available to the vast majority of people with CF.

1.9. The impact of CFTRm on the CF airway microenvironment and *Pa* infection

It remains unclear what impact CFTRm will yet have on the CF lung environment, particularly when started in those with established or advanced CF lung disease. Assuming CFTR functional restoration will lead to an altered environment that is better hydrated, with more neutral pH, the impact of these changes on how chronically infecting bacteria, such as *Pa*, will behave is of particular interest. It is tempting to consider that these changes will lead to improved mucociliary clearance, inflammatory cell function and antimicrobial host response, triggering changes to biofilm stability and reverting of chronic *Pa* phenotype to more motile and virulent forms.

At the time of designing this project, limited data from use of IVA, as the earliest available CFTRm in people with gating variants, was available to suggest potential impact on the CF airway environment. What has been seen is that IVA can restore mucus viscosity, mucociliary clearance, ASL and PCL layers to normal in a CF rat model [94]. Similarly, mouse models have shown reduced macrophage and lymphocyte infiltration in the airway when treated with IVA [95]. No data is published on the effect of IVA or other available CFTRm on airway pH or mucus plug hypoxia, but airway inflammation might be affected [96]. At a population level, sputum samples from before and after IVA initiation in those with at least one *G551D* variant showed decrease in sputum inflammatory markers (NE, IL-1 β and IL-8) within one week of initiation and continued to decrease overtime. However, another study showed no difference in any sputum inflammatory markers in a similar cohort, but those that continued to spontaneously expectorate sputum had persisting higher inflammatory markers compared those who did not [97].

Evidence of the impact on Pa from IVA use in those with *G551D* variants is also, so far, conflicting, with 2 small studies suggesting no change in airway infection [98,99]. One study in those chronically infected with Pa, however, suggested significantly decreased Pa sputum density over the first year of treatment, with a rebound in Pa density by the second year [96]. Where Pa density increased in the second year, strain persistence was seen, rather than new infecting Pa strains entering the airway. Another had shown that use of IVA in those who were Pa negative at baseline had reduced odds of new Pa acquisition, of both non-mucoid and mucoid phenotypes, and those previously intermittently growing Pa were more likely to become negative according to Leeds chronic infection criteria [100]. A further study suggested reduced isolation frequency and abundance of mucoid, but not non-mucoid Pa in clinical samples, and that antimicrobial susceptibility did not change, when tested across a broad range of anti-Pa antibiotics [101]. Younger patients may see greater shifts in bacterial community diversity in the airway than older [97].

Sputum culture and Pa detection using 16S RNA and metagenomic sequencing in patients starting LUM/IVA showed a similar, if not significant, reducing and then rebounding trend in Pa abundance over 12 months [102]. Use of LUM/IVA treated monocyte derived macrophages in the presence of Pa suggested reduced ability of Pa to form biofilms [103]. Untargeted sputum metabolomic analysis by the same group demonstrated a mirrored pattern of metabolic change between 3 and 9 months after LUM/IVA initiation, with return to baseline at 12 months, leading to the suggestion that the microbiome (and hence Pa) might be metabolically adapting to the new 'modulated' CF lung environment.

Post approval studies have demonstrated decreased prevalence of Pa sputum cultures, in one case Pa positivity reduced from 62% at baseline to 44% at 6 to 18 months following Ivacaftor initiation and this decreased further to 39% at 4.5 to 5.5 years [104]. Examining culture data from the US CF registry for 151 enrolled participants, odds of Pa culture positivity reduced by 35% within the first year of starting IVA [105]. There was also reduced odds of mucoid Pa, but no association between changes in Pa prevalence and changes in ppFEV₁, BMI or hospitalisation rate. However, in the same study, those taking IVA experienced reduced or absent sputum expectoration, which could have confounded this observation, if the ability to test sputum was less frequent [105]. This also highlighted how the use of CFTRm will further compound the ability of conventional airway surveillance methods to detect the presence of Pa.

1.10. Existing and evolving challenges of Pa detection

Conventional methods of Pa detection are reliant on culture of expectorated sputum provided at the time of PEx or through routine surveillance sampling during clinic visits. CF Standards of Care recommend a minimum of four surveillance samples of airway microbiology to be performed each year [77]. For those who cannot expectorate sputum, culture can be performed from throat swabs (thus assuming upper airway colonisation is a surrogate of lower airway microbiology) or cough

swabs (where the patient coughs on a cotton bud held in the oropharynx). Both can be more challenging in younger children [106]. However, sensitivity for detecting Pa as compared to sputum or BAL is highly variable (35-71%), making it likely that Pa airway infection is missed [107–110]. In children <18 months old, comparison to BAL suggested a sensitivity of just 44% for Pa [110]. Predictive value of Pa infection of positive cough swabs compared to BAL in patients who could not expectorate was 83%, but negative predictive value was lower at 70% [109]. This suggests that a single negative cough swab could miss Pa infection 30% of the time. Use of sampling methods and laboratory standards for culture methods is set out in the UK CF laboratory standards document [111].

The gold standard of airway culture is BAL, but this is resource intensive and carries specific procedural risk as a sedated procedure in adults or with general anaesthesia in children; it cannot be repeated with useful frequency and has not shown to be of benefit when used with regularity [106,112]. Sputum induction with ultrasonic nebulisation of hypertonic saline is possible and well utilised. Evidence suggests that sputum induction can be as sensitive in detecting lower airway infection as standard 3-lobe lavage protocols and bettered only by 6-lobe lavage [113]. Currently, however, it remains resource intensive and may be poorly tolerated or cause bronchoconstriction. It is not a tool that can be employed with regularity in the clinic setting.

Confirmation of presence of Pa in BAL, sputum and cough swab samples rely on standard culture techniques in an accredited CF microbiology laboratory. This occurs according to protocol, systematically using different solid media agar plates that support the growth of different bacteria, leading to identification based on differential growth. Culture based methods with agar can take up to 48 hours to rule out presence of bacteria in an airway sample. Antimicrobial susceptibility testing (AST) can take additional time beyond this.

Pa antibody serology is an attractive non-sputum based method of detecting Pa but requires the unproven assumption that antibodies are produced in early acute infection and become

undetectable once Pa is eradicated [104–106]. Examining 1791 serum samples from 375 pwCF with confirmed respiratory infection status, sensitivity for a combination of 3 antibodies used together only reached 43% for new onset Pa infection. [114]. Although titres did decrease when Pa was successfully treated, providing one option of monitoring response to therapies, another study found that age specific titre cutoffs could improve specificity and sensitivity of testing too [115]. Evidence reports a wide range of positive and negative predictive values, meaning Pa serology is not yet adopted into routine care at all CF centres, including Royal Brompton Hospital (RBH) [114–116].

These challenges mean prompt and reliable detection of Pa is dependent on spontaneous sputum expectoration, but this is often a problem in people with early-medium stage disease. Whilst historically a limitation in paediatric practice, it is now an issue for people reaching adulthood with well-preserved lung function. To further compound matters, the covid-19 pandemic introduced a rapid shift to CF clinical care with the majority of clinic contacts occurring remotely over video and systems set up to allow patients to post cough swab and sputum samples from home. Once these mechanisms were introduced to clinical service, it was expected that an ever-healthier CF population may continue to have a high proportion of care delivered remotely [117]. Development of reliable, rapid, non-sputum based and remotely collectable methods of detecting infection in an increasingly large group of patients will, therefore, be essential.

1.11. Alternative Pa detection methods: Polymerase Chain Reaction and Mass Spectrometry

Alternatives to culture based methods of Pa detection have been explored. Mass spectrometry (MS) methods like matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) and DNA based methods like polymerase chain reaction (PCR) have both shown high specificity for identifying Pa and other CF pathogens, and in much less time than standard culture [118–123]. Sensitivity of each method however remains unclear and testing to date has typically relied on expectorated sputum or cultured sputum as a substrate.

MALDI-TOF MS allows rapid identification of bacteria from a single cultured bacterial colony or directly from relevant sample materials, such as blood, cerebrospinal fluid or urine [124]. MALDI-TOF combines a whole cell bacterial sample with a Cyano-4-hydroxycinnamic acid (HCCA) matrix [123]. Ultraviolet laser then ionises the matrix compound and this is passed by electric field through a TOF-MS analyser, a mass spectrogram is produced and compared to a database of standardised bacterial signatures. MALDI-TOF is already used in clinical practice in hospital microbiology labs for confirmation of bacterial identification and may have the added advantage of predicting AST profiles [123]. Use is recommended, where identification by standard culture techniques is unclear, by CF microbiology laboratory standards [111]. Sensitivity is probably limited to 10^4 to 10^5 CFU as sizeable bacterial pellet or colony is required as the substrate [123].

PCR is a temperature dependent reaction that uses a polymerase enzyme (*Taq* polymerase) and specific primers to allow amplification of the target DNA, within a thermal cycler. Using validated *Pa* specific primers, *Pa* can be identified in adult CF sputum by PCR with 100% concordance to standard culture techniques and reduces the time taken to identify by over 24 hours [120]. In one setup a sensitivity of 50 CFU/ml for detecting *Pa* in cultured CF sputum has been reported but this required specific sample processing including DNA extraction and using quantitative PCR (qPCR) approach, [125]. These techniques are not often available outside of the research setting for detecting *Pa* in pwCF, and it is unclear if non-sputum based substrates could achieve the same results. The rise in rapid point-of-care testing in emergency departments for common seasonal viruses, like influenza and respiratory syncytial virus from a simple throat swab, could be considered an aspirational design for any future PCR test for *Pa* [126]. However, PCR is commonly used in the assessment of the CF lung microbiome in research. Interpreting the microbiome of CF airway samples, examining classically pathogenic organisms in the context of all airway bacteria, has also been proposed as a way to better understand clinically important infection [127,128]. Loss of microbiome diversity measured by PCR also correlates with lung function decline, perhaps offering another measure of CF disease progression [129].

Loop-mediated isothermal amplification (LAMP) is another DNA based method that uses an alternative polymerase enzyme capable of very large amplification of DNA, in general requires few processing steps and has been shown to detect Pa in clinical samples from a non-CF setting [16,17]. It has not been used to detect Pa in CF before.

1.12. Alternative Pa detection methods: Sniffing out the scent of Pa

Beyond protein or DNA based methods, it has long been recognised that Pa has a distinct scent that is recognisable to the human nose when growing in sufficient concentration. Dogs have a vastly superior sense of smell to humans and Medical Detection Dogs (MDDs; <https://www.medicaldetectiondogs.org.uk/>) have trained sniffer dogs to detect, with high mean sensitivity (94.2%) and specificity (98.5%) compared to controls, the scent of Pa in the filtered supernatant from clinical strains cultured in nutrient broth [130]. The dogs can discriminate between Pa and other common gram-negative CF organisms, such as *Achromobacter xylosoxidans* (Ax), *Burkholderia cepacia complex* (Bcc), *Stenotrophomonas maltophilia* (Smt) and *Serratia marcescens* (Sma), albeit with some reduction in specificity with Bcc (76.9%). Sensitivity to detect Pa dropped below 90% when bacterial concentration went below 10^5 CFU/ml.

Adapting the approach of detecting the scent of Pa, it has been shown possible to use mass spectrometric analysis of volatile organic compounds (VOCs) from the headspace above sputum samples from pwCF, to detect Pa from healthy controls [131] and discriminate between Pa and other common CF pathogens like Sa and Bcc [132]. Identification of VOCs from BAL samples have also been shown to identify Pa infection and exclude Sa infection, with a hope to using these VOCs to examine exhaled breath too [133]. One study has since shown that exhaled breath collected from CF patients can reliably detect Pa in grouped VOC samples, although was insufficiently sensitive to detect Pa on an individual basis [134]. By utilising Pa breath VOC signature, point of care 'electronic noses,' such

as SpiroNose, use signal recognition with electronic sensors that can discriminate between CF patients with and without Pa [135]. Currently, for both dogs and breath analysis by MS, conventional methods to confirm Pa positive status are still required.

These are a number of promising, non-culture based detection methods that require further evaluation to assess suitability for clinical practice. Whilst the specific need in the era of CFTRm is to detect Pa with substrate other than sputum, it's clear that most of these alternative methods have yet to be well evaluated in the absence of sputum production or with a specific focus on rapid Pa detection with minimum sample processing steps.

1.13. The Strategic Research Centre sample repository

The Strategic Research Centre for Personalised approach to *Pseudomonas aeruginosa* (SRC-PAPA), directed by the primary supervisor of this project, has built significant expertise in studying the virulence mechanisms of Pa, with specific focus on pwCF. At the time of project design, the SRC-PAPA repository had collected and stored over 6 years' worth of clinical Pa and other bacterial isolates from cultured sputum samples provided by CF patients attending the clinical service at RBH. These are stored for use in experiments/collaborations and were utilised for a number of experiments in this project. The group collectively has extensive skills to examine Pa infection and those relevant to this project include selective Pa culture, mass spectrometric and PCR detection of bacterial strains, as well as established and practiced standard operating procedures (SOPs) for virulence and persistence assays.

1.14. Hypotheses

The introduction of CFTRm for the majority of pwCF constitutes a massive shift in the way CF is treated. Understanding of the consequences of CFTRm on common CF complications, like infection with Pa, is in its infancy, and the impact on current practice for infection surveillance in CF clinical care is unclear. With this in mind, the following hypotheses were tested in this MD(Res) research project:

- Treatment with CFTRm will lead to a reduction in sputum Pa culture frequency, density and shifts in bacterial virulence/persistence behaviours.
- Alternative, non-invasive and operationally practical infection surveillance approaches can be developed to guide treatment for people on CFTRm who will be less likely to expectorate sputum.

1.15. Aims and Objectives

To evaluate these hypotheses, three aims, each with specific objectives were designed:

Aim 1

To determine the clinical impacts of CFTRm on lower airway Pa in a large patient cohort.

- Assess up to 5 years pre- and up to 2 year post-CFTRm initiation for isolation frequency and abundance of Pa positive airway culture from electronic patient records (EPR). This cohort included all RBH CF patients with chronic Pa infection receiving CFTRm through trials, managed access programmes or clinical service.

Aim 2

To determine the phenotypic response of Pa to CFTRm by virulence and persistence assay of both Pa samples collected from pwCF pre- and post-CFTRm initiation and Pa subjected to *in vitro* environments that mimic some of the *in vivo* airway changes expected following CFTRm initiation.

- Evaluate Pa behaviours using phenotypic assays that assess growth rate, virulence (pyoverdine production) and persistence (mucoidy), for patients in the same cohort from Aim 1, with relevant Pa isolates stored in the SRC-PAPA repository before and after their CFTRm initiation.
- Create *in vitro* broth based culture environments at three physiological pH levels (pH 6.5, 7.4 and 8.0) and two oxygen states (21% and 6% ambient) for serial passage of 11 clinical Pa isolates and one reference strain (Pa14).
- Perform phenotypic assays for these isolates to assess growth rate, virulence (pyoverdine production) and persistence (mucoidy), and antimicrobial susceptibility to tobramycin following exposure to the *in vitro* environments.

Aim 3

Utilise a novel, non-sputum based airway sampling method, to develop, optimise and compare alternative, non-culture based and low complexity Pa surveillance methods.

- Develop a laboratory model to investigate the suitability of 'huff tubes' for collection of airway samples on gauze.
- Assess the validity of rapid identification of Pa, cultured in the huff tube lab model, using MALDI-TOF (with Dr Gerald Larrouy-Maumus, Imperial College London), PCR and novel DNA based methods (LAMP), and sniffer dogs (with MDDs charity), compared to conventional culture.
- Develop a pilot study of 'huff tubes' to test suitability for Pa detection using some or all above detection methods.

Chapter 2 – General methods and methodology development

2.1. General methods

In this section, established methods of culture and microbiological laboratory techniques used throughout to address the aims of this project are outlined. Specific details of their application in each aim are discussed in the following results chapters in turn.

2.1.1. The SRC-PAPA repository and isolate storage

The SRC-PAPA was set up to investigate how *Pa* infection in pwCF behaves and find novel ways of detecting and treating it. To examine different clinical isolates of *Pa* from pwCF, a clinical isolate repository was established within the lab group, with collection beginning in 2012-2013. To date, >10,000 clinical isolates have been collected and stored within this repository.

Collection of isolates occurred from clinically provided samples and isolates were stored on plastic beads within Microbank microbial storage vials and stored at -80°C. Patients attending routine CF clinic providing surveillance airway samples, or during admission for inpatient treatment produced samples according to clinical need, and these would be processed according to CF standard microbiological culture methods in the RBH microbiology laboratory. Once organisms were identified, instead of being discarded, agar plates growing the identified bacteria were collected and transported to the SRC research laboratory.

Under a Class II microbiological safety hood, using a sterile inoculation loop, a single sweep of bacterial colonies was taken. This loop was submerged into the cryopreservative of the Microbank vial, the loop agitated within the beads, the bacterial mass detached from the loop and the loop then removed and disinfected using 1% Distel solution. The Microbank vial was capped and inverted 5 times and let to stand for 2 minutes, before re-opening and removing the cryopreservative (850µl) using sterile pipette. The Microbank vial was then closed, labelled with non-identifiable coding and

stored according to protocol, and log made of the storage position within the freezer. A full SOP document is available for full procedural steps (appendix 1). Code labelling links to patient identifiers were securely stored on clinical database within the hospital electronic file system. Access to clinical data relating to samples stored in the repository could be accessed through specific ethics processes, outlined below (section 2.3).

Initially sample collection only included isolates of mucoid and non-mucoid Pa but later expanded to include all common CF pathogens including, but not exclusively: *Staphylococcus aureus* (Sa), *Burkholderia cepacia complex* (Bcc), *Serratia marcescens* (Sma), *Achromobacter xylosoxidans* (Ax) and *Stenotrophomonas maltophilia* (Smt).

Samples were collected on a weekly basis from 2012 until the onset of the covid-19 pandemic in March 2020. At this point, due to research lab closures and unknown infection control risk, sample collection was suspended for approximately 12 months, when clinical laboratory infection risks regarding covid-19 transmission were better understood, and patients providing samples were screened for covid-19 symptoms prior to clinic attendance.

2.1.2. Broth and agar culture media

Methods used in this project utilised revival of bacterial isolates directly from Microbank beads on solid agar or within liquid media. A range of base media for broth and agar, determined by specific experiment requirements, were used and included Luria Bertani (LB), Ceftrimide, Muller-Hinton Broth 2 (MHB2) and Brain-Heart Infusion (BHI), all from Sigma Aldrich. Broth and agar were reconstituted according to the manufacturer's instructions, and recipes scaled to quantities required. Broth and agar base were prepared using deionised water (dH₂O) and autoclaved at 121°C for 15 minutes. Agar plates were poured within a Class II microbiological safety hood and left to set and cool before use. Aliquots of broth were also decanted using sterile pipette under Class II hood, to preserve sterility of test and control media.

2.1.3. Revival of stored isolates

Isolates stored on Microbank bead were revived either on agar or directly into broth. Required vials were removed from the freezer and kept cold on ice during use. Again, under Class II hood, and without allowing the vial to thaw, a single bead was removed using a 19G microlance needle. For agar revival, the bead was deposited onto the agar and allowed to thaw for 2 to 5 minutes. Using a sterile inoculation loop, the bead was dragged across the surface, to inoculate the agar with bacteria captured on the bead surface. Agar plates were then incubated at 37°C overnight (12-24 hours). For broth revival, the bead was dropped directly into 5-10ml aliquot of broth (volume dependent on specific experimental design) and vortexed to circulate the bead in the media. Inoculated tubes were then incubated at 37°C overnight (12-24 hours) on a shaking platform at 200rpm. A full SOP document is available for full procedural steps for revival of stored isolates (appendix 2). Single colonies of bacteria successfully revived on agar were also similarly inoculated into broth by taking a single colony with inoculation loop from the agar surface and immersing into an aliquot of broth. This was then vortexed and incubated as above.

Bacterial isolates revived on agar or in broth in this fashion were then available for use in experiments throughout this project. Stores of non-clinical Pa isolates, like reference strains Pa01 and Pa14, were also held on beads in Microbank vials, and revived in the same process as stored clinical isolates, when needed. Isolates were typically revived fresh for every experimental run.

Control samples of sterile broth and agar were incubated alongside test samples, to ensure sterility of culture media.

2.1.4. Growth measurement by serial dilution and colony counting

Colony counting was used throughout as an estimate of bacterial growth of revived isolates and is reported as outcome measure in a number of experiments. The established Miles and Misra method of colony counting was followed (full SOP, appendix 3). Using bacteria cultured in broth, following

required incubation period, the sample was spun in a centrifuge at 3214g, the supernatant discarded by pouring and replaced with 10ml (or equal volume if less than 10ml broth in initial culture) of sterile phosphate buffer solution (PBS). The sample was then vortexed to resuspend the bacterial pellet. A ten-fold (log10) dilution series was created by adding 1 x quantity of suspension to 9 x quantity of PBS; typically, 100µl of bacterial suspension was added to 900µl PBS, then vortexed and the pipette tip discarded into 1% Distel. Using a fresh pipette tip, this dilution continued in series to produce each concentration step from neat suspension to 10⁻⁸. For ease, a 6x6 grid square petri dish, with general LB agar was used for colony counting purposes. To provide three technical repeats, 3 x 20µl volumes of each dilution step were spotted onto agar, one spot in each grid space, and allowed to dry upright, before inversion and incubation overnight at 37°C. Control sample spots were also added to the agar to ensure sterility of the original broth.

The next day colonies were counted. High concentration of bacteria merged and formed indistinct numbers of colonies, that are 'too many to count' (TMTc). Very low concentration bacteria produced <2 visible colonies and were labelled 'too few to count' (TFTc). In between, at certain dilution factors, 2-20 colonies in each grid square were visible. These were counted, a mean taken across the three technical repeats, and number of colony forming units (CFU) per ml (CFU/ml) was calculated:

$$CFU/ml = \text{mean number of colonies for a dilution} \times 50 \times \text{dilution factor}$$

At times of high throughput sample processing during the experiments in this project, and in order to maximise efficiency, the first steps of bacterial pellet formation and resuspension in PBS was replaced with vortexing the broth sample to achieve even distribution of bacterial content in broth, and performing the dilution series with additional sterile broth, which differs from the original Miles and Misra protocol.

2.1.5. Growth measurement by Optical Density

Quantity of growth of bacteria in broth was, at times, estimated using measurement of optical density (OD) at 600nm wavelength (OD_{600}) in an optical plate reader. As bacterial quantity increases, the increased bacterial cellular mass make a suspension cloudier and in turn the OD_{600} increases. Although OD_{600} is determined by specific factors relating to the strain and isolate, experience within the laboratory group shows that typically an OD_{600} of close to 1.0 equates to bacterial concentration of 10^8 to 10^9 CFU/ml. This was confirmed in this study by pairing OD_{600} and colony count (CFU/ml) data from 10 different Pa isolates, over 30 separate repeats in various early experiments, and standardised to an OD_{600} of 1.0 (figure 2.1). Excluding obvious outliers, an OD_{600} of 1.0 gives a median concentration of 1.1×10^9 CFU/ml.

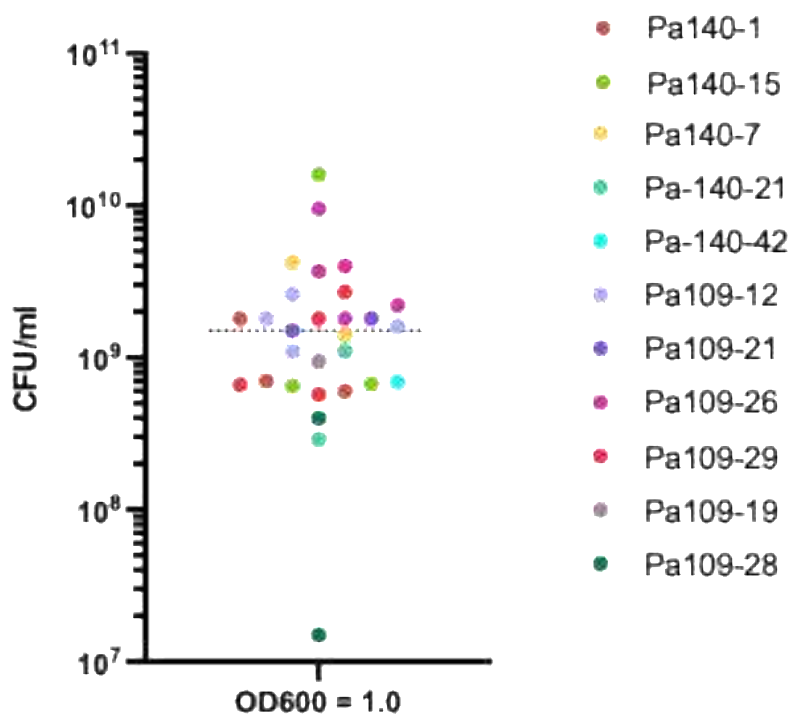


Figure 2.1: Comparison of OD_{600} and CFU/ml measurements of clinical Pa isolates

Paired results of OD_{600} and colony counts (CFU/ml), for 10 different clinical Pa isolates, over 30 separate repeats, were standardised to OD_{600} of 1.0. Obvious outliers were excluded manually and

median CFU/ml calculated, indicated with the horizontal bar above. An OD_{600} of 1.0 equates to 1.1×10^9 CFU/ml.

To measure OD_{600} , bacterial broth suspension following incubation was spun in centrifuge at 3214g to form bacterial pellet, supernatant discarded and replaced with equivalent volume of sterile PBS and vortexed to resuspend the bacterial pellet. 200µl aliquots of required samples were added to an optically clear 96-well plate in triplicate, providing three technical repeats, alongside PBS control (blank). OD_{600} was measured using the plate reader, and blank-adjusted values calculated by subtracting the OD_{600} for PBS control from all test sample OD_{600} values.

Again, at times of high throughput sample processing during the experiments in this project, and in order to maximise efficiency, the first steps of bacterial pellet formation and resuspension in PBS was replaced with vortexing the broth sample to achieve even distribution of bacterial content in broth, and OD_{600} measured directly in the broth media against sterile broth control (blank). Blank-adjusted values were then calculated by subtracting the OD_{600} for sterile broth control.

Sequential measurement of OD_{600} allowed measurement of change in OD overtime to produce a growth curve and illustrate comparative growth rate of different isolates or different growth conditions for example. To do this, 20µl of cultured broth samples, at desired concentration or condition, were added to 180µl of fresh sterile broth within in a 96-well plate. Number of technical repeats were practically limited by space within the 96-well plate for each run, and space for control broth sample (blank) needed to be included too. The plate was sealed with a breathable membrane and incubated in the plate reader at 37°C, with in-built shaking platform at 200rpm, for 24 hours.

Serial OD_{600} measurements were taken at the start ($t=0$) and then every 30 minutes for 24 hours, at the end of which, mean OD_{600} for each technical repeat of each test sample at each time point was calculated and blank-adjusted by subtracting the mean OD_{600} of control samples at that time point. Plots of OD_{600} over time produce a growth curve.

To compare growth curves at difference conditions, doubling times were calculated using GraphPad Prism 10 statistical analysis software. A doubling time represents the length time needed for any given bacterial population to double in size. For any desired growth curve, the time points of exponential phase of growth were determined using non-linear regression analysis (Malthusian model). The OD₆₀₀ values of the start and end of the exponential phase were then used as constraints in repeat non-linear regression, which calculated the doubling time of each growth curve analysed in that time period. Doubling times at difference conditions were then compared using appropriate statistical methods.

2.1.6. Measurement of Relative Fluorescent Intensity

Relative fluorescent intensity (RFI) is a measure of brightness of a fluorescent sample, standardised against a blank control or pre-determined fluorescent brightness standard. It can be measured in a plate reader and was required in a number of assays used in this project. To do this, 200µl test samples, with required technical repeats, were added to 96-well plate along with control/standardisation sample. In setting the plate reader to measure RFI, the control/standardisation samples needed to be identified as a point of reference to measure RFI. Assay specific excitation and emission wavelengths were used. RFI measurement was used as part of resazurin assay in methodology development of the huff tube lab model (section 2.2.2 below) and also as a surrogate measure for pyoverdine production of cultured Pa isolates when assessing Pa virulence (chapter 4).

When RFI of a cultured isolate in broth was measured, RFI was also standardised against bacterial abundance in each sample (for example the same samples CFU/ml calculated by colony counting). This was to compensate for cumulative fluorescent intensity as bacterial density increased.

2.1.7. Measurement of Minimum Inhibitory Concentration

Antimicrobial susceptibility testing (AST) was assessed by measurement of minimum inhibitory concentration (MIC) of antibiotics. This methodology is well established and outlined in peer-

reviewed publication [136]. MIC is the lowest concentration of applied antibiotic to inhibit growth of bacteria in broth culture and is considered the gold standard for AST of bacteria; a standard which other methods of AST are measured against.

In chapter 4, MIC to tobramycin is used as an outcome measure for assessing impact of different growth conditions on clinical Pa isolates. A suitable range of concentrations to test against each isolate was chosen from previous work within the group [137], where median MIC for a well characterised group of 11 clinical isolates from the SRC-PAPA repository, and Pa14, had already been determined. For these experiments, three antibiotic concentrations were to be assessed on each isolate: the previous median MIC and one concentration (2-fold) above and below (table 2.1).

Isolate	Median MIC (mg/L)	Minimum concentration to test (mg/L)	Maximum concentration to test (mg/L)
750	1	0.5	2
899	1	0.5	2
550	1	0.5	2
294	2	1	4
982	2	1	4
422	8	4	16
519	16	8	32
461	16	8	32
490	16	8	32
065	32	16	64
072	512	128	1024
Pa14	1	0.5	2

Table 2.1: Median MIC for examined isolates and expected testing ranges

Minimum inhibitory concentrations (MICs) for the 11 clinical isolates and Pa14 that have previously been examined in work within the lab group. Due to the number of samples being tested, in order to design a manageable workflow, one concentration (2-fold) above and below the previously determined median MIC were tested. A concentration of 1024mg/L was the expected maximum across all isolates that needed to be tested against.

In the protocol, the highest required concentration of tobramycin to be made is double that of the highest expected to be tested (1024mg/L), and so was determined as 2048mg/L.

Stock solution of antibiotic was prepared using the formula:

$$1000/P \times V \times C = W$$

Where:

P is the potency of antibiotic powder product given by manufacturer (µg/mg)

V is volume required (ml) of sterile water

C is final concentration of solution, in multiple of 1000 (mg/L)

W is the weight of antibiotic powder (mg) to be added to V

Potency for Sigma Aldrich Tobramycin stock is Lot specific, and sourced from the manufacturer's website, which in the case of stock used was 960µg/mg. From this the required weight (W) in 5ml (V) of sterilised water was calculated, to produce a stock antibiotic solution at concentration of 10,000mg/L. This was then stored in the fridge and further diluted for use when needed.

To produce the highest required concentration of 2048mg/L, dilution volumes were calculated using the formula:

$$C_1 \times V_1 = C_2 \times V_2$$

Where C_1 was concentration of stock solution (10,000mg/L) and C_2 is desired concentration (2048mg/L), and V_2 was required total volume for use in any experimental run, required volume of stock solution (V_1) to be added to diluent was calculated and prepared. This was filter sterilised prior to use, as per advised protocol [136]. A two-fold (log2) dilution series was produced, with further sterile water diluent, from starting concentration of 2048mg/L down to 1mg/L. This dilution series was at twice the desired final testing concentrations. MIC was assessed with MHB2 broth culture adjusted to a starting OD₆₀₀ of 0.05. Therefore, the OD₆₀₀ of the broth culture outputs from the workflows in chapter 4 were measured, and test samples adjusted to an OD₆₀₀ of 0.1, using additional sterile broth.

In the final step, antibiotic stock at twice desired testing concentration was combined with broth culture adjusted to OD₆₀₀ of 0.1 in 1.5ml Eppendorf tube in a ratio of 1:1 so that final antibiotic testing concentration and starting OD₆₀₀ of 0.05 were achieved. Tubes were vortexed and 200µl aliquots added to 96-plate, along with sterile MHB2 broth as control, sealed with a breathable membrane and incubated for 24 hours at 37°C, with agitation at 200rpm.

MIC was assessed by visually examining wells of the plate and looking for bacterial growth; turbidity indicating resistance and clarity indicating sensitivity to antibiotic at any given concentration. The MIC is defined as the lowest concentration tested to inhibit growth and leave the well clear after incubation.

2.2. Methodology development

Aims 2 and 3 included exploratory areas of research into changes in lung microenvironment and novel methods of detection of Pa respectively; with limited background research available, some methods required development before application. In this section, methodology development for some phenotypic assays and novel collection methods is detailed with discussion of reproducibility of technical and biological repeats.

2.2.1. Congo red and mucoidy scoring

Mucoid phenotype is a hallmark of chronic Pa infection, and its presence or absence was assessed in experiments examining the effect of CFTRm on Pa phenotype, with results presented in chapter 4 [51,52]. In clinical practice mucoid phenotype is usually reported subjectively by laboratories, by visually examining the agar plate for presence of apparent mucoid alginate slime around the Pa colonies. Congo red agar can provide a degree of objective assessment of the presence of mucoid alginate production by bacteria, as it contains a dye that turns dark red to black in the presence of EPS present in mucoid alginate, whereas non-mucoid colonies remain pink [138]. Established protocols for use of congo red were available and followed [139].

To prepare congo red agar, congo red stain (Sigma Aldrich) solution was prepared according to manufacturer's instructions to a concentration of 20mg/ml and filter sterilised. LB agar was prepared as previous and autoclaved. Before pouring the agar plates and when the agar solution was cooled to 55°C, congo red solution was added at a ratio of 1.5µl/ml of agar and gently mixed by inverting the capped agar flask. Plates were poured in the Class II hood and allowed to cool. To assess broth cultured Pa with the congo red agar, 3 x 15µl spots were dropped onto the agar, to provide three technical repeats, and allowed to dry for 15 minutes under class II hood. The plates were then incubated overnight at 37°C and read at 20-24 hours. Plates were viewed from above the agar surface and inverted and viewed from below over a light box, looking for presence of dark staining.

In order to try to grade degrees of mucoidy, a subjective scoring system based on the appearance of colonies (muroid: dark red and black vs. non-muroid: pink staining) from both sides of the plate, and also the appearance of the surface of colonies from the top (muroid: shiny, wet and expansive vs. non-muroid: dry and cracked), was developed (figure 2.2).

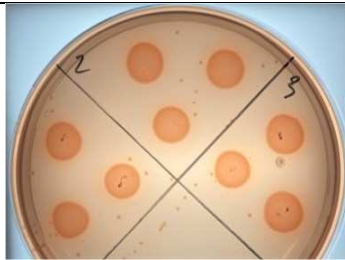
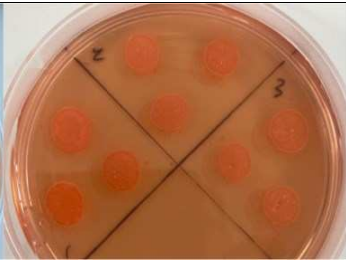
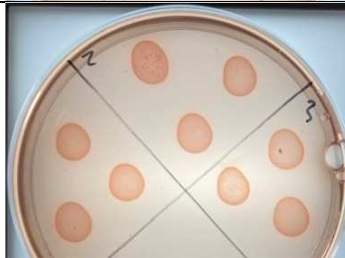
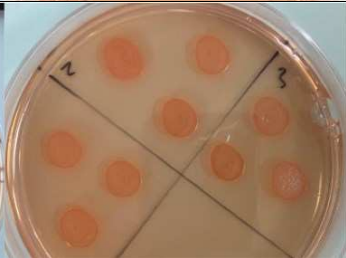
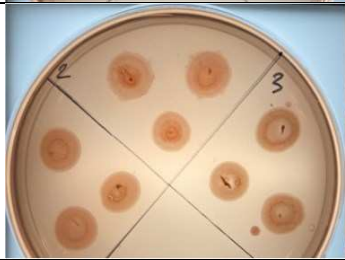
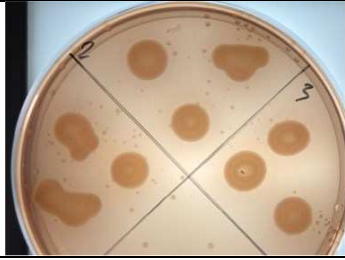
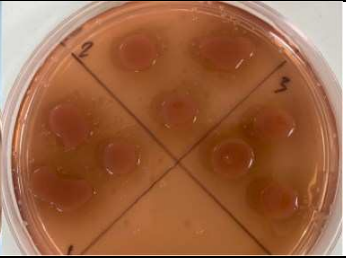
Score	Below:	Above:	Description
--			Bright pink colonies within the spot Dry/desiccated/cracked surface viewed from above
-			Pale pink colonies within the spot Mostly dry, but not desiccated/cracked, surface viewed from above
+			Areas of dark staining within the spot Shiny, wet surface around the edge of the spot, viewed from above
++			Dark staining throughout the spot and spreading beyond original spot boundary Shiny, wet surface all over the spot, loss of definition of circular spot shape, viewed from above

Figure 2.2: Subjective scoring system for mucoidy on congo red agar

A subjective scoring system was developed to grade degrees of mucoidy for Pa using congo red agar. Typically, the surface of muroid alginate production looks shiny and wet, and when large quantities are produced, can spread beyond the confines of the original spot place on agar. Congo red agar stains dark red to black in the presence of exopolysaccharides from muroid alginate. Combining the

staining and surface appearances allowed development of a 4 grade scoring system (- - / - / + / ++), as discussed in the description column of the table.

The system was trialled in Aim 2, initially when examining the phenotype of clinical isolates from pwCF stored in the SRC-PAPA repository, and comparing isolates taken before and after CFTRm initiation. Technical and biological reproducibility of the scoring system is presented within results chapter 4 showed good concordance between technical and biological repeats (table 4.5, chapter 4).

The system was then used in the second part of Aim 2, scoring mucoidy of Pa isolates that were subjected to different oxygen and pH conditions during incubation, mimicking expected changes to the CF microenvironment before and after CFTRm. Biological reproducibility is presented in results chapter 4 and again shows high concordance between biological repeats (figure 4.28, chapter 4).

2.2.2. Developing a lab model for huff tubes

Before designing a clinic pilot of huff tubes, a lab model was developed to:

1. demonstrate whether bacteria revived from the SRC-PAPA repository of clinical isolates, could be grown on sterile gauze in broth.
2. identify the minimum starting concentration of bacteria (CFU/ml) that could be inoculated onto gauze and produce detectable growth of bacteria following 24 hours incubation of the inoculated gauze in broth
3. identify the most appropriate growth conditions, including type of broth and duration of incubation, to achieve this.
4. determine whether OD₆₀₀ measurement alone was sufficient to detect and estimate the quantity of bacteria grown from inoculated gauze in broth after incubation

Figure 2.3 outlines the preliminary workflow, that would later be used to generate mock samples for a range of alternative detections methods (chapters 5 and 6). Pa isolates used in developing this

model came from the SRC-PAPA repository, as described in section 2.1.1. The development of this model is described as follows:

Preliminary workflow

Day1: 4-6 Pa isolates were selected from the repository at random and Microbank bead inoculated directly into 5ml LB broth, as outlined in section 2.1.3, and a second bead from the same vial streaked directly onto LB agar to look for growth a single bacterial species. Both plate and broth were incubated overnight. Successfully grown plate isolates were stored in new Microbank vials, as in section 2.1.1, to provide sub-repository stock for future experiments.

Day 2: Successfully grown broth specimens were identified by the presence of cloudiness within the broth. Beads were removed and samples centrifuged 3214g, resuspended in PBS and serial 10-fold (log 10) dilution, in sterile cetrimide broth, was performed from neat (0) to $\log^{-8}$ as outlined in section 2.1.4. OD_{600} was measured with the plate reader for every Pa strain from 0 (PBS suspension) and -1 to -8 (cetrimide), and blank-corrected, as outlined in section 2.1.5. This represented the **starting OD** for each Pa strain. 30 minute serial OD_{600} was then measured in the plate reader for 24 hours, as outlined in section 2.1.5, using samples from dilution steps (-1 to -8). Growth curves produced would help identify which starting concentrations were sufficient to initiate growth in cetrimide broth and at what time point after inoculation. In parallel, the dilution series was used for colony counting on LB agar, as outlined in section 2.1.4, to provide the **starting colony count**. Finally, separate 5cm x 5cm, 2 ply pieces of sterile gauze were inoculated with 1 x 20 μ l of each of the 0 to -8 starting dilutions for each isolate under the class II hood. Forceps sterilised in 1% Distel and rinsed in sterile dH₂O were used to place gauze into test tubes containing 5ml sterile cetrimide broth. These were incubated overnight at 37°C, on a platform shaker at 200rpm.

Day 3: the 24-hour growth curve was concluded, **starting colony counts** completed for each isolate and gauze tubes removed from the incubator. 200 μ l of each post-incubation gauze/broth mixture

was added to a 96-well plate to provide **post-incubation OD₆₀₀** measurement, in attempt to use OD₆₀₀ to estimate CFU/ml in post-incubation gauze samples. 3 x 20µl of each post-incubation gauze/broth mixture were spotted on to LB agar (**post-incubation spots**) to provide a binary test as to whether each starting concentration inoculated onto gauze was sufficient to achieve growth after 24 hours incubation on agar, and these were read on **day 4**.

At the end of the four-day workflow, the following information is available for isolates tested:

1. **Starting colony counts** (CFU/ml) and corresponding **starting OD₆₀₀** of each isolate culture (and thus calculate the starting CFU/ml of each starting dilution by dividing by factors of 10).
2. **Post-incubation spots** indicate the threshold of the minimum starting concentration achieving growth on gauze.
3. **Post-incubation OD₆₀₀** measurement for each starting dilution to determine if CFU/ml following incubation of gauze sample can be estimated from OD₆₀₀ measurement alone.
4. 24-hour growth curves, providing secondary evidence of Pa growth potential at different starting concentrations.



Figure 2.3: Preliminary workflow of huff-tube lab model development

Outline of preliminary workflow for huff tube lab model. Four-day process, with multiple steps, allowing stored clinical bacterial isolates to be cultured overnight on appropriate agar (A), then single colonies selected and cultured overnight in broth media. Samples are centrifuged and re-suspended in sterile Phosphate Buffer Solution (PBS), and a serial dilution performed. Starting optical density (OD_{600}) is measured, as a method of estimating starting concentration in CFU/ml, for each starting dilution (B). These starting dilutions are used to create colony counting plates, to confirm starting CFU/ml, create a serial dilution in cetrimide broth for overnight growth curves, and inoculated onto gauze patches that were incubated in cetrimide broth (C) overnight. Post-incubation broth was also spotted onto agar (D) to determine minimum starting concentration threshold for growth on gauze (E). LB = Luria broth (a general-purpose media), Cetrimide = Pa selective media, RPM = revolutions per minute

Repetition of this early workflow identified pitfalls and preliminary results to modify proposed methodology; a number of observations and modifications were, in turn, considered.

Observations and modifications

OD₆₀₀ is insufficiently sensitive to detect growth of bacteria in incubated gauze-broth (figure 2.4).

When examining the agar plates of **post-incubation spots**, it was noted that growth was detected at lower starting concentrations inoculated onto gauze than could be identified by **post-incubation OD₆₀₀** measurement ($OD_{600} < 0.1$). OD cannot be used to accurately estimate the CFU/ml of post-incubation gauze samples at lower concentration inoculating doses. Performing colony counting on the lowest starting concentration that is most likely to achieve growth on gauze was, therefore, necessary, provided the lowest starting concentration was identifiable.

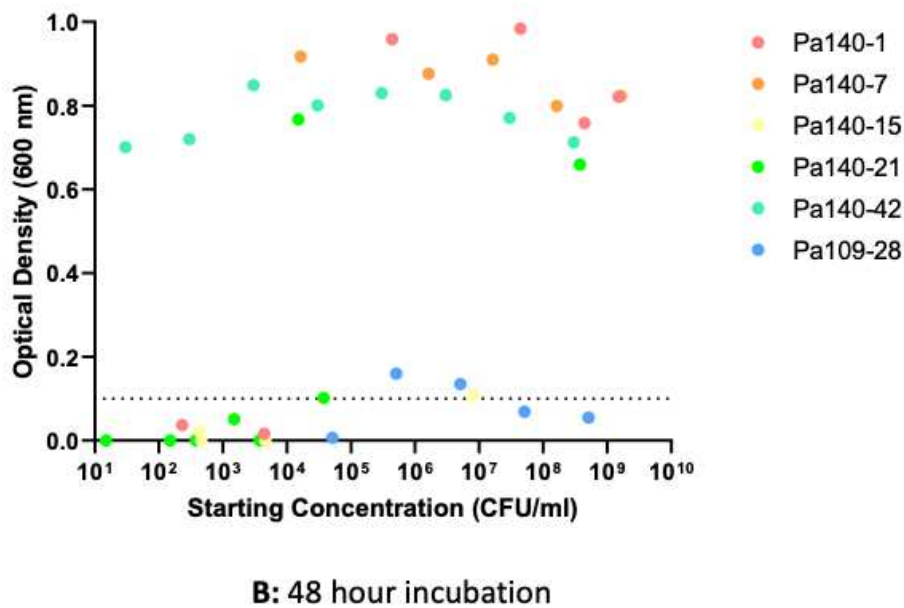
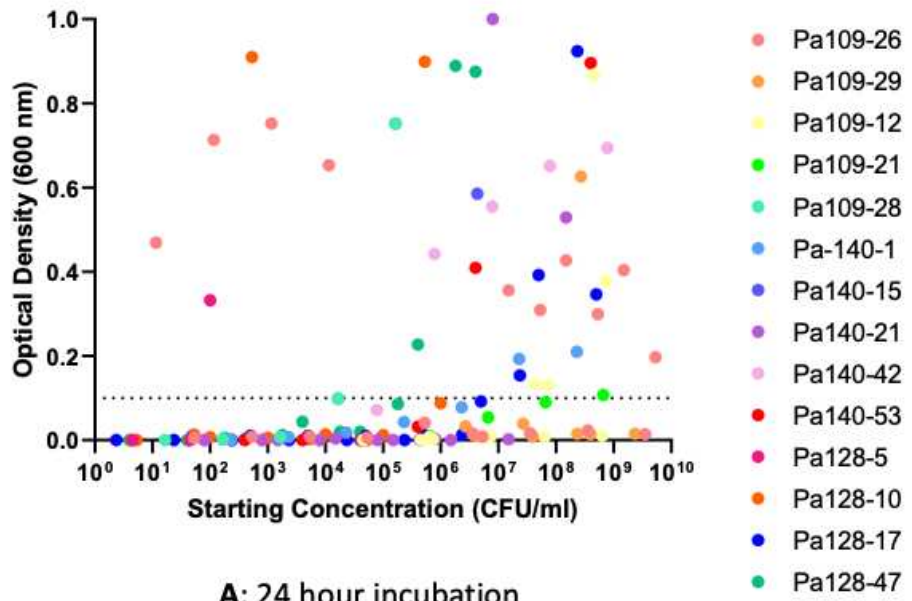


Figure 2.4: Starting concentration of inoculum onto gauze and post-incubation OD

A: shows all starting concentrations inoculated onto gauze, and incubated, that then achieved growth on agar (positive post-incubation spots) at 24 hours incubation; with the dotted line indicating $OD_{600}=0.1$, below which estimation of CFU/ml is not reliable. **B:** shows the same for strains cultured on gauze for up to 48 hours. In both incubation periods, isolates can produce growth on agar that would not be deemed detectable by OD_{600} measurement alone ($OD_{600}<0.1$).

Using resazurin (a viability assay) was also insufficiently sensitive to detect growth from post-incubation gauze samples: With post-incubation OD₆₀₀ measurement proving insufficiently sensitive, a resazurin viability assay (appendix 4) was trialled to determine the viability threshold for each strain by starting CFU/ml. Resazurin is actively metabolised by live bacteria, changing the chemical colour from purple to pink (figure 2.5).



Figure 2.5: Example of resazurin assay colour changes

A resazurin assay is a viability assay, and demonstrates colour change from purple to pink when metabolised by living bacteria. In this example, strains 1 to 4 are shown in concentration (CFU/ml) dilution series from neat (left) to more dilute (right). The threshold between pink and purple for each strain demonstrates whether live bacteria are detected at lower dilution steps by assay. Controls are appropriately purple.

Post-incubation gauze samples were added in 200µl aliquots to 96-well plate from each starting concentration, and the resazurin assay applied as per protocol (appendix 4). The minimum starting concentration to produce 'viable' growth on gauze was indicated by the threshold between pink and purple colour change. Pink fluorescence can be measured as RFI using a plate reader, setting excitation at 544nm and emission at 590nm (544/590). When **post-incubation spots** on day 4 (indicating growth of bacteria on agar) were compared to resazurin assay threshold, it showed poor

sensitivity with the assay missing growth of bacteria at starting concentrations of $<10^3$ CFU/ml (figure 2.6). Sensitivity was better at 48hrs incubation, but this could be because longer incubation has allowed greater endpoint CFU/ml.

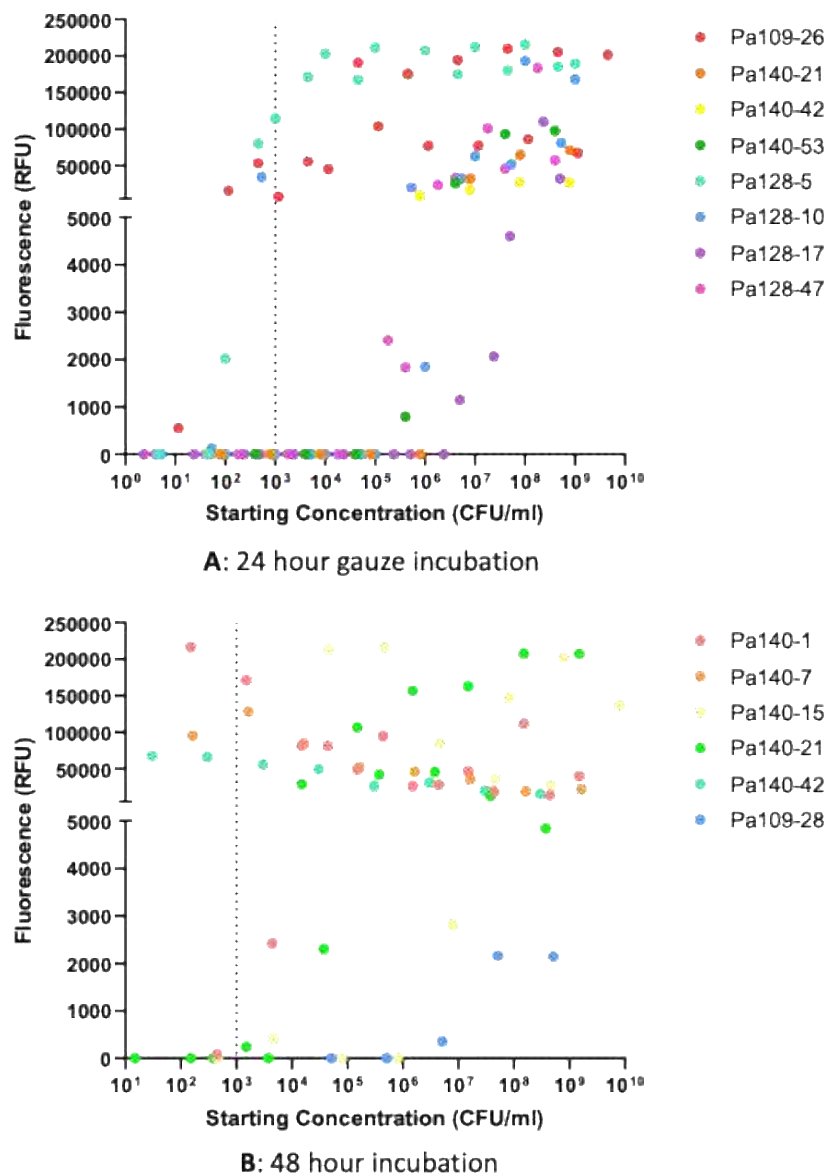


Figure 2.6: Resazurin viability assay to detect the minimum starting concentration to produce viable growth on gauze

A: Plots of post-incubation gauze samples at 24hrs with positive post-incubation spots at day 4 at their relative starting concentrations and the fluorescence (Relative fluorescent units, RFU) measured with the assay, indicating insufficient sensitivity of the assay at lower starting concentrations ($<10^3$, dotted line) at 24 hour incubation. **B:** Shows the same plot for isolates incubated for 48hrs.

Incubating gauze for 48 hours produces detectable growth from lower starting concentrations but was deemed impractical for the purpose of this project and growth at 24 hours deemed sufficient (Figure 2.7): Examining growth curves of Pa isolates, some growth take-off occurs in the 20-24 hour period for lower starting dilutions, suggesting gauze incubation for 24-48 hours would yield better post-incubation growth of gauze samples. This was trialled for several runs of the protocol and did yield growth from both lower starting concentrations and to higher end-point concentrations. However, following discussion, **it was felt that 48-hour incubation went against the aim of the study to produce rapid detection tests**. Ideally, minimal or no incubation time for novel sample collection methods would be preferred, but it was recognised that some amplification of sample quantity was likely to be required to improve sensitivity in early stages. A compromise of aiming for up to 24-hour incubation was agreed for initial experiments.

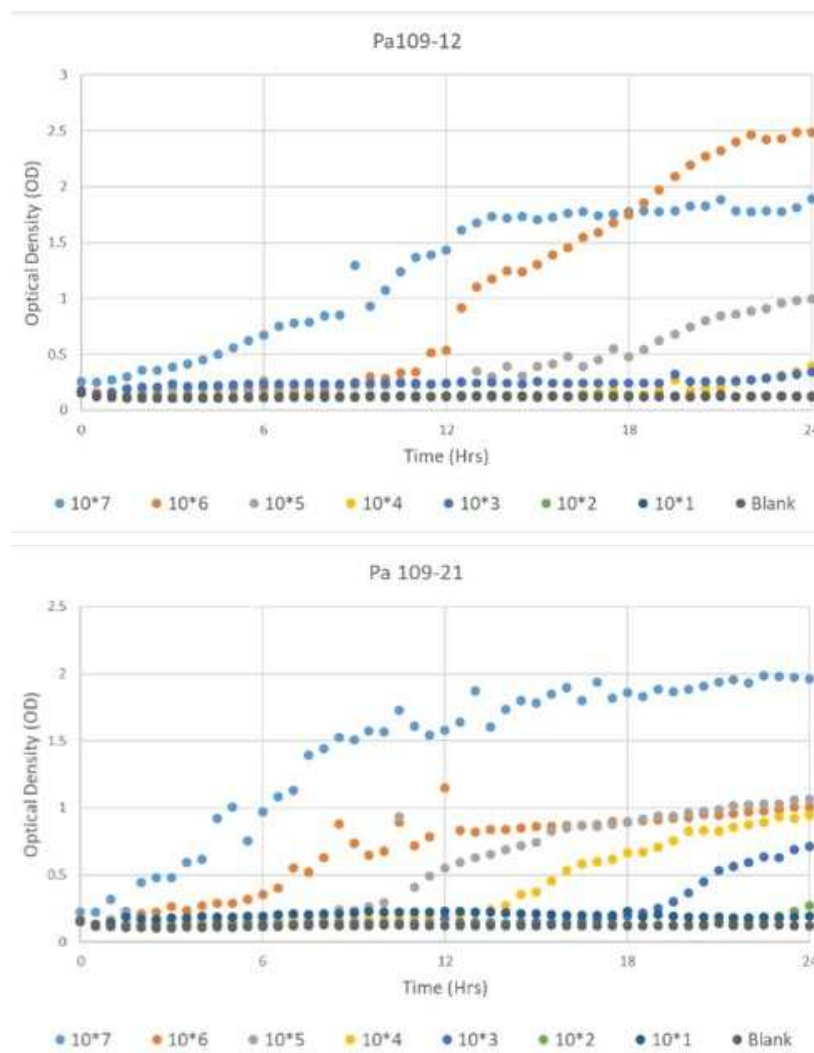


Figure 2.7: Growth curves demonstrating late growth of Pa isolates at very low starting concentrations

Examples of two Pa isolates (Pa109-12 and Pa109-21) that demonstrate growth from lower starting concentrations in the 20-24hr period. Key at the base of each shows the starting concentration of bacteria (CFU/ml).

Growth from gauze can be optimised at under 24 hours of incubation: Anecdotally, using 60ml wide-based pots with 10ml cetrimide, instead of narrow test tubes and 5ml cetrimide, allowed movement of gauze during incubation and increased liquid/air interface for aerobic respiration. The pots were also standard size for presentation to the sniffer dogs in the MDDs testing rigs (chapter 6) and were in turn utilised for all huff tube lab model experiments.

OD₆₀₀ was still useful to estimate and prepare starting inoculating concentrations: OD₆₀₀ and resazurin assays are not sufficiently sensitive to measure potentially low concentration output of this huff tube lab model, and instead colony counting was required to accurately measure post-incubation growth of gauze samples. Targeting starting concentrations of $\sim 10^2$ CFU/ml is clinically useful as this is a very low concentration; any new test that can detect presence of bacteria provided in a clinical sample at this concentration would prove to be highly sensitive. Experience within the lab group that was confirmed above in analysis in section 2.15, an OD₆₀₀ of 1.0 gives a median of 1.1×10^9 CFU/ml (figure 2.1). Therefore, to minimise steps in preparing starting inoculum for the gauze at low concentrations, measurement of starting OD₆₀₀ before performing dilution series could identify which dilution step contained 10^2 CFU/ml. For example, if the starting OD₆₀₀ was around 1.0, it contained approximately 1×10^9 CFU/ml, then the 7th 10-fold (log₁₀) dilution step would contain approximately 10^2 CFU/ml, and application and colony counting of all starting inoculum could be avoided. So, whilst OD₆₀₀ was not a useful outcome measure in this model, it was used to reduce the

range of starting inoculum concentrations applied to gauze, and the confirmatory steps needed to confirm their concentrations.

Cetrimide, a Pa selective broth, was considered beneficial over a generic broth for incubation of inoculated gauze: As outlined above, Pa detection was the priority of the SRC-PAPA over some other common CF related organisms. Whilst Pa has been shown to grow more readily in non-selective broths, such as LB, there was concern that potential competitive inhibition by non-Pa strains could drown out Pa signal. Cetrimide has shown good selectivity for Pa, although Bcc and some other gram-negative organism may grow [140]. It was therefore chosen as the media for incubation of gauze inoculated samples at the start of this project.

Taking into account these observations, the workflow was amended as outlined in figure 2.8. A full SOP was produced, detailing all procedural steps (appendix 5).

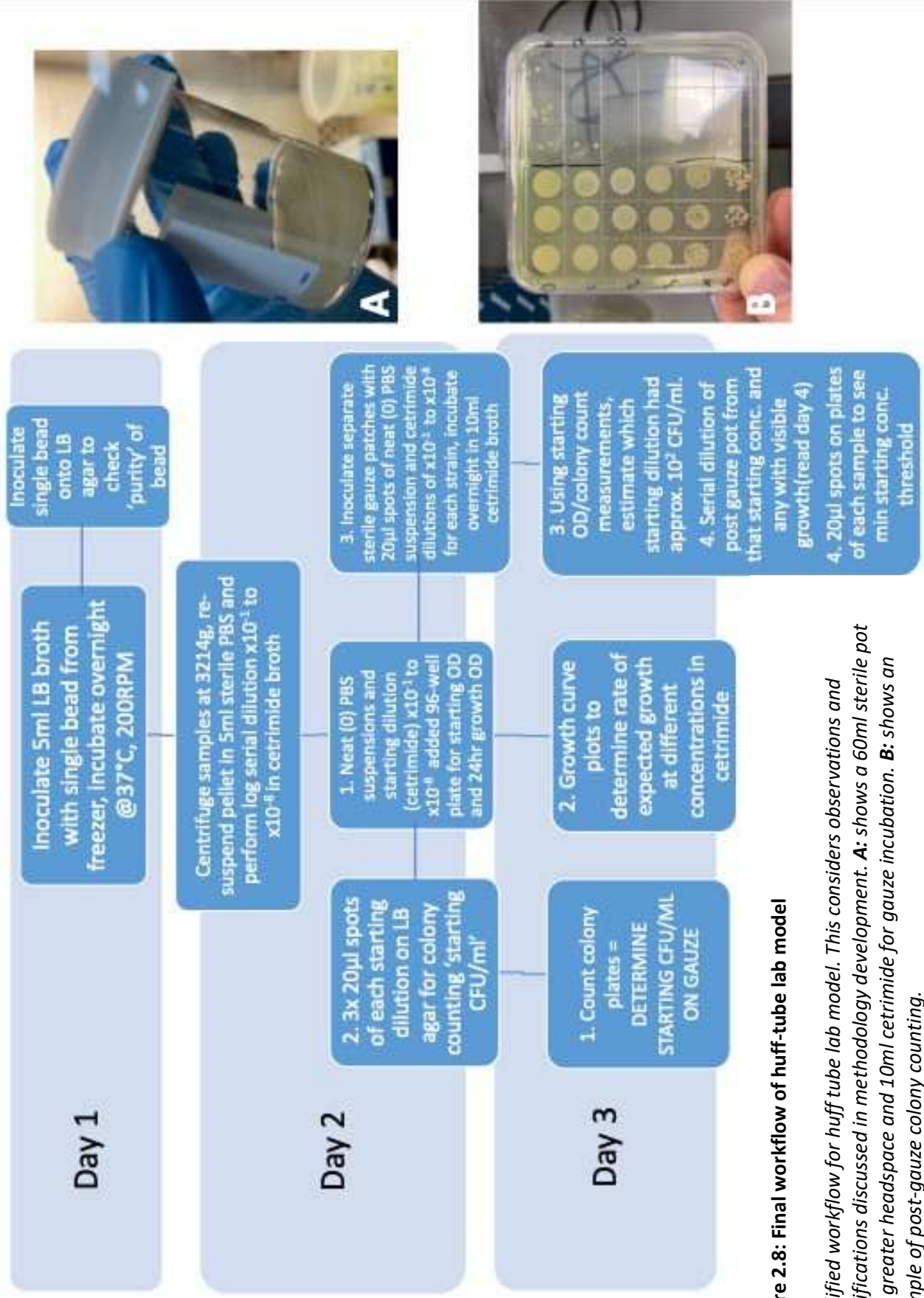


Figure 2.8: Final workflow of huff-tube lab model

Modified workflow for huff tube lab model. This considers observations and modifications discussed in methodology development. **A:** shows a 60ml sterile pot with greater headspace and 10ml cetrinide for gauze incubation. **B:** shows an example of post-gauze colony counting.

Sensitivity of the huff tube lab model

15 Pa strains were evaluated, each with three technical repeats. For those samples incubated on gauze for <24 hours, 7 of 8 Pa isolates (88%) demonstrated $>10^5$ CFU/ml growth (previous threshold for correct identification of Pa by sniffer dogs [57]) from starting inoculating concentrations onto gauze of $<10^2$ - 10^3 CFU/ml (figure 2.9).

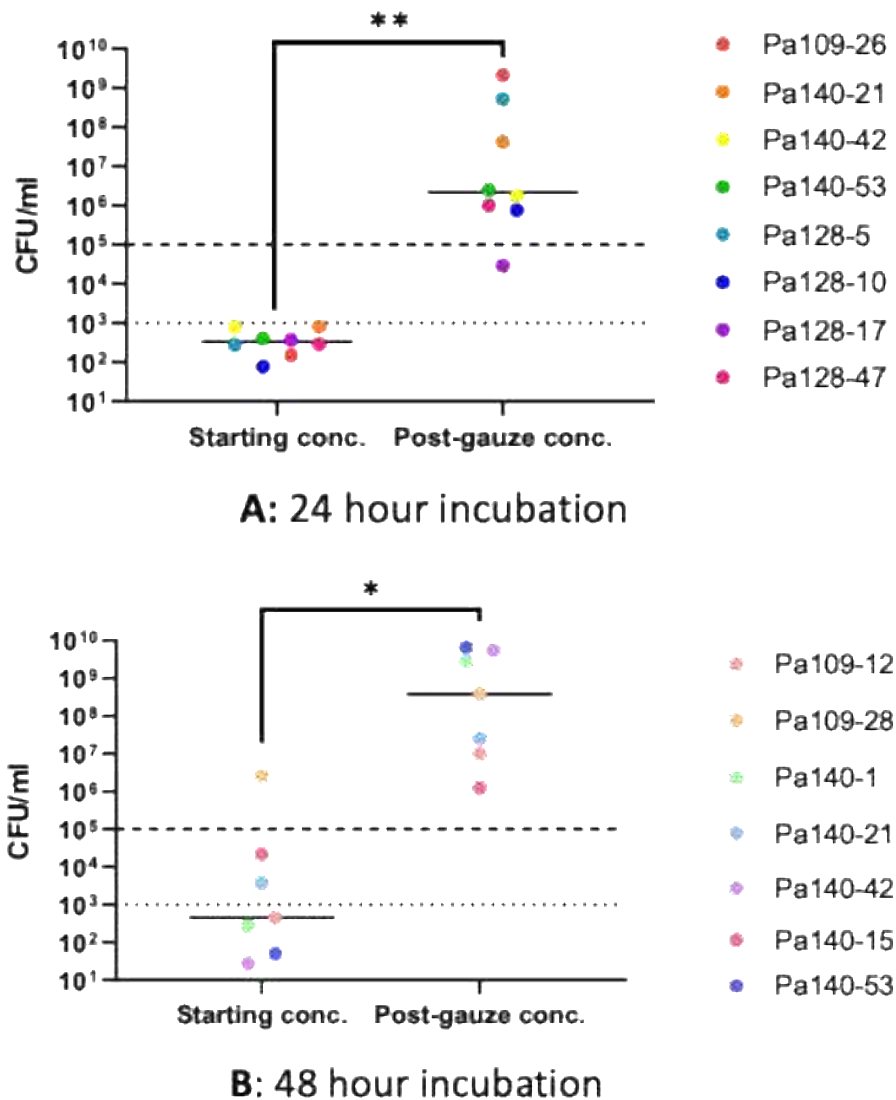


Figure 2.9: Sensitivity of the huff tube lab model

Pa strains examined through the huff tube lab model, with different isolates indicated by colour according to the legend. Latter runs were for up to 24 hours (A), where earlier runs were for 24-48hrs (B) as discussed above. Dotted lines indicate the ideal 'starting threshold' concentration of $<10^2$ - 10^3

CFU/ml, where the dashed line indicates the target endpoint CFU/ml of $>10^5$ CFU/ml, the previously identified threshold of sensitivity for sniffer dogs. Comparisons by non-parametric t-test (Wilcoxon), with ** is $p<0.01$ and * is $p<0.05$.

Specificity of the huff tube lab model

To test the selectivity of cetrимide, 13 strains across seven non-Pa species were tested in a similar way. Growth curves suggests that one of three Bcc strains tested were able to grow in cetrимide (figure 2.10), as expected from the literature [140]. Unexpectedly, one of three Sa strains tested also appeared to show growth in cetrимide (figure 2.11)

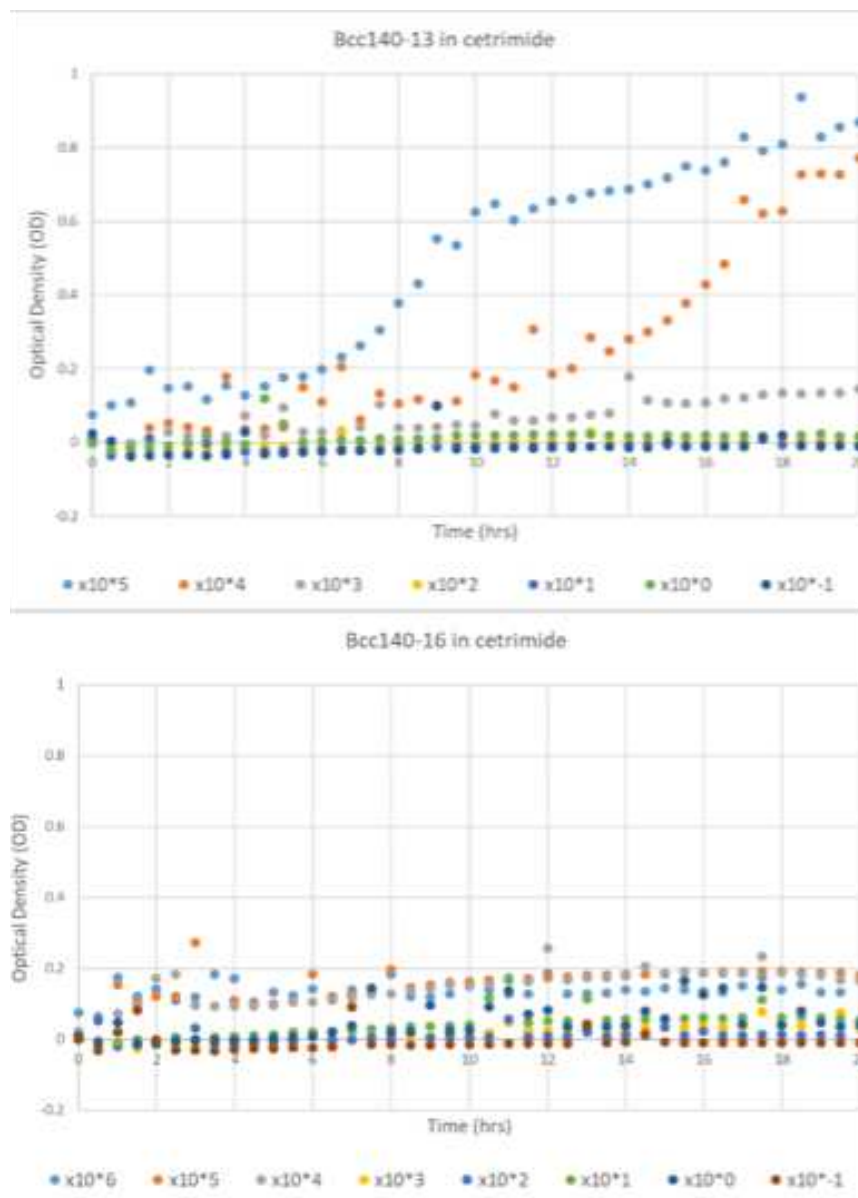


Figure 2.10: Growth curves of Bcc isolates in Pa selective broth

Examples of two different Bcc strains, Bcc140-13 showing growth in cetrимide over 24 hours at a range of starting concentrations, compared to Bcc140-16, which does not.

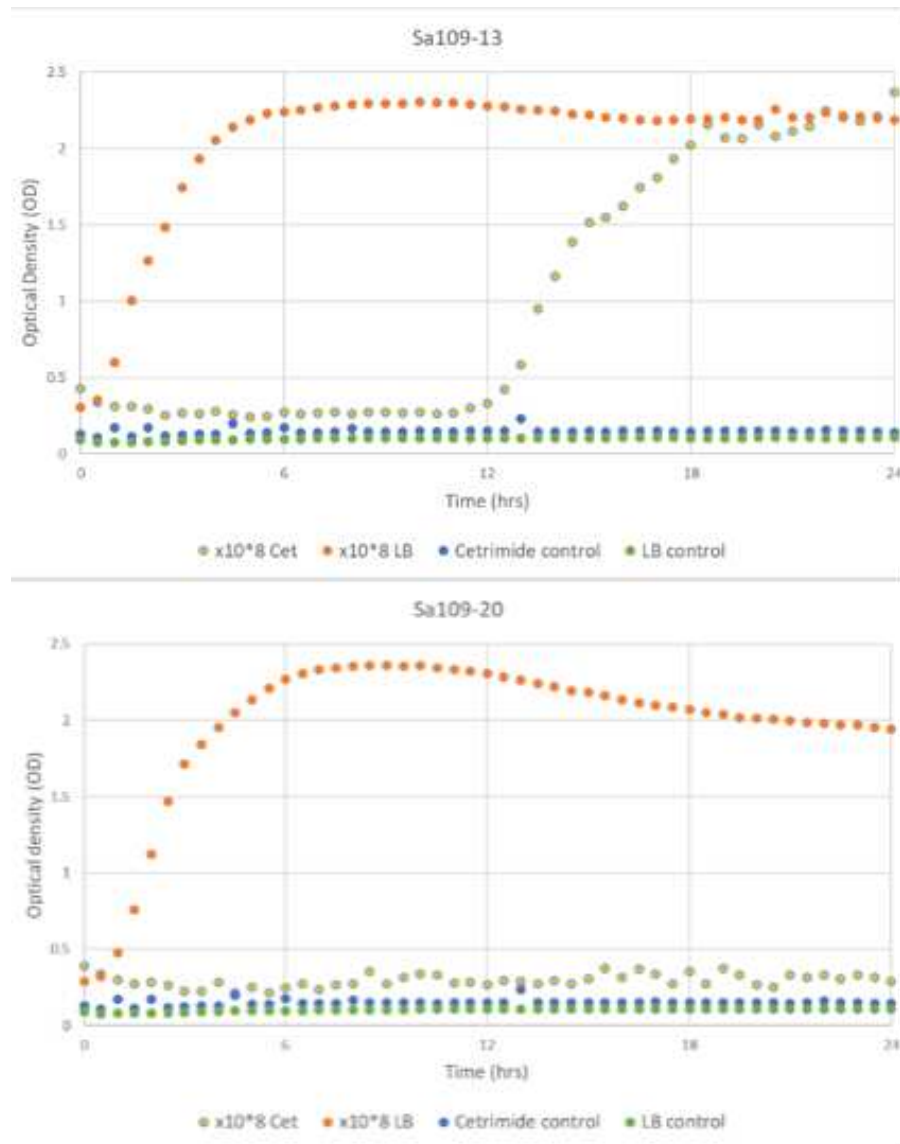


Figure 2.11: Unexpected growth of a single Sa isolate in Pa selective broth

Examples of two different Sa strains, grown in LB and cetrимide over 24hrs. Sa109-13 shows growth in cetrимide after 12 hours, as indicated by rising OD₆₀₀, compared to Sa109-20 that does not.

Subsequently, high (10^8 - 10^9), medium (10^5) and low (10^2) starting CFU/ml (estimated by OD₆₀₀) of non-Pa isolates were inoculated on gauze and incubated for 24 hours (table 2.2). Initial non-Pa

isolates included Bcc, Sa, and Smt, and this was extended to include other Gram-negative rods. The majority of non-Pa strains did not grow on gauze cultured in cetrimide. However, adding to preliminary growth curve data (figure 2.10), two of three Bcc isolates demonstrated some survival on gauze in cetrimide, as does Ax. The same single strain of Sa that again demonstrated survival on gauze, but only once in three repeats, and only from a high starting concentration (10^8). Whilst cetrimide was chosen to discourage growth of competing organisms, knowing about the presence of Bcc or Ax is not clinically irrelevant.

Isolate	Biological repeat	Survival in cetrimide (Y/N)	Lowest inoculating concentration to achieve growth (CFU/ml)
Bcc109-5	1	N	
	2	N	
Bcc140-13	1	Y	10^2
	2	Y	10^5
Bcc140-16	1	N	
	2	Y	10^8
Sa109-13	1	N	
	2	Y	10^8
	3	N	
Sa140-34	1	N	
	2	N	
Sa140-20	1	N	
Smt109-9	1	N	
	2	N	
Smt109-33	1	N	
	2	N	
Kp132-51	1	N	
Ax132-67	1	Y	10^5
Ax136-38	1	Y	10^8
Ecc138-73	1	N	
Eco140-6	1	N	

Table 2.2: Specificity - summary of non-Pa strain survival in the huff tube lab model

Table showing the ability of 13 different non-Pa isolates to grow on gauze in cetrimide, including biological repeats where applicable. Where growth on gauze in cetrimide has occurred, lowest inoculating concentration onto gauze to achieve growth (CFU/ml) is reported. Y=Yes, N= No, Bcc = *Burkholderia cepacia* complex, Sa = *Staphylococcus aureus*, Smt = *Stenotrophomonas maltophilia*, Kp

= *Klebsiella pneumonia*, Ax = *Achromobacter xylosoxidans*, Ecc = *Enterobacter cloacae*, Eco = *Escherichia coli*.

Application of the huff tube lab model for alternative detection methods

The huff tube lab model was used to produce samples for chosen methods of rapid detection of Pa, including PCR, LAMP, MALDI-TOF-MS and with sniffer dogs from MDDs (figure 2.12). Application and specific methods for each alternative test are discussed within results chapters 5 and 6. **Alternative detection methods were developed and tested only with samples produced in the huff tube model and so success or failure of each method examined is considered in this context.**

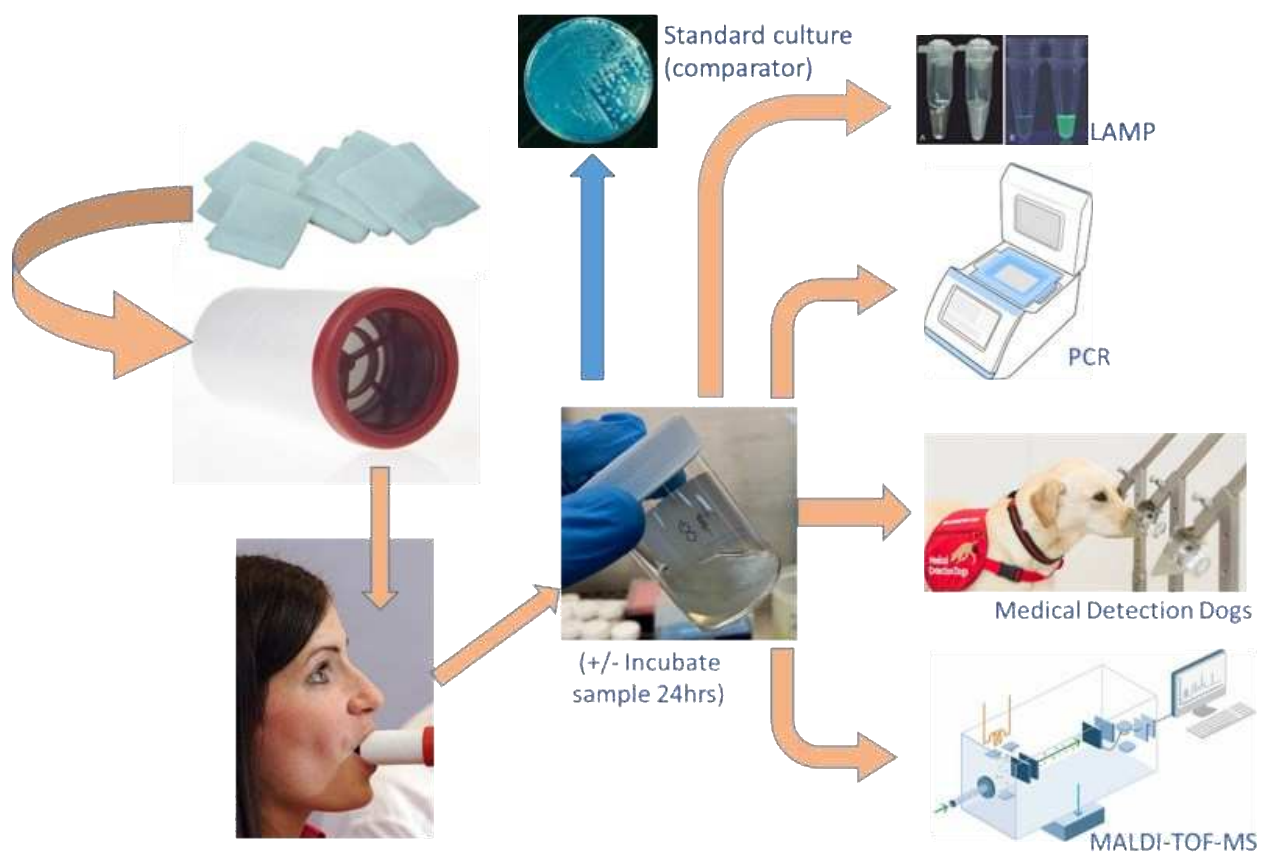


Figure 2.12: The huff tube lab model used with proposed alternative Pa detection methods

A huff tube is illustrated with sterile gauze packed into a sterile mouthpiece. A patient performs a forced expiratory manoeuvre (huff) onto the gauze, which captures their moist breath. This is then

incubated briefly in Pa selective broth (cetrimide) before testing with alternative methods of Pa detections including polymerase chain reaction (PCR), Loop-mediated isothermal amplification (LAMP), Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and sniffer dogs from the Medical Detection Dogs charity and compared to culture as standard comparator.

2.2.3. Polymerase chain reaction

Validated PCR primers exist to detect Pa with high sensitivity and specificity [122]. PAL1 and PAL2 target the *oprL* gene, and sequences have been made available for public use:

PAL1: 5'-ATGGAAATGCTGAAATTCGGC-3'

PAL2: 5'-CTTCTTCAGCTCGA CGCGACG-3'

Stocks of PAL1 and PAL2 existed within the laboratory, purchased from Life Technologies.

As discussed, PCR is a well validated tool for detecting the presence of Pa in sputum and BAL from pwCF [120,122,125,141]. However, in the era of CFTRm it was hypothesised that these substrates are likely to be far less available. Also, in validated tests to date, DNA extraction and purification steps are usually used and such methods may not be widely available and affordable in all clinical microbiology laboratories. In order to design a widely practicable PCR test on substrates other than sputum, the scope of this project specifically looked at performing PCR **directly** on the broth produced following incubation of inoculated gauze from the huff tube lab model, outlined above.

The results of this development process formed part of iterative methodology development and so this is discussed as part of the results section of chapter 5, however the principals of PCR reaction were agreed as basis for this experiment, using experience with prior Pa PCR protocol within the lab group. The pilot process used a 50µl reaction mixture, consisting of:

- 25µl TAC 'mastermix' (Q5 Mastermix, New England Biolabs Ltd)
- 2.5µl of each primer (PAL1 and 2)
- 19µl PCR water
- 1µl of broth samples (or a single colony from an agar plate).

The reaction mixture was added to a PCR thermocycler, heated for 5 minutes at 94°C for in-situ DNA extraction then cycled through 30 repeats of:

- 20 seconds at 94°C
- 20 seconds at 57°C
- 20 seconds at 72°C.

Gel electrophoresis was used to detect presence of amplified DNA in the reaction mix. 1.2% agarose gel was prepared by adding 1.2g agarose powder (Sigma Aldrich) to 100ml Tris Borate EDTA buffer solution and heating in the microwave until fully dissolved. 5µl of Gel Red DNA-staining dye was added whilst the solution was still warm, and then gel poured into the required gel tray, sample well comb placed, and the gel allowed to cool for 30 minutes, and the comb removed. 6µl of each sample, along with 2µl gel loading buffer were loaded into the wells, along with DNA ladder at appropriate spacing and then gel electrophoresis run at 100V potential difference for 30 minutes before viewing in UV image viewer.

2.3. Ethics approval

Patient data collection for Aim 1 was possible because all participants had provided informed consent to the CF registry to use their clinical data for research purposes (Project ID 07/Q0104/2). As part of this specific project, IRAS approval was requested and granted to link registry and microbiological data (IRAS Project ID 314624).

The collection, repository storage, and experimental use of clinic bacterial isolates from pwCF aged 12 years is approved by local research and ethics committee (REC), Health Research Authority (HRA) and RBH research and development department (Project ID 17/C4213/1). IRAS amendments were sought and approved by REC, HRA and RBH research and development department in May 2021 to support experiments in this project (IRAS 233578 SA2). Amendments included permission of the following:

- Ability to take consent remotely via video call
- Collection of samples at home and return of samples by post
- Updates of all relevant participant information sheets
- Expansion of age range to include children age 6-12years
- Inclusion of PCR, MS and sniffer dog rapid detection tests for samples provided.

Isolates stored under this ethics approval were used in Aims 2 and 3.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism 10. Specific statistical methods are discussed in individual chapter methodologies. Support for graphical production of Sankey charts, and data collection and analysis of use of anti-Pa inhaled therapies in Aim 1 was provided by Dr Dominic Fenn. Bootstrapping analysis, also used in Aim 1 and discussed with the result chapter, along with statistical advice was provided by Dr Mark Rosenthal. Some statistical analysis and power calculation for work in Aim 3 with MDDs was performed in conjunction with Mr Steve Morant, MDDs in-house statistician.

Chapter 3 – Results: Changes in airway sampling and *Pseudomonas aeruginosa* isolation after the introduction of Elexacaftor/Tezacaftor/Ivacaftor

This chapter, in its entirety, has been peer reviewed and accepted for publication as an original research article in Annals of the American Thoracic Society journal. Print and online publication are due shortly after thesis submission date. Some figures within the text will be incorporated within an online supplement only in the publication.

3.1. Introduction

Elexacaftor/Tezacaftor/Ivacaftor (ETI) is the first highly effective CFTRm available for the majority of pwCF. In clinical trials and real-world data, ETI improves ppFEV₁ and PEx frequency [91,92,142,143]. However, the impact of ETI on chronic airway infection, especially Pa, has been less well studied. Emerging data suggest that Pa is isolated less frequently and possibly in lower abundance from airway samples, following ETI [144–148].

PwCF taking ETI produce less sputum[147,149] and sputum accounts for a lower proportion of airway samples provided post-ETI [144]. In paediatrics, spontaneous sputum production has long been challenging, so clinicians routinely use surrogate samples such as cough swabs. These can be used in adults, but in all scenarios, cough swab sensitivity for Pa infection is much lower than sputum [106,108–110]. It is essential to learn if perceived reduction in Pa isolation frequency is, in part, an artefact of reduced frequency, quality and quantity of sampling, if we are to understand the impact of ETI on airway infection.

Pre-ETI, chronic Pa infection was associated with poorer outcomes in ppFEV₁, morbidity and mortality; the mucoid phenotype was associated with even worse outcomes [45–48,52]. Single airway samples can grow more than one Pa subtype, with colonies exhibiting both mucoid and non-

mucooid phenotypes present. Treatment of Pa to prevent chronic infection required detection and, where eradication was not possible, chronic suppression with long term inhaled anti-Pa treatments forms part of Standards of Care [77]. Presence of any mucooid phenotype is suggestive of chronic infection that is more resistant to eradication or suppression [52].

Chronic Pa infection is most commonly defined by Leeds criteria; an individual is classified as chronic infection if >50% of samples per year grow Pa, non-chronic in those growing Pa in >1 sample but <50% of total samples, and negative if not growing [43]. Leeds criteria were based on a minimum of 4 airway samples per year, in accordance with Standards of Care, although provision of <4 samples per year did not exclude someone from categorisation in the original study. The impact of ETI and changes to CF care during the covid-19 pandemic on the frequency of sample provision, compared to current Standards of Care or chronic infection definitions has not been well documented.

Given the clinical significance, and associated treatment burden, of chronic Pa infection in pwCF pre-ETI, it is important to know its relevance post-ETI, with specific focus on ensuring Pa infection is not missed, chronic infection definitions are still valid, and whether new opportunities to eradicate are possible.

In aim 1, the impact of ETI on airway sampling and infection status in pwCF defined as chronically infected with Pa pre-ETI was examined. A retrospective large cohort analysis was performed to achieve the following:

1. Identify changes in airway **sampling frequency and type of sample** pre- and post-ETI; assess any impact of this on chronicity definitions
2. Examine changes in Pa **isolation frequency** and Pa **mucooid and non-mucooid phenotypes** following ETI
3. Examine changes in the **abundance** of mucooid and non-mucooid Pa following ETI

3.2. Methods

PwCF at RBH meeting Leeds defined chronic Pa infection in at least one of the two years (termed -1 and -2) prior to individual ETI start date were identified in February 2023. At this time, people aged ≥ 6 years of age were eligible for ETI in the UK. Those CFTRm naïve or previously taking LUM/IVA or TEZ/IVA were eligible for inclusion. Those previously taking IVA monotherapy (n=24) were excluded as it is generally regarded similarly 'highly effective' as ETI. Patients gave signed informed consent for the collection of clinical data for research and inclusion in the national CF registry, with separate approval from the regional ethics committee to link registry and microbiology data [150]. Data were then collected from local CF registry database, EPR and hospital microbiology laboratory system; the latter reported phenotype (mucoid/non-mucoid) and abundance based on visual inspection of culture plates. The collection period included each of 5 years pre- (-5 to -1) and 2 years post- (+1, +2) each individual's ETI start date. For each year the following were collected:

- number of airway samples per person per year (pppy) and type (sputum/cough swab) provided from any encounter including acute admissions, clinic attendance and remote testing;
- all airway sample Pa microbiology results (no growth/Pa positive/other, non-Pa) from which Pa isolation frequency was calculated (pppy; total, non-mucoid Pa, mucoid Pa);
- Pa abundance (log-transformed median CFU/ml; non-mucoid Pa and mucoid Pa).

Change in Leeds-defined chronic infection status, use of inhaled anti-pseudomonal treatments and ppFEV₁ pre- and post-ETI initiation was examined.

For nearly all our patients, year -1 coincided with the covid-19 pandemic, and changes to routine in-person clinic attendance and sample provision. During this time, some airway samples were returned by post; it is not possible to identify which ones retrospectively. For the majority, return to standard care occurred during year +1 and in all patients by year +2. To minimise potential confounding by pandemic clinic restrictions, comparisons of year -2 (baseline) with years +1 and +2

(post-ETI) were the focus, with separate sub-analysis of year -1. The fuller pre-ETI period years -5 to -2 were also assessed to confirm stability or evolution of signal.

Those with complete time-point data (i.e. those under active clinical care at RBH for all 7 years examined) were the **primary focus group**; sensitivity analysis of only **sputum** microbiology data (cough swab results excluded) and sub-analyses by genotype (homozygous *F508del* vs heterozygous *F508del*/other) as surrogate for prior modulator use, sex (male vs female) and, where complete data available, baseline ppFEV₁ (<50%, 50-65%, >65%, n=79) and change in ppFEV₁ from baseline (<10% vs >10%, n=76) were performed.

Paired statistical analysis was performed using GraphPad Prism 10. Normality was assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests. Mean[SD] for demographic data of age, baseline ppFEV₁, BMI and proportions by ethnicity, pancreatic status, CF-related diabetes (CFRD), genotype and prior modulator use was calculated. Sex difference in demographic baselines was assessed using t-test (continuous) and Chi² (discrete). Difference in sample number and type, bacterial growth and mucoid phenotype were analysed with two-way ANOVA and Tukey's multiple comparison test. Annual median Pa abundance was log transformed and compared with non-parametric ANOVA (Friedman test) and Dunn's multiple comparison test. As data were paired throughout, no correction for missing values was required.

To assess change in specified paired parameters from before to after ETI initiation, the median change and its 95% confidence interval from year -2 to +2 was calculated by bootstrapping [151]. Bootstrapping is a robust statistical method for smaller grouped data sets that uses multiple resampling, with replacement of the original data set to calculate the median for each sample and uses the distribution of these medians to estimate median change (with confidence intervals) between two groups.

3.3. Results

Patient population and baseline demographics

At the time of analysis, 510 people started ETI, of whom 211 (41.4%) met Leeds criteria for chronic Pa infection in at least year -1 or -2, and 168 (32.9%) met criteria in both years (figure 3.1). Of these, 148 (70.1%) had clinical data available for every year around their individual ETI start date and made up the **primary focus group**. Of 148, 136 (91.9%) provided ≥ 1 airway samples every year, 94 (63.5%) gave ≥ 1 sputum samples every year. For Pa abundance analysis, of 136 providing ≥ 1 airway sample per year, 97 (71.3%) and 63 (46.3%) had paired data for mucoid Pa and non-mucoid Pa abundance respectively for years -5, -2 and at any time post-ETI start. Missing data was mostly years -1 and/or +1, which coincided with changes to care delivery during the pandemic. Of the primary focus group, 72 (49%) were male. Mean[SD] age at the time of ETI commencement was 36.5[12.6] years (table 3.1). Mean baseline ppFEV₁ pre-ETI start was 58.6[21.8]%; increasing in paired samples after ETI to 66.4[20.2]% ($p < 0.001$; $n = 148$).

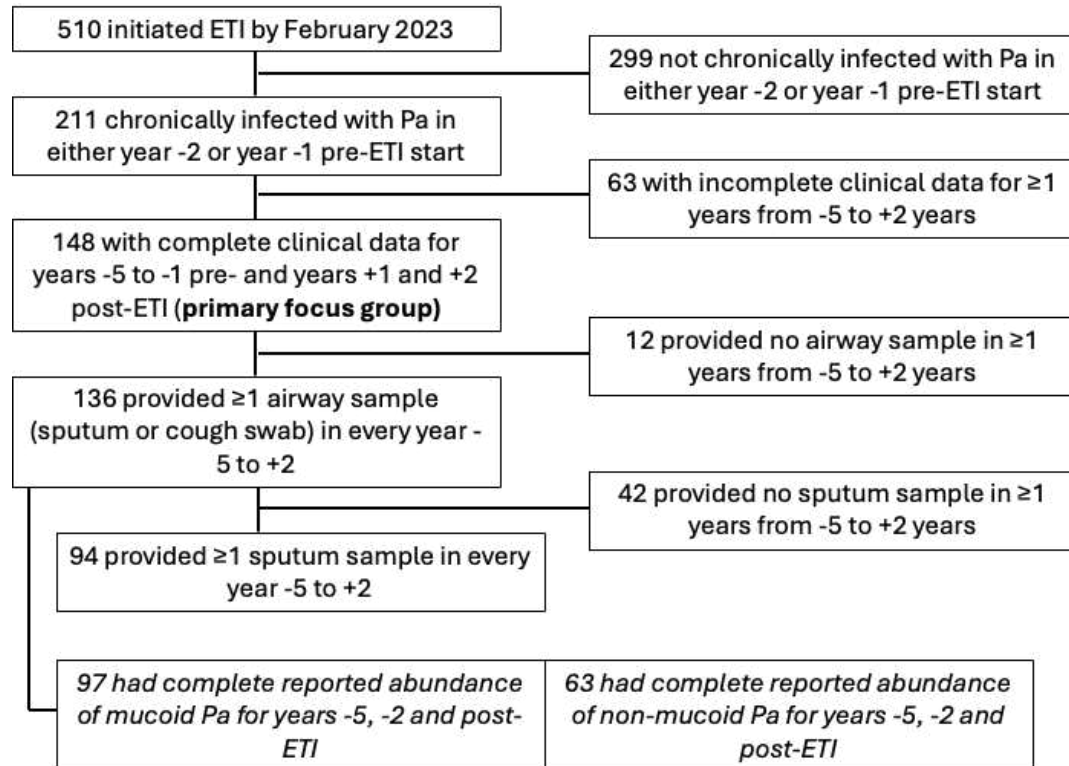


Figure 3.1: Diagram of included study population and subgroups

Study population and breakdown into subgroups for sensitivity and sub-analyses.

Demographics	Total	Male	Female	Male/Female difference	
Number [%]	148 [100]	72 [49]	76 [51]		
Mean age [SD]	36.5 [12.6]	39.7 [12.9]	33.5 [11.6]	p<0.01	[t-test]
Age interquartile range	26.4 - 45.6	30.8 - 49.9	23.2 - 41.6		
Mean ppFEV1 [SD]	58.6 [21.8]	59.5 [24.0]	57.7 [19.5]	ns	[t-test]
Mean BMI [SD]	22.4 [3.3]	23.2 [3.0]	21.7 [3.4]	p<0.01	[t-test]
Mean BMI z score [SD]	-0.1 [0.9]	0.0 [0.9]	-0.2 [0.8]	ns	[t-test]
Caucasian [%]	142 [95.9]	72 [100.0]	70 [92.1]	p<0.05	[Chi ²]
Pancreatic insufficient [%]	127 [85.9]	62 [86.1]	65 [85.5]	ns	[Chi ²]
CFRD [%]	59 [39.9]	26 [36.1]	33 [43.4]	ns	[Chi ²]
Genotype [%]					
F/F	94 [63.5]	45 [62.5]	49 [64.5]	}	ns [Chi ²]
F/MF	36 [24.3]	20 [27.8]	16 [21.2]		
F/RF	14 [9.5]	6 [8.3]	8 [10.5]		
Other/not known	2 [1.4]	0 [0.0]	2 [2.6]		
Modulator before ETI [%]					
TEZ/IVA	87 [58.8]	42 [58.3]	45 [59.2]	}	ns [Chi ²]
LUM/IVA	3 [2.0]	0 [0.0]	3 [3.9]		
None	58 [39.2]	30 [41.7]	28 [36.8]		

Table 3.1: Baseline demographics

Baseline demographics for 148 people with paired data for year -5 to -1, +1 and +2 years relative to ETI start date. Males had higher mean age, whilst there was no sex difference in body mass index (BMI) z-score, pancreatic exocrine function or CF related diabetes (CFRD). There was no sex difference in baseline ppFEV1, despite females being younger. There was no sex difference in proportions of F508del homozygotes (F/F), F508del and minimal function heterozygotes (F/MF), F508del and residual function heterozygotes (F/RF). Two females received ETI for genotypes with no copies of F508del; one G85E homozygote (MF/MF) and one I502T homozygote (RF/RF). There was no sex difference in prior modulator use. Statistical methods indicated in the table.

As expected, fewer samples were provided pppy in year -1 (5.9[4.5]), the year which coincided with pandemic related clinic changes, compared to year -2 (8.0[4.5]); p<0.0001). As discussed, year -2 was used as baseline to compare with years +1 and +2, with differences and changes (mean[SD], mean% and median[95%CI] where applicable) **following ETI** discussed under the following headings.

Fewer sputum samples pppy were provided, with no increase in cough swabs

Fewer total samples were provided pppy in year +1 (3.0[2.5]) than year -2 (8.0[4.5]; $p<0.0001$, $n=148$, figure 3.2A), and this effect was sustained in year +2 (2.9[2.2]; $p<0.0001$). Pre-ETI and pandemic, between years -5 and -2, there had been no significant change in total sample number provided pppy. In years -5 to -2, 86-91% of sample provided were sputum, the remaining being cough swabs. Post-ETI, there was no difference in number of cough swabs pppy, despite substantial reduction in number of sputum pppy (year -2, 7.4[4.5]; year +1 2.6[2.2] $p<0.0001$; year +2, 2.5[2.2] $p<0.0001$). Calculating the paired median change [95%CI] by bootstrapping, the nearest whole number of samples pppy from year -2 to +2, reduced by -5[-5 to -4] total samples; -5[-5 to -4] sputum and 0[0 to 0] cough swabs (figure 3.2B) [151].

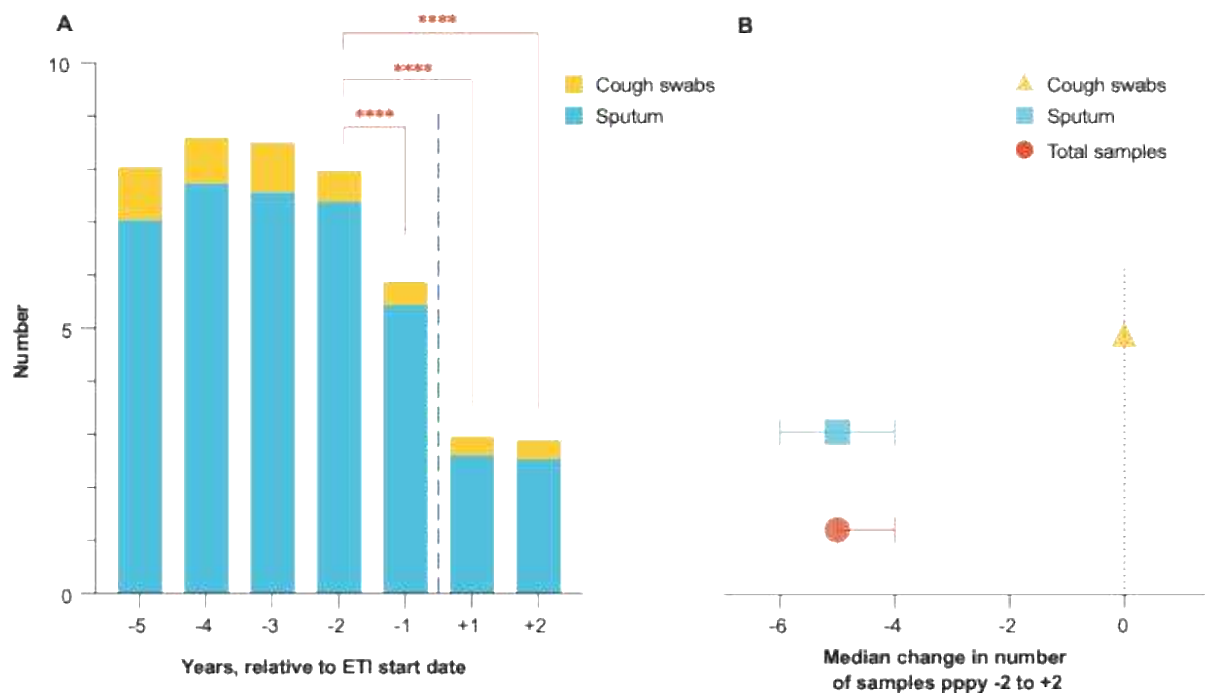


Figure 3.2: Change in paired airway samples (number and type) per person per year

A: Mean number of airway samples provided pppy ($n=148$), with breakdown of sample type (sputum or cough swab). Blue dashed line represents ETI start date. There was reduction in mean number of samples pppy from baseline at year -1 (during covid-19 pandemic) and further, sustained reduction in year +1 and +2 following ETI introduction. Selected p -values represent difference in total number of

samples pppy from year-2 to year-1, +1 and +2 (**** is $p < 0.0001$, two-way ANOVA with Tukey's multiple comparison). There was no difference in the number of cough swabs taken over time. **B:** Median change in number of samples (cough swab, sputum and total samples, rounded to whole numbers) from year -2 to year +2, as calculated by bootstrapping methodology[151]. Error bars represent 95% CI.

Number of samples provided each year was the same irrespective of baseline ppFEV₁ or change in ppFEV₁ (figure 3.3).

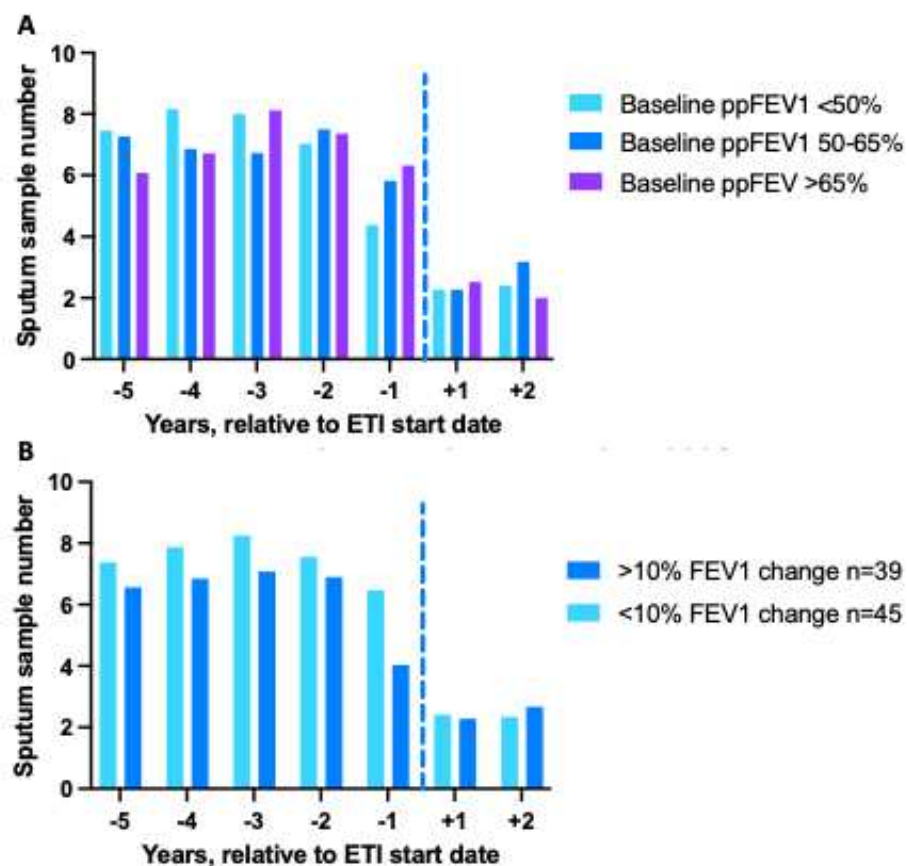


Figure 3.3: Sub-analysis of number of sputum samples provided pppy by ppFEV₁

Sub-analysis of number of sputum samples provided per person per year, by: **A:** baseline (year -2) ppFEV₁, in categories of ppFEV₁ <50%, 50-65% and >65% and **B:** change in ppFEV₁ of < or >10% from baseline. No significant difference, by any sub-analysis category, is seen (Two-way ANOVA). Blue dashed line represents ETI start date.

In reference to CF Standards of Care, 90% of patients provided ≥ 4 samples pppy in year -2; this reduced to 31% and 30% in year +1 and +2 respectively ($p < 0.0001$, figure 3.4).

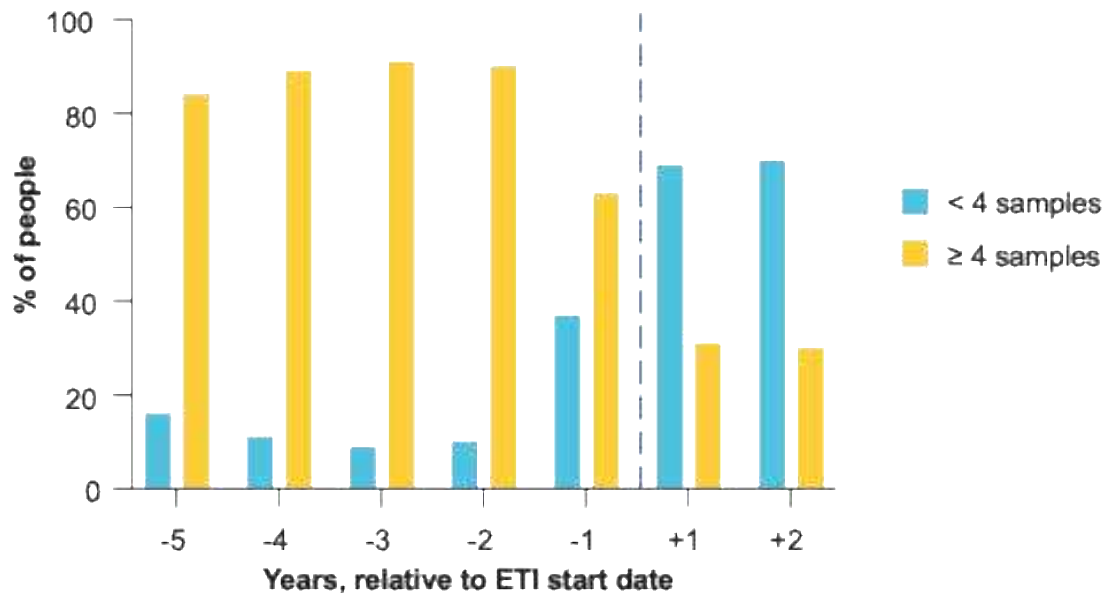


Figure 3.4: Proportion of people meeting CF Standards of Care pre- and post-ETI

Blue dashed line represents ETI start date. Prior to the introduction of ETI, in year -2, 90% of patients provided 4 or more airway samples (cough swab or sputum) per year ($n=148$). Following ETI, fewer than 35% of patients are meeting CF standards of care, providing fewer than 4 samples per year, a significant reduction ($p < 0.0001$, χ^2).

Chronic infection status, defined by Leeds criteria, was mapped over time with two contrasting Sankey plots: one plotted irrespective of number of samples provided pppy (figure 3.5A), and showing the same change when those providing < 4 samples pppy are excluded from Leeds classification (figure 3.5B)

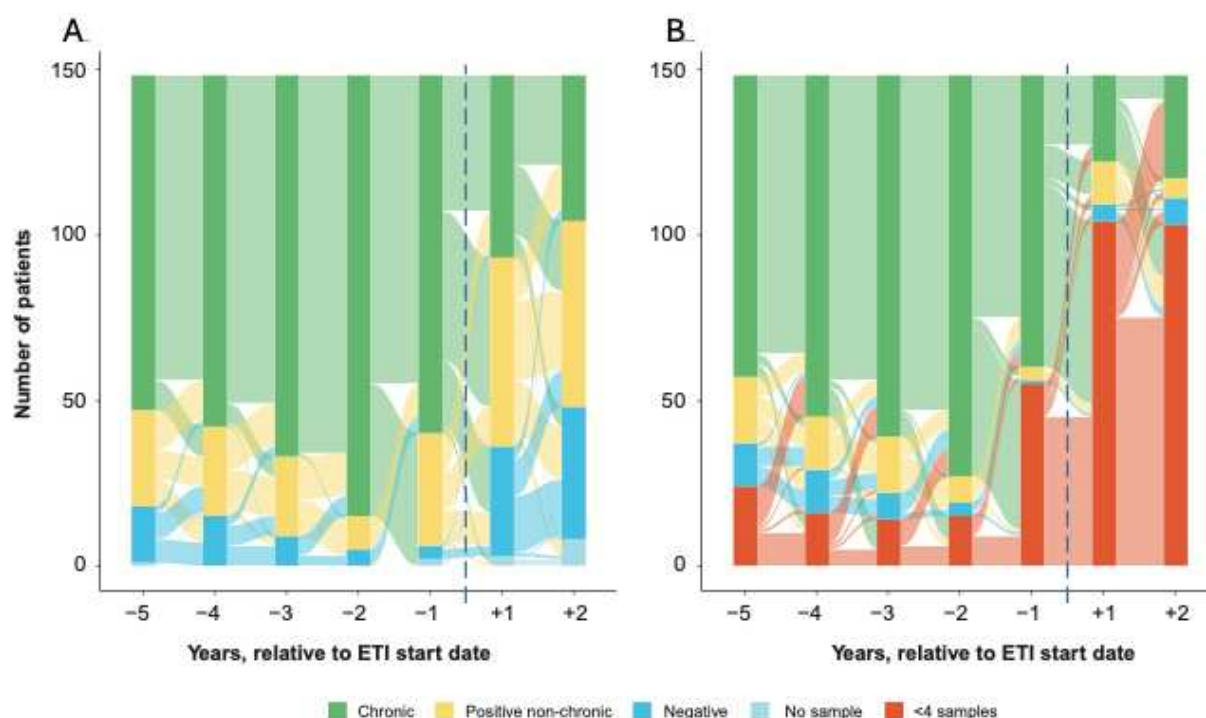


Figure 3.5: Change in paired Leeds defined chronic infection status over time

Sankey plots show the change in Leeds defined airway infection status over time ($n=148$). Blue dashed line represents ETI start date. Leeds criteria: an individual is classified as having chronic infection if $>50\%$ of samples per year grow *Pa*, non-chronic in those growing *Pa* in >1 sample but $<50\%$ of total samples, and negative if not growing at all [43]. **A** presents classification of any year where ≥ 1 sample pppy provided. **B** presents classification if those providing <4 samples per year are excluded from categorisation, to demonstrate the impact of significantly decreased sample provision pppy following introduction of ETI. If CF Standards of Care were enforced as a requirement for Leeds criteria, then the majority of those losing chronic infection status following ETI are now unclassifiable.

The proportion of *Pa* positive cultures decreased

Post-ETI, the proportion of *Pa* positive samples pppy (of anyone providing ≥ 1 sample pppy) decreased from year -2 (84.1%) to year +1 (66.1%; $p<0.0001$) and +2 (58.3%; $p<0.0001$), as shown in figure 3.6A ($n=136$). From year -2 to +1, there was increased proportion of other, non-*Pa* bacteria

(year -2, 8.1%; year +1, 19.4%; $p<0.01$) and no difference in proportion of negative cultures (year -2, 7.8%; year +1, 14.5%; $p=0.07$). From -2 to +2, the increase in proportion of other, non-Pa bacteria was sustained (17.7%; $p<0.01$) and proportion of negative culture increased (24.0%; $p<0.0001$).

From year -2 to +2, the paired median change [95%CI] in proportion of samples pppy, calculated by bootstrapping, that were: Pa positive was -25% [-33.3% to -16.7%]; growing other, non-Pa bacteria was +0% [0% to 8.3%]; that showed no growth was +11.7% [0% to 16.7%] (figure 3.6B) [151].

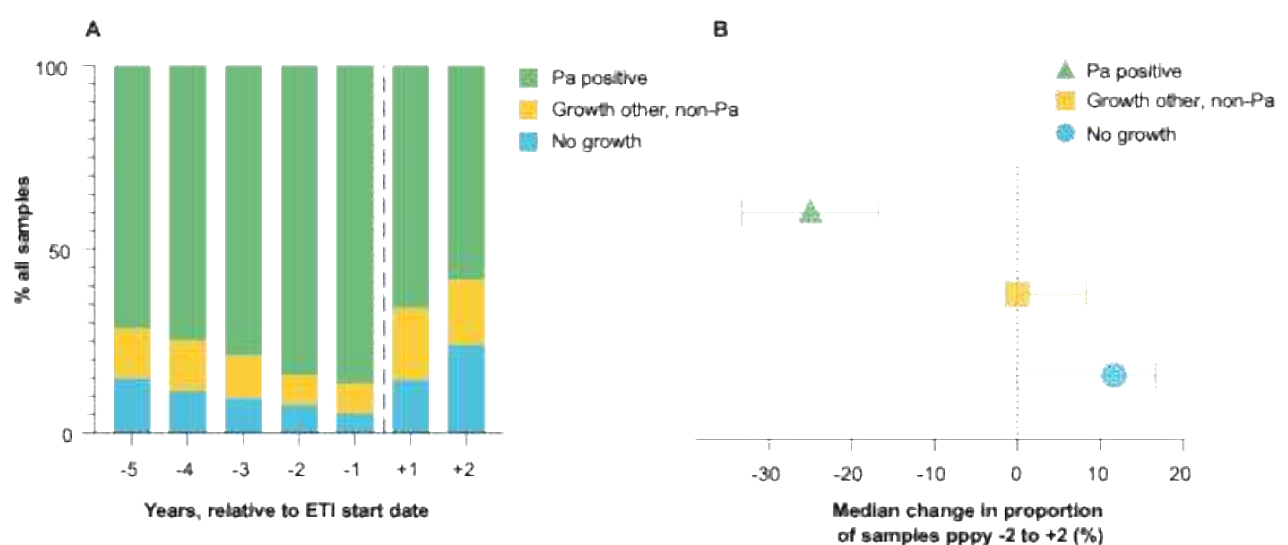


Figure 3.6: Changes to bacterial growth from paired airway samples pppy

A: Airway sample culture results were analysed and for each person the proportion of samples growing any Pa, any other bacteria, or negative on culture were calculated for each year of the study ($n=136$). Blue dashed line represents ETI start date. Mean proportions for each culture result category across all participants are represented (analysis by two-way ANOVA with Tukey's multiple comparison) and shows decreases in proportion of Pa positive cultures seen in each of the two years following ETI. **B:** Median change in the proportion of samples growing Pa; growing other, non-Pa bacteria or no growth, from year -2 to year +2, as calculated by bootstrapping methodology [151]. Error bars represent 95% CI.

The same findings of Pa isolation frequency in all airway samples (figure 3.6A) were robust to sensitivity analysis of paired sputum-only samples (n=94, figure 3.7).

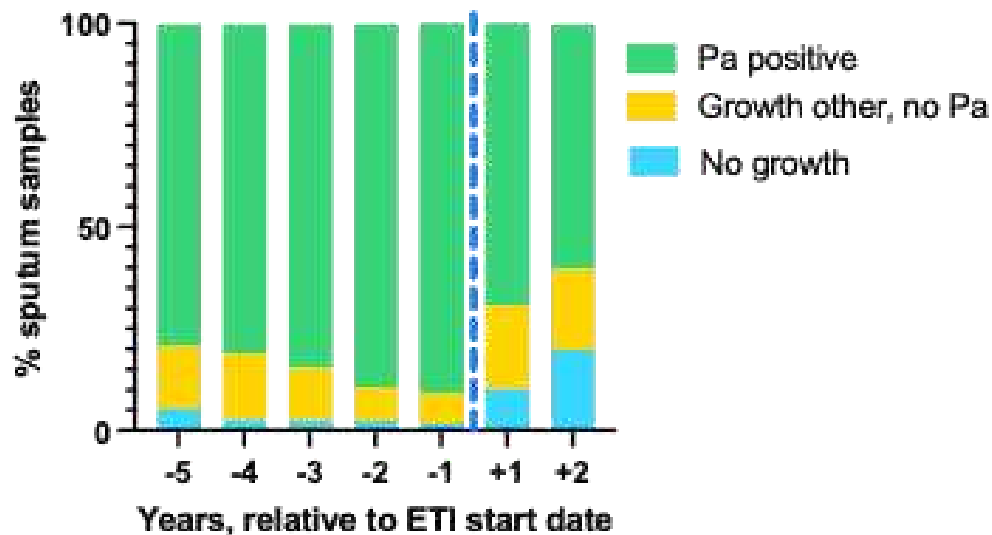


Figure 3.7: Sensitivity analysis of bacterial growth from paired sputum samples pppy

Sensitivity analysis of sputum only (cough swabs excluded) culture results (n=94). For each person the proportion of samples growing any Pa, any other bacteria, or negative on culture were calculated for each year of the study. Blue dashed line represents ETI start date. Mean proportions for each culture result category across all participants are represented (analysis by two-way ANOVA with Tukey's multiple comparison) and showed decrease in the proportion of Pa positive cultures seen in each of the two years following ETI, similar to analysis of all airway samples.

The mean proportion of Pa positive samples pppy was the same irrespective of sex, genotype, baseline ppFEV₁ or change in ppFEV₁ (figure 3.8).

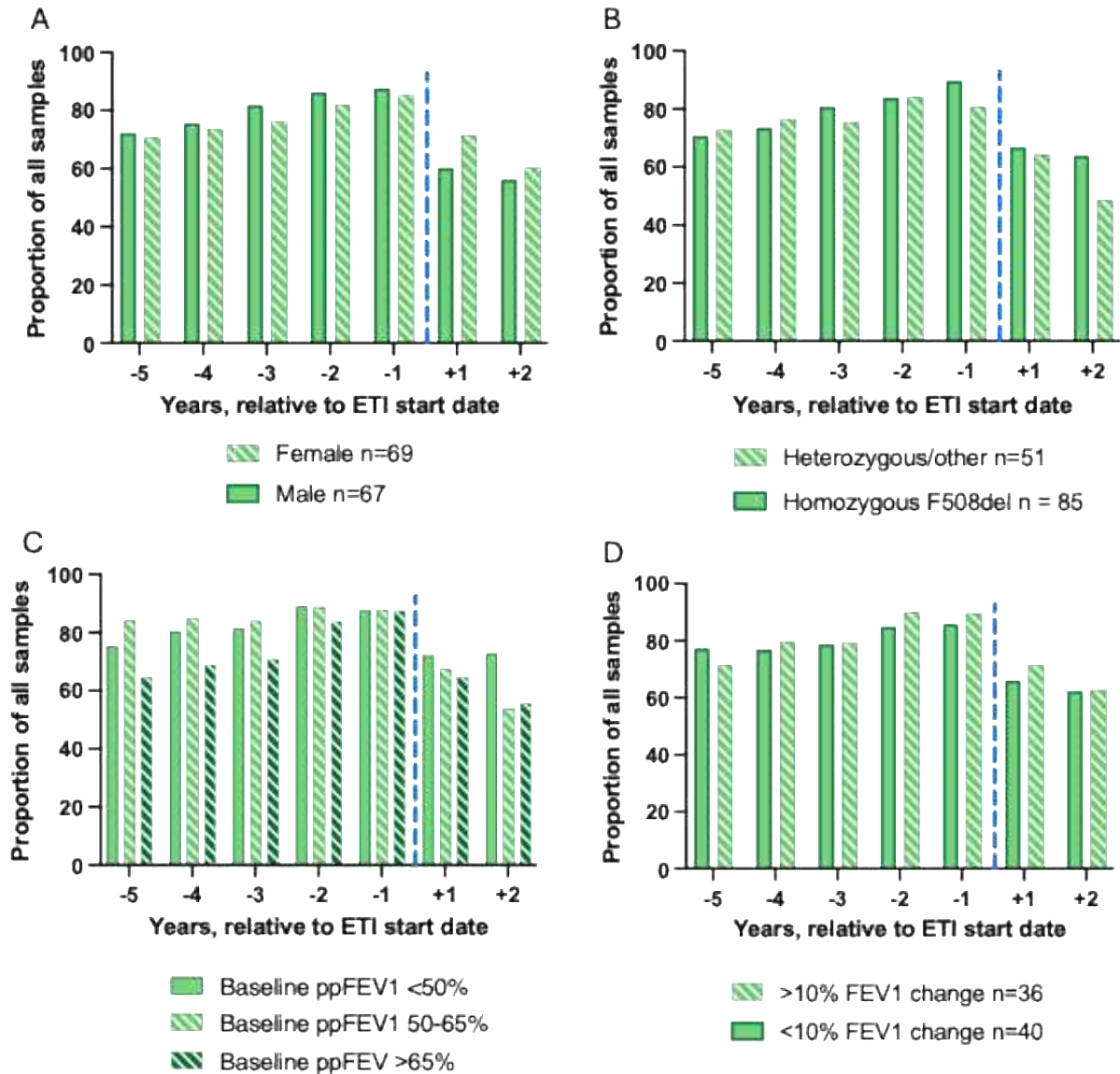


Figure 3.8: Sub-analysis of *Pa* isolation frequency by sex, genotype, pre-ETI baseline ppFEV₁ and change in ppFEV₁

Pa isolation frequency for each year, when sub-analysed by sex (A), genotype (B), pre-ETI baseline (year -2) ppFEV₁ (C) and change in ppFEV₁ (D). Blue dashed line represents ETI start date. The mean proportion of *Pa* positive samples pppy was the same irrespective of sex (male, n=69 vs female, n=67; t-test), genotype (homozygous F508del, n=85 vs heterozygous/others n=51; t-test), baseline ppFEV₁ (<50%, n=32 vs 50-65%, n=22 and >65%, n=25; ANOVA), or change in ppFEV₁ from baseline (<10%, n=40 vs >10%, n=36; t-test).

The proportion of Pa positive cultures exhibiting mucoid phenotype decreased

Post-ETI, the proportion of mucoid Pa containing samples reduced (figure 3.9A, n=136): year -2 (62.1%), year +1 (48.4%; $p<0.001$) and year +2 (43.6%; $p<0.0001$). There was no difference in proportion of samples with only non-mucoid Pa, and the proportion of Pa negative samples increased.

From year -2 to +2 the paired median change [95%CI] in proportion of samples pppy, calculated by bootstrapping, that were growing any mucoid Pa was -13.9%[-20.8% to -8.3%]; growing only non-mucoid Pa was -5%[-8.5% to 0%]; Pa negative was +25%[16.7% to 33.3%] (figure 3.9B).

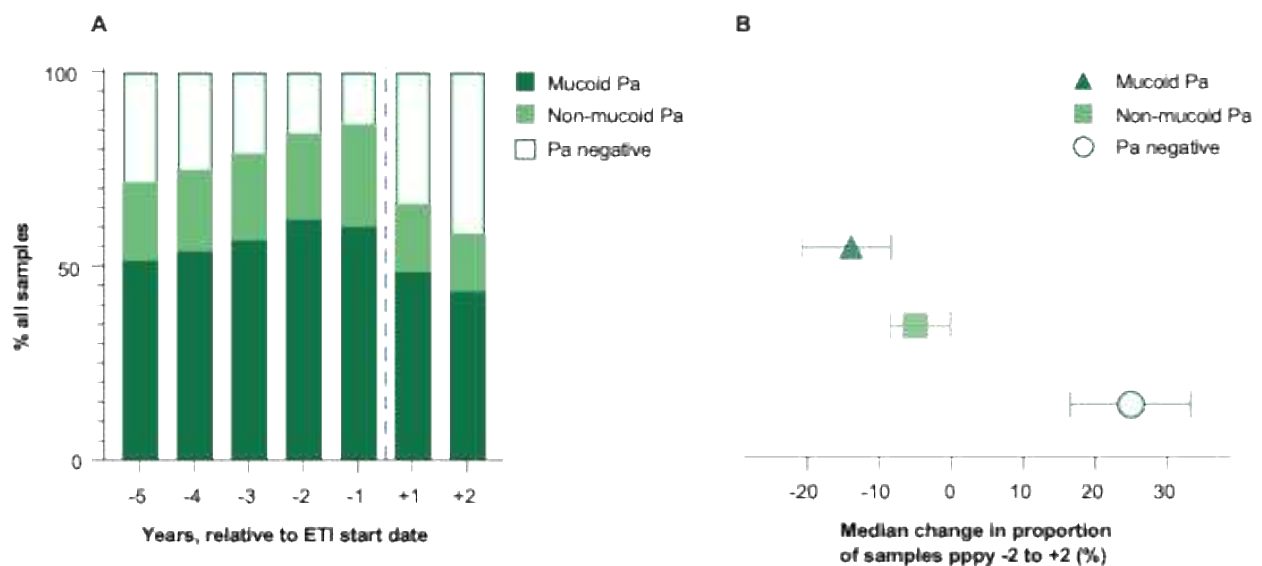


Figure 3.9: Changes in mucoid Pa phenotype from paired airway samples pppy

A: Airway sample culture results were analysed and for each person the proportion of samples growing mucoid Pa, growing only non-mucoid Pa, or growing no Pa (negative or other non-Pa bacteria) were calculated for each year of the study (n=136). Blue dashed line represents ETI start date. Mean proportions for each culture result category across all participants are represented (analysis by two-way ANOVA with Tukey's multiple comparison) and shows a decrease in proportion of growth of mucoid Pa seen following ETI. **B:** Median change in the proportion of samples growing no Pa, growing only non-mucoid Pa, or growing mucoid Pa, from year -2 to year +2, as calculated by bootstrapping methodology [151]. Error bars represent 95% CI.

The same findings of mucoid Pa isolation frequency in all airway samples (figure 3.9A) were robust to sensitivity analysis of paired sputum-only samples (n=94, figure 3.10).

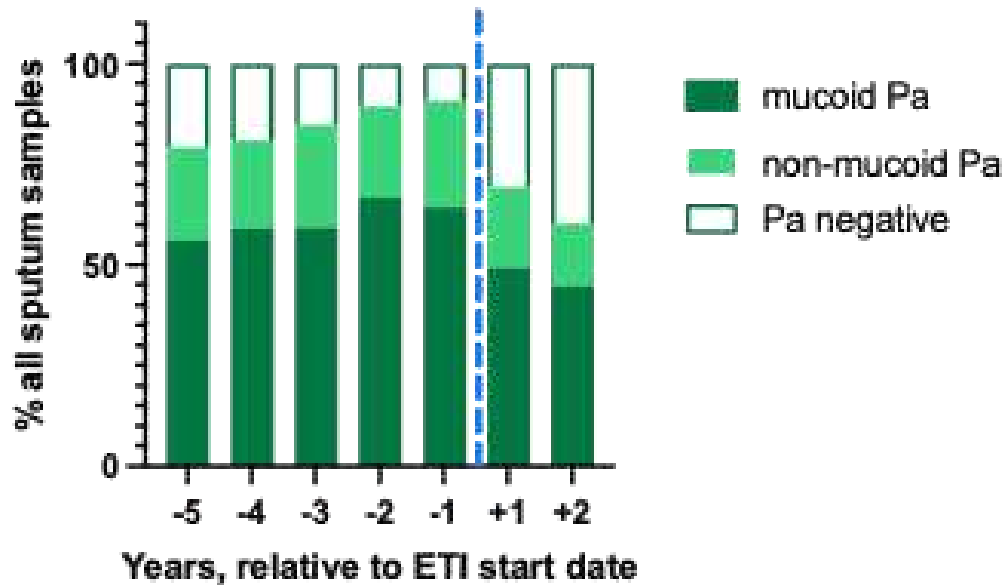


Figure 3.10: Sensitivity analysis of mucoid Pa phenotype from paired sputum samples pppy

Sensitivity analysis of sputum only (cough swabs excluded) culture results (n=94). For each person the proportion of samples growing mucoid Pa, growing only non-mucoid Pa, or growing no Pa (negative or other non-Pa bacteria) were calculated for each year of the study. Blue dashed line represents ETI start date. Mean proportions for each culture result category across all participants are represented (analysis by two-way ANOVA with Tukey's multiple comparison) and shows a decrease in proportion of growth of mucoid Pa seen following ETI, similar to analysis of all airway samples.

The proportion of mucoid Pa positive samples pppy was the same irrespective of sex, genotype, baseline ppFEV₁ or change in ppFEV₁ (figure 3.11).

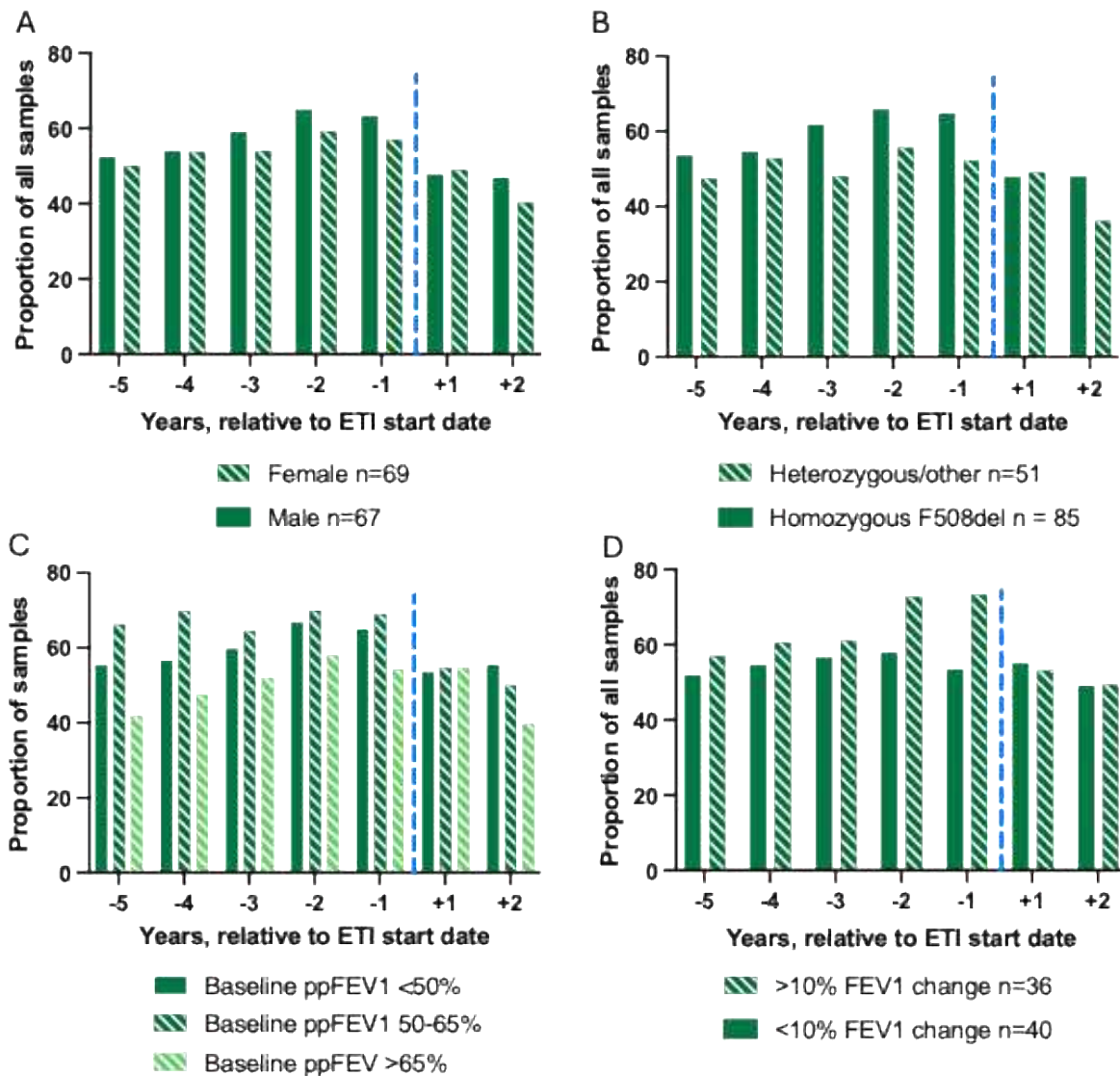


Figure 3.11: Sub-analysis of mucooid *Pa* isolation frequency by sex, genotype, pre-ETI baseline ppFEV₁ and change in ppFEV₁

*Mucooid *Pa* isolation frequency for each year, when sub-analysed by sex (A), genotype (B), pre-ETI baseline (year -2) ppFEV₁ (C) and change in ppFEV₁ (D). Blue dashed line represents ETI start date.*

*The mean proportion of *Pa* positive samples pppy was the same irrespective of sex (male, n=69 vs female, n=67; t-test), genotype (homozygous F508del, n=85 vs heterozygous/others n=51; t-test), baseline ppFEV₁ (<50%, n=32 vs 50-65%, n=22 and >65%, n=25; ANOVA), or change in ppFEV₁ from baseline (<10%, n=40 vs >10%, n=36; t-test).*

Median mucoid and non-mucoid Pa abundance reduces

Estimated CFU/ml abundance is not reported on all Pa positive cultures in our laboratory; as fewer samples were provided pppy post-ETI, abundance was reported less frequently. Complete data sets across all 7 years were only possible for a small number (non-mucoid Pa, n=22; mucoid Pa, n=42). To better assess change in Pa abundance over time, paired samples reporting CFU/ml at year -5, -2 and across year +1 and +2 combined (post-ETI) were examined. Those no longer growing Pa were recorded as CFU/ml=0.

Median CFU/ml of cultures containing only non-mucoid Pa (figure 3.12A, n=63) post-ETI (1×10^5) was lower than in either year -2 (1×10^6 ; $p < 0.0001$) or year -5 (5.5×10^5 ; $p < 0.01$). Median CFU/ml of cultures containing mucoid Pa (figure 3.12B, n=97) post-ETI initiation (1×10^4) was lower than both year -2 (3.25×10^6 ; $p < 0.0001$) and year -5 (1×10^6 ; $p < 0.0001$).

From year -2 to post-ETI, the paired median change [95%CI] in abundance, calculated by bootstrapping, of mucoid Pa was $-2[-2.87 \text{ to } -2]$ log CFU/ml and non-mucoid Pa was $-1.64[-2 \text{ to } -1.5]$ log CFU/ml (figure 3.12C).

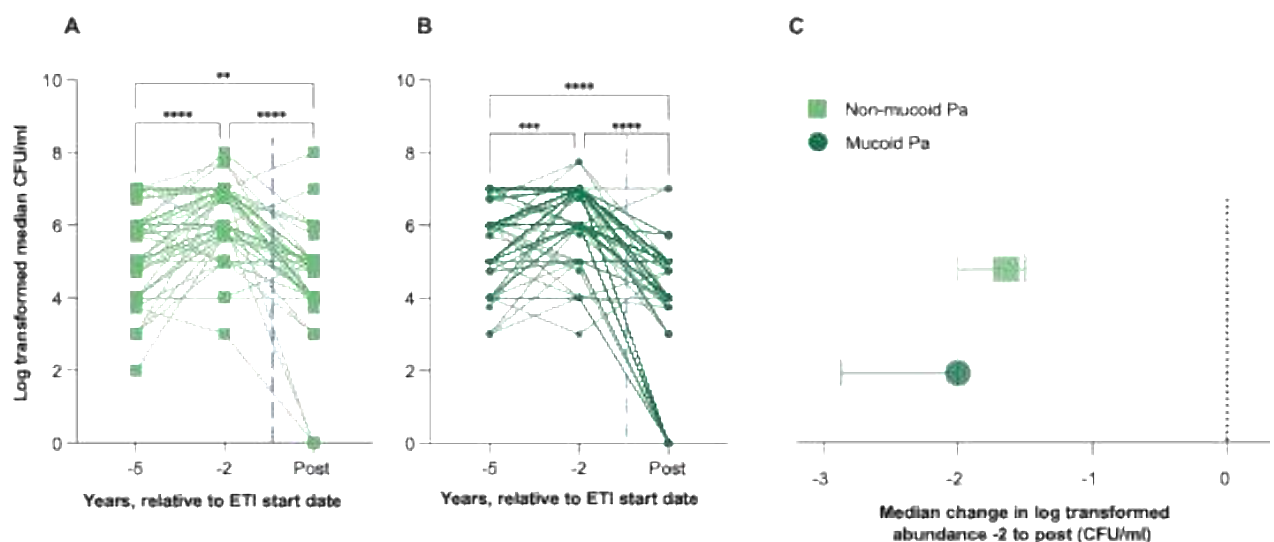


Figure 3.12: Change in median Pa abundance in Pa positive paired airway samples pppy

A and B: Pa abundance data is reported on a minority of Pa positive samples pppy. Sub-analysis of those with paired CFU/ml reported for both non-mucoid (A, n=63) and mucoid Pa (B, n=97) in year -5,

-2 and at some point, in either year +1 or +2 following ETI is shown. Blue dashed line represents ETI start date. Those no longer growing Pa reported as CFU/ml=0. Log transformed median CFU was calculated and compared using Friedman test with Dunn's multiple comparison test. There is reduction in log transformed median abundance of both non-mucoid (A) and mucoid Pa (B) following ETI when compared to both year -2 to baseline and year -5 (**** is $p<0.0001$, *** is $p<0.001$, ** is $p<0.01$). C: Median change in the log transformed abundance of non-mucoid Pa and mucoid Pa, from year -2 to year +2, as calculated by bootstrapping methodology [151]. Error bars represent 95% CI.

Changes seen in isolation frequency and abundance do not coincide with differences to inhaled anti-Pa prescriptions

At baseline (year -2), the number of people in the following inhaled anti-pseudomonal treatments prescription groups were: none (18 [13.2%]), single (66 [48.5%]) and double (52 [38.2%]). These proportions did not change in year +1 (none, 8 [5.9%]; single, 68 [50.0%]; double, 60 [44.1%]) or year +2 (none, 12 [8.8%]; single, 67 [49.3%]; double, 57 [41.9%], χ^2).

Changes in sample provision and Pa isolation frequency persist when the pandemic year is used as baseline

Year -1, coinciding with the covid-19 pandemic, was excluded a priori as a baseline comparator given its unique conditions for clinical care. **From year -2 to year -1**, mean[SD] total samples (8.0[4.5] to 5.9[4.5]; $p<0.0001$) and sputum (7.4[4.5] to 5.4[4.5], $p<0.0001$) pppy reduced, whilst cough swabs remained unchanged. Despite fewer samples pppy during the pandemic, the proportion of samples showing no growth, growth of other (non-Pa) bacteria or growth of Pa, including breakdown by mucoid phenotype, remained unchanged.

Comparing year -1 to +1 and +2, fewer total samples (+1: 3.0[2.5], $p<0.0001$; +2: 2.9[2.2], $p<0.0001$) and sputum (+1: 2.6[2.2], $p<0.0001$; +2: 2.5[2.2], $p<0.0001$) were provided pppy than year -1, and cough swabs remained unchanged. There was increased proportion of other, non-Pa bacteria (-1:

8.1%; +1: 19.4%, $p<0.001$; +2: 17.7%; $p<0.05$) and negative cultures (-1: 5.4%; +1: 14.5%, $p<0.01$; +2: 24.0%; $p<0.0001$). There was decreased proportion of Pa positive samples pppy (-1: 86.5%; +1: 66.1%, $p<0.0001$; +2: 58.3%, $p<0.0001$) and decreased proportion of mucoid-Pa positive samples (-1: 60.1%; +1: 48.4%, $p<0.05$; +2: 43.6%, $p<0.0001$). There was insufficient data to analyse year -1 Pa abundance in isolation.

3.4. Discussion

Larger registry and similarly sized real-world studies have demonstrated post-ETI reduction in the proportion of all airway samples that are sputum [144,147]. This study goes further, showing specifically that absolute numbers of total samples and sputum pppy have dropped dramatically since the pandemic and coinciding with ETI initiation. In this single centre, data suggests that routine sampling is not being replaced with alternatives as number of cough swabs did not increase.

CF Standards of Care recommend ≥ 4 airways samples pppy and are the standards that Leeds criteria for chronic Pa infection are defined by [77]. Although Leeds definition does not exclude those providing <4 samples pppy, only 6 of 143 (4%) in the original study did not meet this standard [43]. A minority of the cohort studied met Standards of Care for sampling after starting ETI; $>60\%$ did not provide ≥ 4 samples, so misclassification by Leeds criteria is probable. This may lead to incorrect treatment decisions, such as premature termination, by current guidance, of suppressive inhaled therapies. If current Standards of Care cannot be met, an argument is made for the need to review the relevance of current definitions of chronic Pa infection.

The cause for sustained reduced sample provision post-pandemic and post-ETI is unclear. A combination of better lung health related to ETI and persistence of pandemic-driven diversification of ways pwCF interact with their clinic (in-person, virtual and hybrid attendance) may be responsible. Irrespective of the cause, it highlights a need to increase non-sputum-based airway sampling to

meet Standards of Care, as highlighted in recent Cystic Fibrosis Foundation position paper [152].

Given poorer sensitivity of cough swabs versus sputum, alternative non-sputum-based sampling is urgently needed.

Dittrich *et al* demonstrated greatest reduction in Pa positivity in those with the higher baseline ppFEV₁; this was not replicated here, however average baseline ppFEV₁ was lower in this study (58.6% vs 63%), which may be explained by the baseline chronic Pa infection status or higher mean age of the cohort examined [144].

Other studies have reported reduction in Pa positivity post-ETI [144,145,147,148,153]. This study demonstrates similar effects, but specifically in a cohort with established chronic Pa infection pre-ETI, in most cases for 5 years prior. Adjusting for the number of samples provided pppy, both mucoid and non-mucoid Pa is isolated less frequently post-ETI. As there is no change in sample type provided, this is not an artefact of increased cough swab use. Neither does it likely reflect an increase in antibiotic use treatment as inhaled anti-Pa prescriptions did not change.

Whilst, decreasing Pa positivity rate post-ETI coincides with increases in proportion of both negative cultures and growth of other, non-Pa bacteria, absolute number and identity of non-Pa bacteria is not known. Further work is needed to document which non-Pa bacteria are grown, explore the mechanisms for this change and whether these organisms are pathogenic in a post-ETI CF lung.

It was not possible to track an individual's change in Pa mucoid status overtime, as isolation frequency had to be adjusted for the variable number of samples provided pppy. However, at a group level, decrease in Pa positivity rate post-ETI was greater in those isolating mucoid rather than only non-mucoid phenotype. This seems contrary to the expectation of mucoid Pa being more persistent and resistant to treatment. Mechanisms for this change, and how an individual's chronic pathogens adapt post-ETI are not known. It can be hypothesised that restoration of CFTR function, leading to normalisation of airway surface environment, prompts conversion of Pa from

mucoïd/biofilm to planktonic state, and resultant increased susceptibility to improved airway clearance and host defences. Further study may offer opportunity for new methods of eradication and reduced disease burden.

Bacterial abundance is reported for some sputum samples, and these are estimated by visual plate inspection. This measure is not reported for all samples that grow bacteria, however there was sufficient paired data to track an individual's Pa abundance over three timepoints. A 1-2 log reduction in median CFU/ml, apparent in both mucoïd and non-mucoïd phenotypes, was observed. This may have implications for the ability to treat and eradicate chronic infection. Whilst mechanisms for this need exploration, these results support findings from CF PROMISE and other studies [146,147,154]

Introduction of ETI coincided with changes to CF-care during the covid-19 pandemic. Video clinics were default, unless physical review was required, and samples were commonly returned by post, though it is not possible to retrospectively determine which. The impact of these changes, where perhaps only the most unwell provided samples in person, the relative yield of postal samples (though perhaps thought to be similar), and where shielding requirements may lead to reduced risk of viral triggered exacerbation, make interpreting the changes in Pa isolation challenging [155,156].

To mitigate the baseline impact of the pandemic, year -2 was chosen as a comparator to years +1 and +2. In year +1 there were likely ongoing pandemic related measures in CF care and shielding/social distancing for some. By year +2, these measures had ended and the majority of pwCF had returned to normal social arrangements; CF care adopted a hybrid model of video and in-person review, and combinations of airway sampling on site and at home. The fact that changes in Pa positivity rate in year +2 were sustained from year +1 suggests a persisting impact of ETI. Sub-analysis suggested fewer samples were provided than in year -1 than -2, but despite this the same trends in Pa isolation frequency persisted until ETI was introduced. Post-ETI, Pa isolation frequency

decreased within the first 12 months, and sample provision again decreased, further separating ETI and pandemic effects.

This study has several limitations. As a single UK CF-centre study, models of pre-, mid- and post-pandemic care, mechanisms of ETI reimbursement and post-ETI care may differ from other countries, limiting generalisability. Whilst expected care practices are known at each timepoint, actual practice is not known, including which samples came remotely and timing of samples in relation to exacerbation or clinical stability. We do not know ETI adherence or tolerance and saw insufficient post-ETI sweat chloride testing to perform analysis ($n < 10$ pairs). Similarly, analysis of anti-Pa inhaled therapies could only be based on prescription practices; collection and use of these medications was not known. A comparator group was not possible due to insufficient numbers of modulator ineligible pwCF with chronic Pa infection at baseline. Sub-analysis by underlying baseline structural lung disease was not possible but could be insightful.

This study provides further evidence that Pa isolation frequency and abundance reduce post-ETI, even in those who have demonstrated persistent chronic Pa infection pre-ETI. Research is required to understand the mechanisms behind these observations. Current Standards of Care for airway sampling are not being met, and this impacts our ability to accurately define and determine ongoing chronic infection. These findings support the argument for new ways to define chronic Pa infection and to develop novel, non-sputum-based sampling methods that may safely support changes to use of burdensome chronic anti-Pa inhaled therapies.

Chapter 4 – Results: Phenotypic impact of CFTRm on *Pseudomonas aeruginosa* isolates from people with Cystic Fibrosis

4.1. Introduction

Compared to normal health, the CF airway is associated with lower surface pH and relative hypoxia within sputum plugs [27,28,30,31,50,63,73]. At the time of project design, the impact on pH and oxygen state from CFTR modulators was not known but restoration of chloride and bicarbonate conductance, ASL hydration and mucociliary clearance suggested potential to normalise pH and oxygen state in the airway [157,158]. The impact of modulators on Pa also remained unclear but evidence from the large cohort study in Aim 1, along with emerging evidence from real-world studies suggests a significant reduction in frequency of Pa isolation and abundance after introduction of CFTRm [144,147]. It has previously been demonstrated that pH can affect Pa antimicrobial sensitivity and that changes in oxygen state (hypoxic environments) can induce mucoid phenotype [50,63,71,73]. Exploring how proposed lung microenvironment changes due to CFTRm affect Pa and its virulence and persistence phenotype was, therefore, the next step to understanding mechanisms for these observed changes in Pa isolation frequency and abundance.

In Aim 2, described in this chapter, the *in vivo* use of modulators and the *in vitro* effect of pH and oxygen state on Pa phenotype including growth rate, virulence/persistence phenotypes and antimicrobial sensitivity of CF clinical isolates of Pa were investigated, to help understand the impact of modulators on CF airway Pa infection. This was addressed in two stages:

1. To compare the phenotypes of serial Pa isolates, developed *in vivo*, from pwCF before and after CFTRm initiation
2. To examine the impact of mimicking the environmental changes expected from CFTRm (pH and oxygen state) on phenotypic characteristics of clinical Pa isolates *in vitro*

Three readily assessable areas of Pa phenotype, growth, pyoverdine production and mucoid status, were chosen to reflect easily available and simple measures of virulence and persistence, as a pilot assessment of changes to Pa phenotype in response to starting CFTRm, with expectation that positive findings could lead to greater exploration in future projects. Additionally, the impact of altered CF lung environment on antibiotic sensitivity of Pa isolates was examined in the *in vitro* experiments, where environment was controlled.

Growth

Environmental conditions in the CF lung encourage Pa to convert from planktonic to biofilm state; biofilm growth rate is known to be slower than planktonic bacteria [44,56,63,159,160]. Restoration of CF lung environment towards normal, could feasibly lead to switching from biofilm to planktonic state as environmental pressures are lifted, and perhaps increase the growth rate of Pa isolates. However, in clinical practice, use of modulators has been shown to reduce the abundance of Pa in samples from pwCF [96,144,147,153]. So conversely, with restoration of lung environmental defences, with improved mucociliary clearance, it could equally be argued that isolates from pwCF after CFTRm initiation may have reduced growth potential as bacteria are more effectively killed or cleared. Enumeration of bacteria and estimation of growth rate is used frequently within the lab group. There are many ways they can be assessed (outlined in methods, sections 2.1.4 and 2.1.5.), including measurement of bacterial density in broth with OD₆₀₀, serial measurement of OD₆₀₀ over time to produce growth curves and calculate doubling times, and colony counting. Most assessment methods are prone to 'noise' with a degree of variability; this was assessed as part of the analysis.

Pyoverdine

Pyoverdine is considered a Pa virulence factor, with increased pyoverdine produced when Pa is in an acute infectious, planktonic state [59,75]. Changes in quantity of pyoverdine produced by Pa isolates from pwCF exposed to CFTRm, compared to their isolates before CFTRm exposure, may therefore

indicate an impact on the virulence of Pa. As pyoverdine is fluorescent in UV light, quantification can occur by measurement of relative fluorescent intensity (methods, section 2.1.6.). The assay is quick, simple and was available in the lab group so was selected for pragmatic reasons.

Mucoidy

Mucoid alginate production is considered a marker of Pa persistence, with greater mucoid alginate production suggestive of more persistent isolates that are resistant to eradication or suppression with antibiotic treatments [44,51,52,159]. Changes in mucoidy status of Pa isolates from pwCF exposed to CFTRm, compared to their isolates before CFTRm exposure, may indicate an impact on the persistence of Pa. Mucoidy can be assessed with a congo red agar assay (methods, section 2.2.1.), again an assay that has been used previously within the lab group.

Antimicrobial sensitivity

Environmental pH can impact the efficacy of antibiotics against Pa [161]. Given the role of CFTR in bicarbonate conductance, the pH of airway surface in pwCF is lower. Hypothesising that restoration of CFTR function with CFTRm may lead to more normal airway pH, then this may in turn impact the effectiveness of antibiotics on Pa in the CF airway. To assess this, the minimum inhibitory concentration (MIC) to tobramycin was examined, using standard UKAST methodology (methods, section 2.1.7.) [136]. Tobramycin was chosen as the primary antibiotic to examine as it is commonly used as an intravenous and nebulised treatment for Pa.

Two separate workflows were designed, and application of phenotypic assays used is discussed within the separated methods in turn.

4.2. Materials and methods

4.2.1. Phenotypic assays for Pa isolates from pwCF before and after CFTRm initiation

To further explore the changes seen in mucoid and non-mucoid Pa isolation frequency and abundance, the SRC-PAPA repository of clinical Pa isolates was utilised to perform phenotypic analysis of Pa samples over time, before and after the pwCF from whom the isolates originated initiated CFTRm (ETI or IVA monotherapy). To identify suitable patient samples, the same list of pwCF under the care of RBH and chronically infected with Pa in at least one of two years before starting CFTRm, as used in Aim 1 (Chapter 3, n=211), and was cross referenced with the SRC-PAPA sample repository database. As routine sample collection for the repository paused during the covid-19 pandemic, and there was gradual return to regular collection, only a minority of patients had serially stored samples over multiple years that would therefore be suitable for inclusion. Eight (of 211) people with serial stored isolates of Pa for up to 24 months pre- and 24 months post- personal CFTRm start date were identified; four on IVA monotherapy and four on ETI (irrespective of prior LUM/IVA or TEZ/IVA use, as previous). Due to the timing when SRC-PAPA repository collection started, no samples prior to -20 months before IVA start date were available. Patients on ETI were included if isolates were available at least 3 months after ETI start, to ensure comparison included samples from after >3 months of taking ETI. Due to the small numbers of both ETI and IVA patients meeting these criteria, it was decided to include both ETI and IVA users to evaluate changes in Pa isolate phenotype before and after CFTRm start date as a whole.

All participants had provided informed consent to the CF registry to use their clinical data for research purposes (Project ID 07/Q0104/2). As part of this specific project, IRAS approval was requested and granted to link registry and microbiological data (IRAS Project ID 314624).

Demographic data for all eight patients was retrieved from electronic patient records (EPR) and included: sex, age, ppFEV₁ prior to starting CFTRm, BMI and BMI z-score, ethnicity, pancreatic exocrine status, CFRD status, genotype and prior modulator usage, and are reported on individual

basis, including number of revivable samples and RBH laboratory reported mucoid status of each sample. Pa isolates within the SRC-PAPA repository were then retrieved and sample data plotted on timeline relative to individual CFTRm start date.

Pa isolates, for each person and for each time point before and after CFTRm start date, were revived on general agar as according to protocol (methods, section 2.1.3.) and incubated overnight at 37°C. Successfully revived isolates were inoculated into 10ml LB broth and incubated for 24 hours and underwent phenotypic analysis in triplicate (three technical repeats, TR) by assessing the following:

- growth measured by OD₆₀₀ and colony count (CFU/ml)
- RFI of broth samples at 24 hours as surrogate marker for pyoverdine (a known Pa virulence factor) production
- subjectively scored mucoidy status using congo red agar

This process was completed three times for each isolate to provide three biological repeats (BR) and mean[SD] or median[IQR] calculated.

Growth

The first measure of growth was made by assessing OD₆₀₀. Following the 24 hour incubation of each isolate in broth, three x 200µl aliquots of were added to 96-well plate and OD₆₀₀ measured in the plate reader and standardised against control (methods, section 2.1.5). Negative values after control standardisation were set to zero. Colony counting was also performed, in triplicate by standard protocol, for each isolate to provide a secondary measure of growth (methods, 2.1.4.).

Pyoverdine

Because pyoverdine is fluorescent under UV light [162], RFI can be used as a surrogate marker for pyoverdine production. After 24 hours incubation, three 200µl aliquots of each isolate was added to 96-well plate and RFI, standardised to control, was measured using a plate reader (methods, section

2.1.6.). Negative values after control standardisation were set to zero. As RFI is dependent on quantity of bacteria in the sample, results were standardised to the CFU/ml count for each biological repeat.

Mucoidy

Mucoidy status was assessed using congo-red agar, a differential media used to demonstrate mucoid alginate production with dark red/black staining seen on the media [138,139]. After 24 hours incubation, three x 15µl spots of each isolate were spotted onto congo red agar plates, which were in turn incubated at 37°C for a further 24hrs (methods, section 2.2.1.). Plates were then photographed and mucoidy was scored subjectively on a four-point scale as illustrated in methods, figure 2.2 (repeated below within the results section, for ease of reference). The results for each technical and biological repeat (to demonstrate variability of scoring system) and median technical and median biological repeat scores were plotted as a heat map.

Statistics

With only 8 patients suitable for longitudinal phenotypic analysis, statistical analysis over time for each individual would lack sufficient power. Raw data was reviewed for obvious outliers or errors (discussed below in results). Mean[SD] of three biological repeats (absolute values of OD₆₀₀, CFU/ml, CFU adjusted RFI) for each isolate was plotted on a timeline for each patient, pivoted around CFTRm start (t=0), and also standardised against baseline (earliest) sample results to show relative change over time. Results for all biological repeats are presented to demonstrate variability in assay results, and coefficient of variance (%) calculated; a coefficient of <10% was deemed acceptable.

Statistical analysis was performed at group level for all results for all patients pre- compared to all results for all patients post-CFTRm start using GraphPad Prism 10. Normality of results was assessed Shapiro-Wilk and Kolmogorov-Smirnov tests. Whilst OD₆₀₀ values were normally distributed, CFU/ml

and CFU adjusted RFI values were not. To present values similarly, median[range] of all BRs pre- and all BRs post- t=0 was calculated, plotted and compared using paired non-parametric (Wilcoxon) t-test. As IQR could not be reported by GraphPad Prism if <5 repeat values included, total range (minimum to maximum) was reported for all values.

4.2.2. Modelling pre- and post- CFTRm treatment changes to the CF airway and their impact on *Pseudomonas aeruginosa* phenotype.

Anticipating a limited number of people with sufficient longitudinal samples in the repository pre- and post-CFTRm initiation, the second part of this aim was designed to examine phenotypic changes seen when clinical Pa isolates were subjected to some of the likely differences in growth environment seen before and after CFTRm initiation. Specifically low pH and hypoxic conditions (found in the CF airway) and neutral/high pH and normoxic conditions (found in the healthy airway), were examined. The pH of exhaled breath condensate has been well studied and suggests airway pH in healthy controls is between 7.4 and 8.1 (although some papers did report slightly lower) and between 5.9 and 6.5 in pwCF [22–26]. pH 6.5, 7.4 and 8.0 were chosen to represent these conditions in the experimental design. Quantifying the amount of oxygen in CF sputum plugs is more challenging, and it is likely that oxygen quantity varies from anoxia to relative hypoxia compared to airways free of mucous obstruction (21% ambient oxygen) [32]. On this basis a pragmatic comparison of normoxia (21%) and hypoxia at 6% ambient oxygen was chosen.

A workflow was designed to allow three-day serial passage of clinical isolates in all combinations of these conditions, prior to phenotypic analysis, similar to section 5.2.1, examining:

- growth by OD₆₀₀, colony count and **additionally** examining growth curves and doubling time
- RFI as surrogate measurement for pyoverdine virulence factor production

- subjectively scored mucoidy status using congo red agar
- and **additionally** examining MIC to tobramycin

Figure 4.1. illustrates the workflow. 11 CF clinical isolates that have previously been interrogated in detail for other research projects within the SRC-PAPA group were again selected from the SRC-PAPA repository (methods, table 2.1) [137]. Pa14 was selected as a reference strain, with stocks held alongside clinical isolates on Microbank storage beads at -80°C. Passage and assay methodology are outlined below:

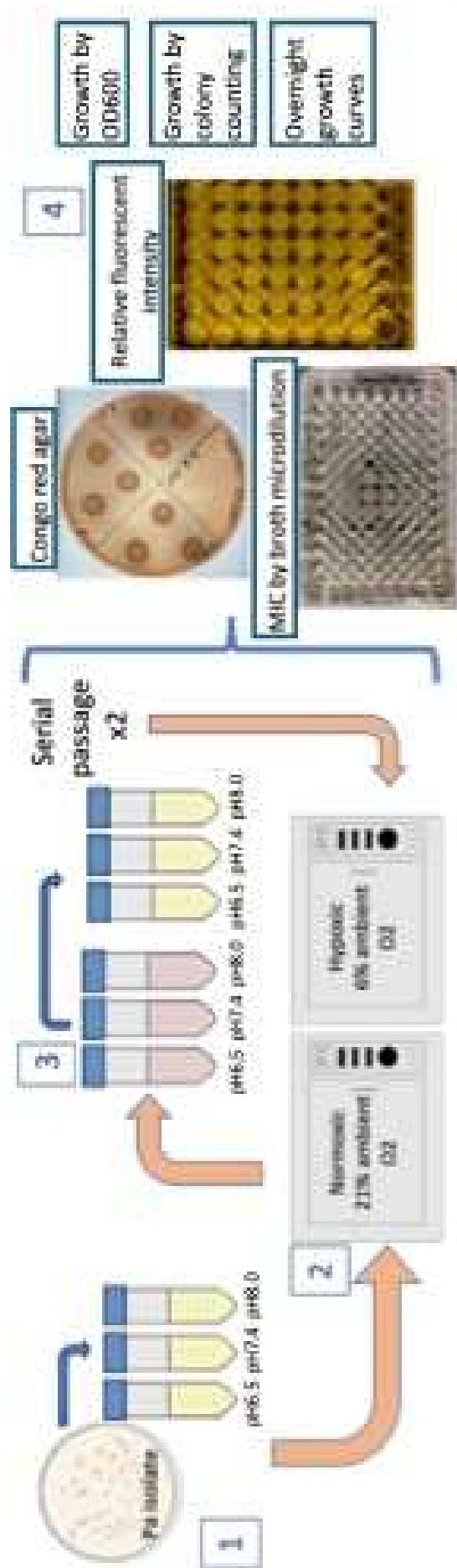


Figure 4.1: Workflow of serial passage through a range of pH and oxygen state environments for 11 *Pa* isolates and phenotype assays

Demonstration of workflow through 4 steps. 1) Revival of isolate on agar and inoculation into pH adjusted MHB2. 2) Incubation in normoxic (21%) and

hypoxic (6%) environments for 24 hours, in parallel for all pH conditions. 3) Serial passage into fresh pH adjusted MHB2 over a further 2 cycles (2 days).

4) Phenotypic assays performed include growth assessment by optical density (OD₆₀₀), colony counting, and overnight growth curve production, relative fluorescent intensity as a surrogate marker for pyoverdine production, mucoidy status using congo red agar, and MIC to tobramycin using standard microdilution methodology.

Passage:

11 CF clinical isolates and Pa14 were revived on LB agar according to previously described methods (section 2.1.3) and incubated overnight at 37°C. Three separate 500ml volumes of Muller Hinton Broth 2 (MHB2) were prepared and pH adjusted to three different pH levels (pH 6.5, 7.4 and 8.0) using standardised preparations of hydrochloric acid and sodium hydroxide. Final pH was confirmed using electronic pH-metre, with the probe cleaned with 1% distel and rinsed in sterile dH₂O before each measurement.

10ml aliquots of each pH adjusted MHB2 were prepared, and colony sweeps of the 12 revived isolates were inoculated (methods, section, 2.1.3) in duplicate at each pH level and sealed with breathable membrane. A hypoxic (6% oxygen) environment was achieved using Oxoid™ CampyGen™ 2.5L sachets (Thermo Fisher Scientific) within a 2.5L sealed tank and confirmed using Oxoid™ Resazurin anaerobic environment indicator strip (Thermo Fisher Scientific). Each isolate at each pH was incubated overnight at both hypoxic (6%) and normoxic (21%) environments at 37°C, on shaking platform at 200rpm, along with sterile MHB2 at each pH to act as control.

Repeated twice over the next two days, 100µl of each aliquot underwent passage under the same conditions for 24 hours, to establish stable generational growth conditions (three-day passage in total).

At the end of serial passage, isolates were analysed to gain information about Pa phenotype in each growth environment (normoxic at pH 6.5, 7.4 and 8.0; hypoxic at pH 6.5, 7.4 and 8.0). The workflow for all isolates at all conditions was repeated three times, to produce three biological repeats, in addition to technical repeats as outlined in each assay section. The pH of broth after 24 hour passage was also measured once during the workflow (after second day of passage), to look for pH changes in culture media environment over the passage process and compared to controls.

Growth

Growth was again assessed by measuring broth optical density (OD_{600}) of 200 μ l aliquots in the plate reader at the end of the third passage, with three technical repeats, and mean[SD] calculated across three biological repeats (methods, section 2.1.5.). Colony counting was performed by standard protocol for each isolate at each condition, with three technical repeats, and mean[SD] calculated across 3 biological repeats (methods, section 2.1.4.).

20 μ l samples of each isolate at each condition were inoculated in fresh 180 μ l aliquots of respective pH adjusted MHB2 broth (pH 6.5, 7.4, 8.0) in a 96-well plate and incubated overnight, at normoxic conditions only (hypoxic conditions could not be created), for 24 hours in a plate reader to serially measure OD_{600} every 30 minutes. A single technical repeat was possible due to limited capacity in the plate reader. Mean[SD] of each biological repeat at each timepoint was calculated and plotted to produce growth curves (methods, section 2.1.5.). Doubling times were calculated for each isolate and mean doubling time across all isolates for each growth condition compared.

Pyoverdine

RFI was again used as a surrogate marker for pyoverdine production. 200 μ l of each isolate at each condition, and control, were added to 96 well plate and RFI, standardised to control, were measured using a plate reader with three technical repeats. RFI values were again standardised to CFU/ml for each isolate. Mean[SD] was calculated across three biological repeats.

Mucoidy

Mucoid alginate production was assessed using congo-red agar, as per previous methods (section 2.2.1.). Median of each biological repeat (to demonstrate variability of scoring system) and median across all three biological repeats was plotted as a heat map.

Antimicrobial sensitivity

The MIC to tobramycin of the 12 isolates used, at neutral pH and normoxic conditions, has been previously defined within the group providing experimental guidance on which MICs to examine in this workflow (methods, section 2.1.7. and table 2.1) [137].

There was capacity in the workflow for two technical repeats for each isolate. Three tobramycin concentrations were chosen for each isolate based the previous work define median MIC for each isolate and using one concentration (2-fold) above and below these pre-assessed values [137].

Where no MIC was found, this was recorded as 'greater than' the highest tobramycin concentration tested. Median MIC for each isolate and each condition in each biological repeat and median across all three biological repeats was plotted as a heat map of relative difference (-2, -1, 0, +1 or +2 log₂ MIC) from each isolate's normoxic pH 7.4 MIC, to adjust for baseline variability in MIC for each isolate.

Statistics

Statistical analysis was performed using GraphPad Prism 10. Normality was assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests. Mean[SD] and Median[5th,95th centile] were calculated from biological repeats. **Absolute** and **relative** (standardised to the results for normoxic pH7.4 conditions) values for OD₆₀₀, CFU/ml and CFU adjusted RFI are presented graphically. Mean[SD] or median[IQR] **relative** results for all isolates at each environmental condition were compared to the normoxic pH7.4 condition result using paired t-test or Wilcoxon test, with Bonferroni correction for multiple t-tests. Correlation between OD₆₀₀ and CFU/ml, for each condition, was assessed using Spearman rank correlation coefficient (r), and slope compared using simple linear regression. Doubling times for growth curves were calculated (methods, section 2.1.5) and compared to normoxic pH7.4 condition results using repeated measures one-way ANOVA with multiple comparisons (Friedman's test).

Mucoidy results are presented in a heatmap, with all biological repeats shown to provide information of variability of scoring method, and no statistical analysis appropriate. MIC to

tobramycin is presented with all biological repeats in tabular form and heat map indicating MIC compared to normoxic pH7.4 conditions. Median MIC across all isolates, compared to median MIC at normoxic pH7.4 is compared using Wilcoxon test.

4.3. Results - Phenotypic assays for Pa isolates in the SRC-PAPA repository before and after their CFTRm initiation

4.3.1. Demographics

Eight pwCF under the care of Royal Brompton CF clinic had sufficient samples stored in the SRC repository around their CFTRm start date, with four having samples stored around their IVA start date and four around their ETI start date (table 4.1). Four (50%) were male, mean age at starting was 38 years, only one was *F508del* homozygous (patient ETI 3), and therefore eligible to prior modulator, taking TEZ/IVA at time of initiation; the rest were modulator naïve. Mean ppFEV1 at the time of starting IVA/ETI was 48.7%. Median number of samples included pre- and post-start date was three. The RBH clinical laboratory reported mucoid appearance in all the samples provided by 6 (75%) of patients included.

Patient	IVA 1	IVA 2	IVA 3	IVA 4	ETI 1	ETI 2	ETI 3	ETI 4
CFTRm start date	Oct 2015	Oct 2015	Oct 2015	Apr 2015	Dec 2020	Sep 2020	Nov 2020	Sep 2020
Sex	Male	Female	Male	Female	Female	Male	Male	Female
Age starting CFTRm (years)	35	41	48	21	32	50	43	37
Genotype	F/MF	F/MF	F/MF	F/MF	F/MF	F/MF	F/F	F/MF
Gene 1	F508del	F508del	F508del	F508del	F508del	F508del	F508del	F508del
Gene 2	G178R	S549N	S549N	S549N	2711delT	1898+1G->A	F508del	R347P
Prior modulator use	N/a	N/a	N/a	N/a	None	None	TEZ/IVA	None
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian
Baseline pre-CFTRm ppFEV1 (%)	63.0	38.8	50.5	31.4	73.4	36.7	45.0	50.9
BMI	22.8	25.3	22.6	21.7	23.9	22.7	23.2	21.7
BMI z-score	-0.06	0.85	-0.14	-0.02	0.57	-0.11	0.10	-0.02
Pancreatic exocrine function	Insufficient	Insufficient	Insufficient	Insufficient	Insufficient	Insufficient	Insufficient	Insufficient
CFRD	No	Yes	No	Yes	No	Yes	Yes	Yes
Samples in repository								
Pre-start total (excluded)	3	3	1	2 (1)	3	3	3	3
Post-start total (excluded)	8 (2)	3	6	7 (1)	2	3	2	2
Total included	9	6	7	7	5	6	5	5
Lab mucoid status of included samples (%)	1 (11)	6 (100)	7 (100)	7 (100)	5 (100)	6 (100)	5 (100)	2 (40)

Table 4.1: Demographics of people providing isolates used in phenotype assays

Demographics of patients providing the clinical samples that were revived from the SRC repository for phenotypic analysis. CFTRm = CFTR modulator, ppFEV₁ = percent predicted forced expiratory volume in 1 second, BMI = body mass index, CFRD = Cystic Fibrosis related diabetes, F/F = homozygous F508del, F/MF heterozygous F508del, TEZ/IVA = tezacaftor/ivacaftor.

For the four IVA patients, revivable isolates were available in the repository from -20 months to +24 months relative to IVA start date. For the four ETI patients, revivable isolates were available for -18 months to +10 months relative to ETI start date. Earliest sample before CFTRm ranged from -20 to -2 months, and latest sample following CFTRm ranged from +4 to +24 months. Timelines are illustrated in figure 4.2, with further breakdown of sample dates and lab reported mucoid status in table 4.2.

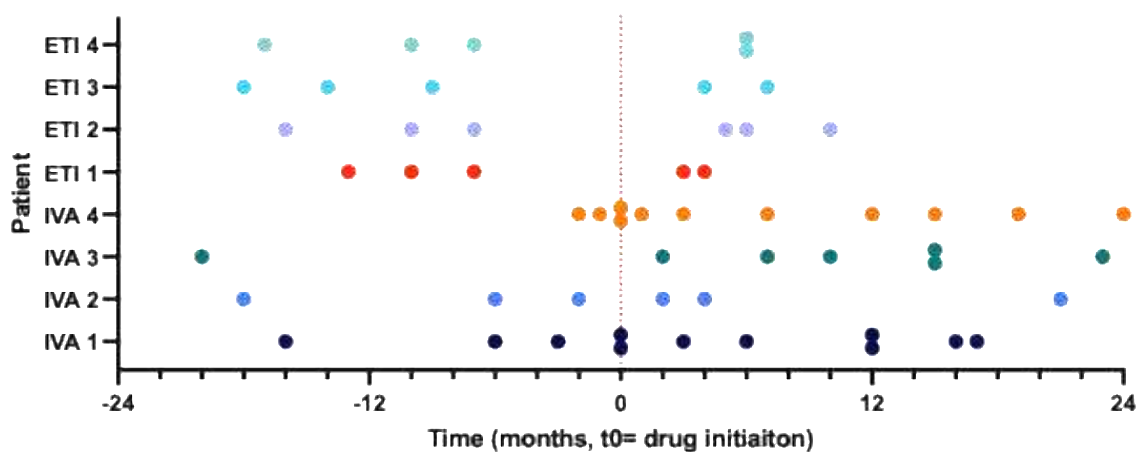


Figure 4.2: Pa isolate timeline relative to CFTRm start date

Timeline illustrating timing of each individuals Pa isolates stored in SRC-PAPA repository, relative to individual CFTRm start date (represented by red dashed line at t=0). Each isolate available represented by green dots. Duplicates were identified using lab serial number and excluded. Those coinciding with t=0 were excluded as it is not possible to know if the sample was provided before or after drug initiation. IVA = Ivacaftor, ETI, Elexacaftor/Tezacaftor/Ivacaftor.

IVA 1			IVA 2			IVA 3			IVA 4		
Sample date	Months	Muc	Sample date	Months	Muc	Sample date	Months	Muc	Sample date	Months	Muc
02/06/2014	-16		03/04/2014	-18					03/02/2015	-2	
13/04/2015	-6		04/04/2015	-6					08/04/2015	0	
13/07/2015	-3		12/08/2015	-2		08/02/2014	-20				
Oct 2015			Oct 2015			Oct 2015			Apr 2015		
12/10/2015	0		11/12/2015	2		08/12/2015	2		21/04/2015	0	
12/10/2015	0		05/02/2016	4		23/05/2016	7		07/07/2015	3	
11/01/2016	3		14/07/2017	21		22/08/2016	10		10/11/2015	7	
11/04/2016	6					17/01/2017	15		11/04/2016	12	
10/10/2016	12					17/01/2017	15		16/09/2016	17	
10/10/2016	12					18/09/2017	23		01/11/2016	19	
27/02/2017	16								07/04/2017	24	
22/03/2017	17										
ETI 1			ETI 2			ETI 3			ETI 4		
Sample date	Months	Muc	Sample date	Months	Muc	Sample date	Months	Muc	Sample date	Months	Muc
28/11/2018	-13		28/05/2019	-16		11/04/2019	-18		20/04/2019	-17	
18/02/2019	-10		24/01/2020	-10		19/08/2019	-14		10/11/2019	-10	
20/05/2019	-7		28/02/2020	-7		18/02/2020	-9		16/02/2020	-7	
Dec 2020			Oct 2020			Nov 2020			Sep 2020		
16/03/2021	3		22/02/2021	5		17/03/2021	4		12/03/2021	6	
07/04/2021	4		15/03/2021	6		14/06/2021	7		12/03/2021	6	
			26/07/2021	10							

Key	
	Pre-start samples
	Samples excluded at t=0
	Start date
	Post-start samples
	Lab reported non-mucoid Pa
	Lab reported mucoid Pa

Table 4.2: Date of Pa isolate provision and clinical reporting of mucoid status by RBH lab

Table illustrating the dates of samples in the SRC repository for included patients, relative to their IVA/ETI start date in months, and associated lab reported mucoid (muc) phenotype of samples.

Data handling

Raw data was reviewed; colony counting results for IVA1 biological repeat 3 at +3 months and IVA2 biological repeat 3 at -2 months were missing due to failed bacterial growth on colony counting plates. CFU adjusted RFI could therefore not be calculated for these repeats and so were also removed from analysis. RFI measurement of ETI1 biological repeat 1 at -13 months did not produce measurable output and so was not included either. It was noted that three values in patient IVA4 had much higher variability in biological repeats than other patients. Examining this data, biological repeat 1 was outlying ($>1.5 \times$ IQR above upper quartile) for CFU/ml at +3 months, and OD₆₀₀ at +7 and +24 months. IVA4 biological repeat 1 at these timepoints was excluded from analysis of OD₆₀₀, CFU/ml and RFI.

4.3.2. Growth measurement by OD₆₀₀

Growth by absolute OD₆₀₀ after 24 hours incubation for each sample at each timepoint was examined (figure 4.3). No specific trends in mean absolute OD₆₀₀ was seen over time for any patient.

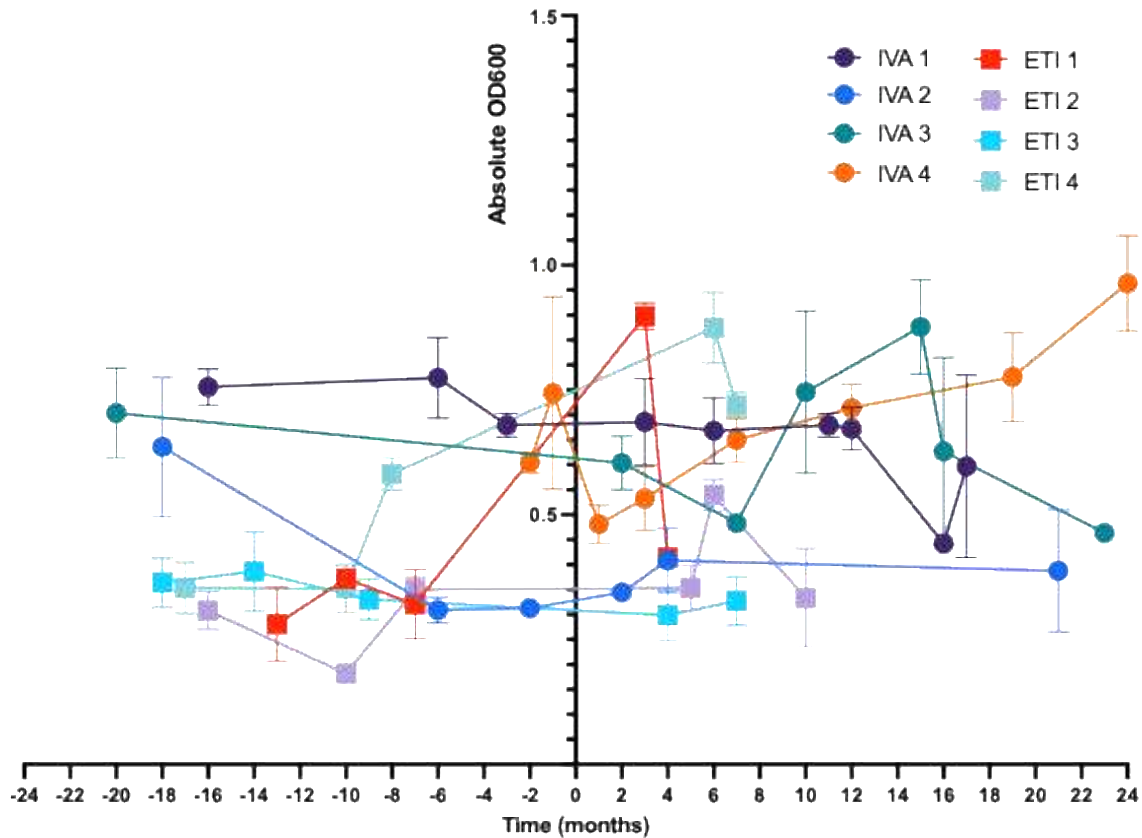


Figure 4.3: Absolute OD₆₀₀ for each patient's isolates over time pre- and post-CFTRm

Absolute OD₆₀₀ following 24 hours incubation in broth. Mean of three biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

To look for trends and account for baseline differences in growth based on individual Pa species, growth by OD₆₀₀ was examined relative to baseline (figure 4.4). In this representation, each biological repeat is expressed as a proportion of the baseline (earliest) sample for the same

biological repeat run and mean[SD] relative OD₆₀₀ calculated and plotted over time. Again, no trend is identified over time, in this representation of the data.

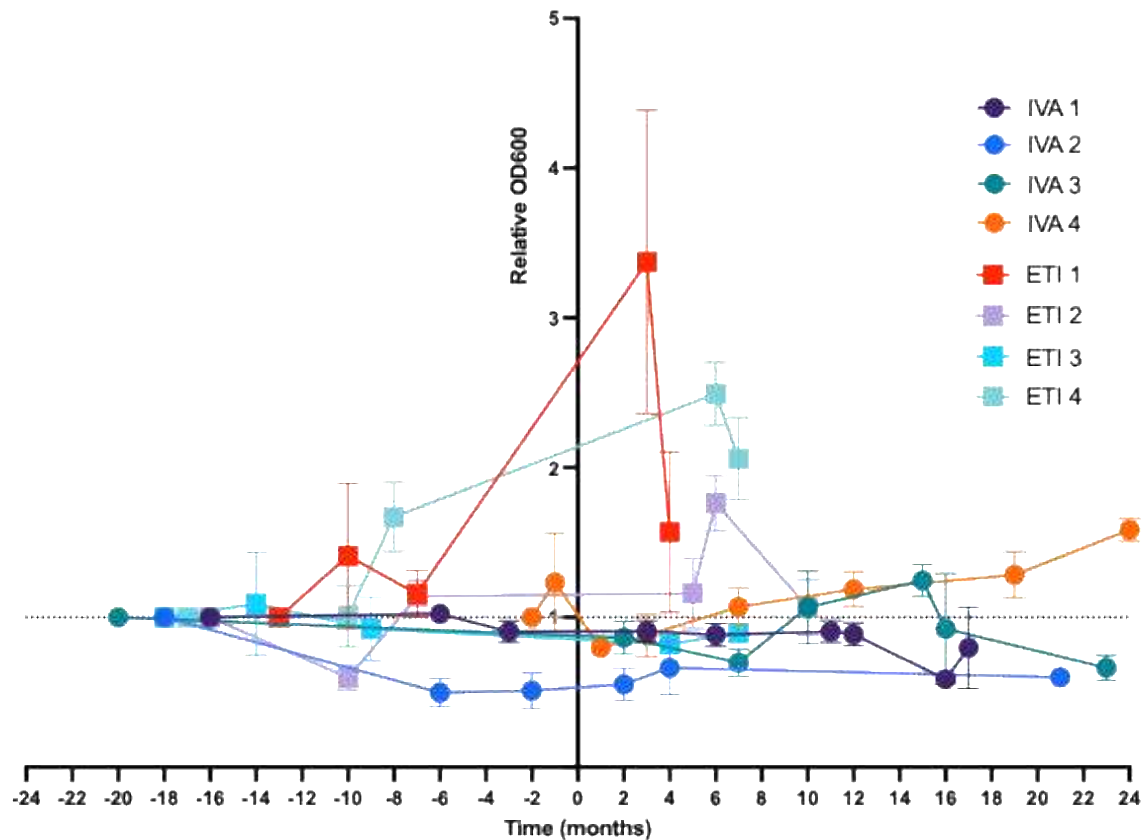


Figure 4.4: OD₆₀₀ for each patient's isolates relative to baseline over time pre- and post-CFTRm

Relative OD₆₀₀ following 24 hours incubation in broth, with results for each biological repeat standardised as a proportion of first available sample biological repeat (OD₆₀₀=1.0, black dotted line). Mean of three relative biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

Figure 4.5 illustrates the median[range] OD₆₀₀ of all isolates for each individual pre-CFTRm compared the same post-CFTRm. No significant difference is seen.

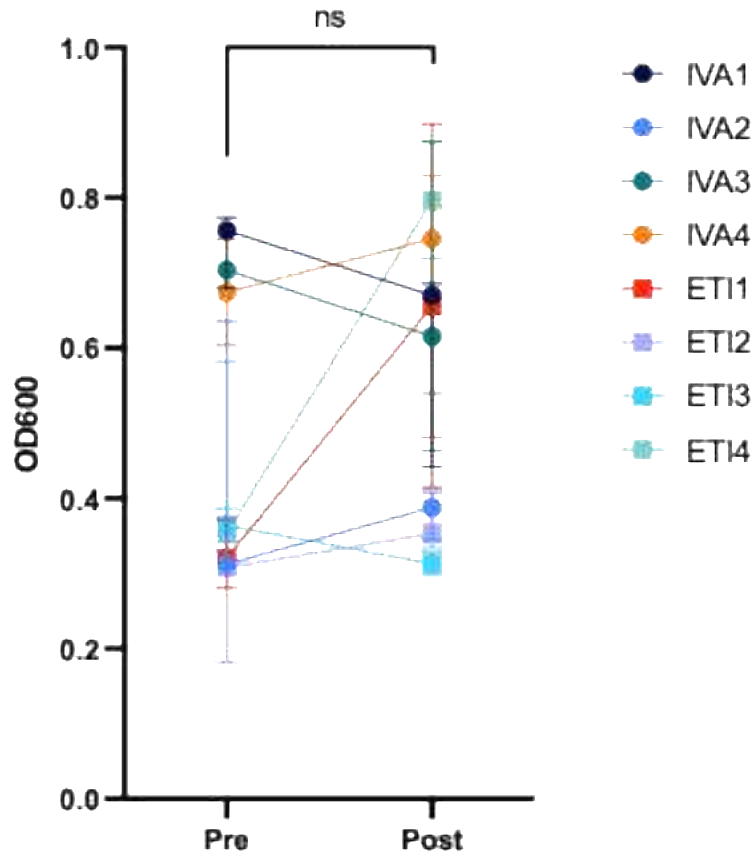


Figure 4.5: Pooled OD₆₀₀ for all isolates pre- compared to all isolates post-CFTRm

Median OD₆₀₀ of each individual's isolates pre-drug initiation compared to all those post-drug initiation onward, with errors bars indicating range. As some patients had <5 isolates either pre- or post-CFTRm, IQR is not presented by GraphPad Prism. Comparison with non-parametric paired t-test (Wilcoxon), ns=non-significant.

4.3.3. Growth measured by colony count

Growth by absolute colony count (CFU/ml) after 24 hours incubation for each sample at each timepoint was examined (figure 4.6). No specific trend in mean absolute CFU/ml is seen over time for any patient.

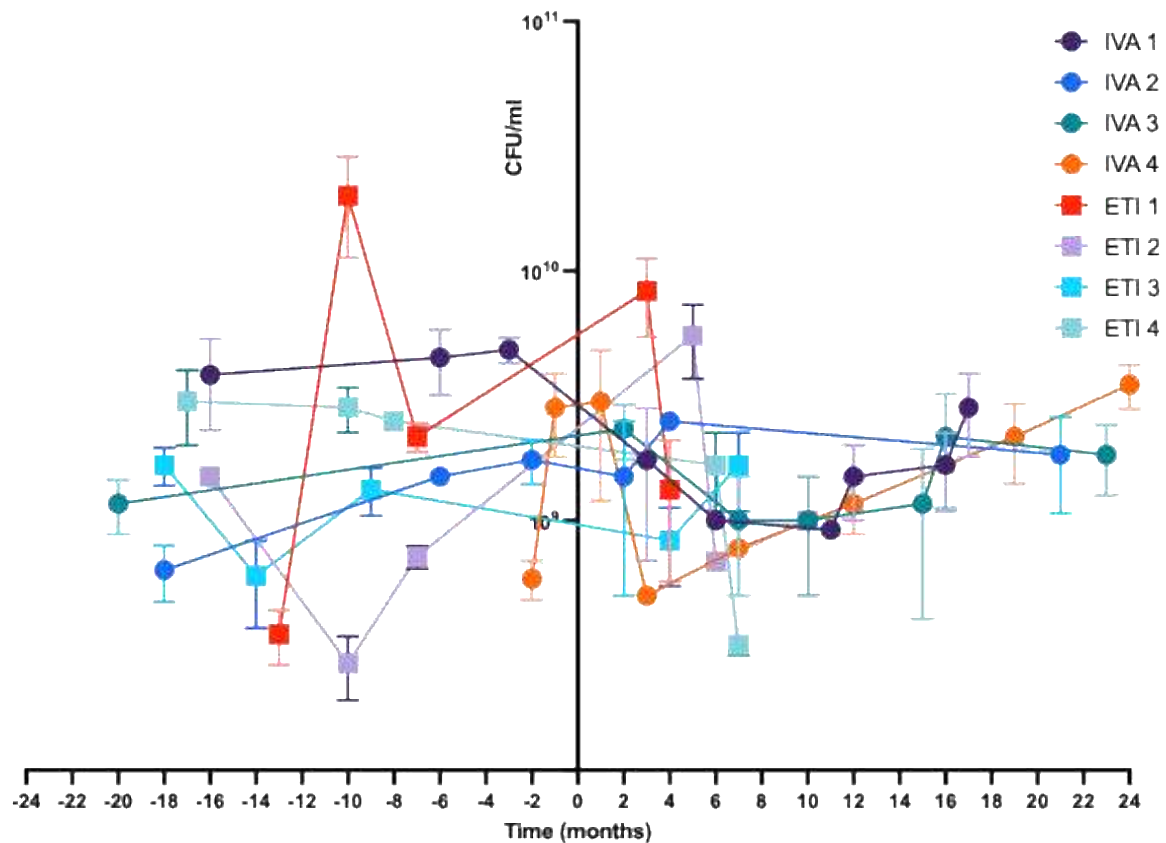


Figure 4.6: Absolute CFU/ml for each patient's isolate over time pre- and post-CFTRm

Absolute colony count (CFU/ml) following 24 hours incubation in broth. Mean of three biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD.

IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

To look for trends and account for baseline differences in growth based on individual Pa species, growth by colony count was examined relative to baseline (figure 4.7). In this representation, each biological repeat is expressed as a proportion of the baseline (earliest) sample for the same biological repeat run, before taking mean[SD] of each relative biological repeat. Again, no trend is identified over time, in this representation of the data.

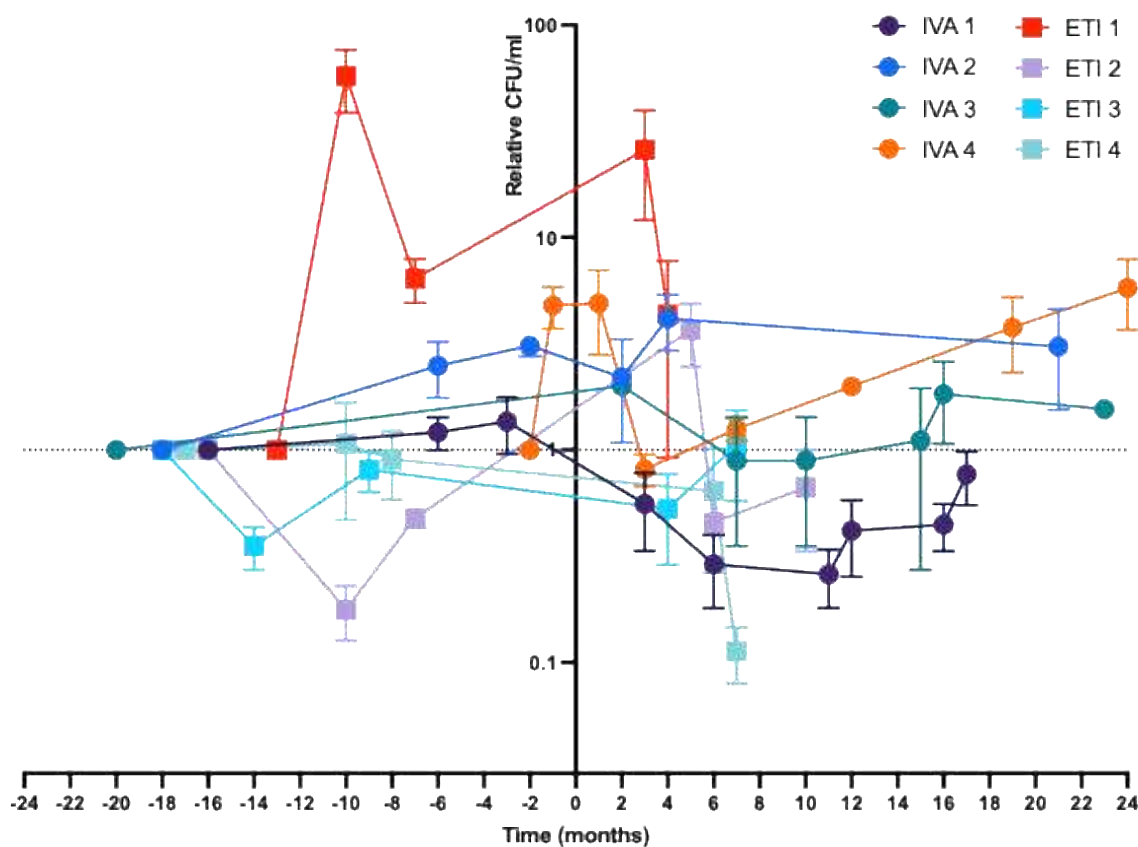


Figure 4.7: CFU/ml for each patient's isolate relative to baseline over time pre- and post-CFTRm

Relative colony count (CFU/ml) following 24 hours incubation in broth, with results for each biological repeat standardised a proportion of first available sample biological repeat (starting relative CFU/ml of 1.0, black dotted line). Mean of three relative biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD on a log₁₀ y-axis scale. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

Figure 4.8 illustrates the median[range] CFU/ml of all isolates for each individual pre-CFTRm compared the same post-CFTRm. No significant difference is seen.

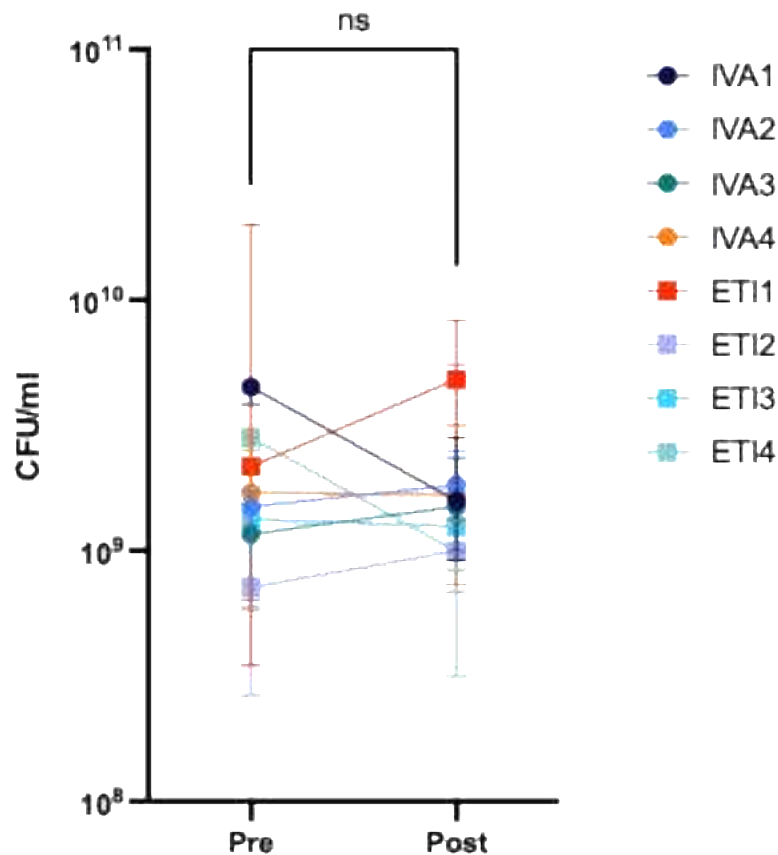


Figure 4.8: Pooled CFU/ml for all isolates pre- compared to all isolates post-CFTRm

Median CFU/ml of each individual's isolates pre-drug initiation compared to all those post-drug initiation onward, with errors bars indicating range. As some patients had <5 isolates either pre- or post-CFTRm, IQR is not presented by GraphPad Prism. Comparison with non-parametric paired t-test (Wilcoxon), ns=non-significant.

There is disparity between some findings as measured by OD₆₀₀ and colony count. Table 4.3 illustrates corresponding raw OD₆₀₀ and CFU/ml values for each biological repeat, and calculated coefficient of variance (%), with a mean coefficient of <10% representing good concordance of repeats. Mean coefficient of variance for measurement of CFU/ml and OD₆₀₀ was 1.4% and 11.8% respectively.

Patient /Month	OD600			Coeff Var %	CFU/ml			Coeff Var %
	BR1	BR2	BR3		BR1	BR2	BR3	
IVA1								
-16	0.74	0.798	0.731	4.8	2.5E+09	3.5E+09	5.5E+09	1.8
-6	0.715	0.866	0.741	10	3E+09	5E+09	5.5E+09	1.5
-3	0.7	0.653	0.685	3.5	4.5E+09	4.5E+09	5.5E+09	0.52
3	0.664	0.781	0.612	13	1E+09	2.5E+09		3.1
6	0.593	0.715	0.697	9.9	1E+09	1E+09	1E+09	0
*12	0.685	0.653	0.7	3.5	8.5E+08	9E+08	1E+09	0.4
12	0.72	0.657	0.639	6.3	1.5E+09	1E+09	2E+09	1.7
16	0.421	0.452	0.454	4.2	1E+09	2E+09	2E+09	1.9
17	0.48	0.503	0.808	31	2.5E+09	2E+09	4E+09	1.6
IVA2								
-18	0.556	0.797	0.555	22	4.5E+08	7E+08	7.5E+08	1.4
-6	0.279	0.322	0.325	8.3	1.5E+09	1.5E+09	1.5E+09	0
-2	0.322	0.293	0.323	5.4	1.5E+09	2E+09		0.96
2	0.336	0.346	0.352	2.3	5E+08	1.5E+09	2.5E+09	3.9
4	0.346	0.404	0.475	16	2.5E+09	2.5E+09	2.5E+09	0
21	0.323	0.529	0.311	32	2E+09	1E+09	2.5E+09	2.2
IVA3								
-20	0.797	0.617	0.698	13	1E+09	1E+09	1.5E+09	1.1
2	0.593	0.556	0.662	8.9	2E+09	2E+09	3E+09	1.1
7	0.483	0.487	0.479	0.83	5E+08	1.5E+09	1E+09	2.7
*10	0.676	0.63	0.931	22	5E+08	1.5E+09	1E+09	2.7
15	0.984	0.838	0.806	11	1E+09	2E+09	5E+08	3.3
15	0.413	0.764	0.704	30	1E+09	2.5E+09	3E+09	2.8
23	0.457	0.45	0.484	3.9	1.5E+09	1.5E+09	2.5E+09	1.4
IVA4								
-2	0.599	0.587	0.628	3.5	5E+08	5.5E+08	7E+08	0.86
-1	0.966	0.627	0.639	26	2.5E+09	2E+09	4E+09	1.60
1	0.454	0.464	0.525	8	1.5E+09	2.5E+09	5E+09	2.8
3	0.572	0.578	0.487	9.3	Excl.	5E+08	5E+08	0
7	Excl.	0.681	0.618	6.9	6.5E+08	7.5E+08	8E+08	0.52
12	0.7	0.767	0.676	6.6	1E+09	1E+09	1.5E+09	1.1
19	0.877	0.705	0.747	12	1.5E+09	3E+09	2E+09	1.6
24	Excl.	0.896	1.031	9.9	2.5E+09	4E+09	3E+09	1.1
ETI1								
-13	0.196	0.332	0.315	26	2.5E+08	4E+08	4E+08	1.4
-10	0.387	0.367	0.362	3.6	1.5E+10	1.5E+10	3E+10	1.7
-7	0.255	0.393	0.313	22	2E+09	2.5E+09	2E+09	0.6
3	0.888	0.877	0.928	3	1E+10	1E+10	5E+09	1.8
4	0.427	0.394	0.423	4.3	2E+09	5E+08	1.5E+09	3.5
ETI2								
-16	0.35	0.295	0.279	12	1.5E+09	1.5E+09	1.5E+09	0
-10	0.178	0.198	0.17	7.9	2E+08	2.5E+08	3.5E+08	1.5
-7	0.361	0.367	0.321	7.2	6.5E+08	7E+08	8E+08	0.52
5	0.349	0.311	0.4	13	4E+09	5E+09	7.5E+09	1.4
6	0.546	0.566	0.506	5.7	6.5E+08	7E+08	7E+08	0.21
10	0.439	0.316	0.245	29	1.5E+09	1E+09	5E+08	2.7
ETI3								
-18	0.42	0.327	0.345	14	1.5E+09	1.5E+09	2E+09	0.78
-14	0.326	0.477	0.356	21	4E+08	5.5E+08	8.5E+08	1.9
-9	0.306	0.377	0.308	12	1E+09	1.5E+09	1.5E+09	1.1
4	0.356	0.26	0.28	17	1E+09	1E+09	5E+08	2
7	0.383	0.302	0.297	15	2E+09	2E+09	1E+09	1.9
ETI4								
-17	0.41	0.309	0.341	15	2E+09	3E+09	4E+09	1.6
-10	0.323	0.325	0.407	14	3.5E+09	2.5E+09	2.5E+09	0.89
-7	0.605	0.596	0.545	5.6	2.5E+09	2.5E+09	2.5E+09	0
6	0.955	0.845	0.824	8	2E+09	2E+09	1E+09	1.9
6	0.742	0.727	0.688	3.9	3E+08	3E+08	3.5E+08	0.45
			Mean	11.8			Mean	1.4

Table 4.3: Comparison of biological repeats of OD₆₀₀ and CFU/ml for each isolate

Table of raw OD₆₀₀ and CFU/ml results for all biological repeats for each patient and every isolate examined, to demonstrate biological repeat variability. BR = biological repeat. Coeff Var = Coefficient of Variance (%), quoted for each set of BRs for OD and log transformed CFU results, with mean Coeff Var reported at the end of the column. Blank cells indicated where no CFU/ml was reported (IVA1 +3month BR3 and IVA2 -2 month BR3). Excl. = excluded values due to extreme outlying value (see methods).

To examine this further, mean[SD] for corresponding OD₆₀₀ and CFU/ml values for each sample were plotted for each patient, and then pooled to perform simple linear regression analysis and correlation across all values, as shown in figure 4.9. There is variable but generally poor correlation for each isolate between sample OD₆₀₀ and CFU/ml. When all patients are grouped, spearman's rank correlation coefficient is $r = 0.18$, with $p=0.22$.

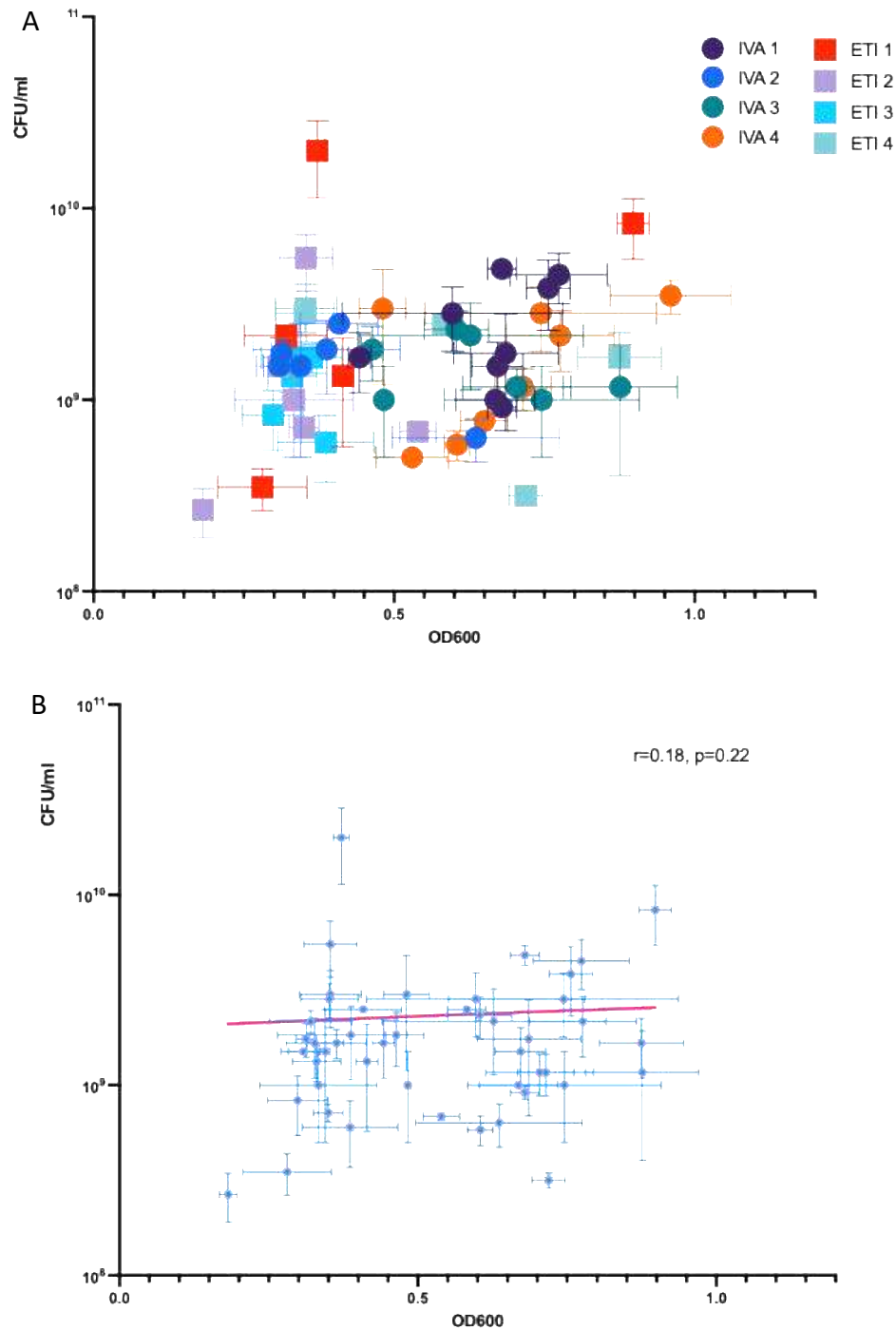


Figure 4.9: Correlation of OD₆₀₀ and CFU/ml for each isolate

A: Each individual's mean[SD] OD₆₀₀ plotted against mean[SD] CFU/ml for each sample analysed, with mean of three biological repeats (except for IVA 4, as previous) consisting of three technical repeats. Error bars indicate SD on a log₁₀ y-axis scale. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor. **B:** Pooled results across all individuals, with simple linear regression (line in pink) indicating no linear relationship, as expected. Poor correlation indicated by spearman's rank correlation coefficient (*r*) of 0.18.

As OD₆₀₀ is the surrogate marker for growth, colony counting is a direct measure and should reflect the true changes in growth in this experiment and no trend is seen. Measurement of CFU/ml is used for any standardisation of results by growth for remaining results analysis.

4.3.4. Relative fluorescent intensity

RFI values were measured using a plate reader, with raw RFI values reported relative to control samples. Typically, there is variation in the amount of pyoverdine each isolate produces. It is also expected that pyoverdine production will be dependent on the number of CFU/ml in any sample. Table 4.4 illustrates corresponding raw RFI values for each biological repeat, and calculated coefficient of variance (%). Mean coefficient of variance for all repeats was 25.7%, indicating poor concordance of biological repeats.

Patient/ Month	Raw RFI			Coeff Var %
	BR1	BR2	BR3	
IVA1				
-16	70667	67020	65042	4.2
-6	73887	77535	56524	16
-3	71001	47173	52795	22
3	53097	69297	58282	14
6	54546	52934	50712	3.7
*12	68193	51273	60338	14
12	68868	59917	47885	18
16	72197	63850	57760	11
17	72413	51465	113995	40
IVA2				
-18	54886	75379	52398	21
-6	34320	30038	27097	12
-2	49470	41236	38047	14
2	64863	72361	61575	8.3
4	49986	54510	67829	16
21	67752	106947	60030	32
IVA3				
-20	94725	54535	62047	30
2	34506	31500	57607	35
7	31236	46501	92954	56
*10	69697	45740	73187	24
15	72893	53792	55953	17
15	96167	72927	56646	26
23	32339	60527	108997	58
IVA4				
-2	69073	52997	47700	20
-1	81814	47993	49567	32
1	51125	39031	48202	14
3	56831	39566	30718	31
7	112379	64741	74492	30
12	80809	70457	65331	11
19	95540	65714	72489	20
24	50525	118999	124017	42
ETI1				
-13		28823	26643	5.6
-10	25071	18495	21490	15
-7	10952	17760	9007	37
3	88574	87747	94585	4.1
4	30119	26593	32029	9.3
ETI2				
-16	42683	32291	31080	18
-10	23564	22159	18755	12
-7	41769	28810	12114	54
5	31340	19117	20009	29
6	974	0	0	173
10	77764	117457	117457	22
ETI3				
-18	40929	31990	44031	16
-14	26749	17166	6067	62
-9	117457	48586	38368	63
4	43348	40454	44901	5.3
7	50309	44739	68408	23
ETI4				
-17	37870	39597	41768	4.9
-10	35406	27401	40525	19
-7	66237	47166	45706	22
6	117438	77594	84719	23
6	54667	53245	51373	3.1
			Mean	25.7

Table 4.4: Comparison of biological repeats of RFI for each isolate

Table of RFI results for all biological repeats for each patient and every isolate examined, to demonstrate biological repeat variability. BR = biological repeat. Coeff Var = Coefficient of Variance (%), quoted for each set of BRs for raw RFI values.

Figure 4.10 shows the correlation, isolate by isolate, of CFU/ml and raw RFI value with associated spearman rank coefficient (r). There is high variability in r, probably as some isolates are higher pyoverdine producers than others. In view of this variability it was confirmed, as per methods above, that RFI would be adjusted for CFU/ml in corresponding sample. As discussed in results above, measurement of CFU/ml biological repeat 1 of patient IVA4 demonstrated outlying results ($>1.5 \times$ IQR above upper quartile) so was excluded in any further analysis.

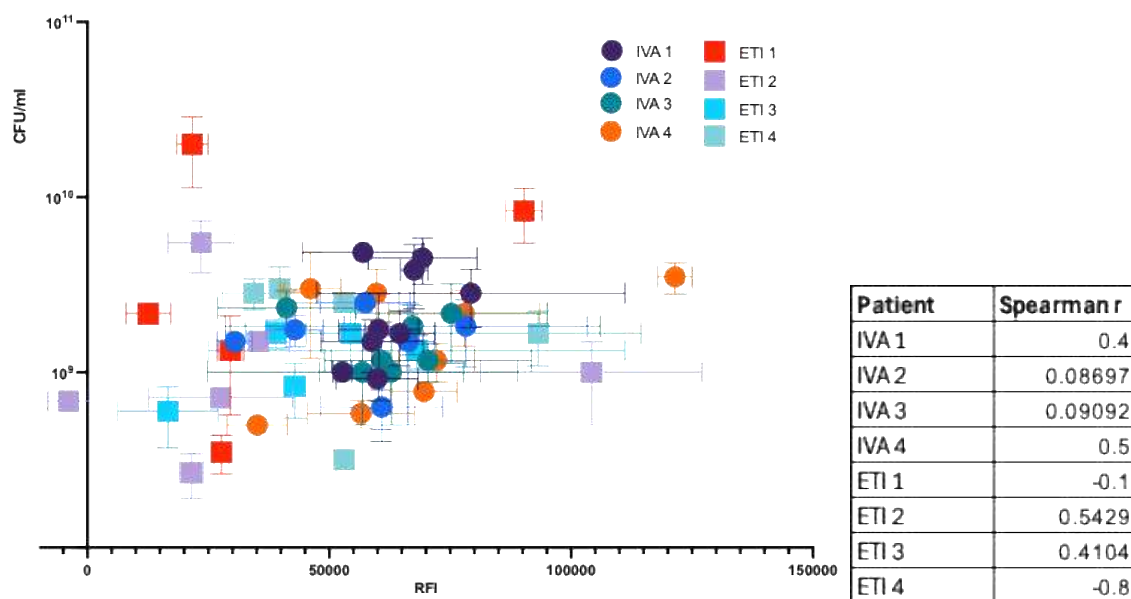


Figure 4.10: Correlation of RFI and CFU/ml for each isolate

Each individual's mean[SD] raw RFI plotted against mean[SD] CFU/ml for each sample analysed, with mean of three biological repeats (except for IVA 4, as previous) consisting of three technical repeats. Error bars indicate SD on a log₁₀ y-axis scale. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor. Table indicates associated Spearman rank correlation coefficients for each patient.

CFU adjusted RFI after 24 hours incubation for each sample at each timepoint was examined, as illustrated in figure 4.11. No specific trends in mean CFU adjusted RFI is seen over time for any patient.

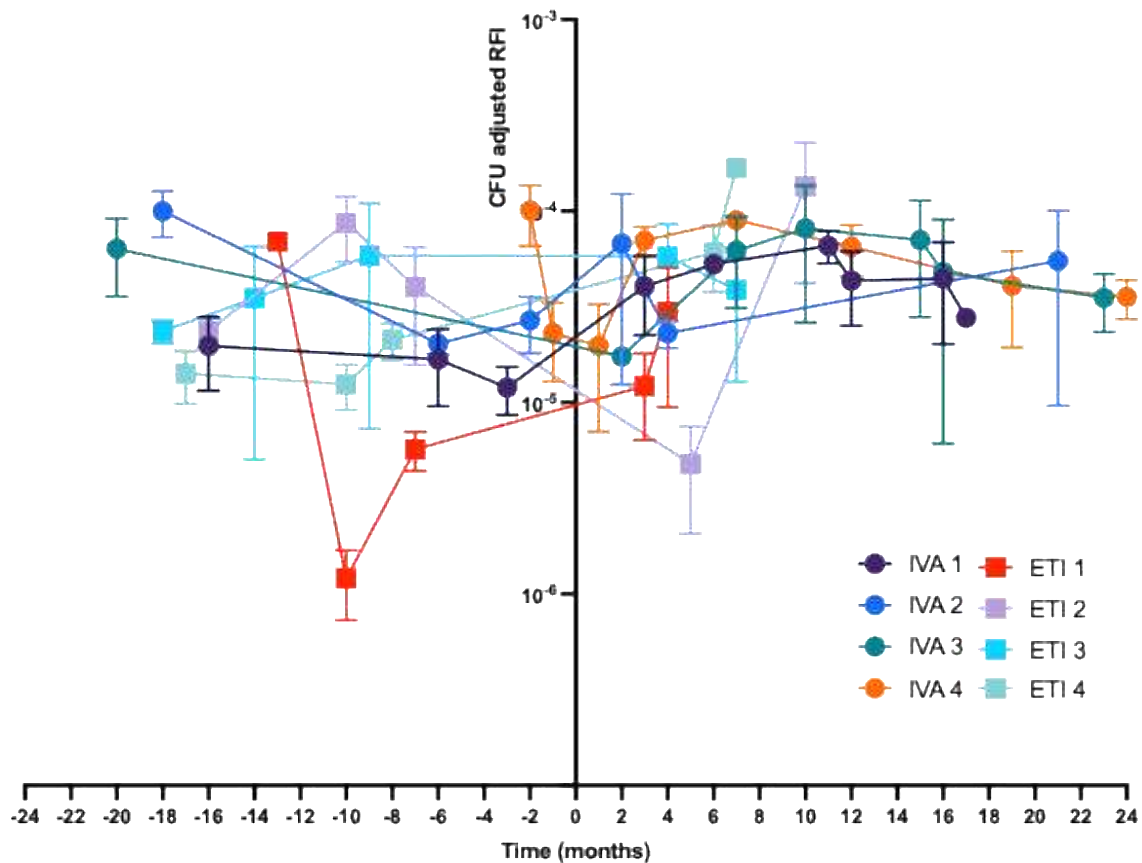


Figure 4.11: Absolute CFU adjusted RFI for each patient's isolate over time pre- and post-CFTRm

Absolute RFI adjust to CFU/ml for each isolate following 24 hours incubation in broth. Mean of three biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

To look for trends and account for baseline differences and isolate-dependent potential to produce pyoverdine, CFU adjusted RFI was examined relative to baseline (figure 4.12). In this representation, each biological repeat is expressed as a proportion of the baseline (earliest) sample for the same biological repeat run, before taking mean[SD] of each relative biological repeat. Again, no trend is identified over time, in this representation of the data.

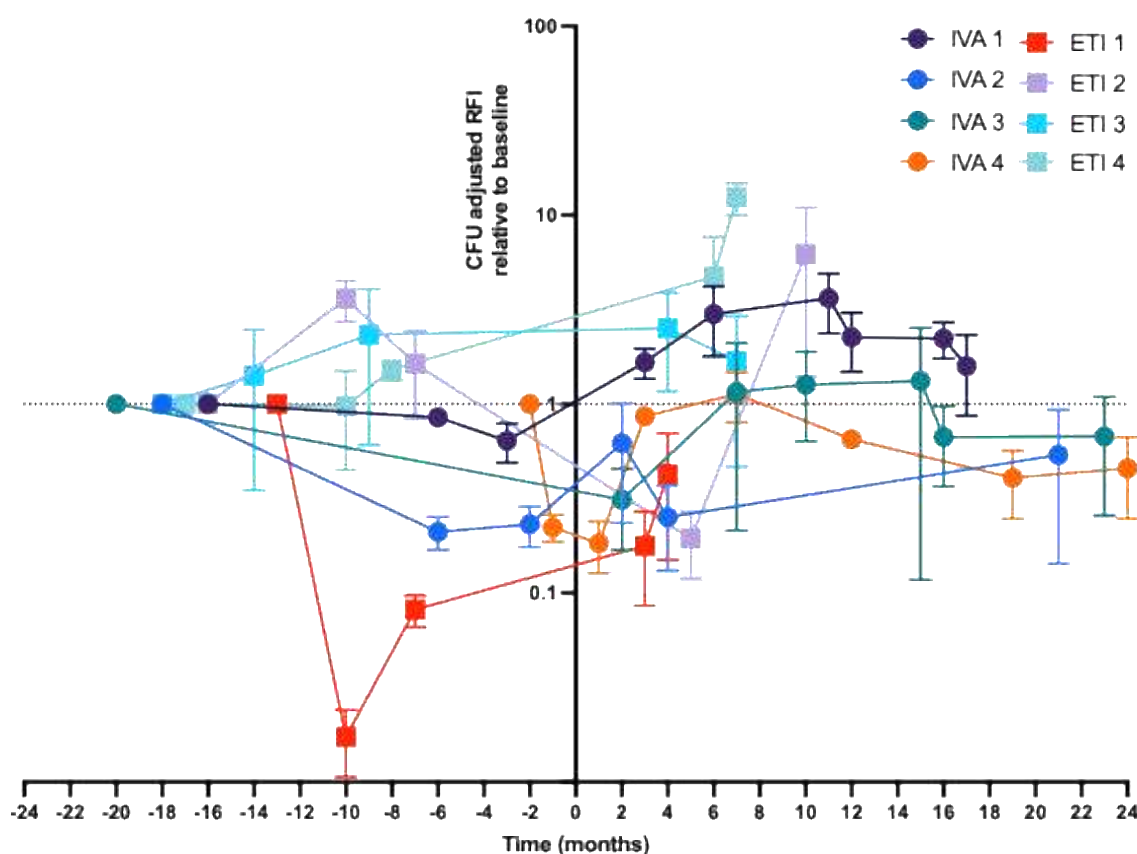


Figure 4.12: CFU adjust RFI for each patient's isolate relative to baseline over time pre- and post-CFTRm

Relative CFU adjusted RFI following 24 hours incubation in broth, with results for each biological repeat standardised as a proportion of first available sample biological repeat starting relative CFU adjusted RFI of 1.0, black dotted line). Mean of three relative biological repeats (except timepoints at 3, 7 and 24 months for IVA 4, where there are two biological repeats), each consisting of three technical repeats, is plotted for each isolate with error bars indicating SD on a log10 y-axis scale. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

Figure 4.13 illustrates the median[range] CFU adjusted RFI of all isolates for each individual pre-CFTRm compared the same post-CFTRm. No significant difference is seen.

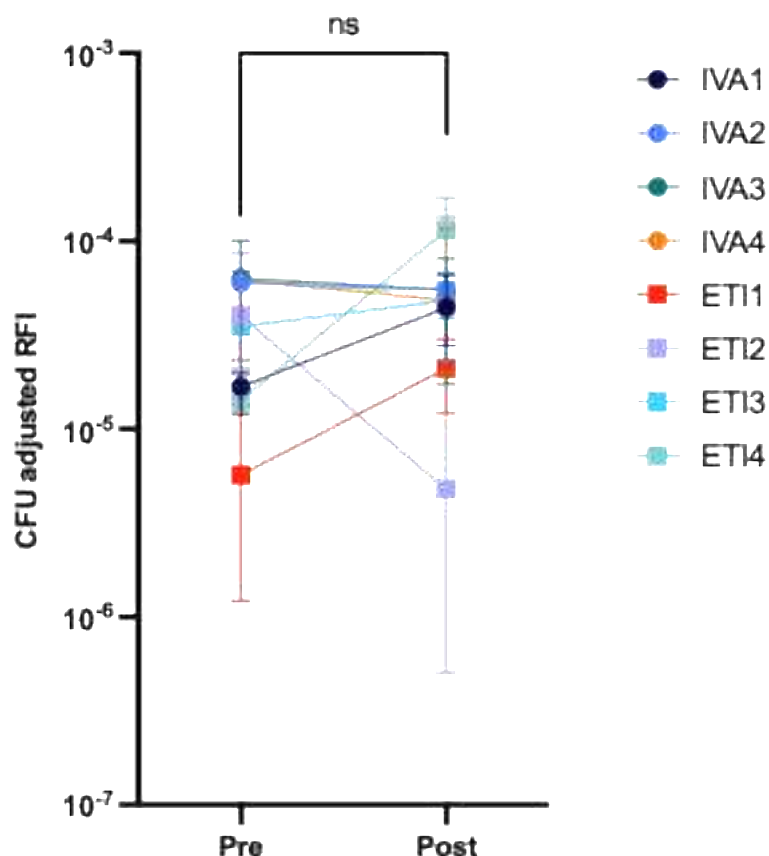


Figure 4.13: Pooled CFU adjusted RFI for all isolates pre- compared to all isolates post-CFTRm

Median CFU adjusted RFI of each individuals isolates pre-drug initiation compared to all those post-drug initiation onward, with errors bars indicating range. As some patients had <5 isolates either pre- or post-CFTRm, IQR is not presented by GraphPad Prism. Comparison with non-parametric paired t-test (Wilcoxon), ns=non-significant.

4.3.5. Muroid phenotype

Muroid phenotype is reported subjectively (visible/not visible) on Pa cultures in RBH laboratory prior to being made available for storage in SRC-PAPA repository. Degrees of muroidy can be assessed after overnight growth on congo red agar and was subjectively scored as --, -, + and ++, using a scoring system developed for this assay (methods, section 2.2.1, and repeated figure 2.2 below for ease of reference when interpreting scoring).

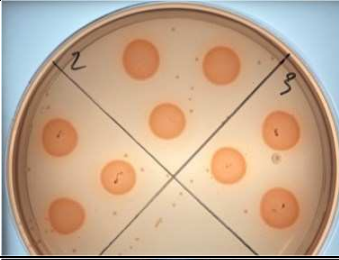
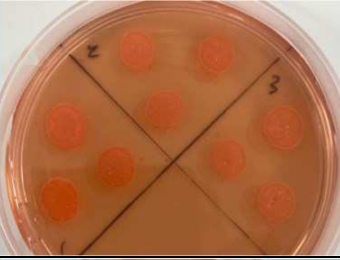
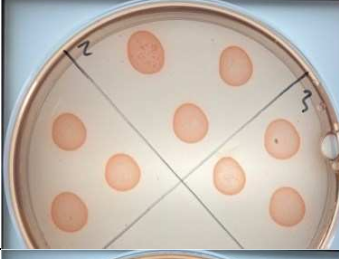
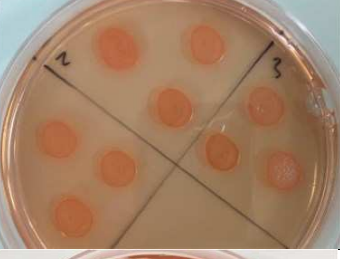
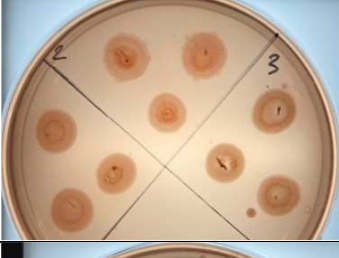
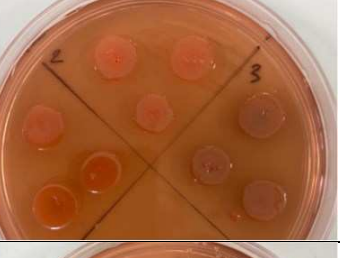
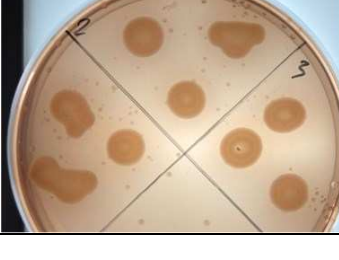
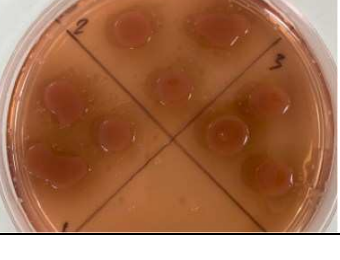
Score	Below:	Above:	Description
- -			Bright pink colonies within the spot Dry/desiccated/cracked surface viewed from above
-			Pale pink colonies within the spot Mostly dry, but not desiccated/cracked, surface viewed from above
+			Areas of dark staining within the spot Shiny, wet surface around the edge of the spot, viewed from above
++			Dark staining throughout the spot and spreading beyond original spot boundary Shiny, wet surface all over the spot, loss of definition of circular spot shape, viewed from above

Figure 2.2 (repeated): Subjective scoring system for mucoidy on congo red agar

A subjective scoring system was developed to grade degrees of mucoidy for Pa using congo red agar, with methodology development discussed in methods (section 2.2.1.)

Scoring was performed by a single operator who was unblinded to scores of technical and biological repeats, with samples photographed for secondary, blinded evaluation if trends emerged. As previous, three biological repeats were performed for each isolate, each biological repeat being the median of three technical repeats. Median scores for each biological repeat were standardised as a change from baseline (earliest) isolate, where relative mucoid score = 0 and change in median score was calculated (figure 4.14). Raw scores of technical and biological repeats are shown (table 4.5)

All technical repeats for each isolate were scored the same across each biological repeat. No specific trend across all 8 patients was identified. Two patients (ETI 1 and 4) saw reduction in relative mucoidy score over time, starting prior to CFTRm start date. One patient (ETI 2) showed reduction in relative mucoid score after 6 months of ETI. Two patients (IVA 3 and 4) showed a variable but generally increased relative mucoidy score after starting IVA. Three patients (IVA 1, 2 and ETI 3) showed static mucoid scores over time.

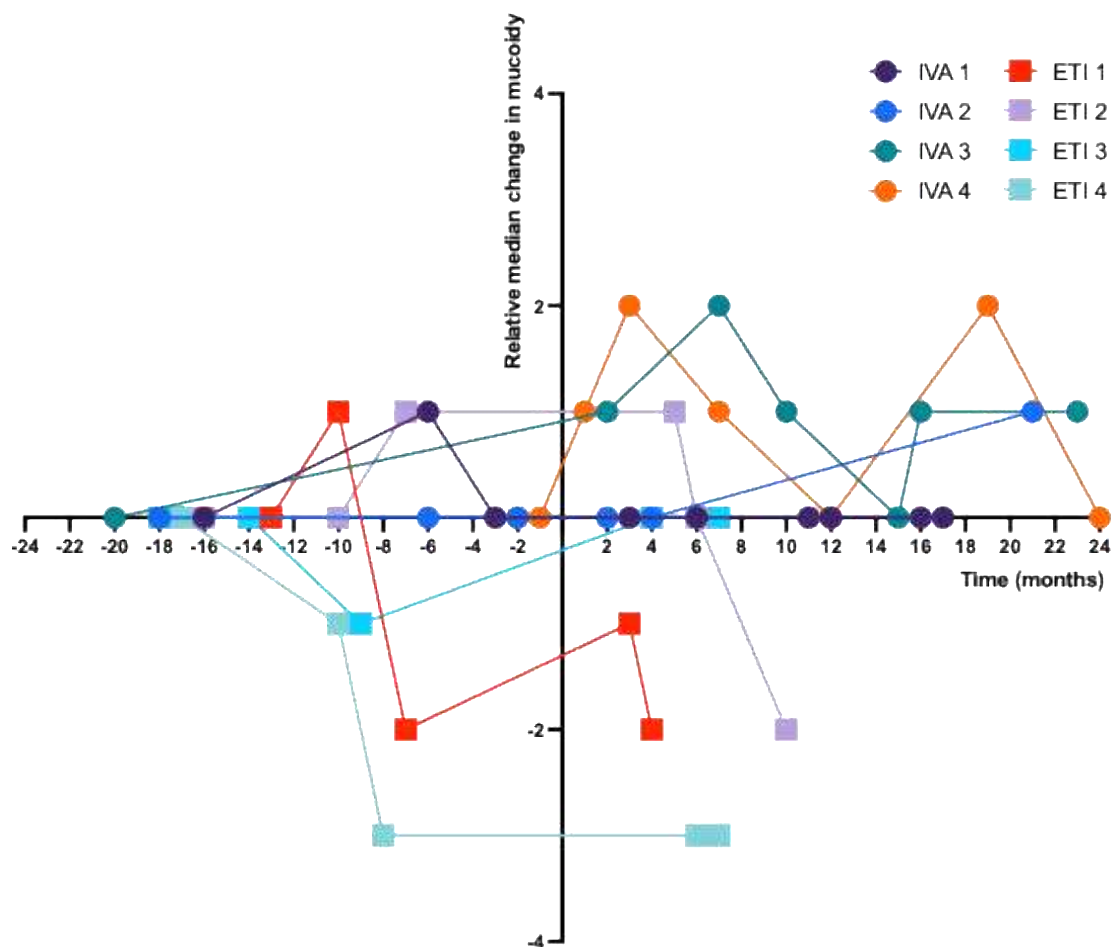


Figure 4.14: Subjective mucoidy score for each patient's isolate relative to baseline over time pre- and post-CFTRm

Change in median mucoid score over time, with results standardised to first available isolate. Median of triplicate repeat plotted for each isolate. IVA = Ivacaftor, ETI = Elexacaftor/Tezacaftor/Ivacaftor.

Patient/ Months	BR1			BR2			BR3			Median of TRs			Median of BRs	Median in numbers	Mucoidy change from baseline
	TR1	TR2	TR3	TR1	TR2	TR3	TR1	TR2	TR3	BR1	BR2	BR3			
IVA1															
-16	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
-6	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
-3	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
3	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
6	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
12	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
12	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
16	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
17	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
IVA2															
-18	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
-6	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
-2	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
2	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
4	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
21	++	++	++	++	++	++	++	++	++	++	++	++	++	3	1
IVA3															
-20	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0
2	+	+	+	+	+	+	+	+	+	+	+	+	+	2	1
7	++	++	++	++	++	++	++	++	++	++	++	++	++	3	2
10	+	+	+	+	+	+	+	+	+	+	+	+	+	3	1
15	-	-	-	-	-	-	-	-	-	-	-	-	-	2	0
15	+	+	+	+	+	+	+	+	+	+	+	+	+	2	1
23	+	+	+	+	+	+	+	+	+	+	+	+	+	2	1
IVA4															
-2	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
-1	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
1	—	—	—	-	-	-	+	+	+	-	-	+	-	1	1
3	+	+	+	+	+	+	+	+	+	+	+	+	+	2	2
7	-	-	-	-	-	-	-	-	-	-	-	-	-	1	1
12	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
19	+	+	+	+	+	+	+	+	+	+	+	+	+	2	2
24	—	—	—	—	—	—	—	—	—	—	—	—	—	0	0
ETI 1															
-13	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
-10	++	++	++	++	++	++	++	++	++	++	++	++	++	3	1
-7	—	—	—	—	—	—	—	—	—	—	—	—	—	0	-2
3	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-1
4	—	—	—	—	—	—	—	—	—	—	—	—	—	0	-2
ETI 2															
-16	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
-10	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
-7	++	++	++	++	++	++	++	++	++	++	++	++	++	3	1
5	++	++	++	++	++	++	++	++	++	++	++	++	++	3	1
6	+	+	+	+	+	+	+	+	+	+	+	+	+	2	0
10	—	—	—	-	-	-	—	—	—	—	-	—	—	0	-2
ETI 3															
-18	++	++	++	++	++	++	++	++	++	++	++	++	++	3	0
-14	++	++	++	++	++	++	++	++	++	++	++	++	++	3	0
-9	+	+	+	+	+	+	+	+	+	+	+	+	+	2	-1
4	++	++	++	++	++	++	++	++	++	++	++	++	++	3	0
7	+	+	+	++	++	++	++	++	++	+	++	++	++	3	0
ETI 4															
-17	++	++	++	++	++	++	++	++	++	++	++	++	++	3	0
-10	+	+	+	++	++	++	+	+	+	+	++	+	+	2	-1
-7	—	—	—	—	—	—	—	—	—	—	—	—	—	0	-3
6	—	—	—	-	-	-	—	—	—	—	-	—	—	0	-3
6	—	—	—	—	—	—	—	—	—	—	—	—	—	0	-3

Table 4.5: Comparison of technical and biological repeats of subjective mucoidy score for each isolate

Table of raw mucoid scores for all technical and biological repeats for each patient and every isolate examined, to demonstrate consistency of scoring. TR = technical repeat, BR = biological repeat. All scoring performed by single, unblinded scorer with technical repeats scored on the same day and biological repeats performed on different days. Subjective scores of --/-/+/>++ allocated as per scoring system in figure 2.2 and outlines in methods, section 2.2.1.

4.4. Results - Modelling pre- and post- highly effective modulator treatment CF airway changes and their impact on *Pseudomonas aeruginosa* phenotype.

4.4.1. Stability of pH during passage

In generating the hypoxic environments, oxygen is chemically displaced for carbon dioxide, which could end up in solution within the broth and may impact pH over time. To provide context for interpretation of results attributable to oxygen state and pH, the pH of all broth aliquots (tests and control sample) was measured after 24 hours incubation, on the second day of passage. Adjusted broth pH is not sustained over time in both normoxic and hypoxic conditions, and controls and bacteria containing test samples are affected differently (figure 4.15).

In normoxic conditions, the mean[SD] pH of test samples (containing bacteria) after 24hrs passage increased from: pH6.5 to 7.5[0.34], $p < 0.001$; pH7.4 to 7.9[0.21], $p < 0.001$; pH8.0 did not change. In normoxic controls: pH6.5 increased to 6.7[0.08], $p < 0.05$; pH 7.4 and pH8.0 did not change (8.0[0.1]).

In hypoxic conditions, the mean[SD] pH of test samples after 24hrs passage from: pH6.5 increased to 7.0[0.20], $p < 0.001$; pH7.4 decreased to 7.3[0.12], $p < 0.01$; pH8.0 decreased to 7.5[0.10], $p < 0.001$. In hypoxic controls: pH6.5 did not change (6.5[0.05]); pH 7.4 decreased to 7.0[0.05], $p < 0.001$; and pH8.0 decreased to 7.2[0.20], $p < 0.01$.

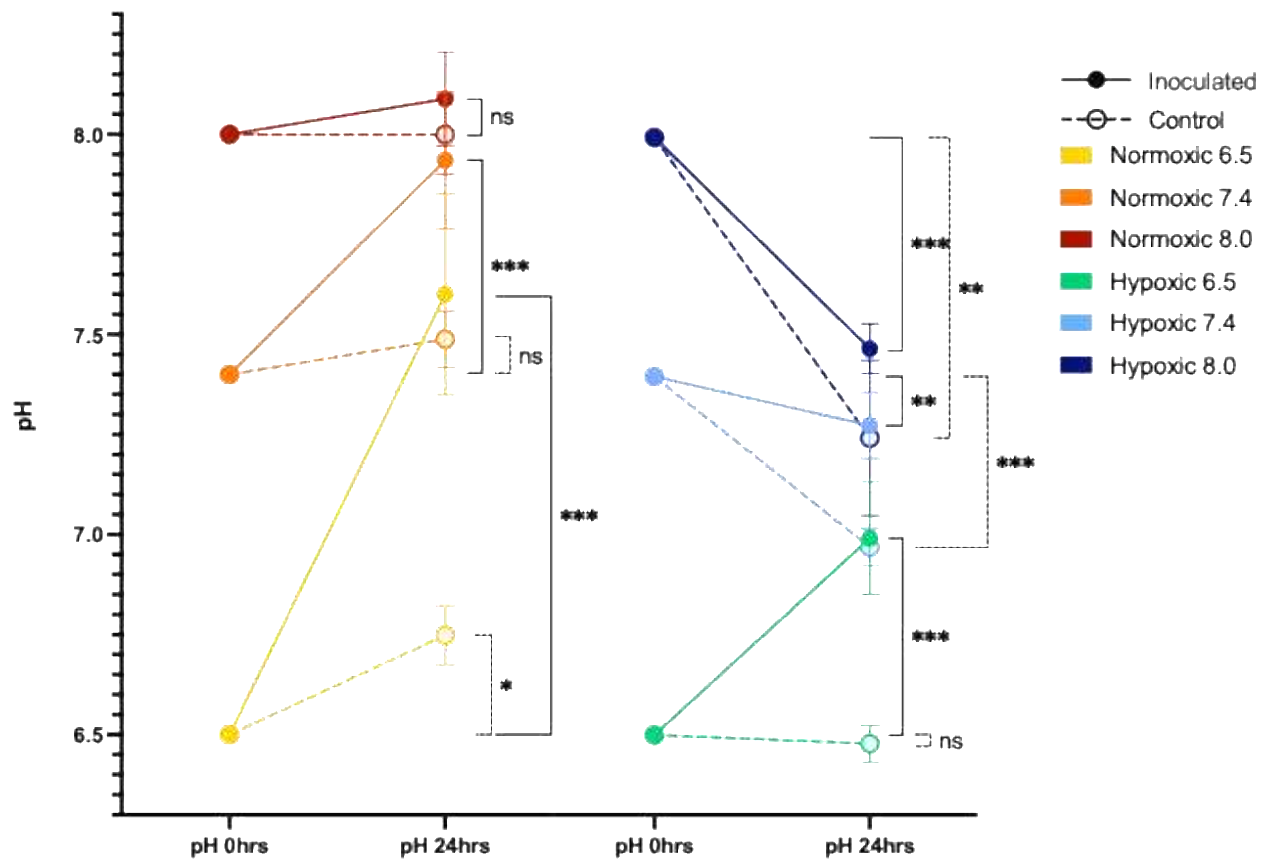


Figure 4.15: Changes in broth pH after 24 hours incubation at different oxygen states

Starting and end pH of broth after second day of passage for normoxic (left) and hypoxic (right) conditions, with mean of triplicate repeats and error bars indicating SD. Solid lines show test samples (with bacterial growth) and dashed lines show controls (plain broth). Statistical analysis with paired t-test with Bonferroni comparing starting pH and pH 24 hours later, on the second day of passage, with solid lines for test sample comparisons, and dashed lines for control comparisons (***= $p < 0.001$, **= $p < 0.01$, *= $p < 0.05$, ns= not significant). In normoxic conditions, the pH increases in Pa containing samples at pH 6.5 and 7.4 over the duration of 24hrs passage, with greatest increase from baseline seen at lower starting pH. In normoxic controls, pH increased from pH 6.5 but did not change at pH 7.4 or 8.0. In hypoxic conditions, for Pa containing samples at starting pH 6.5 the pH increases but at starting pH 7.4 and 8.0, pH decreases over 24hrs passage. In hypoxic controls, pH decreased from pH 8.0 and 7.4 but did not change at pH 6.5.

Examining change in pH from baseline over 24 hours incubation based on the presence of bacteria in broth (controls vs. tests, mean[SD], two-way ANOVA with multiple comparisons) at the same environmental conditions, we see bigger increase in pH when bacteria are present at low pH environments, irrespective of oxygen state: normoxic pH 6.5 $+0.25[0.04]$ vs. $+1.10[0.07]$ ($p<0.01$) and hypoxic pH 6.5 $-0.02[0.02]$ vs. $+0.49[0.03]$ ($p<0.001$). At hypoxic pH 7.4, bacteria present coincided with smaller decrease in pH ($-0.43[0.03]$ vs $-0.12[0.03]$, $p<0.001$).

Examining change in pH from baseline of infected (test) samples over 24 hours incubation based on environmental oxygen state (normoxic vs. hypoxic, mean[SD], two-way ANOVA with multiple comparisons) at the same pH conditions, we see bigger increase in pH at normoxic conditions: pH 6.5 $+1.10[0.07]$ vs. $+0.49[0.03]$ ($p<0.01$), pH 7.4 $+0.53[0.11]$ vs. $-0.012[0.03]$ ($p<0.05$) and pH 8.0 $+0.09[0.09]$ vs. $-0.53[0.02]$ ($p<0.05$).

The results of these experiments need to be interpreted in the context that pH did not stay constant throughout each 24 hour passage process, that presence of bacteria in low pH environment coincided with greater increase in pH, and that hypoxic conditions coincided with smaller increase in pH at starting pH 6.5 and decrease of pH at pH 7.4 and 8.0. Hypoxic state and pH are not independent variables in this process.

4.4.2. Growth and growth rate

Figure 4.16 illustrates median[5th,95th centile] absolute OD₆₀₀ values for three biological repeats, each consisting of three technical repeats, for all isolates, in each condition, at the end of third day of passage. This representation of the data was done for visual purposes only, with statistical analysis below in the grouped data only. A trend for higher OD₆₀₀, indicating more growth, is seen in normoxic compared to hypoxic conditions and irrespective of pH, after 3-day passage, in the majority of isolates. No obvious pattern of different growth between different pH at the same oxygen conditions is seen.

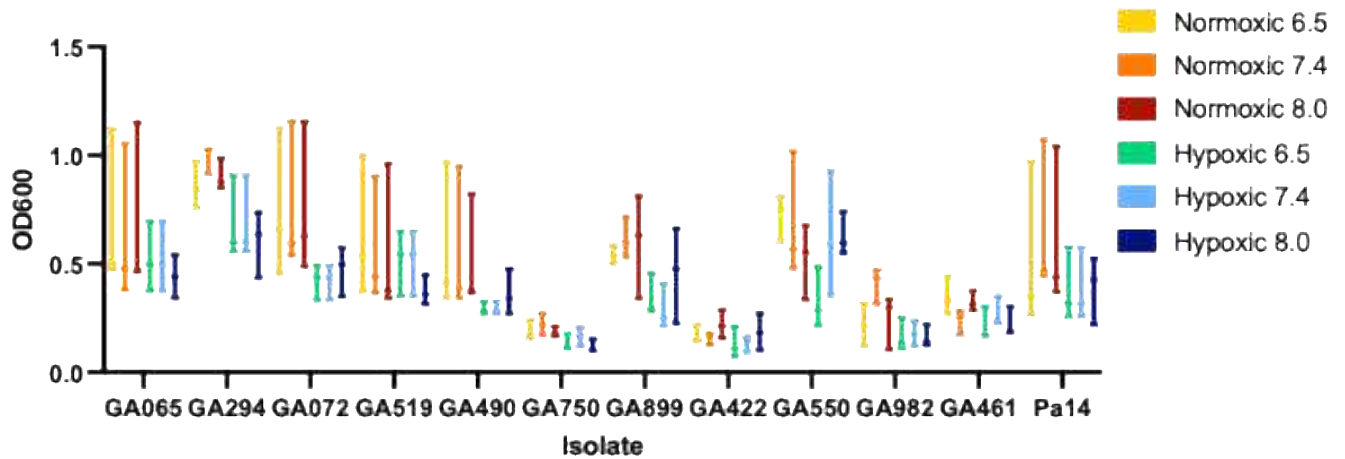


Figure 4.16: Absolute OD₆₀₀ for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference isolate Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Median OD₆₀₀ of three biological repeats, each consisting of the mean of three technical repeats, is plotted with error bars representing 5th and 95th centile (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). A trend for higher OD₆₀₀ (implying more growth over 24 hours incubation) is seen in normoxic conditions compared to hypoxic conditions in most isolates.

Because each isolate has inherently different growth rate, results were standardised as a proportion of the OD₆₀₀ for normoxic neutral pH 7.4 (i.e. typical laboratory conditions and presumed normal airway conditions) growth for each isolate (figure 4.17). A similar pattern is seen with the majority of isolates showing higher relative OD₆₀₀, indicating more growth, after incubation in normoxic than hypoxic conditions, irrespective of pH after 3-day passage. Again, no obvious pattern of different growth between different pH at the same oxygen conditions is seen.

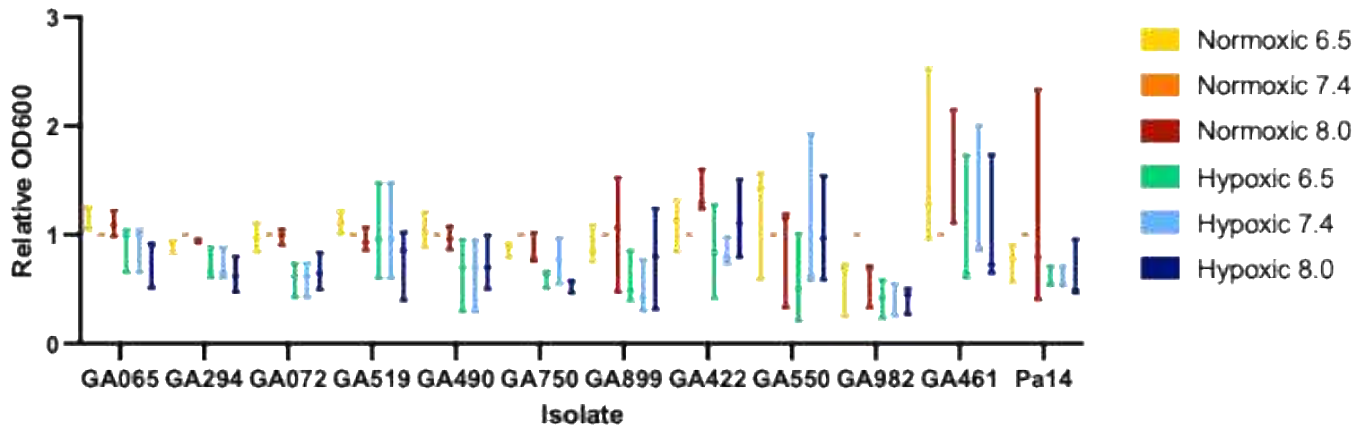


Figure 4.17: Relative OD₆₀₀ for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference strain Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Relative OD₆₀₀ of three biological repeats, each consisting of the mean of three technical repeats, presented as relative to each isolate's OD₆₀₀ at normoxic pH 7.4 conditions. Median OD₆₀₀ is plotted with error bars representing 5th and 95th centile (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). A trend for higher relative OD₆₀₀ (implying more growth over 24hrs incubation) is seen in normoxic conditions compared to hypoxic conditions in most isolates.

Figure 4.18 demonstrates comparison of pooled OD₆₀₀ of each isolate at each condition, relative to pooled OD₆₀₀ for all isolates at normoxic pH 7.4 condition. Relative OD₆₀₀ was lower in all hypoxic conditions (median[IQR]: pH 6.5, 0.64[0.51,0.88], $p < 0.001$; pH 7.4, 0.70[0.55,0.93], $p < 0.01$; pH 8.0, 0.67[0.50,0.95], $p < 0.01$; Wilcoxon test) compared to normoxic pH 7.4. There was no difference in relative OD₆₀₀ by pH, within the two oxygen conditions.

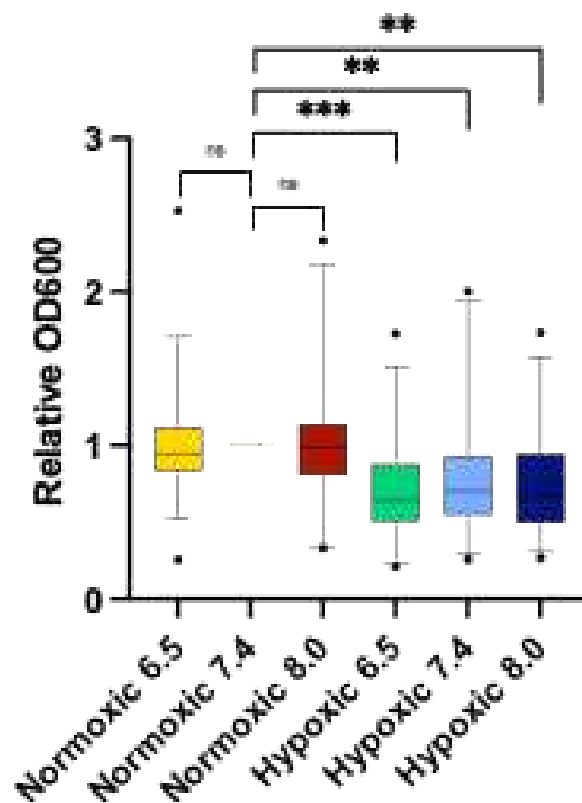


Figure 4.18: Pooled OD₆₀₀ for all isolates at each condition, relative to results for normoxic pH 7.4

Box and whisker plot of OD₆₀₀ for all isolates at each condition, relative to OD₆₀₀ for all isolates at normoxic pH 7.4 condition, with whiskers indicating 5th and 95th centiles and outliers as black dots. Comparisons by Wilcoxon test to normoxic pH 7.4 conditions (ns=not significant, **= $p < 0.01$, ***= $p < 0.001$), with Bonferroni correction applied for multiple t-tests. Hypoxic conditions show lower relative OD₆₀₀ (and implied growth) at 24hrs incubation, irrespective of pH. There was no difference in relative OD₆₀₀ between different pHs within each oxygen state.

Similar trends were less evident when assessing end point growth after 3-day passage with colony counting. Figure 4.19 illustrates median absolute colony count values (CFU/ml) for three biological repeats, each consisting of three technical repeats, for all isolates, in each condition, at the end of third day of passage. This representation of the data was done for visual purposes only, with statistical analysis below in the grouped data. No consistent trends of differential growth by oxygen or pH state are identifiable.

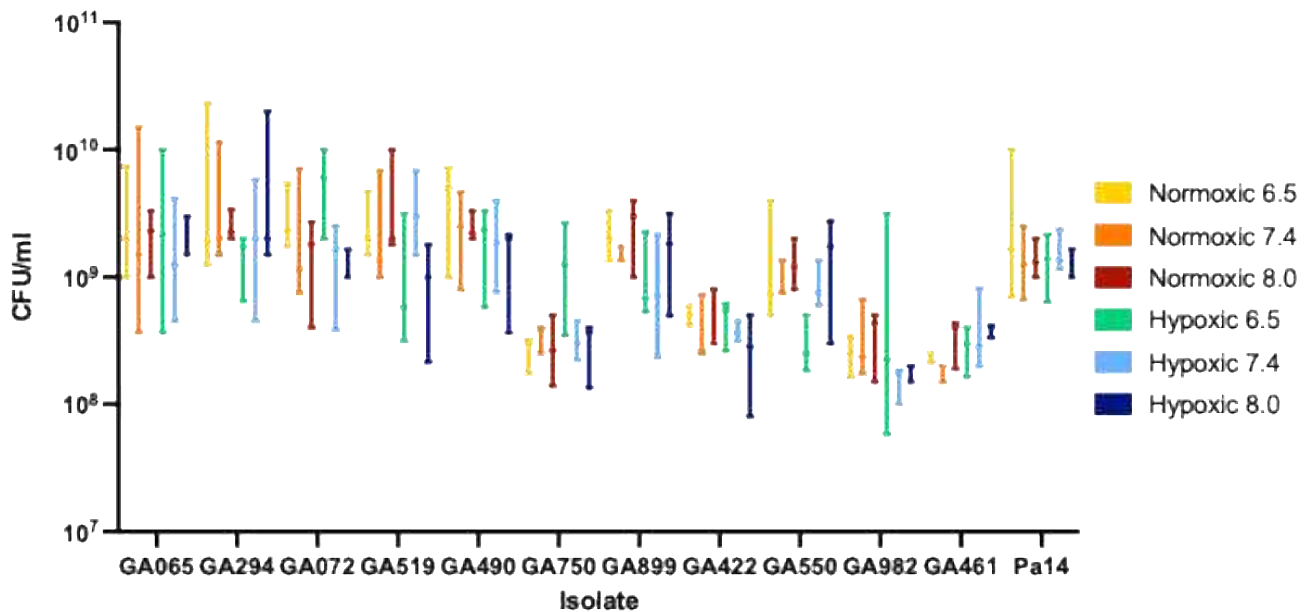


Figure 4.19: Absolute CFU/ml for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference strain Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Median CFU/ml of three biological repeats, each consisting of the mean of three technical repeats, is plotted with error bars representing 5th and 95th centile (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). No consistent trends of differential growth by oxygen or pH state are identifiable across the different isolates.

Again, because each isolate has inherently different growth rate, results were standardised as a proportion of the CFU/ml for normoxic neutral pH 7.4 (i.e. typical laboratory conditions) growth for each isolate (figure 4.20). No consistent trends of differential growth by oxygen or pH state are identifiable.

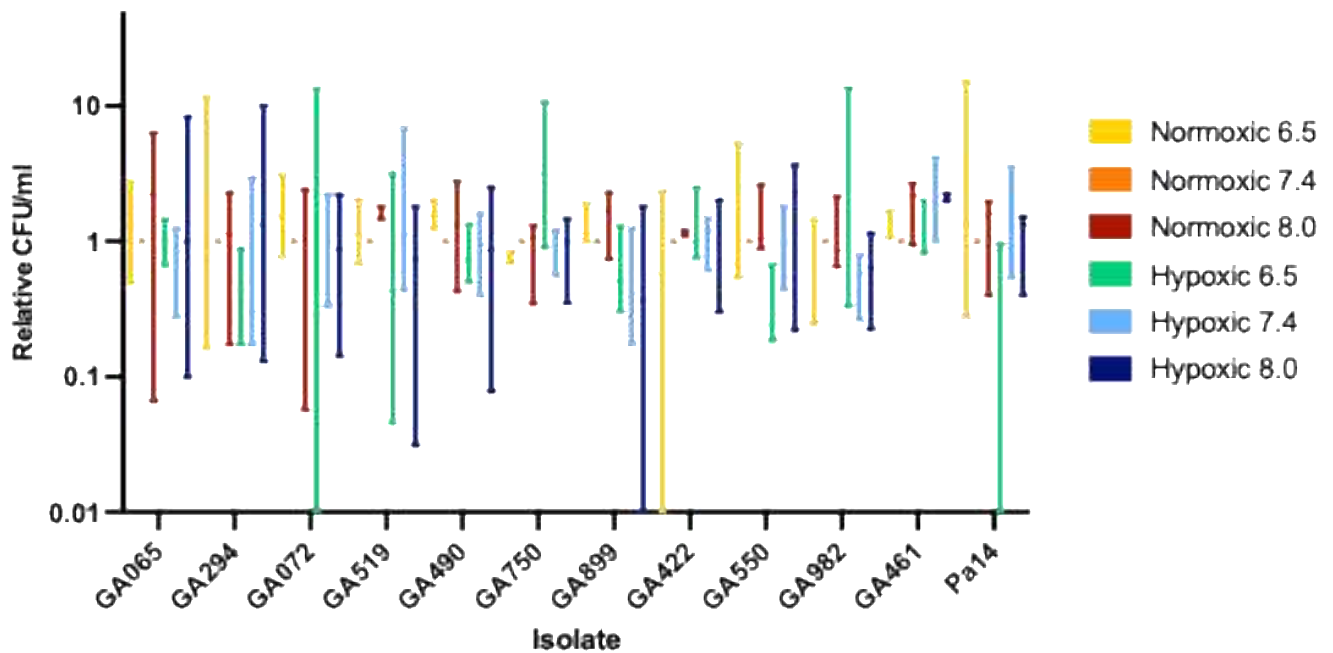


Figure 4.20: Relative CFU/ml for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference strain Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Relative median of three biological repeats, each consisting of three technical repeats, each relative to the median CFU/ml for each isolate at normoxic pH 7.4 conditions is plotted with errors bars representing 5th-95th centiles (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). No consistent trends of differential growth by oxygen or pH state are identifiable across the different isolates. Note log10 y-axis scale.

Figure 4.21 demonstrates comparison of pooled CFU/ml of each isolate at each condition, relative to pooled CFU/ml for all isolates at normoxic pH 7.4 condition. Relative CFU/ml was the same in all hypoxic conditions compared to normoxic pH 7.4 (Wilcoxon test). Relative CFU/ml was higher in normoxic pH 8.0 (median[IQR]: 1.3[0.87,2.2], $p < 0.05$; Wilcoxon test) compared to normoxic pH 7.4. There was no difference in relative CFU/ml in the other pH conditions (normoxic pH 6.5 and hypoxic pH 6.5, 7.4 and 8.0, Wilcoxon test).

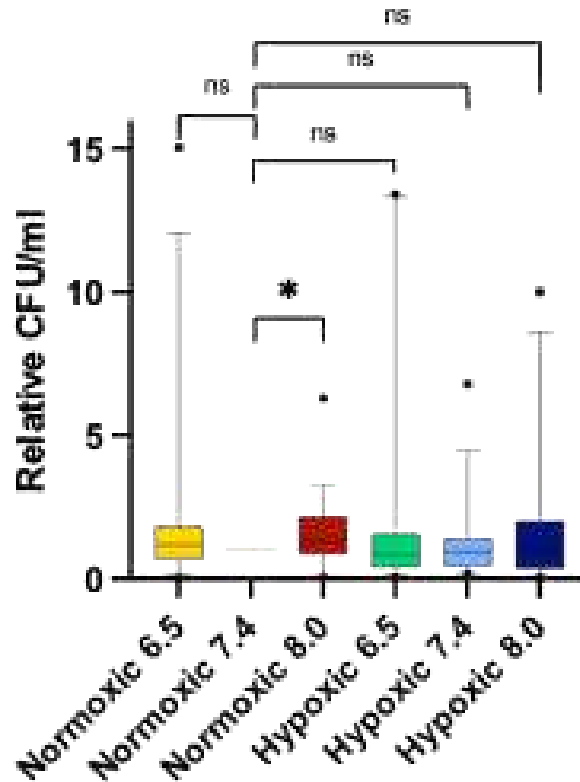
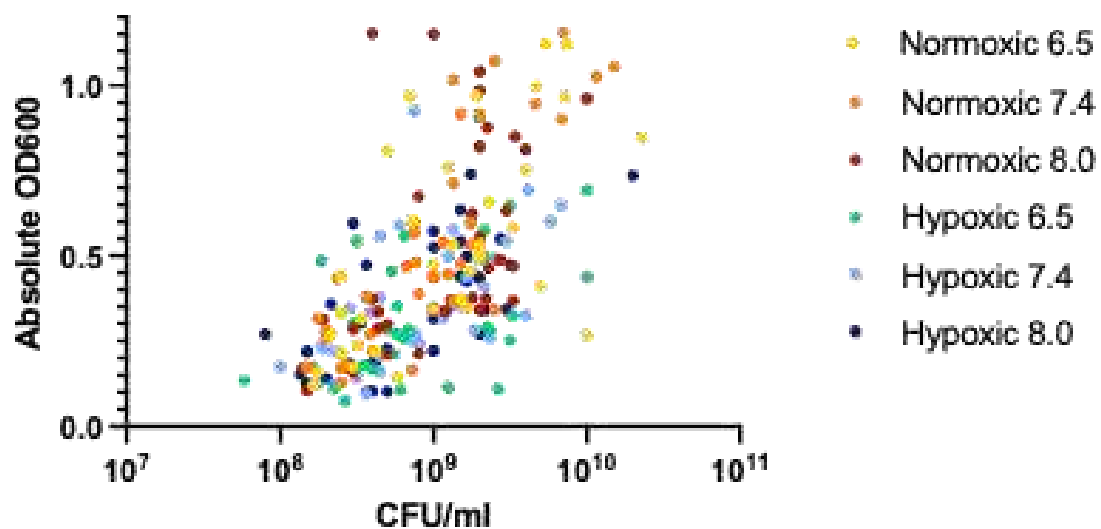


Figure 4.21: Pooled CFU/ml for all isolates at each condition, relative to results for normoxic pH 7.4

Box and whisker plot of CFU/ml for all isolates at each condition, relative to mean CFU/ml for all isolates at normoxic pH 7.4 condition, with whiskers indicating 5th and 95th centiles, with outlier as black dots. Comparisons by Wilcoxon test to normoxic pH 7.4 conditions (ns=not significant, *= $p < 0.05$), with Bonferroni correction applied for multiple t-tests. Normoxic pH 8.0 has a higher mean CFU/ml than standard laboratory growth conditions (normoxic pH 7.4). No other significant differences are seen.

Unable to replicate the same trends in growth measured by colony counting, as with optical density, the correlation between OD₆₀₀ and CFU/ml, at the end of the third day of passage was examined (figure 4.22). Correlation for the whole cohort (all conditions combined) by Spearman's rank calculation gives r [95%CI] of 0.65[0.56-0.72]. There is variable correlation by conditions, with Spearman's rank correlation coefficient reported, as per table within figure 4.22. Note there is good

correlation at normoxic pH 7.4 conditions, which are typical lab conditions. Correlation is weaker at both lower and higher pH, and in relative hypoxic conditions. Correlation is the weakest at hypoxic pH 6.5 condition. There is a significant difference between slopes at each condition when compared using simple linear regression ($p < 0.05$). This may suggest that the environmental conditions themselves explain the difference seen between growth results shown by optical density and colony counting.



Condition	Spearman r	95%CI
All conditions combined	0.65	0.56 to 0.72
Normoxic 6.5	0.66	0.42 to 0.82
Normoxic 7.4	0.81	0.64 to 0.90
Normoxic 8.0	0.64	0.39 to 0.81
Hypoxic 6.5	0.41	0.07 to 0.66
Hypoxic 7.4	0.69	0.46 to 0.83
Hypoxic 8.0	0.58	0.30 to 0.77

Figure 4.22: Correlation between CFU/ml and OD₆₀₀ values at different conditions

Graph showing correlation between the mean post-third day of passage OD₆₀₀ value and corresponding mean colony count (CFU/ml) for triplicate repeat in each environmental condition. The table shows Spearman rank correlation coefficient for each condition separately, and 95% CI. Correlation is strongest at normoxic pH 7.4 conditions, and weakest at hypoxic pH 6.5 conditions.

Lower pH and hypoxic conditions appear to weaken correlation between OD₆₀₀ and CFU/ml measurements. Slopes of correlation at each condition are different when compared using simple linear regression model ($p < 0.05$).

Growth curves were created for each isolate following 3-day passage in each condition by plotting the mean[SD] of serial OD₆₀₀ measurements (every 30 minutes) for 24 hours for three biological repeats (each being a single technical repeat due to capacity in the plate reader overnight), as shown in figure 4.23. Each isolate demonstrates different rates of change of OD₆₀₀, and variability is apparent in the different conditions applied, but with no consistent pattern. Doubling times were calculated (methods, section 2.1.5.) for each isolate, and mean provided across all isolates, in each condition, were compared using repeated measures one-way ANOVA (figure 4.24). Hypoxic pH 8.0 had a longer mean[SD] doubling time of 2.1[0.5] compared to normoxic pH 7.4 of 1.9[0.44] ($p < 0.05$). No other difference was seen in doubling time by different condition.

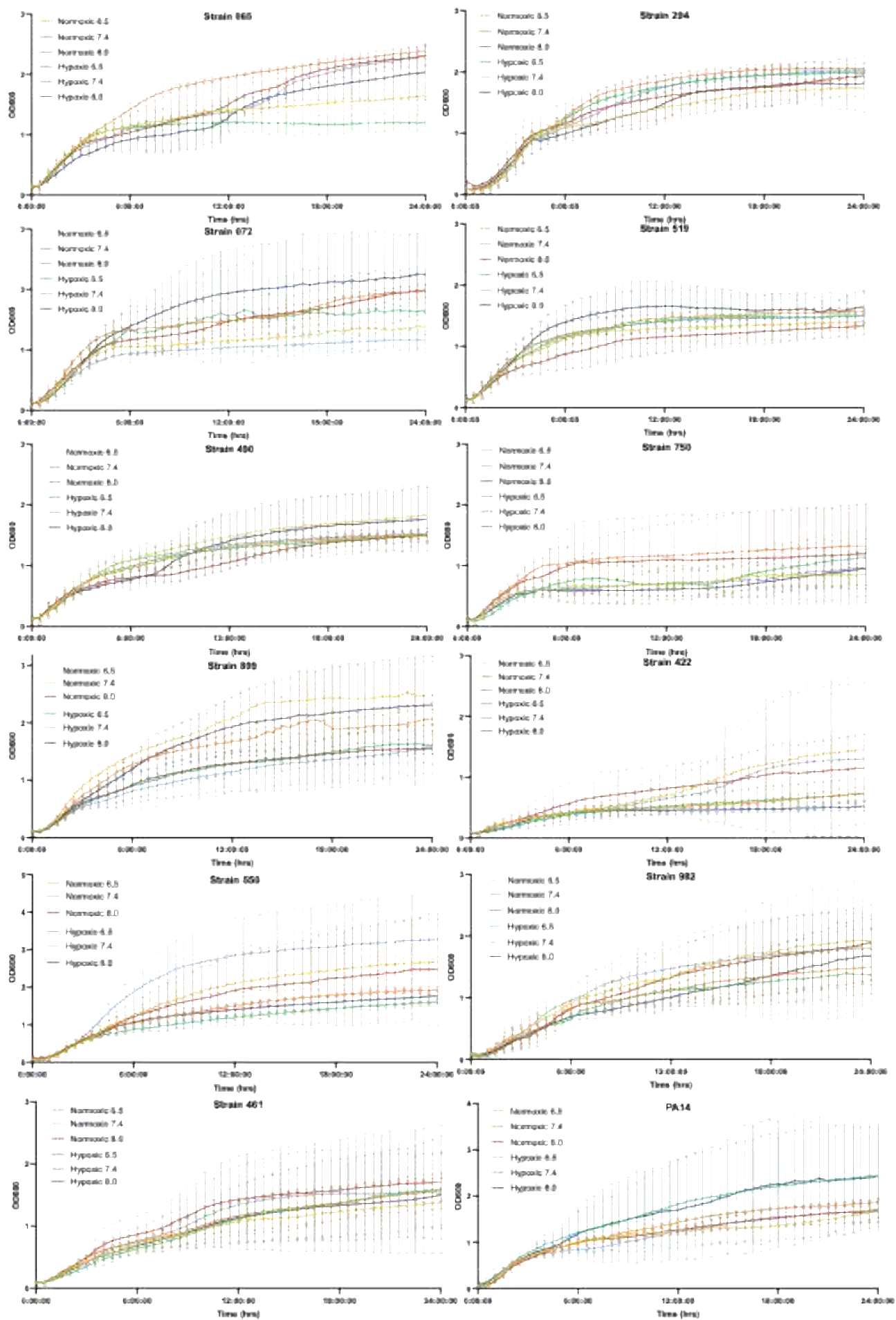


Figure 4.23: Growth curve plots of serial OD₆₀₀ measurements used to calculate doubling times

Serial plot of mean OD₆₀₀ of three biological repeats, every 30 minutes, over 24 hours, following 3-day passage in each growth condition. Error bars indicate SD. All 11 isolates and reference isolates Pa14 are shown. Growth curves were used to calculate doubling times for each isolate at each condition (figure 4.24).

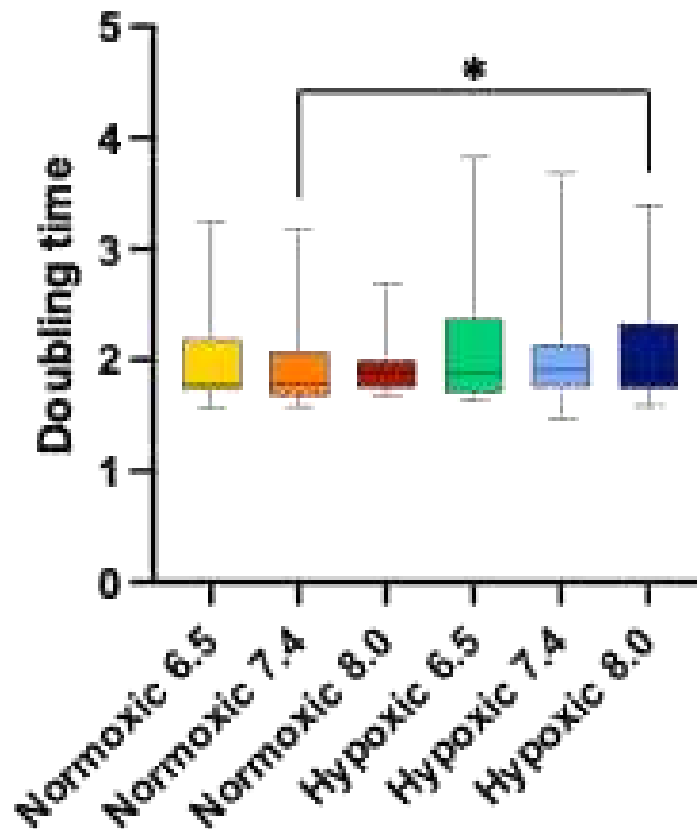


Figure 4.24: Comparison of doublings times at each environmental condition

Box and whisker plots of the calculated doubling time across all isolates, for each growth condition, with whiskers indicating 5th and 95th centiles. Comparison performed using repeated measures one-way ANOVA, with no difference seen in doubling time across all conditions.

4.4.3. Pyoverdine production

Pyoverdine production will in part be dependent on quantity of bacteria in the sample and so RFI values are adjusted for CFU/ml values for each isolate (figure 4.25). Some isolates show higher CFU adjusted RFI, suggesting greater pyoverdine production at normoxic and pH 7.4 growth conditions.

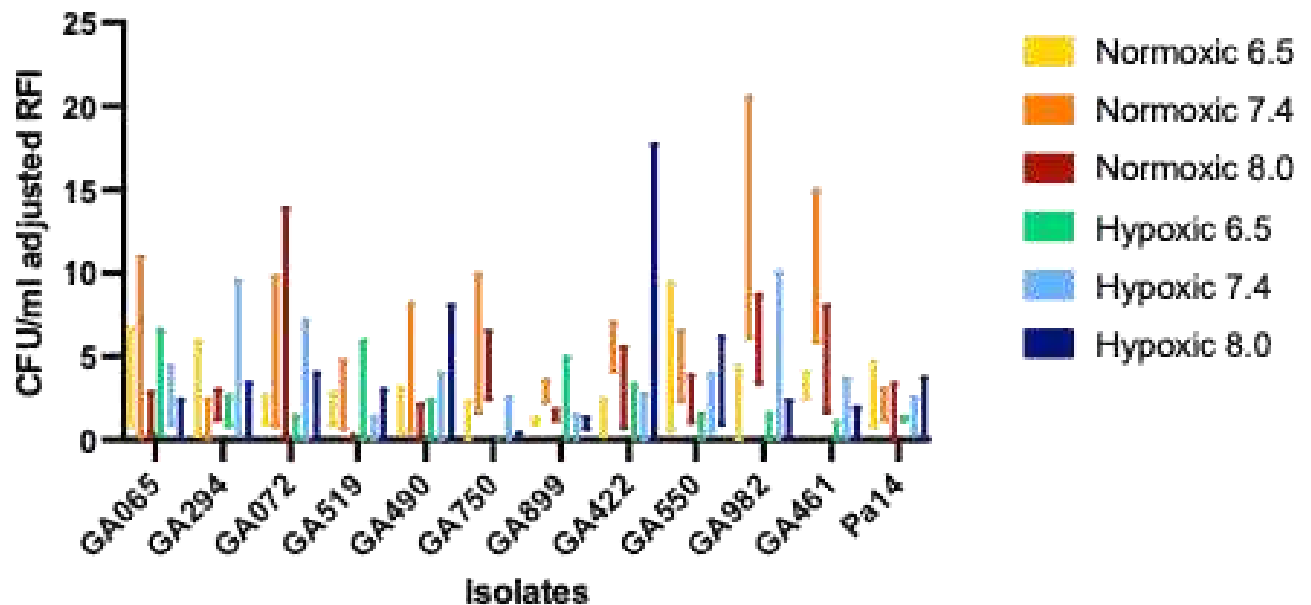


Figure 4.25: Absolute CFU adjusted RFI for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference strain Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Median RFI adjusted for respective CFU/ml of three biological repeats, each consisting of three technical repeats, is plotted with error bars representing 5th-95th centiles (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). Negative values are adjusted to zero. Some isolates appear to show a higher CFU/ml adjusted RFI, indicating greater pyoverdine production, in normoxic conditions and at neutral pH7.4.

Because each isolate has inherently different tendency to produce pyoverdine, results were then standardised as a proportion of the RFI at normoxic neutral pH 7.4 (i.e. typical laboratory conditions and presumed normal airway conditions) for each isolate (figure 4.26). At this adjustment, no apparent isolate-by-isolate trends in different growth conditions are seen.

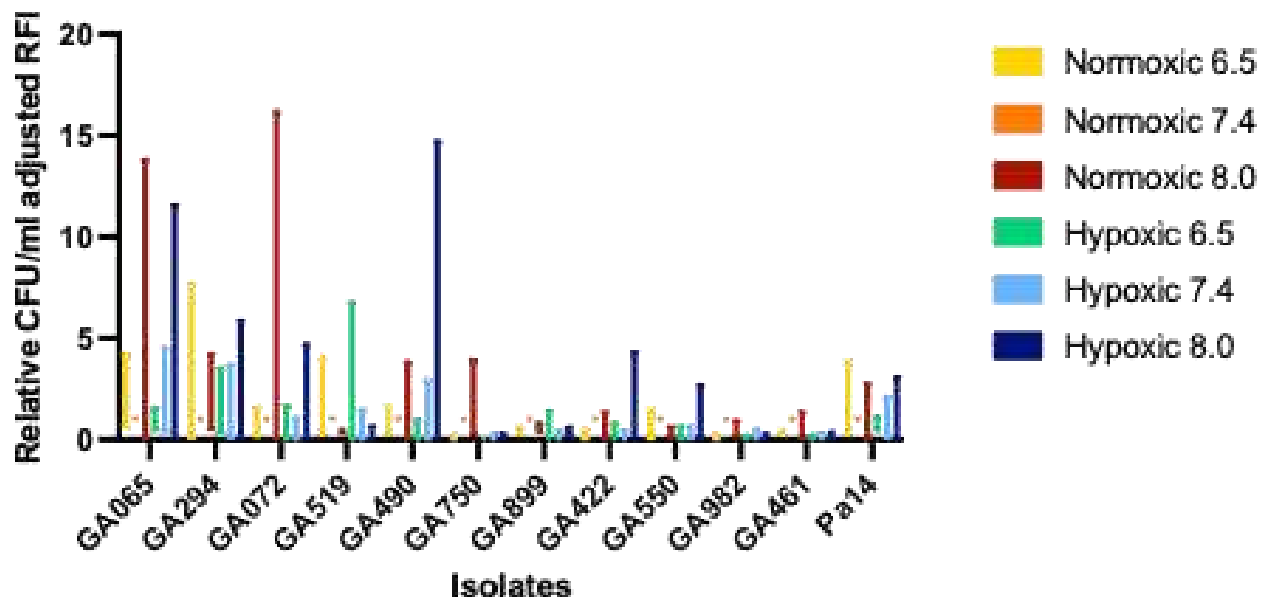


Figure 4.26: Relative CFU adjusted RFI for all isolates at each pH and oxygen state

The 11 isolates are indicated, along with reference strain Pa14, at 6 different conditions, pH 6.5, 7.4 (neutral) and 8.0 in both normoxic and hypoxic incubation environments. Relative median of three biological repeats, each consisting of three technical repeats, each relative to the CFU/ml for each isolate at normoxic pH 7.4 condition is plotted with error bars representing 5th-95th centiles (IQR in box/whisker is not presented by GraphPad Prism if <5 repeats). Negative values are adjusted to zero. Following adjustment to normoxic pH7.4 conditions, no trends are visible.

In order to perform statistical analysis, relative CFU adjusted RFI values for each isolate, at each condition were pooled and compared to normoxic pH 7.4 conditions (figure 4.27). Relative CFU adjusted RFI was lower at hypoxic pH 6.5 ($p < 0.01$) and hypoxic pH 7.4 ($p < 0.05$), suggesting pyoverdine production, as a marker of acute virulence, is lower in the reduced oxygen and more acidic conditions presumed in the pre-CFTRm environment.

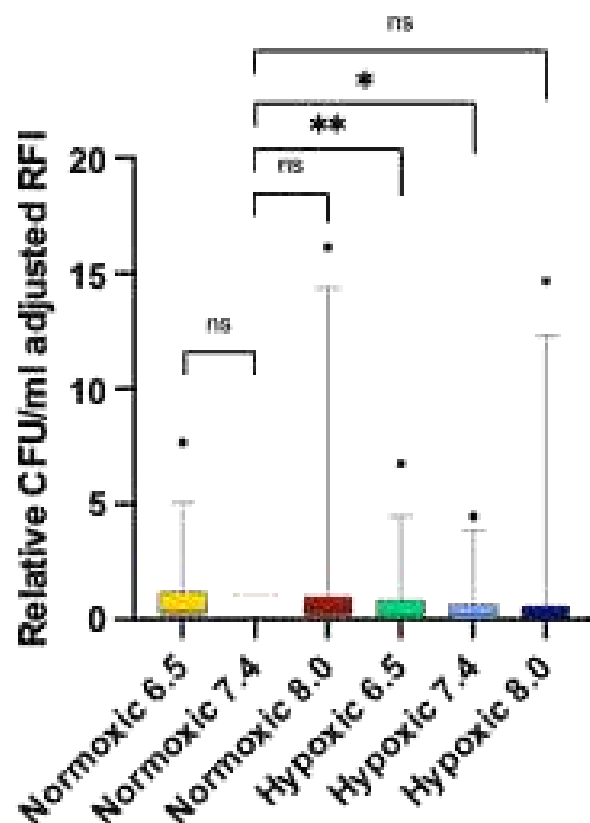


Figure 4.27: Pooled CFU adjusted RFI for all isolates at each condition, relative to results for normoxic pH 7.4

Box and whisker plots of CFU/ml adjusted RFI for all isolates at each condition, relative to CFU/ml adjusted RFI for all isolates at normoxic pH 7.4 condition, with whiskers indicating 5th and 95th centiles. Comparisons by Wilcoxon test to normoxic pH 7.4 conditions, with Bonferroni correction for multiple tests. Hypoxic pH 6.5 and pH 7.4 have a lower relative CFU/ml adjusted RFI than standard laboratory growth conditions (normoxic pH 7.4). * = $p < 0.05$ and ** = $p < 0.01$.

4.4.4. Mucoidy

Mucoid alginate production was subjectively scored from appearances of each isolate after third day passage in each environmental condition and grown on congo-red agar. The same subjective scoring system was used to identify differences in degree of mucoidy, as illustrated in figure 2.2 above (and in methods, section 2.2.1). Each condition for each isolate had 3 biological repeats, each consisting

of three technical repeats. Scoring occurred by single, unblinded person. Median score was applied to a heat map (figure 4.28). Due to the subjective scoring system, no statistical analysis was thought appropriate. Technical repeats were consistent for each isolate at each condition in each biological repeat. At all pH values, there appears to be trend for mucoid alginate production in hypoxic conditions, compared to normoxic. Within normoxic conditions, there may be a trend to more mucoid alginate production at pH 6.5, compared to pH 7.4 and 8.0. Within in hypoxic conditions, there is no apparent trend of difference between pH 6.5, 7.4 and 8.0.

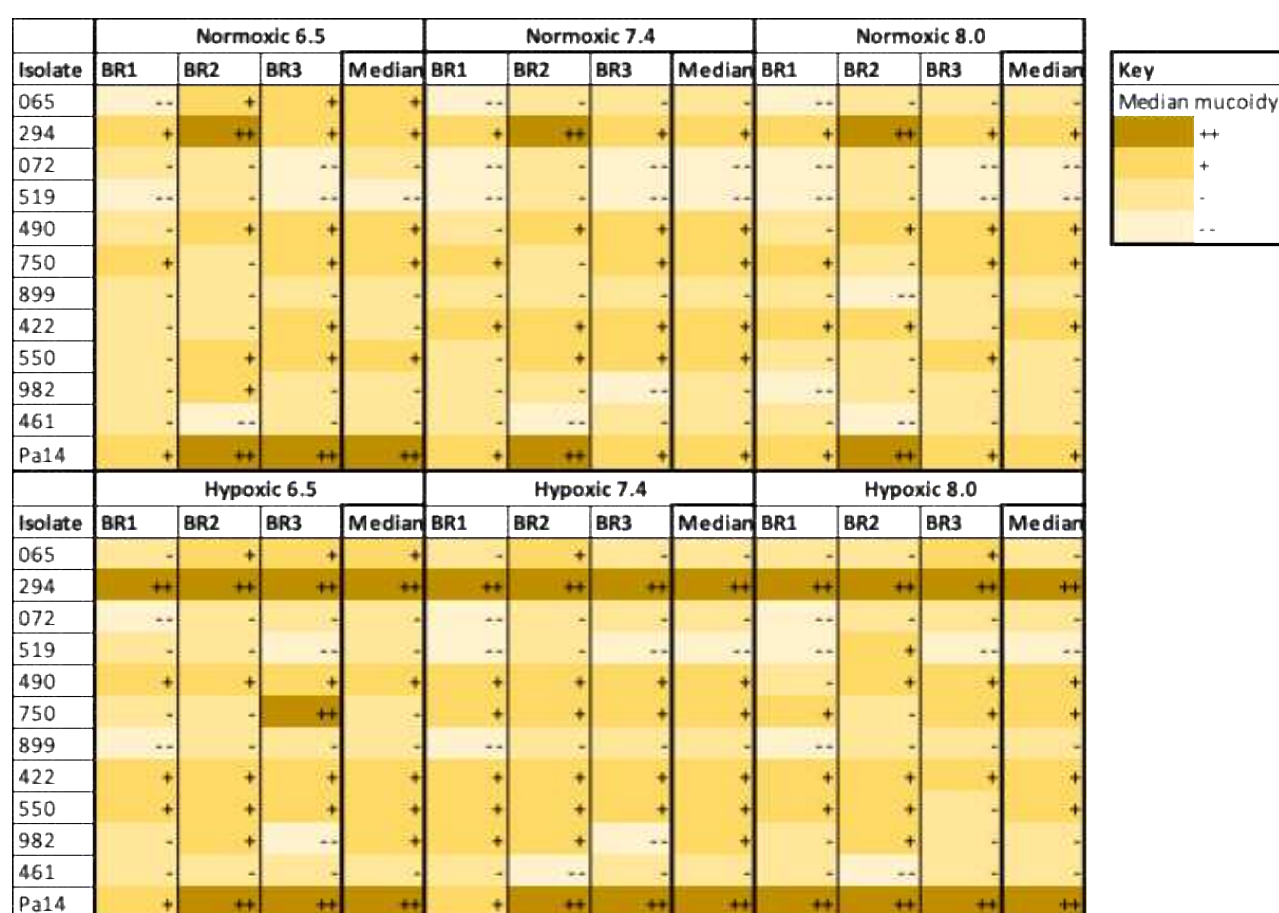


Figure 4.28: Mucoid scores for all biological repeats for isolates at each environmental condition

Heat map showing each biological repeat and median of 3 biological repeat mucoid score (--, -, + or ++, as per figure 2.2) after third day of passage in each of 6 environmental conditions and examined on congo-red agar. Hypoxic conditions subjectively appear to lead to more mucoid alginate production. Within normoxic conditions, low pH subjectively appears to lead to more mucoid alginate production.

4.4.5. Antimicrobial sensitivity testing

MIC to tobramycin was measured for each isolate after third day passage. Absolute MIC appears higher as environmental pH becomes lower, so that more acidic pH appears to lead to higher MIC and reduced tobramycin susceptibility. This appears to be case in both normoxic and hypoxic conditions (figure 4.29).

A	Normoxic 6.5				Normoxic 7.4				Normoxic 8.0			
	BR1	BR2	BR3	Median	BR1	BR2	BR3	Median	BR1	BR2	BR3	Median
065	>64	>128	>128	>128	>64	>128	128	128	64	>64	128	128
294	4	>4	4	4	4	>4	8	8	4	4	8	4
072	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
519	32	>32	64	64	16	32	32	32	16	32	16	16
490	>32	>64	64	64	32	32	32	32	32	32	32	32
750	4	>4	>4	>4	2	2	4	2	2	2	2	2
899	>4	>4	>4	>4	4	>4	>4	>4	4	>4	4	4
422	16	8	16	16	4	<4	8	4	<4	<4	<4	<4
550	>4	>4	>4	>4	4	>4	>4	>4	4	4	4	4
982	8	8	8	8	4	8	8	8	4	4	8	4
461	64	64	>64	64	32	32	32	32	16	32	32	32
Pa14	>2	4	4	4	2	2	2	2	2	2	2	2

Isolate	Hypoxic 6.5				Hypoxic 7.4				Hypoxic 8.0			
	BR1	BR2	BR3	Median	BR1	BR2	BR3	Median	BR1	BR2	BR3	Median
065	>64	>128	>128	>128	>128	>128	>128	>128	64	32	128	64
294	4	>4	8	8	4	>4	8	8	4	>4	8	8
072	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024	>1024
519	>32	>64	>64	>64	>32	64	32	64	16	16	16	16
490	>32	>64	>64	>64	32	>32	>64	64	16	32	>64	32
750	>4	4	>4	>4	2	2	4	2	2	2	2	2
899	>4	>4	>4	>4	>4	>4	>4	>4	4	4	4	4
422	16	16	<4	16	4	<4	<4	<4	<4	<4	<4	<4
550	>4	>4	>4	>4	>4	>4	>4	>4	4	4	4	4
982	8	8	>8	8	4	4	8	4	4	4	4	4
461	>64	>64	>64	>64	32	64	16	32	16	16	16	16
Pa14	>2	4	2	4	2	2	2	2	2	2	2	2

B	Normoxic			Hypoxic		
	6.5	7.4	8.0	6.5	7.4	8.0
065	>128	128	128	>128	>128	64
294	4	8	4	8	8	8
072	>1024	>1024	>1024	>1024	>1024	>1024
519	64	32	16	>64	64	16
490	64	32	32	>64	64	32
750	>4	2	2	>4	2	2
899	>4	>4	4	>4	>4	4
422	16	4	<4	16	<4	<4
550	>4	>4	4	>4	>4	4
982	8	8	4	8	4	4
461	64	32	32	>64	32	16
Pa14	4	2	2	4	2	2

Key	
Median MIC relative to Normoxic 7.4	
+2	
+1	
Normoxic 7.4	
-1	
-2	

Figure 4.29: MIC to tobramycin for isolates at each environmental condition

A: Median MIC ($\mu\text{g/ml}$) for each biological repeat of each isolate after 3-day passage in each of 6 environmental conditions. environmental condition tested, illustrates inter-biological repeat variability. **B:** Heat map showing median MIC ($\mu\text{g/ml}$) to tobramycin of 3 biological repeats for each isolate at each condition. Heat map colours are standardised against the MIC for each isolate at normoxic pH 7.4 conditions, to account for baseline differences in MIC of each isolate. Lower pH (more acidic) conditions subjectively appear to lead to higher MIC, in both normoxic and hypoxic conditions.

As a range of three different MICs could be tested for each strain, where no MIC was found, the MIC is reported as '>' the maximum MIC tested. In order to perform statistical analysis, these values were assumed to be the next MIC, so that MIC >64 $\mu\text{g/ml}$, becomes MIC =128 $\mu\text{g/ml}$. Median MIC for each condition was then compared to that of normoxic pH7.4, but after Bonferroni correction for multiple tests, no difference was seen (figure 4.30).

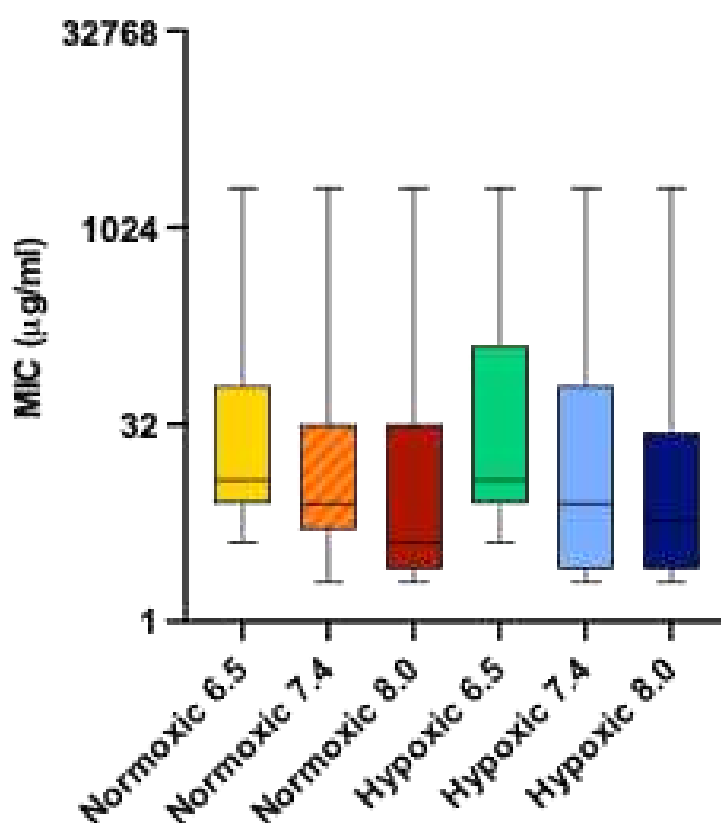


Figure 4.30: Pooled median MIC at each environmental condition

Box and whisker plot of median MIC for all isolates at each condition with whiskers indicating 5th and 95th centiles. Comparisons by Wilcoxon test with Bonferroni correction for multiple tests, to normoxic pH7.4 conditions, with no significant difference found.

4.5. Discussion

As outlined, aim 2 has been addressed in two parts. The first looked at applying phenotypic assays to stored isolates of Pa from pwCF with chronic Pa infection, before and after they started CFTRm, to see if *in vivo* use of CFTRm has affected an individual's Pa isolates over time. Assays looked at growth potential, pyoverdine production as a marker of acute virulence, and mucoidy as a marker of persistence. Isolates came from the SRC-PAPA repository and unfortunately sample collection was significantly reduced during the covid-19 pandemic, a period of time that coincided with the national provision of ETI. Cross referencing the same cohort examined in Aim 1 (chapter 3), with the sample repository database, only four candidates starting ETI in this time had sufficient stored isolates over the desired timeframe. Expanding the search to those taking IVA monotherapy (to look at two modulators and populations considered similar), which started nationally circa 2012, an additional four candidates could be added. This cohort was also limited as far fewer pwCF were eligible for IVA monotherapy, based on genotype, and the SRC-PAPA repository only started collecting samples around the time of prescribing rollout, meaning limited availability of pre-IVA isolates.

In the context of a much more limited than anticipated number of suitable candidate patients, no significant differences and no discernible trends were seen in markers of growth, pyoverdine production or mucoidy status, before and after CFTRm. Effects may have been further diluted by the presence of two CFTRm groups, which whilst similar in clinical impact, act on genotypically distinct CF sub-groups and different pharmacological mechanisms. This design is also limited in its approach

because isolates stored from patients are left exposed, on agar, to external laboratory conditions outside of the host airway for several days before being collected and stored in the repository. An untested assumption that airway specific phenotype is preserved in this time could have undermined ability to detect differences.

Larger studies, aggregating isolates stored from even larger CF populations would be needed to see if increasing sample number can identify differences, using this approach and in assessing the immediate 2-year time frame post-ETI introduction. Assuming any changes to Pa persist following ETI use, which is an unproven theory, it may be possible to prospectively collect samples from pwCF at RBH and compare them to their pre-ETI stored isolates in the repository. No preliminary findings from this cohort can guide which assays to pursue further.

Driven in part by the very small sample numbers in the above, the second part of this aim looked at testing the phenotypic response of clinical Pa isolates and Pa14 to growth conditions that mimic predicted changes in the CF airway from use of CFTRm. pH adjusted broth was used to serially passage Pa isolates at three different pH levels: 6.5, 7.4 and 8.0 and two different environmental oxygen states: normoxic (21%) and hypoxic (6%). Lower pH and hypoxic states represent the CF airway and mucous plugs before CFTRm, and the higher pH and normoxic state the theorised post-CFTRm environment. Isolates were passaged in fresh pH-adjusted broth every 24 hours for 3 days. Measurement of broth pH at 24 hours (on day 2) indicated that pH did not remain stable. In normoxic conditions, low and neutral pH became more alkaline in the presence of bacteria. In hypoxic conditions, low pH became more neutral in the presence of bacteria, but neutral and high pH became more acidic, in high ambient CO₂ environment. Continuous pH monitoring throughout passage was not possible and so it is not known how long pH was sustained. The effect of oxygen state on pH means attributing observed changes to one or the other more challenging, and results are interpreted in that context.

When measured by OD₆₀₀, Pa growth rate appears to be slower in hypoxic conditions relative to growth of Pa in normoxic and neutral pH conditions, but not to be affected by pH. However, these results were not confirmed when growth was measured by colony counting. A slightly higher relative growth, when measured by colony counting, was seen at normoxic pH 8.0 only. This discrepancy was unexpected as optical density is typically considered a good approximation for CFU/ml measurement in typical laboratory conditions. In these experiments however, typical laboratory conditions (i.e. normoxic and neutral pH) were purposefully altered. Optical density measures total bacterial content, including alive or dead bacteria, bacterial fragments and other particulate matter, where colony counting requires a second step of bacterial revival for them to grow in the serial dilution and colony count plating. If the more hypoxic and more acidic conditions are limiting bacterial survival, then there will be increased discrepancy between OD₆₀₀ and CFU/ml results for the same condition. Mucoid alginate production, producing cloudier broth, will also impact the OD₆₀₀ value. Measuring growth rate and doubling time with OD₆₀₀ would be impacted by accumulation of dead bacteria and mucoid alginate and so the only significant result of longer doubling time at hypoxic pH 8.0 condition should be interpreted with caution. Profiling growth in this model does not replicate clinical findings from aim 1, and other real-world studies, suggesting reduced abundance following the introduction of CFTRm [96,144,147].

RFI, adjusted for CFU/ml was used as a surrogate measure for pyoverdine production, an acute virulence factor. When analysed relative to results for normoxic pH 7.4 conditions, it was lower at hypoxic pH 6.5 and 7.4 conditions, suggesting perhaps less pyoverdine production in low pH and low oxygen state expected in traditional, CFTRm unexposed CF airway. This is consistent with the expectation that Pa adapts to the CF lung environment by down regulating acute virulence factors [44,76,160]. It can be hypothesised that restoration of normoxia and normal airway pH may lead to upregulation of acute virulence factors again in the same isolates. Pyoverdine and other virulence factors have not yet been well characterised in Pa isolates that have been exposed to CFTRm in current literature.

Mucoidy was assessed using congo red agar, and a subjective scoring system developed. There was apparent high concordance between technical and biological repeats, although scoring was unblinded by a single person (myself). No statistical analysis was performed on this novel, unvalidated scoring system as this was not felt appropriate. However, a trend toward more mucoid alginate production in hypoxic conditions seemed evident from heat map analysis. This would fit with expectation that a mucoid production is increased, as a persistence factor, in traditional, CFTRm unexposed CF airway [52]. It can be hypothesised that restoration of normoxia and normal airway pH may lead to downregulation of persistence factors in the same isolates, as hostile environmental conditions change. However, since the design of these experiments, published data in one study does not support change in Pa mucoid status or antimicrobial resistance, despite specific Pa strain persistence in chronically Pa infected pwCF, following ETI [163].

The antimicrobial sensitivity of Pa is thought to be decreased in acidic environments [71,161]. If CFTRm leads to restoration of more neutral pH then Pa may become more susceptible to eradication. Testing MIC to tobramycin, heatmap trend suggested increasing susceptibility at higher pH compared to low. However, when compiled and analysed this did not reach statistical significance. Aminoglycoside antibiotics have also shown pH dependent action, with reduced activity in the presence of cations, and so effects should be explored with a range of non-aminoglycoside, anti-Pa antibiotics as well [164].

There are a number of limitations to consider. The model used in this aim was limited in its ability to replicate the complexity of CF lung environment. As evidenced by the changes to pH over the duration of 24 hour passage, stable conditions could not be guaranteed. Presence of mucous, airway surface liquid, inflammatory cells are also missing, and so true effect may need a more complex model. Use of models using CF artificial sputum media (ASM) or more complete model of infection in lung environment such as airway liquid interface (ALI) culture might be better suited for this purpose, though not chosen from the outset, as these experiments were designed to explore

potential differences that could lead to more complex experimental design if signal emerged. A limited range of assays were performed, partly on the basis that the model required testing, and so an expansive range of assays was not considered in case the model was found to be insufficient. An attempt was made to assess the reproducibility of chosen assays and whilst measurement of CFU/ml by colony counting produced a very low coefficient of variance (1.4%), measurement of growth by OD₆₀₀ and RFI was above 10% (11.8% and 25.7% respectively). Alternative assays may have provided better assessment of virulence and persistence phenotypes. There is also a possibility that the handling and storage following-sample collection could mean isolates lose whatever phenotype they had; using fresh, prospectively collected samples, or analysing sputum directly for pyoverdine concentration, for example, should therefore be considered.

Future experiments could look at other markers of acute virulence including motility assays, and persistence, like biofilm crystal violet assay that could better quantify change than the mucoid congo red agar assay. An overriding limitation is the changes to the CF environment, post CFTRm, and at the time of experimental design were assumed and unproven. One study, in 44 people, has since failed to measure any difference in exhaled breath condensate pH of pwCF following ETI initiation, compared to baseline [157]. No additional evidence looks at airway oxygen state, whilst a number of studies have shown differences in sputum rheology and inflammatory markers, which could also impact how Pa behaves in the airway [146,157,165]. Experiments that examine the changes in CF sputum oxygen state and airway pH are needed to support further development of Pa phenotype assessment in response to its post-CFTRm environment, and this is discussed further in the main discussion in chapter 7.

Chapter 5 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: PCR, LAMP and MALDI-TOF

5.1. Introduction

Chapter 3 outlined how provision of sputum samples by pwCF has significantly reduced since the initiation of ETI in people with chronic Pa infection in our centre. This is supported by evidence from real world studies [144,147]. Non-sputum based airway infection surveillance is possible with cough swabs however we know that these are much less sensitive to detecting Pa infection [106,108]. Chapter 3 also outlines how post-ETI, Pa isolation frequency and abundance decreases, even when accounting for reduced total and sputum sample provision. This is also supported in real world studies [144,147]. However, Pa is not eradicated in most people, and despite restoration of CFTR function and anticipated improvement in mucociliary clearance, monitoring of ongoing or new infection is required. This most likely will need to occur in the absence of sputum samples to help define chronic infection, determine its impact in CF airways that are responding to modulators, and guide treatments decisions.

Airway surveillance can be performed by BAL, which is the gold standard for excluding lower airway infection [107]. However, it is invasive, requires a general anaesthetic in children, and is not practically useful in routine regular surveillance, which should be at least four times per year, according to current CF Standards of Care [77]. Sputum can be generated by induction using hypertonic saline, is less invasive, and has been shown to detect CF pathogens with comparable sensitivity to standard bronchoscopy (3-lobe lavage) protocols [113]. Whilst there is also ongoing evaluation of sputum induction at home for those already using hypertonic saline regularly as part of an airway clearance routine [166], for most clinical services sputum induction is a labour intensive process performed by highly specialised physiotherapists, making its regular use for airway surveillance similarly challenging.

Infection surveillance from cough swabs, spontaneously expectorated or induced sputum and BAL all still rely on standard culture techniques on agar to detect CF pathogens. This takes at least 48 hours to confirm absence of bacteria in a sample and longer still for AST. Methods, such as PCR and MALDI-TOF have the potential to identify CF pathogens much more quickly – though still typically to identify a colony from a cultured sample rather than directly detect presence in the sample itself – and accelerate decision making [120,123]. Both have demonstrated very high specificity for Pa [118–122]. Sensitivity is less clear though and is dependent in each case on bacterial load. MALDI-TOF is already used in clinical practice in hospital microbiology labs for confirmation of bacterial identification and may have the added advantage of predicting AST profiles [123]. Sensitivity is probably limited to a minimum quantity of 10^4 to 10^5 CFU as sizeable bacterial pellet or colony is required as the substrate. In the case of PCR, sensitivity is likely dependent on sample processing, but a lab model using cultured CF sputum containing Pa demonstrated that, with multiple processing steps, DNA extraction, and quantitative PCR (qPCR) approach, a lower limit of Pa detection of 50 CFU/ml could be achieved [125]. Whether this is replicable in everyday clinical practice and if there is sufficient substrate in non-sputum based tests is unclear. Loop-mediated isothermal amplification (LAMP) is an alternative DNA based method that allows massive DNA amplification from very small substrate concentrations and can be used with minimal resources and sample processing steps [16,17].

Aim 3 looked at a novel sample collection method which was called a “huff tube,” which was conceived within the lab group, and I then developed the prototype design. The huff tube consisted of pieces of sterile gauze held in place at the end of a sterile cylindrical mouthpiece to collect the exhaled breath and droplets of a patient during a forced expiratory manoeuvre (huff). The rationale is that gauze provides a larger surface area for sample collection, compared to cough swabs, resulting in more bacteria per sample. It was anticipated that the gauze would then need to be incubated in Pa selective broth overnight to increase substrate quantity and select out oral commensal organisms or other CF pathogens. This could theoretically optimise, in less time than

standard culture, sensitivity and specificity of alternative methods of rapid, non-culture based tests that were applied. The aim was also designed to be able to apply non-culture based methods with minimal further processing steps to the samples, to reduce test complexity and increase future practical application in the clinic, and possibly even point-of-care, setting.

In chapter 2 (methods, section 2.2.2), the development of a lab model representing 'huff tubes' is explained. In summary, these preliminary experiments led to the development of a lab model of the desired 'huff tubes,' mimicking inoculation of different starting quantities of bacteria onto gauze. Clinical isolates from the SRC-PAPA repository were revived from Microbank beads in general broth (methods, section 2.1.3.), concentration (CFU/ml) established, serial dilutions made to create a range of starting concentrations to be inoculated onto gauze and incubated in 10ml of a Pa selective broth (cetrimide). 15 Pa clinical isolates from the SRC-PAPA repository were examined. Seven of eight (88%) incubated for <24hrs were able to grow from starting concentrations $<10^3$ to $>10^5$ CFU/ml. Whilst 48-hour incubation probably yielded higher quantity of bacterial substrate for testing, it was not felt useful for the purpose of this aim, where the focus was on producing a diagnostic test that was minimally dependent on bacterial culture and ideally quicker than standard culture techniques. The use of Pa selective broth (cetrimide) demonstrated good specificity across 13 isolates of seven non-Pa species tested. Two of three (66%) *Burkholderia cepacia* complex (Bcc) and two of two (100%) *Achromobacter xylosoxidans* (Ax) isolates tested were able to survive in cetrimide. Otherwise, there was good selective inhibition of *Staphylococcus aureus* (Sa), *Stenotrophomonas maltophilia* (Smt), *Klebsiella pneumoniae* (Kp) and *Escherichia coli* (Eco) growth.

This huff tube lab model was the platform for examination of alternative detection methods outlined in this chapter (PCR, LAMP, MALDI-TOF) and chapter 6 (medical detection dogs).

5.2. Materials and methods

As this chapter focusses on development of ways to use recognised techniques (PCR, LAMP and MALDI-TOF), the results are iterative and so discuss the developmental process at each step.

Methods here are outlined to show the starting materials and processes used to develop these diagnostic tools further, in addition to general methods set out in chapter 2.

5.2.1. Polymerase chain reaction

Validated PCR primers for PAL1 and PAL2 are readily available for detecting *Pa* genes following DNA extraction by targeting the *oprL* gene and have shown high sensitivity and specificity for *Pa* [120,122]. Typically, multiple steps are needed for isolation of pure DNA, confirming of *Pa* presence in test samples, while avoiding false positives from environmental contamination. For the purpose of this aim, however, the key principle was to develop DNA based methods that could be performed with minimal processing steps, directly from the samples produced by the huff tube lab model and detect DNA at very low ($<10^3$ CFU/ml) quantity.

For the first DNA based method a pilot process for PCR was developed and from this, iterative development and associated results are discussed. The pilot process was designed to take an aliquot of sample directly from the output of the huff tube lab model. As outlined previously (methods, section 2.2.2.) this was produced after reviving bacterial isolates from Microbank beads in LB broth (methods, section 2.1.3), 20µl of resultant broth culture was then inoculated onto sterile gauze under class II hood, gauze submerged into 10ml of ceftrimide broth, in 60ml sterile containers, vortexed briefly and incubated overnight at 37°C on shaking platform at 200rpm.

Samples were then used in a pilot process for PCR testing. DNA extraction occurred in situ in the PCR thermocycler. Typically, a 50µl reaction was made, using 25µl *Taq* polymerase mastermix, (GoTaq, Promega Corporation used initially), which contains both polymerase and dNTPs, 2.5µl of each

primer (PAL1 and PAL2), 19µl of PCR grade water (Sigma Aldrich) and 1µl of completed huff tube lab model broth sample (containing cetrimide). Reaction mixture was added to the PCR thermal cycler and run as outlined in table 5.1.

Step	Temperature (°C)	Time (seconds)	Cycles
DNA extraction	94	300	1
1	94	20	30
2	57	20	
3	72	20	

Table 5.1: Pilot PCR thermal cycler settings

DNA extraction occurred in situ for 5 minutes at 94°C, then the sample cycled through 30 repeats of 20 seconds at 94°C, 20 seconds at 57°C, 20 seconds at 72°C.

Presence of DNA in each sample was tested using gel electrophoresis. 6µl of each sample, mixed with 5µl Gel Red, along with DNA ladder, were loaded into 1.2% agarose gel (methods, section 2.2.3.), and run at 100V for 30 minutes, before being photographed using UV image viewer. Samples tested initially were reference strains of Pa (Pa14 and Pa01) and known extracted Pa DNA as positive control, along with use of clinical isolates from the SRC-PAPA repository.

5.2.2. Loop-mediated isothermal amplification

LAMP is a single tube reaction method of DNA amplification. First developed in 2000, LAMP uses *Bst* polymerase, instead of *Taq* polymerase used in PCR, derived from *Geobacillus stearothermophilus*, and the background is well reviewed [167,168]. It is recognised for high DNA strand displacement capabilities and stable reaction at consistent and moderate temperatures (suggested 65°C). Reaction can occur in a heat block on the bench top and does not require access to a thermal cycler, unlike

PCR. Reaction mix can be prepared in a single tube with the substrate added directly, with good tolerance of presence of non-DNA material in the reaction mix.

LAMP uses 4-6 primers to target 6-8 distinct regions of desired DNA, making it highly specific. The *Bst* polymerase displaces target DNA strands and two specifically designed primers form loop structures that act as multiple focus sites of subsequent repeat amplification. DNA produced is very long, variable and formed of numerous repeats of the short different target sequences desired by the primers in different combinations. Amplified DNA is therefore not suitable for downstream manipulation, but is well suited to rapid detection methods, which include colorimetric and fluorescent indicators as well detection on gel electrophoresis.

Standard kits for LAMP are available (New England Biolabs), containing *Bst* polymerase with and without in-built colorimetric indicator. In the colorimetric indicator version, a colour change from pink to yellow occurs when DNA amplification occurs and this was designed to be examined by visual inspection. This version offered an attractive option for a simple, bench top test with minimal sample processing, and so was explored first. Validated LAMP primers for Pa were purchased (Life Technologies) using published sequences [169]. There are 4 primers (F3, B3, FIP and BIP) that make up the primer stock, with the following sequences:

F3: 5'- CAAGCGCAAGATAGTCGCC – 3'

B3: 5' – TCCGCTTGAACAGGCTGGTG – 3'

FIP: 5' – GAAGATATCCGGCTGGTTGCTTTTCAAGAGGGAATGCCGCAGT – 3'

BIP: 5' – AACGGATCATCGGCATCCTGGTTTTTCATCGCCGTCCACAGGTAGA – 3'

Whilst use of LAMP in detecting Pa in airway samples from pwCF has not previously been reported, research that examined detection of Pa from contaminated water supplies in the food industry suggested lower limits of detection of just 16 CFU/ml [170].

Primer stock recipe and LAMP reaction protocol was prepared according to manufacturer's instructions provided with LAMP reaction materials (New England Biolabs, appendix 6). Primer stocks were prepared using nuclease free water according to manufacturer's recommendation. Components were thawed and kept on ice during preparation. Reaction mix recipe combined 12.5µl LAMP mastermix, 2.5µl primer stock and 9µl deionised water. 1µl of test samples were taken directly from the huff-tube lab model broth samples and added to 1ml Eppendorf tubes and closed. These were placed in the heat block and heated at 94°C for 5 minutes to denature the sample. 24µl of reaction mix was then added, vortexed for 20 seconds, and returned to a heatblock at 65°C, for a minimum of 30 minutes. Detection of amplified Pa DNA was explored using colorimetric and fluorescent indicators, development of which is discussed iteratively in the results section, and confirmed against gel electrophoresis, as used with PCR (section 5.2.1). Of note, as multiple variations of length of amplified DNA can be produced in LAMP, so a single band may not necessarily be seen on gel electrophoresis but instead the presence of several bands, like ladder, or long columns of dye uptake, is evidence of DNA amplification [171].

5.2.3. Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

This work was performed in conjunction with a MSc student from a collaborating lab with experience and technical setup for MALDI-TOF, therefore, samples were prepared for processing, and results analysed externally. MALDI-TOF takes a target whole cell bacterial sample - in this case bacterial pellet from centrifuged broth samples from the huff tube lab model - and co-crystallises this with α-Cyano-4-hydroxycinnamic acid (HCCA) matrix [123]. This target is then desorbed and ionised by the MALDI ion source such as an ultraviolet laser. These ionised molecules including bacterial peptides and proteins are accelerated through an electric field to be analysed by TOF mass spectrometry analyser. Mass spectrum analysis of peptide/protein signatures are produced and matched to a library of recognised pathogen signatures, such as that for Pa.

To test the entire huff tube lab model, 10 different *Pa* isolates were revived and cultured in cetrimide broth to high concentration ($>10^7$ CFU/ml) (methods, 2.1.3. and 2.1.4.). Dilution in sterile cetrimide was performed to produce three different starting concentrations (high, medium and low) so that 20µl samples of each inoculated onto gauze contained approximately 10^6 , 10^3 and 10-100 CFU respectively. The samples were cultured in the huff tube model as previously stated (methods, 2.2.2.). 1ml aliquots of the resulting broth were transferred by pipette to 1.5ml Eppendorf tubes taken for MALDI-TOF analysis. Samples were centrifuged at 3214g for 15 minutes to generate bacterial pellet and supernatant discarded by pouring away. Only samples where adequate pellet was generated, that was visible on visual inspection, were suitable for analysis in the MALDI-TOF mass spectrometer. The pellet was scraped from the base of the Eppendorf tube using sterile inoculation loop and mixed with HCCA matrix within the testing wells of MALDI-TOF mass spectrometer. The mass spectrometer was run and mass spectrograms interpreted according to collaborating lab protocol (not presented). Of the remaining 9ml of the tested huff tube lab model samples, 1ml was used to perform colony counting (Methods, section 2.1.4.) and determine final, post-incubation, sample concentration and the rest saved for use in another workstream (chapter 6). Access to the collaborating laboratory MALDI-TOF spectrometer was limited to an initial single exploratory biological repeat of all 10 isolates, with plans for further repeats if results indicated useful limits of detection. MALDI-TOF results output was **Pa detected vs Pa not detected**, with those samples that did not generate adequate bacterial pellet reported as 'Pa not detected.'

5.3. Results

5.3.1. PCR

PCR can detect *Pa* directly from *Pa* cultured in cetrimide broth

In the first run of the model, for proof of concept and to investigate degree of sensitivity, 2 clinical isolates (labelled A and B) from the SRC-PAPA repository were revived on agar (Methods, section

2.1.3.) and single colonies subsequently inoculated into cetrimide broth and incubated overnight. A two-fold (log₂) dilution of each post-incubation bacterial culture was produced. PCR was run as outlined above (section 5.2.1.) with test samples including a single colony from culture plates of A and B added directly to PCR mastermix, 1µl of A and B dilutions series from neat (1) to weakest concentration (1/32 dilution), with 1µl known extracted Pa01 DNA (Sigma Aldrich, 10ng/µl) as positive control, and sterile cetrimide and mastermix alone as negative controls. Positive controls were detected as expected against DNA ladder, and cetrimide and mastermix negative controls were correctly excluded (Figure 5.1). Whole colony test samples were successfully detected by PCR and sample A was detected from neat (1) to 1/16, but negative at 1/32 dilution, whereas sample B was detected from neat to 1/2, but negative thereafter.

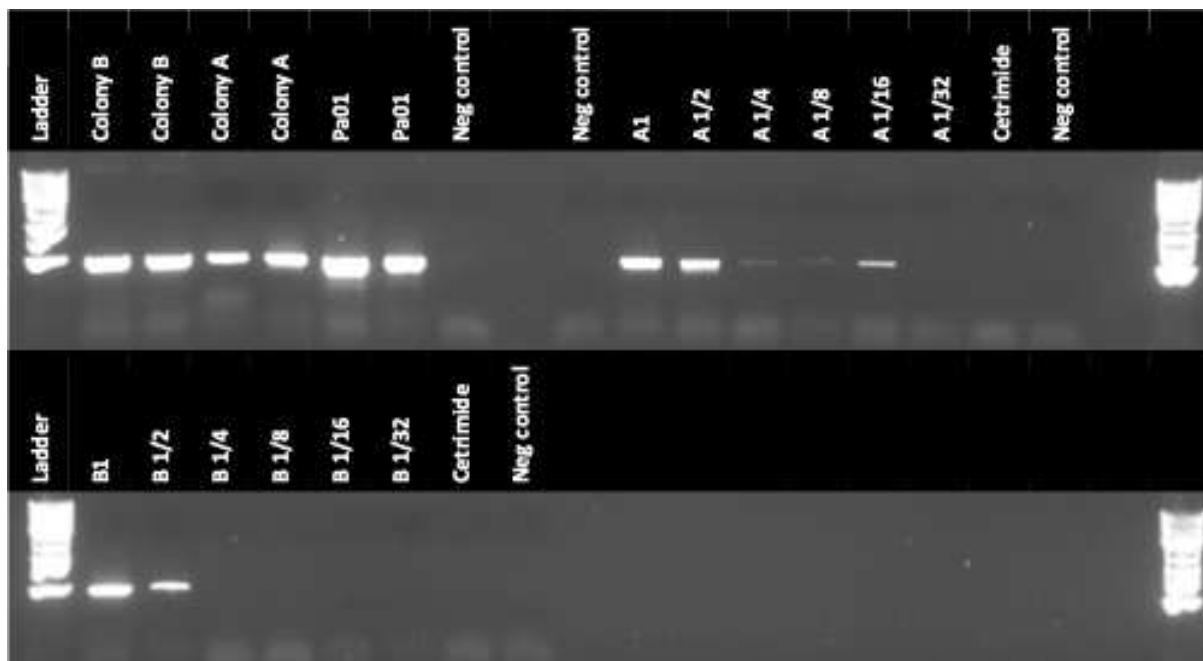


Figure 5.1: First attempt to detect Pa directly from bacteria cultured in cetrimide broth

Gel-electrophoresis following first run of PCR. Two clinical isolates (A and B) were revived on agar and subsequently single colonies from agar inoculated into cetrimide broth and incubated overnight to produce broth culture. Single colonies of A and B taken from agar, and a two-fold dilution series of A and B suspended in cetrimide broth were added to PCR mastermix as described in methods.

Positive control used 10ng Pa01 DNA (Sigma Aldrich), negative controls provided by sterile cetrimide broth, and mastermix alone. In situ DNA extraction in the thermal cyclor was possible, as demonstrated by single colonies A and B showing DNA present on electrophoresis bands. Samples suspended in cetrimide showed positivity with A detected at a 1/16 dilution and B at 1/2 dilution.

This pilot study illustrated the feasibility of performing DNA extraction in situ in the thermal cyclor, with no separate DNA extraction step needed.

Cetrimide appears to significantly reduce the sensitivity of PCR

The next trial looked to explore limits of sensitivity and confirm if cetrimide, which contains high concentrations of magnesium and potassium salts, might inhibit the PCR process. Whilst not directly demonstrated in literature, cetrimide salt compounds act as a cationic detergent, which can bind with DNA and interfere with primer annealing during PCR [172].

Two clinical isolates (A and B, as used above) were again revived on agar and then inoculated in 10ml cetrimide broth. Following incubation overnight at 37°C on shaking platform at 200rpm, samples were each split into 5ml aliquots and centrifuged at 3214g for 15 minutes to form a pellet. One pellet of each isolate was resuspended in 5ml fresh cetrimide and the other in sterile PBS. Each then underwent ten-fold (log₁₀) serial dilution using cetrimide or PBS respectively. Colony counting (methods, section 2.1.4.) was performed to establish the CFU/ml. PCR was performed using the same protocol (section 5.2.1.), with 1µl test samples against sterile cetrimide (C), PBS (P) and mastermix (M) as negative controls (figure 5.2). For isolate A, when diluted in cetrimide, a positive PCR band was only seen at neat, 10⁹ CFU/ml, (10⁶ CFU in 1µl test volume). In contrast, for the PBS suspension, a positive band was seen from neat until 10⁵ CFU/ml (10² CFU in 1µl test volume), and a faint band was seen at 10⁴ CFU/ml (10¹ CFU in 1µl test sample). For isolate B, again when diluted in cetrimide, a positive PCR band was only seen at neat, 10⁹ CFU/ml, (10⁶ CFU in 1µl test sample). In PBS suspension, whilst a positive band was missing at neat (10⁹ CFU/ml), which was likely an error in

sampling, a positive band was seen from 10^8 CFU/ml down to 10^5 CFU/ml (10^2 CFU in $1\mu\text{l}$ test sample). This suggests that cetrимide broth does inhibit PCR process at most concentrations, but when suspended in PBS, sensitivity of this model could be as low as 10^4 to 10^5 CFU/ml (10^1 to 10^2 CFU in $1\mu\text{l}$ test volume).

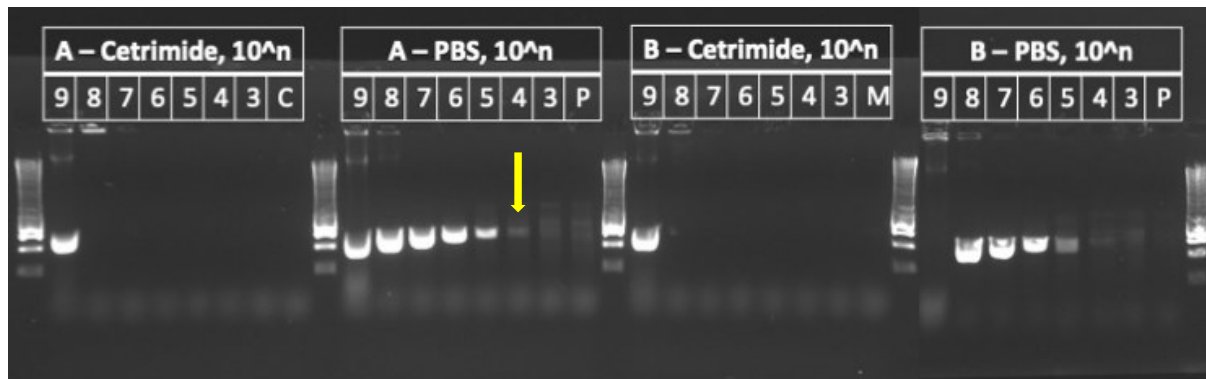


Figure 5.2: Comparison of PCR performed on samples directly from cetrимide broth against those resuspended in PBS.

Image of gel-electrophoresis of PCR comparison of Pa clinical isolates (A and B) in dilution series, with bacterial pellets suspended in cetrимide or PBS. Numbers represent CFU/ml of sample dilution series (10^n CFU/ml). $1\mu\text{l}$ samples were used in the PCR reaction mix. Limits of sensitivity are better in PBS suspended samples, suggesting direct sampling from cetrимide broth partially inhibits the PCR process. Yellow arrow indicates a faint band suggesting lower limit of detection 10^4 CFU/ml (10^1 CFU in $1\mu\text{l}$ sample in reaction mix) for isolate A. Note absent band in sample B in PBS at 10^9 CFU/ml, likely due to gel loading error. Sterile cetrимide (C), PBS (P) and mastermix (M) as negative controls.

Taq polymerase can be selected to improve sensitivity in this model when sampling directly from cetrимide

In the context of the huff tube lab model, resuspending all samples in PBS was initially considered not to be a viable option. This was because the aim was to detect low concentration growths with minimal (<24 hours) incubation times. The size of bacterial pellets from low concentration (< 10^3 CFU/ml) gauze-broth samples following incubation are too small and the resuspension process

would lead to further loss of sensitivity due to washing away of bacteria in the discarded supernatant. Additional processing steps would also impact the rapidity of the desired test.

Whilst all *Taq* polymerase is derived from *Thermus aquaticus*, manufactures can alter properties of their *Taq* products by adding stabilisers or altering purity of the polymerase, which could impact efficacy. Therefore, to overcome the inhibition of cetrимide, two different *Taq* polymerases were compared, to investigate whether tolerance of high density salts in the solution was better with alternative brands of *Taq* polymerase. GoTaq (Promega Corporation) was compared to SigmaTaq (Sigma Aldrich) mastermix. Using 10-fold serial dilution in cetrимide broth of isolates A and B, 1µl test samples were run against sterile cetrимide (C), and mastermix (M) as negative controls (figure 5.3). In this comparison, greater sensitivity was seen with SigmaTaq (10^7 and 10^6 CFU/ml with isolates A and B, respectively) than with GoTaq (10^8 CFU/ml with isolate A and not detected with isolate B). This suggests that certain *Taq* products can achieve better sensitivity than others, although still likely insufficient for the proposed huff tube lab model to detect a low concentration of bacteria.

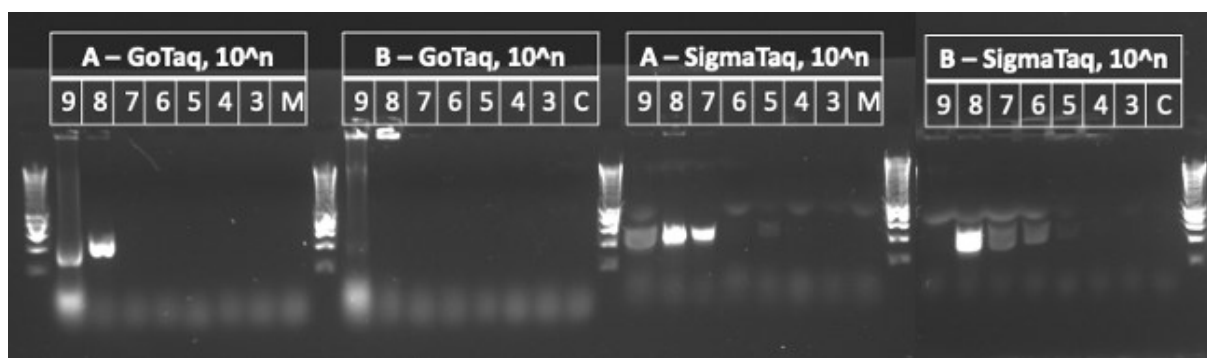


Figure 5.3: Comparison of different *Taq* polymerase brands when performing PCR with samples taken directly from cetrимide broth

Image of gel-electrophoresis of PCR comparison of Pa clinical isolates (A and B) in dilution series in cetrимide, using different brands of Taq polymerase, GoTaq and SigmaTaq. Numbers represent CFU/ml of sample dilution series (10^n CFU/ml). 1µl samples were used. Limits of sensitivity are lower

with SigmaTaq compared to GoTaq, suggesting different Taq brands may have better ability to overcome cetrimide inhibition of PCR. Sterile cetrimide (C) and mastermix (M) as negative controls.

Concentrating test samples by centrifuge prior to PCR can improve sensitivity but this method lacks reproducibility and adds processing time

In the design of the huff tube lab model, the amount of bacteria present in a sample, after the inoculated gauze was incubated, was expressed in CFU/ml as bacterial quantity was examined by colony counting. However, the low volumes needed for PCR reaction mean that, even at high CFU/ml concentrations, when 1 to 5µL samples are used in the reaction mix, only very small absolute numbers of CFU will be present. For example, when gauze-broth samples of $<10^5$ CFU/ml are tested using 5µl samples then <100 CFU will be present in the reaction mix. In order to improve sensitivity further, concentrating bacteria in the gauze-broth sample was attempted. To avoid washout of bacteria when transferring from one container to another, concentration of the sample occurred within the PCR plate. 90µl of gauze-broth samples after overnight incubation were added into the wells of a 100µl PCR plate. The plate was spun in centrifuge for 20 minutes at 3214g (maximum). Using a multichannel pipette, the majority of the supernatant was drawn off the top of each well. The PCR reaction mix, using SigmaTaq, was added directly on top of each well. This method had the potential added benefit of reducing the volume of cetrimide (and associated inhibitory salts) from the reaction mix. Testing again with isolate A and B in this scenario, against cetrimide negative control. In this scenario it appeared that Pa DNA was detected down to 10^3 CFU/ml, with a faint line also seen at 10^2 CFU/ml, suggestive of a highly sensitive test, occurring rapidly and directly from the gauze-broth sample pot (figure 5.4).

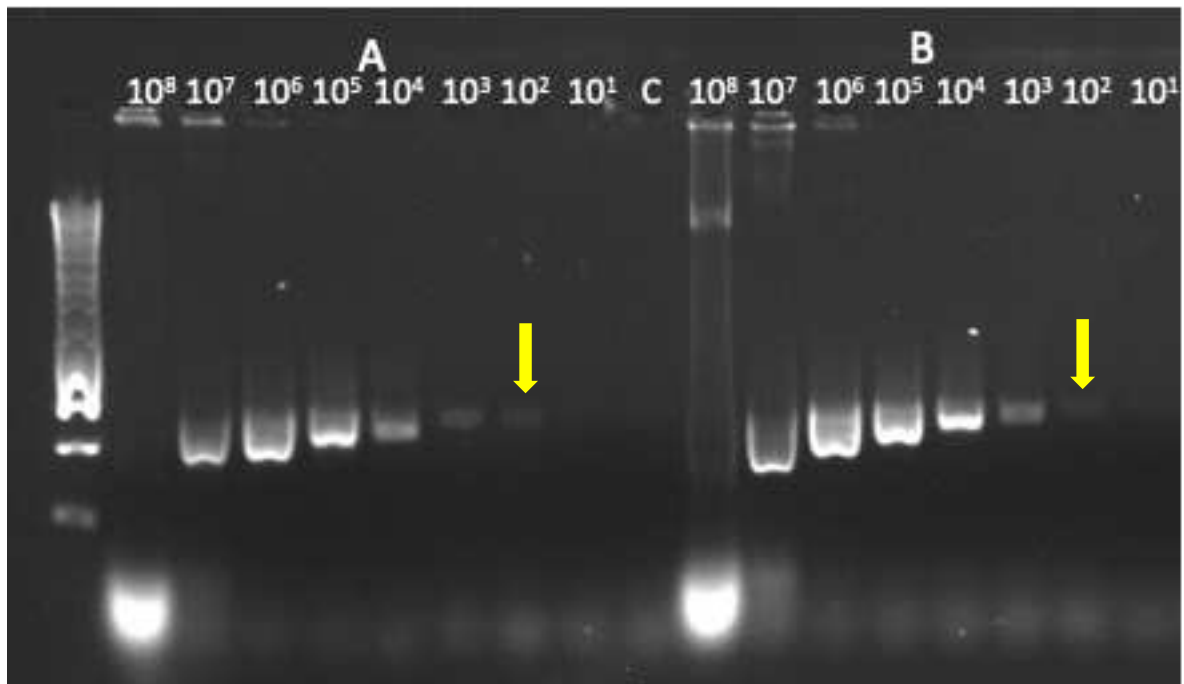


Figure 5.4: Concentrating bacterial samples in a multi-well plate for PCR to attempt to improve sensitivity when sampling directly from cultured cetrимide broth

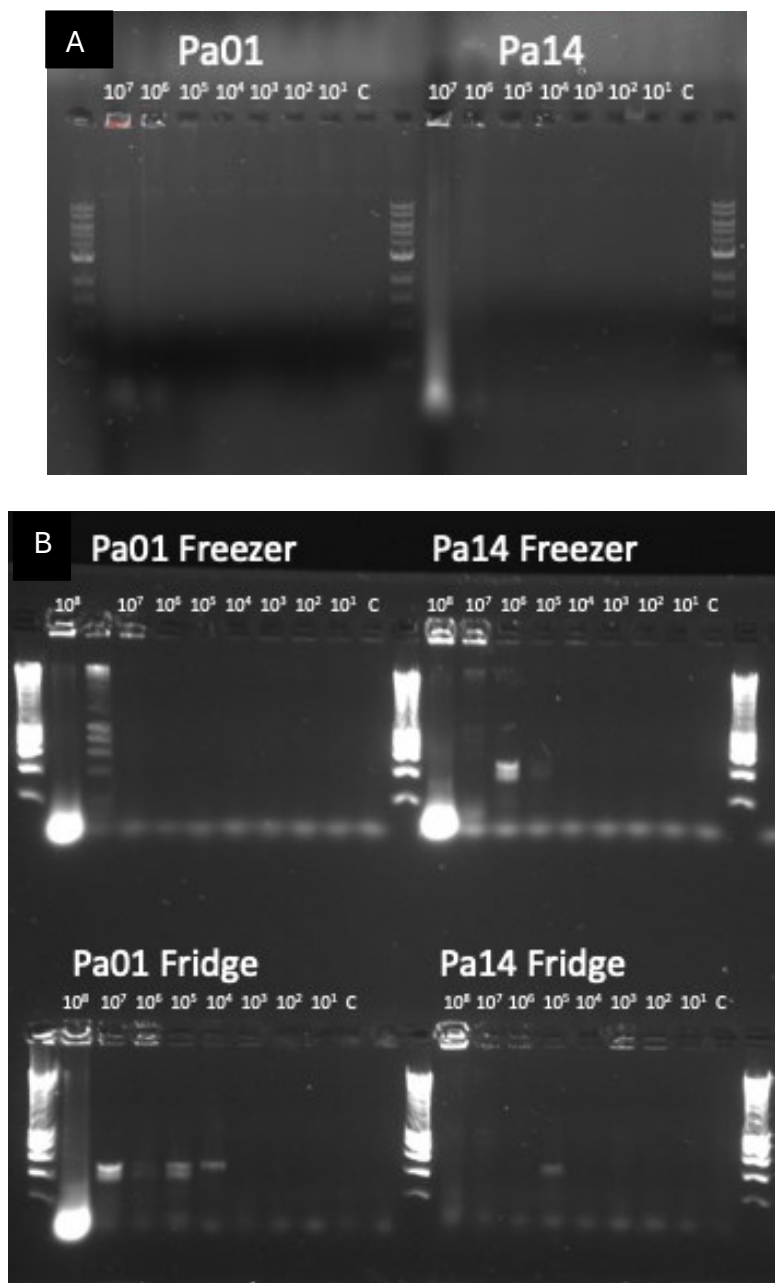
Gel-electrophoresis of PCR comparison of Pa clinical isolates (A and B) in dilution series, in cetrимide.

Numbers represent CFU/ml of sample dilution series (10^n CFU/ml). 90 μ l samples were added directly to the PCR plate and plate centrifuged. Majority of supernatant was removed using multi-channel pipette and PCR reaction mix added directly on top of presumed pellet at the base of each well.

Absolute quantity of CFU in each well is not known, but limits of sensitivity appear to be as low as 10^2 CFU/ml based on starting concentration tested (yellow arrow indicating faint band). Sterile cetrимide (C), as negative control.

However, after initial successful trial of this method as above, repeating the process in the same conditions showed poor reproducibility either with failure to detect any DNA present in the Pa containing samples, or demonstrating lower sensitivity than the original run, with lower limit of detection around 10^5 CFU/ml (figure 5.5A, as example). To examine this further, a series of single repeat experiments explored simple technical variables within the lab that could explain this. Firstly, the use of fresh and frozen gauze-broth samples was examined (figure 5.5B), as both had been used

in prior runs. Pa01 demonstrated greater sensitivity from fresh (refrigerated) samples to level of 10^4 CFU/ml, compared to frozen which was not detectable in this run. Pa14 demonstrated similar limit of detection of 10^5 CFU/ml in both frozen and fresh (refrigerated) samples. Comparison was also made of the lab stocks of DNTP (in-house prepared DNTP 1) and branded Sigma Aldrich stock (DNTP 2), and PCR primers (two version of in-house prepared PCR primers, A and B), shown in figure 5.5C. The use of primer stock B appeared to give greater sensitivity for detecting Pa14 (10^4 CFU/ml compared to 10^5 CFU/ml) but made no difference to Pa01 (10^5 CFU/ml) and DNTP stocks made no difference in any combination with primers.



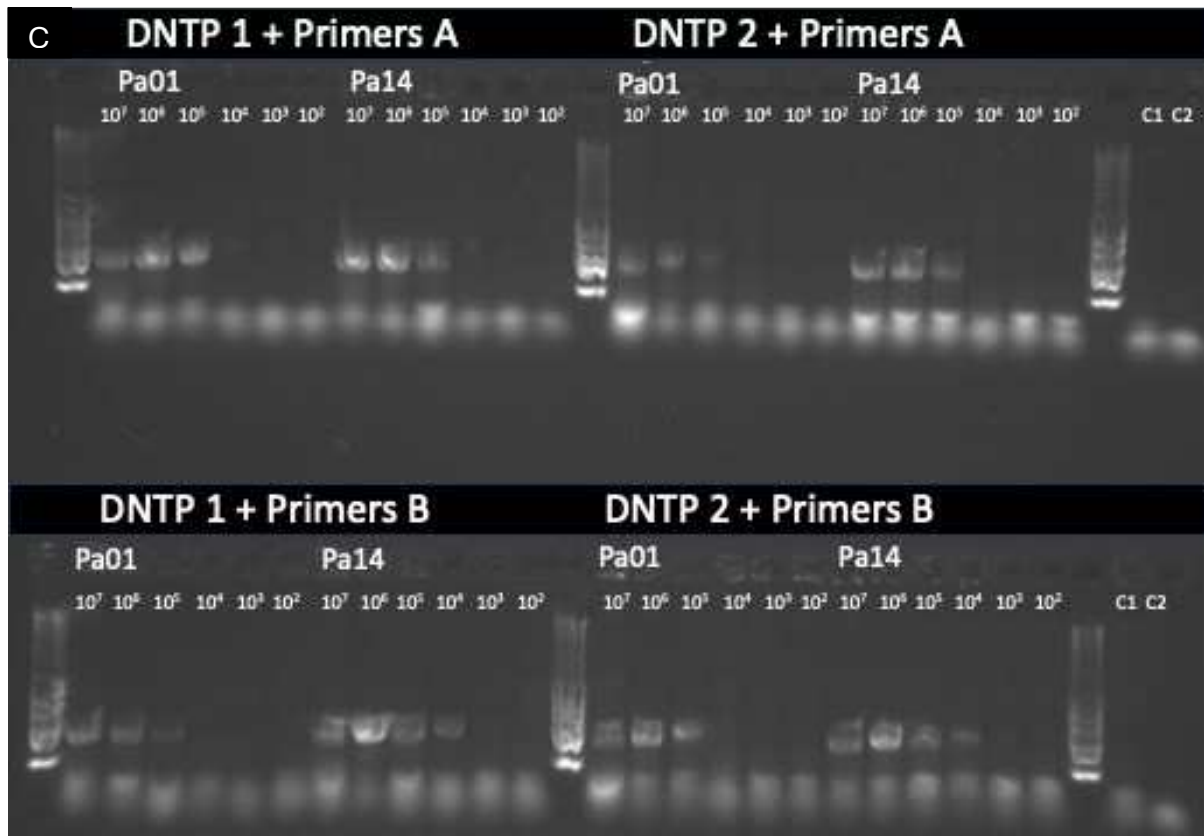


Figure 5.5: Exploring failures to reproduce results when attempting PCR by concentrating samples prior to analysis

Three examples of gel-electrophoresis from PCR experiments investigating poor reproducibility of previous successful method (figure 5.4). **A:** an example where the same methodology was followed but no DNA bands were detected. **B:** Comparison of fresh versus frozen samples of bacteria, suggesting better limit of detection with fresh compared to frozen samples of Pa01, but no difference in Pa14. **C:** Systematic comparison of two different stocks of DNTP (1 and 2) and two different stocks of primers (primers A and B); primer B seems to produce better sensitivity than primer A, but no difference based on stocks of DNTP used.

It was felt that, on balance, these factors alone were unlikely to be the cause of poor reproducibility seen with this methodology and was not explored further. It was considered that perhaps centrifuge efficacy (in producing a condensed bacterial pellet) and penetration of the pellet with PCR mastermix, and also practical inconsistency in method (taking broth supernatant off using a

multichannel pipette) leading to variable reaction volume and possible ongoing ceftrimide inhibition may also explain these findings.

In a final attempt to overcome this, the model was re-designed to maximise the quantity of bacteria (absolute CFU) in the sample by concentrating broth cultured bacteria in 1/10 volume, before serial dilution, and using PBS to resuspend samples to remove ceftrimide contamination. To do this, 10ml samples of cultured ceftrimide broth (approximately 10^8 CFU/ml, determined later by colony count) from the huff tube lab model were centrifuged at 3214g and resuspended in 1ml PBS, and dilution series created using 100 μ l of sample in 900 μ l of PBS, with serial steps diluting to approximately to 10^1 CFU/ml of starting, unconcentrated broth (methods, section 2.1.4). 5 μ l of each dilution step was then added to the PCR reaction mix. Despite this attempt, limit of sensitivity remained around 10^5 CFU/ml (figure 5.6).

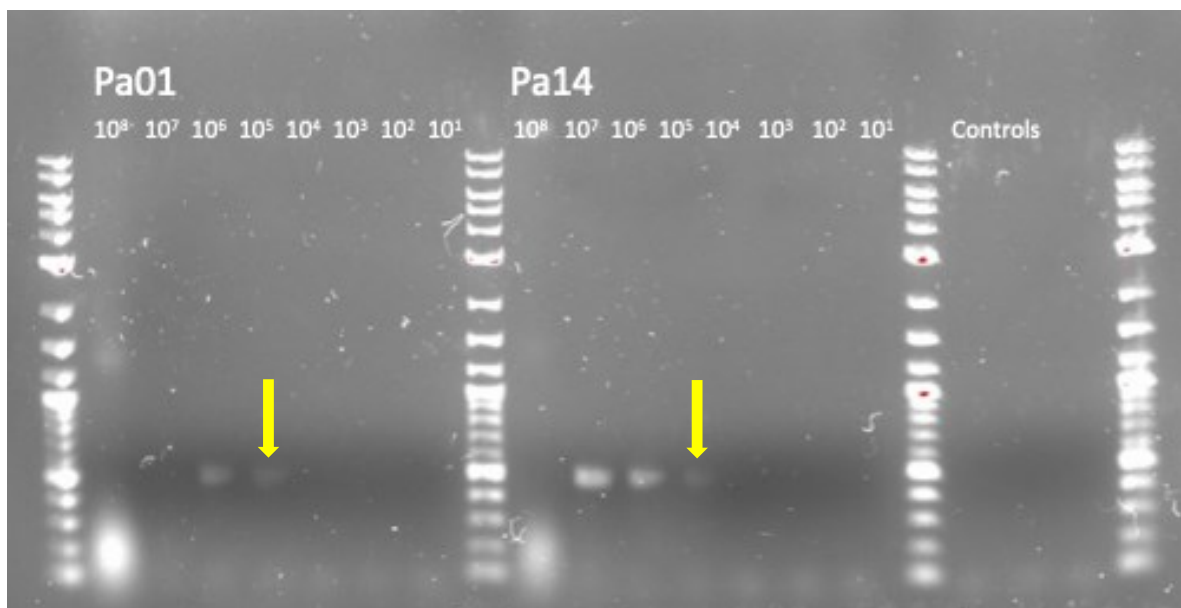


Figure 5.6: Final attempt to concentrate bacterial samples for PCR to attempt to improve sensitivity when sampling directly from cultured ceftrimide broth

Gel-electrophoresis of PCR comparison of Pa01 and Pa14 in dilution series in PBS. Each 10ml dilution series sample was concentrated by centrifuge and resuspension in 1ml PBS. Numbers indicate the concentration in CFU/ml at the start of process. Sensitivity remained around 10^5 CFU/ml (faint bands indicated by yellow arrows).

At this point, further exploration of PCR in this model was stopped, as it was expected that cetrимide inhibition and the imprecise natures of 'quick and easy' methods were leading to variable success and limited sensitivity. An alternative DNA based method called Loop-mediation isothermal amplification (LAMP) was explored, as it was expected that attributes of this method may help overcome some of the issues identified with PCR in the huff tube lab model.

5.3.2. LAMP

LAMP colorimetric indicators do not appear to change in presence of cetrимide

The first experiments employed the colorimetric dye tags, which meant reaction mixture should change from pink to yellow when the desired amplification of DNA occurred (i.e. if Pa DNA was present). Initially, required sample quantity, and effects of cetrимide being present were investigated. Manufacturer's instructions stated that a reaction time of 30 minutes is sufficient, though longer may be required in the presence of 'inhibitory substances.' In the first attempts high concentration samples (approximately 10^8 CFU/ml) were used, to examine necessary reaction quantities and reaction times, before limits of sensitivity were explored. Samples of Pa01 and Pa14 were cultured in the huff tube lab model in 10ml cetrимide broth and reaction volumes of 1 μ l, 5 μ l and 10 μ l, added directly into the reaction mix. The remaining gauze-broth samples were then centrifuged at 3214g and resuspended in equal volumes (10ml) of PBS, vortexed for 20 seconds, and 1 μ l, 5 μ l and 10 μ l added to reaction mix for comparison. As the reaction took place in a bench top heat block, samples could be removed at 30, 45 and 60 minutes to examine for colour change at each timepoint.

No colour change was seen by eye at 30 minutes (table 5.2). At 45 minutes some intermediate colour change (not complete yellow colour) occurred and at 60 minutes colour changes appeared more consistently. At 60 minutes, colour change was evident in PBS re-suspended positive samples, but not evident in samples taken directly from cetrимide broth, which suggested that cetrимide had

the same inhibitory effect on DNA amplification as it did in PCR. Smaller volumes of test sample (1µl) in the reaction mix, amongst PBS suspended samples, showed colour change where larger volumes (5µl) did not. PBS and cetrimide controls both showed intermediate colour change at 60 minutes.

Strain/Control	Suspension	Sample Volume (µl)	30 min	45 min	60 min
Pa01	Cetrimide	1			
Pa01	Cetrimide	5			
Pa01	Cetrimide	10			
Pa01	PBS	1			
Pa01	PBS	5			
Pa01	PBS	10			
Pa14	Cetrimide	1			
Pa14	Cetrimide	5			
Pa14	Cetrimide	10			
Pa14	PBS	1			
Pa14	PBS	5			
Pa14	PBS	10			
Negative control	Cetrimide	5			
Negative control	PBS	5			
Mastermix control	N/a	N/a			

	Negative
	Positive
	Intermediate

Table 5.2: First attempt at using LAMP with colorimetric indicators to detect presence of Pa DNA in the huff tube model

Results of first LAMP experiment, using Pa01 and Pa14 cultured in the huff-tube model in cetrimide.

Samples were taken and added to the reaction mix at different volumes (1, 5 and 10 µl), and

resuspension of the same cultures in PBS were also tested at the same sample volumes for comparison. Reaction colour change was observed at 30, 45 and 60 minutes. No colour changes occurred in the presence of bacteria and cetrimide, but colour change was visible at 60 minutes reaction in PBS at lower sample volumes. Intermediate colour change occurred in some samples, including negative controls of cetrimide and PBS, the interpretation of which was unclear.

Cetrimide inhibits LAMP colorimetric indicators but does not inhibit DNA amplification

The differential colour changes in cetrimide broth compared with PBS suspended Pa positive samples, suggested either inhibition of DNA amplification by cetrimide again, or differential colour change based on presence of cetrimide or PBS. The presence of intermediate colour changes in negative controls however made it more likely that the colorimetric indicator was being impaired by a process separate to DNA amplification, or that the sample preparation process was open to DNA contamination. To examine this further, 1µl samples of Pa01 and Pa14 (approximately 10⁸ CFU/ml) in cetrimide from the huff tube lab model, and 1µl of the same cultures resuspended in equal volume PBS were examined again using the LAMP protocol (section 5.2.2.), with up to 60 minutes allowed for reaction time. At the end of the process, each sample was run on gel electrophoresis (as per PCR methods, section 2.2.3) to confirm presence of amplified DNA in the reaction mix (figure 5.7). Of the positive samples, amplified DNA was confirmed present in both neat cetrimide and resuspended PBS samples, by gel electrophoresis. However, of these positive samples, colour change only occurred in those suspended in PBS. Mastermix alone and cetrimide negative controls did not change colour and were DNA free on electrophoresis. PBS negative control appeared contaminated with Pa DNA and showed colour change and a band on the gel.

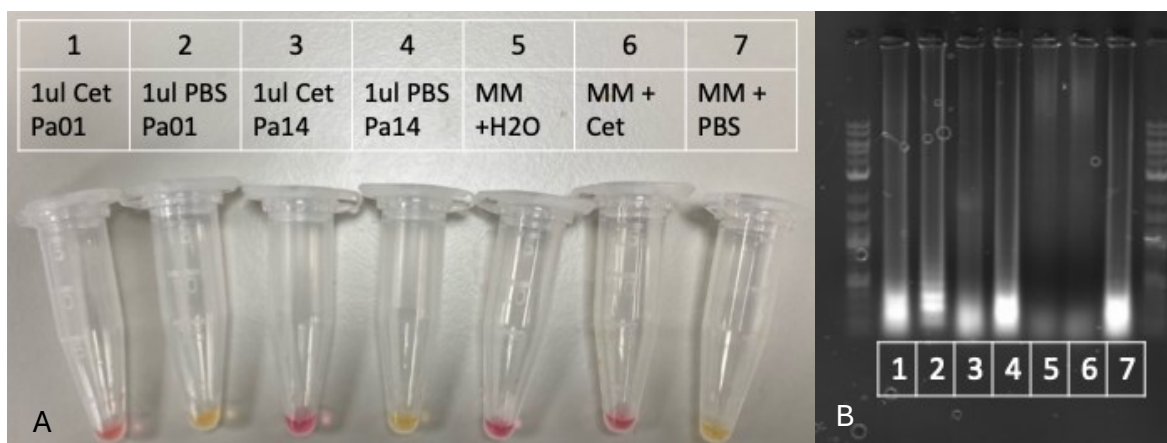


Figure 5.7: Comparing use of LAMP colorimetric indicators to detect Pa in samples taken directly from cultured cetrinide broth and samples resuspended in PBS

A: photo of reaction tubes (1-7) indicating colour change for different conditions, with pink being negative (no DNA amplification) and yellow being positive (DNA amplification occurred). Cet= cetrinide, PBS=Phosphate Buffer Solution, MM=mastermix. **B:** image showing corresponding gel-electrophoresis using the sample following LAMP process, to look for presence of amplified DNA.

Note, multiple variations of length of amplified DNA can be produced in LAMP, so presence of several bands or a column of bright dye or ladder is evidence of DNA amplification [171]. Pa containing samples (1, 2, 3, 4) appropriately showed amplification on gel-electrophoresis, but only those suspended in PBS (2, 4) showed appropriate positive colour change. Water and cetrinide controls (5, 6) showed no DNA amplification (just residual loading gel in the well) and appropriately no colour change. PBS control (7) appeared contaminated, showing positive colour change and amplified DNA on gel-electrophoresis.

At this point it was clear that cetrinide did not inhibit the amplification process but did affect the colour change indicator. Further reading highlighted that the colour change in the product was pH dependent and highly buffered and high pH samples (as may be assumed in high magnesium salt containing cetrinide) can inhibit colour change.

LAMP with fluorescent indicators were not a suitable alternative to colorimetric indicators.

At this point, alternative LAMP rapid detection strategies were investigated. Separate fluorescent intercalating dye tags (New England Biolabs) can be added that bind to double stranded DNA, fluorescing when DNA amplification has occurred. A wide range of fluorescent dye quantity was suggested in the manufacturer's specifications (0.05 to 0.5 μ l per 25 μ l LAMP reaction mix). Despite attempts across a range of four quantities within the manufacturers specifications, no visual or quantified (measured by relative fluorescent intensity in the plate reader) difference was seen between Pa positive and negative controls (results not presented). Fluorescent dye-tags can be linked to strong primer-dimer interactions, which can occur in LAMP in negative controls [173]. Further exploration of this methodology was not performed.

Issues with sample testing with PCR and LAMP relating to use of cetrimide may be resolved by using non-selective culture media instead

With both PCR and LAMP, it was clear that use of Pa selective broth like cetrimide was challenging for simple and minimal step sample processing. The PCR reaction was inhibited by the high concentration of complex salts in cetrimide, when compared to samples resuspended in PBS. In LAMP, pH dependent colorimetric indicators were buffered by the presence of the same salts in the reaction mix. Whilst a selective broth had been favoured for the gauze model, as a way to improve specificity of any subsequent detection test, given PCR and LAMP should have very high levels of specificity as DNA based tests, it was considered appropriate to examine both methods with a general media broth (LB) to see if these obstacles from cetrimide could be overcome.

Samples were produced in the same huff-tube lab model, with Pa14, but using the same volume of LB broth instead of cetrimide. Further steps were taken to reduce contamination of controls with ultra sterile sample processing. Purchased guaranteed DNA-free water stocks (as opposed to sterilised deionised water or PBS produced in the lab) were used and mastermix preparation

occurred in a clean hood environment, rather than bench top. Broth solutions were UV treated overnight before use. Test samples were prepared as follows:

- Pa14 was revived and cultured in 15ml LB broth (methods, section 2.1.3.) The sample was briefly vortexed to distribute bacterial suspension and split into 3 x 5ml aliquots using pipette.
- From the first 5ml aliquot, a 10-fold dilution series was produced in sterile LB from a concentration of approximately 10^7 CFU/ml down to 10^2 CFU/ml (methods, section 2.1.4.).
- The second 5ml aliquot was centrifuged at 3214g, supernatant discarded by pouring and then resuspended in 5ml of DNA free water and 10-fold dilution series produced in DNA-free water, also from a concentration of approximately 10^7 CFU/ml down to 10^2 CFU/ml (methods, section 2.1.4.).
- The third 5ml aliquot was centrifuged at 3214g and resuspended in 0.5ml of DNA free water (to see if concentration improved sensitivity) and a ten-fold dilution series was produced as above.
- A 5 μ l sample of each dilution step from each group was added to PCR reaction mastermix and run as outlined previously (section 5.2.1.).

Limits of sensitivity in LB, DNA free water and 1/10 concentrated DNA free water were similar, approximately 10^5 CFU/ml (500 CFU) (figure 5.8). In LB, a faint band was seen at 10^4 CFU/ml.

Controls were appropriately negative. LB did not appear to inhibit PCR in the same way cetrimide did and achieved similar sensitivity to early experiments where cetrimide cultured samples were resuspended in PBS. Resuspending LB cultured samples in same volume or 1/10 volume of DNA free water conferred no further gain in lower limit of detection.

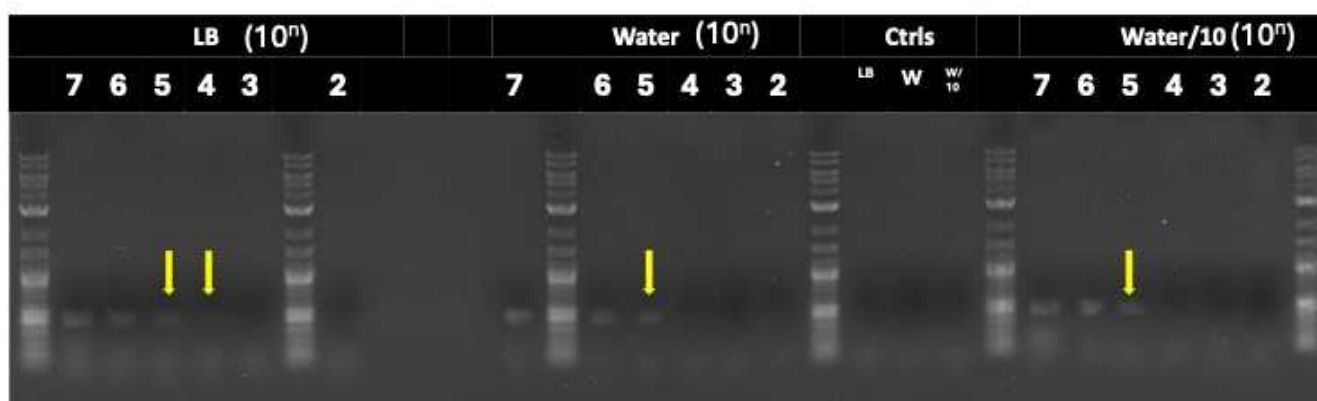


Figure 5.8: Using PCR to detect Pa in the huff tube lab model when LB is used as culture media instead of cetrimide

Gel-electrophoresis of PCR comparison of Pa14 in LB (Luria Bertani) broth, resuspended in DNA-free water at same volume (water) and at 1/10 volume (water/10). Numbers represent CFU/ml of sample dilution series (10^n CFU/ml). Limits of sensitivity at 10^4 to 10^5 CFU/ml again (yellow arrows indicating faint bands), with no improvement in sensitivity with concentration of sample resuspension. PCR appears more tolerant of LB than cetrimide in previous experiments. Controls are negative.

This was repeated with 5µl of the same concentrations in LB, DNA free water and 1/10 concentrated DNA free water using the LAMP methodology with colorimetric indicators. LAMP reaction was run for 30 and 45 minutes, with colours noted at each time point. Gel electrophoresis was run after 45 minutes of LAMP reaction time.

Interpreting positive amplification of DNA on gel electrophoresis was challenging in this experiment. As discussed, because multiple lengths of DNA fragments can be produced, amplification can be indicated by a column or ladder of bands of DNA on the gel. Whilst easy to distinguish from residual loading gel in the wells in the previous run (figure 5.7), in this experiment two possible patterns of amplification were seen (figure 5.9). One showed a clear band halfway up the run, and the other a column of dye dragged up from the loading well. The latter appeared brighter than just residual

loading dye in the loading well. Both of these patterns were considered to be positive DNA amplification, but results should be interpreted with caution. On this basis it was found that:

- DNA was detected by electrophoresis in all LB samples, down to 10^2 CFU/ml. A positive colour change occurred at all concentrations from 10^6 CFU/ml and below, at 30 minutes, however, at 45 minutes the colour change became intermediate. LB control is appropriately negative for colour change and electrophoresis.
- In water resuspended samples, Pa DNA is detected at all concentrations by gel electrophoresis. Colour change occurred in all samples, except at 10^4 CFU/ml at 30 minutes. Water control (W) colour change was negative at 30 minutes but became positive at 45 minutes and DNA was detected on electrophoresis, meaning results cannot be meaningfully interpreted.
- In 1/10 concentrated water resuspended samples, DNA was detected at all concentrations by gel electrophoresis. Colour change was only confirmed at 10^7 CFU/ml at 30 minutes but detected to 10^3 CFU/ml at 45 minutes. The control (W/10) was negative by colour change at 30 minutes, but positive at 45 minutes and DNA was detected on electrophoresis, meaning results cannot be meaningfully interpreted.

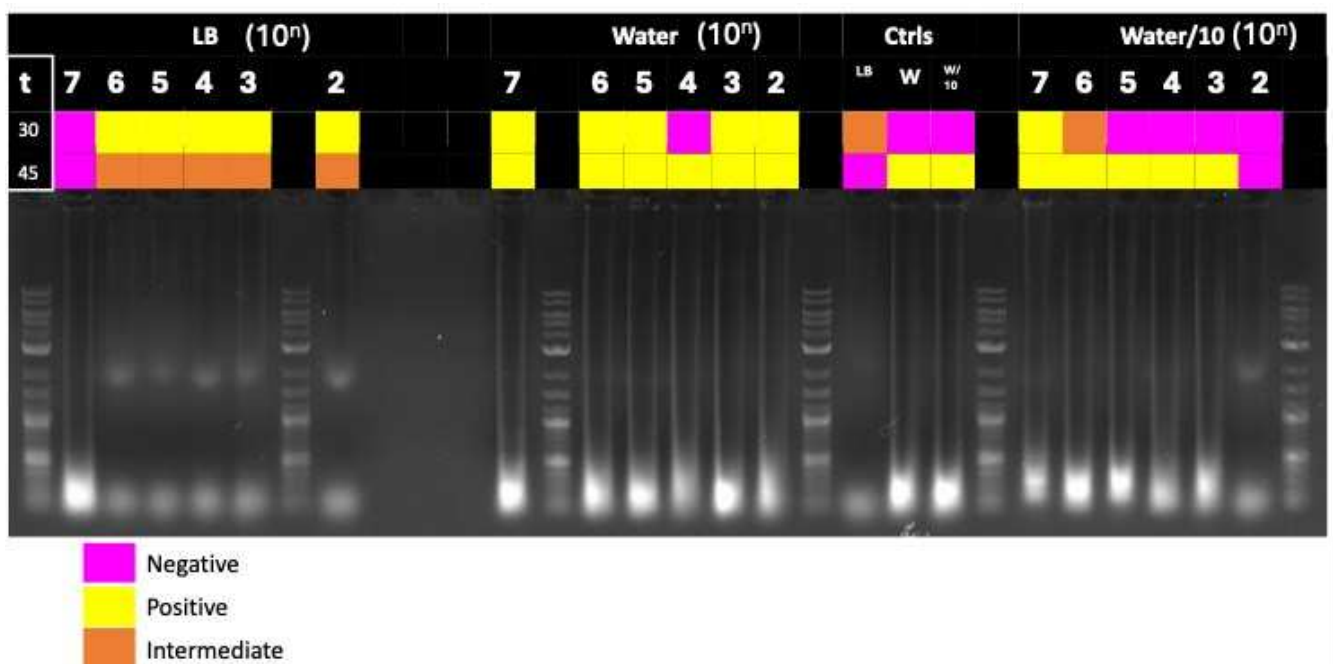


Figure 5.9: Using LAMP to detect Pa in the huff tube lab model when LB is used as culture media instead of cetrimide

Gel electrophoresis of LAMP comparison of Pa14 in LB (Luria Bertani) broth, resuspended in DNA-free water at same volume (water) and at 1/10 volume (water/10). Numbers represent CFU/ml of sample dilution series (10^n CFU/ml). Colour change is indicated at time (t) 30 mins and 45 mins and resulting DNA bands/column/ladder on electrophoresis seen below. On the basis that a band or bright column/ladder indicated DNA amplification, DNA was detected by electrophoresis at all concentrations in LB, and LB control was negative. Positive colour change occurs in all LB concentrations at $t=30\text{min}$ except 10^7 CFU/ml. Water controls (W and W/10) appeared to contain DNA and could suggest contamination, making further interpretation of these results difficult.

Two further attempts to modify this method were made, examining whether shorter reaction time or removing the colorimetric indicator from the reaction mix made a difference, however no solution was found, with inconsistent colour change and intermittent control contamination remaining an issue when gels were run (results not shown). It was concluded that, in this experimental design, LAMP colour change was too temperamental, too easily contaminated or impacted by external factors to be clinically useful, and too easily contaminated or affected by primer-dimer interactions, to provide a rapid test with minimal sample preparation.

5.3.3. Sensitivity of PAL1 and 2 PCR primers in the huff tube lab model when used with LB

Acknowledging that LB may be a better choice of media in testing the huff tube lab model with PCR, specificity of PCR primers was considered in a final step. To do this, direct PCR using $5\mu\text{l}$ of bacteria grown in LB broth (without resuspension in water/PBS), undiluted at high concentration (approximately 10^8 CFU/ml), using the same PCR methodology (section 5.2.1.), was performed on Pa01, Pa 14, 5 clinical isolates of Pa, 5 non-Pa clinical isolates, and a combination of Pa and non-Pa bacteria, cultured separately but mixed together in equal volume in the same test sample. This

methodology detected Pa01, Pa 14 and all 5 clinical Pa isolates (figure 5.10). Non-Pa isolates of Sa, Bcc and Smt were appropriately negative. Faint DNA bands were seen in Pa isolates in combination with Smt and Bcc but were not seen in combination with Sa. Sa was cultured in BHI (due to poor revival in LB), and BHI is known to inhibit PCR [174], so this is likely an artefact. LB, BHI and mastermix controls were negative.

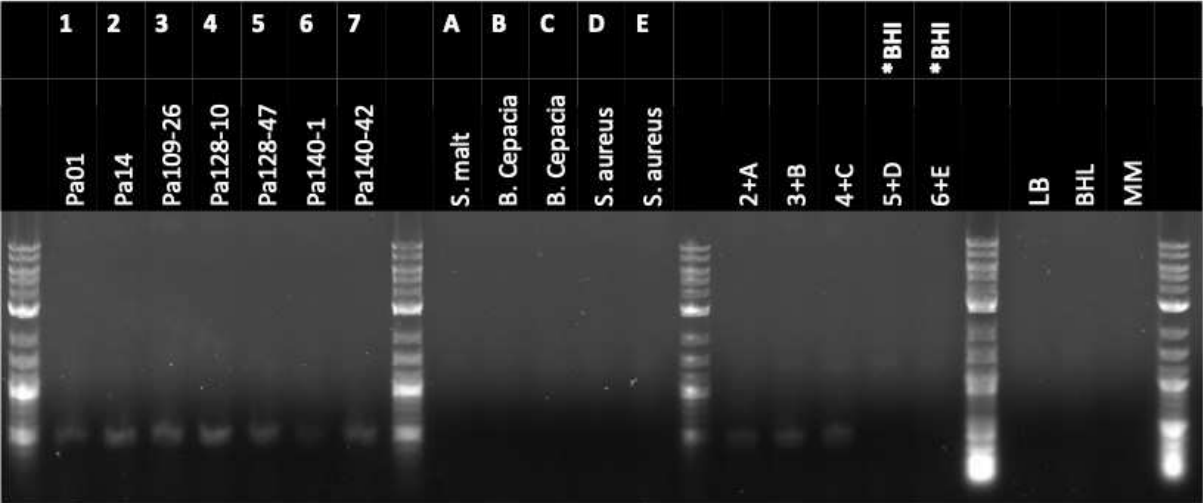


Figure 5.10: Examining specificity of PCR primers in the huff tube lab model

Gel-electrophoresis of PCR used to examine specificity of the PAL1 and PAL2 primers. A range of Pa isolates were used (1 = Pa01, 2 = Pa14 and 3-7 = five clinical isolates from pwCF, from the SRC-PAPA repository), which were all correctly positive. A to E are clinical isolates of other, non-Pa, CF pathogens including B. cepacia, all were correctly negative. Isolates were grown in LB, unless needing to contain S. aureus, which required Brain-heart infusion (BHI) broth. When Pa and other-non-CF pathogens are used in combination in LB, Pa is correctly identified. When used in combination with S. aureus in BHI, PCR appears to be inhibited. Controls were correctly negative.

Results indicate that use of PCR primers PAL1 and 2 are specific for Pa cultured in LB when sampling directly from broth culture, even in the presence of 2 different bacteria in the sample. Given limits of sensitivity reported above, it is possible that PCR of samples taken directly from cultured broth may be a useful rapid diagnostic test in the huff tube model.

5.3.4. MALDI-TOF Mass Spectrometry

20µl samples of Pa, from 10 different clinical isolates, each at three approximate starting quantities of high ($\sim 10^6$ CFU), medium ($\sim 10^3$ CFU) and low (~ 10 -100 CFU) were inoculated onto gauze and cultured in the huff tube model as previously stated (methods, 2.2.2.). 1ml aliquots of the resulting broth were transferred by pipette to 1.5ml Eppendorf tubes and taken for MALDI-TOF analysis at a collaborating laboratory. Final concentration (CFU/ml) of the test samples was measured by colony counting (methods, 2.1.4.). Access to the collaborating laboratory MALDI-TOF spectrometer was limited to an initial single exploratory biological repeat of all 10 isolates.

Irrespective of starting inoculating quantity, isolates achieving final post-incubation concentration of $>10^8$ CFU/ml were consistently detected by MALDI-TOF (figure 5.11). Of the five isolates that achieved final concentrations $>10^7$ but $<10^8$ CFU/ml, three were detected and two were not. Of the 11 isolate with a final concentration $<10^7$ CFU/ml, one, with a final concentration of 10^3 CFU/ml, was detected by MALDI-TOF. All samples with a high ($\sim 10^6$ CFU) inoculating quantity were detected, irrespective of final concentration. Four of 10 medium (10^3 CFU) starting quantities were detected, and three of 10 low (10-100 CFU) starting quantities were detected.

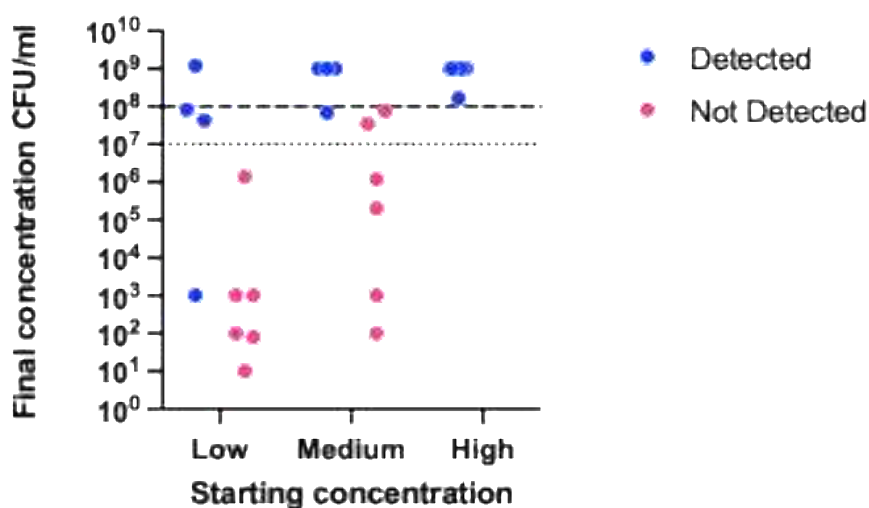


Figure 5.11: Limits of detection of Pa in the huff tube lab model using MALDI-TOF

Limits of detection of MALDI-TOF mass spectrometry in detecting 10 different isolates of Pa (single biological repeat). Each isolate was cultured in the huff tube lab model from 3 different inoculating

bacterial quantities, high ($\sim 10^6$ CFU), medium ($\sim 10^3$ CFU) and low (~ 10 -100 CFU) and end CFU/ml calculated by colony counting. Blue dots represent samples that were identified as Pa by MALDI-TOF and pink dots those that could not be. Limits of detection for MALDI-TOF were between 10^7 and 10^8 CFU/ml.

MALDI-TOF does not work with sufficient sensitivity for the aims set for Pa detection in this model, as either a high immediate sample collection bacterial load ($>10^6$ CFU) or high post incubation concentration ($>10^8$ CFU/ml) is needed.

5.4. Discussion

In the absence of reliable sputum production in the CF clinic, alternative methods of infection surveillance are needed. Non-culture based identification of bacteria from airway samples may be useful, if they can help overcome issues of sensitivity with currently available non-sputum based tests, like cough swabs, and support use of other non-invasive tests. To be useable on a large scale, in all CF clinics, and repeatable multiple times a year to meet Standards of Care, a test would ideally require minimal sample processing. Ideally any test would need to improve on turnaround times of standard culture methods, which currently take at least 48 hours to confirm a negative sample.

As part of the development of this aim, a novel model of non-sputum based airway sampling was considered, a huff tube, that uses gauze packed into a mouthpiece, to capture moist breath from the patient as they perform a forced expiratory manoeuvre (a huff). With this design greater capture of bacteria was expected than with a cough swab, due to increased surface area to absorb breath and droplets. A lab model mimicking this was devised and demonstrated ability to grow clinical isolates of Pa from starting concentrations of less than 10^3 CFU/ml to reliably over 10^5 CFU/ml in less than 24 hours (methods, section 2.2.2.). In this chapter, DNA-based methods and mass spectrometry were

explored to test for Pa following the bacterial expansion stage from brief culture in this huff tube lab model.

PCR was examined first. PCR for Pa is not routinely used in clinical practice, but multiple authors have demonstrated the ability to detect Pa with PCR with very high specificity [120–122,125]. All have performed separate DNA extraction from the sample, and it is acknowledged that detection is largely dependent on the concentration or quantity of substrate going into the reaction mix. In the huff tube lab model, a brief culture step was used to increase bacterial abundance prior to diagnostic methods being applied. Over a series of iterative experiments, it was demonstrated that PCR can detect Pa in samples produced in the huff tube lab model, and samples can be taken directly from the post-incubation gauze-broth without the need for complex processing or DNA extraction. However, cetrimide broth, used for selective Pa growth, appears to actively inhibit the PCR reaction, therefore, sensitivity was limited when this was used, and attempts to manipulate methodology to overcome this were not successful. Use of a selective broth in the huff tube model was originally chosen to help isolate Pa and improve specificity of an overall test process from collection to diagnosis. However, given the extremely high specificity of PCR and DNA based tests, use of a more general broth was later considered. Using LB broth in the model instead, the lower limit of detection appears to be around 10^5 CFU/ml, which if using a 1 μ l test sample, meant detecting approximately 100-1000 CFU, without the need to wash and change the bacterial buffer. The huff tube lab model could generate bacterial concentrations of $>10^5$ CFU/ml and so this method could be acceptable. However, it would still be dependent on an unknown factor which is the number of CFU that can be captured in the huff model from a single huff. On this basis though, this methodology is promising and potentially worth testing further with clinical samples.

Despite validated PCR primers being available for over a decade, PCR remains predominantly a research tool, supporting lower airway culture (sputum and BAL) diagnostics and assessment of the lung microbiome. However, multiple studies have examined the use of PCR on lower airway samples

in clinical practice. In a 3 year prospective cohort study, 96 patients with no evidence of chronic *Pa* infection at the start were enrolled and had sputum samples collected at each clinic visit [141]. Sputum samples were examined by standard culture and PCR, with 36 becoming *Pa* culture positive over the course of the study. Evidence of *Pa* infection was detected by PCR in 20 of 26 patients **before** culture positivity, with a median detection time gain of 8 months. In another 3 year study of 20 adult and 20 paediatric pwCF who were clear of *Pa* infection for 12 months before enrolment, standard culture of lower airway samples found *Pa* present in 9% of samples, but PCR detected presence of *Pa* DNA in 61% of samples. [175]. These are massive discrepancies, and have significant implications for classification of chronic infection, by current definitions, as well. Unlike culture, PCR will also detect the presence of dead bacteria in a sample, so the relevance of these results also needs to be considered in this context. Whilst the focus of using non-culture based methods like PCR is admirable, studies to date still rely on an abundance of sputum as substrate. The realities of post-ETI CF symptomatology mean that this cannot be relied upon and examining the literature to date, research on the use of PCR in those that do not produce sputum is lacking.

LAMP offered an alternative DNA based strategy for detecting *Pa*. The purported advantages of LAMP as a DNA amplification method was that *Bst* polymerase was better at tolerating complex media and substrates and could be used without DNA extraction and purification [167,168]. It can also be performed in a simple heat block, without need for an expensive thermal cycler, as needed for standard PCR. LAMP produces huge quantities of amplified DNA, and whilst this DNA cannot be manipulated or used beyond its amplification, as a diagnostic tool it should be sufficient. To date, no published literature shows the use of LAMP in detecting *Pa* from CF clinical isolates or being used in clinical practice. However, one paper examining *Pa* detection from other, non-CF, *Pa* isolates showed the validation of LAMP primers for *Pa*, with high specificity [169].

Again, iterative experiments were performed to explore use of LAMP as a diagnostic tool for the huff tube lab model. A colorimetric indicator was first explored, as the option of a visible-by-eye output

was very attractive. However, presence of cetrимide salts in the reaction mix likely affected the pH dependent colour change. In the final experiment using LAMP, switching Pa culture media to LB, with a run time of 30 minutes, was probably helpful and could offer route for further exploration, but was not possible within the time constraints of this project. This method did show sensitivity as low as 10^2 CFU/ml, however wider issues of suspected recurrent DNA contamination of some controls needs to be considered too and so these results, without time for further repeats, should be interpreted very cautiously. In addition to complications of false positives and negatives from likely pH-related effect on the colorimetric indicator, it has since become apparent that false positive DNA amplification can occur when viewed on gel electrophoresis due to primer-dimer interactions that readily occur in LAMP due to presence of larger number of primers[173]. Other colorimetric tags can be used, including fluorescent dye tags, though these can be linked to primer-dimer interactions leading to false positives. Another colorimetric indicator, hydroxynaphthol blue, which is not pH dependent or linked to DNA, but instead is calcium dependent secondary to the amount of DNA amplification occurring, could still be explored. Also not examined was the use of lateral flow tests for amplified DNA, which have been used with LAMP amplification products [167]. Underlining the importance of making tests simple and replicable around clinical labs, recent evidence has shown a way to use modified CRISPR/Cas12b in a single tube LAMP assay at isothermic conditions (55°), to detect Pa from non-CF clinical isolates with high specificity and sensitivity [176]. Such methods could be examined further to extend this explorative LAMP work for Pa detection from patients with CF.

MALDI-TOF is used in clinical settings for confirmation of culture diagnostics in the microbiology lab and has been used for CF patients. MALDI-TOF has shown to be highly specific and can be sensitive enough to detect Pa in samples with 10^4 to 10^5 CFU [119,123]. MALDI-TOF was examined as a diagnostic output for the huff tube lab model as well, with work performed in conjunction with an MSc student from the Larrouy-Maumus lab at Imperial College. This lab group have significant experience with detecting and analysing lipid structures within Pa [177]. In this model, cetrимide was still used as the broth for culture of gauze inoculated samples. This investigation demonstrated

disappointingly poor sensitivity for Pa, with only isolates being applied to gauze at high quantity ($\sim 10^6$ CFU), being consistently detected. End concentration of bacteria after incubation in the huff tube lab model needed to be $>10^8$ CFU/ml to guarantee detection. In this assay, a sample volume of 1ml was centrifuged into a pellet for analysis, therefore, at this concentration, the number of CFU per pellet needed to achieve detection was much higher than 10^4 to 10^5 CFU. However, it is possible that salts within cetrimide can act as surfactant and form adducts with matrix molecules, complicating observed spectra [178]. It may be that only at higher bacterial concentrations could this effect be overcome. Further work investigating MALDI-TOF combined with Pa cultured in LB instead of cetrimide was not possible in the time and resources available to the project but could be an important next step for future work.

These experiments have a number of limitations. Firstly, committing from the outset to examine the test directly from the huff tube lab model meant commitment to certain aspects, such as use of cetrimide broth, that took time to overcome. Approaching these variables separately might have allowed stronger conclusions about the correct approach for each technique and determined levels of sensitivity more clearly. Whilst the pursuit of a detection method with minimal processing was part of the aim, use of DNA extraction methods could have been explored to confirm that Pa DNA can be detected at similar levels of sensitivity to other PCR studies. Addition of steps such as lysozyme incubation or bead beating, to help lyse the bacterial cell surface and release DNA, could have been explored. Absence of adequate or consistent cell lysis by heat treatment alone may have been another reason for the sometimes variable and limited DNA detection sensitivity seen in both PCR and LAMP experiments.

Reviewing literature relating to this work, it is concerning how the focus for assessing non-culture based diagnostics is limited to sputum and BAL. Whilst these tests are clearly able to provide diagnostic certainty with high specificity and sensitivity, and some cases sooner than standard culture based methods, the role of DNA and mass spectrometry detection tests on alternative

airway samples needs to be explored urgently. Given the proven sensitivity of some DNA-based techniques, finding alternative substrate to represent lower airway infection that can be tested easily and regularly is essential. All candidate techniques here could be further explored, as outlined above, but establishment of a novel test substrate, independent of invasive lower airway sampling is needed as well.

With these limitations in mind, the principal next step in this field should focus on testing and validating alternative methods of airway sample collection for detecting Pa infection in pwCF in an era where sputum production is significantly reduced. A clinical pilot of huff tubes was performed and results presented in chapter 6. Extending the work performed in this chapter, PCR offers the most promising sensitive test of the three non-culture based methods explored and next steps could include exploring methods of DNA purification from minimally cultured samples, to identify the minimum amount of sample processing that can be achieved whilst optimising sensitivity from current bacterial substrates. However, given the reduction in bacterial density in samples from the airway post-CFTRm, alternative, non-airway samples like urine or skin secretions and sweat, for example, could also be explored for potential biomarker of infection, and are discussed within the thesis discussion (chapter 7).

Chapter 6 – Results: Alternative, rapid, non-sputum based, low complexity Pa surveillance methods: sniffer dogs and a clinical pilot of huff tubes

6.1. Introduction

When considering airway microbiological surveillance in CF in the era of CFTRm, where spontaneous sputum expectoration is significantly reduced, current methods of confirming the presence or absence of lower airway bacteria may be considered too invasive (such as by bronchoscopy) or unpleasant (sputum induction) to perform with any regularity. Therefore, the goal of any alternative, non-invasive test does not necessarily need to be completely diagnostic. The last chapter highlighted the challenges of developing lab based tests, such as PCR and MALDI-TOF that can be used with minimal processing steps of samples straight from the clinic with potentially very low bacterial concentrations. If airway infection surveillance can be performed as a screening tool instead, it may be possible to identify those deemed at risk of lower airway Pa infection and select out those who then need to go on to have the more invasive tests performed.

Pa has a distinct scent that is apparent to the human nose, and different strains can produce more scent than others [179]. Attempts have been made to use this to clinical advantage by analysing volatile organic compounds (VOCs) produced by Pa using different types of mass spectrometry. The characteristic odour is related to VOCs such as 2-aminoacetophenone and hydrogen cyanide amongst others; analysis of this in exhaled breath of pwCF using selected ion-flow tube mass spectrometry (SIFT-MS) is able to discriminate at a group level between those with and those without chronic Pa infection [134]. Similarly, it has been shown to be possible to use breath VOC patterns to track changes in chronic Pa phenotype over time and distinguish between Pa infection in children compared with adults with CF [180,181]. MS work has also shown that VOCs can detect Pa in the headspace of cultures from pwCF [182], and can identify those with Leeds defined chronic Pa infection from the headspace of sputum samples alone, though is not successful at detecting new Pa

infection, producing significant false positives and negatives [183]. It has also been proven possible to detect specific VOCs using sensor containing 'electronic noses' (eNose and SpiroNose), differentiate specific breath signatures for Pa and Sa infection, and differentiate from those clear of infection, at a group level [184]

Rather than work to define the exact VOCs that give highly specific and sensitive diagnosis of Pa infection and then design electronic sensors to detect them in breath or other substrates, we could consider using animals with trainable and highly sensitive senses of smell to discriminate Pa infection from other bacteria and non-infected samples. Medical Detection Dogs (MDDs) is a registered charity in England and Wales (No. 1124533, www.medicaldetectiondogs.org.uk) that trains dogs for two purposes: medical alert assistance dogs, used to support and raise alert to people with, for example, fragile hypoglycaemia in type-1 diabetes and pre-empting onset of seizures, and; bio detection dogs, who are trained to detect the scent of pathogens or certain types of cancer in samples from humans [185–187]. This lab group has previously worked with MDDs and shown that dogs can be trained to detect, with high specificity and sensitivity, the presence of Pa in the headspace of filtered supernatant from broth cultured clinical isolates [130]. Within this work, dogs were trained and tested with broth culture supernatant headspace, presented to them in a customised testing rig with arm that places the sample pot just below head height. A period of training involved presenting up to four samples, with maximum of one Pa positive sample, at a time along with Pa negative (containing other bacteria) and sterile controls. Positive detection of the Pa scent was signalled by the dog sitting or standing still by the chosen rig, and the handler interpreting this signal and rewarding correct identification with an auditory click and low calorie treat. An example video of sample presentation to dogs and sample identification by dog and handler at the MDDs centre can be found within supplementary material of work published on the detection of malaria [188].

Following training, the dogs were tested to discriminate samples that did and did not contain Pa, using a similar process to training, however the handlers in the room with the dogs would be blinded to Pa positive samples, with this information only being revealed once the dog and handler had declared their choice. At high concentration, the dogs could identify Pa from other CF pathogens, which they had been trained on, with 94.2% sensitivity and 98.5% specificity. Two of three dogs maintained sensitivity >90% when identifying Pa from other CF pathogens, which they had not encountered before. However, sensitivity for Pa discrimination reduced when concentrations were <10⁵ CFU/ml.

In this section of Aim 3, the use of dogs in detection of Pa was explored again, and this time in the context of the huff tube lab model (methods, section 2.2.2.). This chapter is presented in two parts: firstly, the methods and results of the training and testing of dogs with the huff tube lab model; secondly the methods and results of a clinical pilot with huff tubes that were presented to dogs for testing.

6.2. Methods – training and testing the huff tube lab model with sniffer dogs

Training dogs with the huff tube lab model

The dogs require training on scent of a specified target; training and testing is performed by trained handlers under protocolled conditions at the MDDs centre in Milton Keynes. Typically, three to four dogs are trained on a single scent project at one time, and group testing using all trained dogs is performed, to provide intra- and inter-dog comparison of sensitivity and specificity. However, several years had passed since the dogs had encountered Pa scent, and the original Pa project dogs had now been retrained to other projects. In a further change to the previous project, testing would be directly from the headspace of cultured samples from the huff tube lab model (methods, section 2.2.2.), instead of filtered supernatant, and thus introducing two additional scents from the piece of

gauze material in the pot and the broth odour. Veterinary advice was sought to confirm there was no risk of safety to the dogs from sniffing directly from cultured Pa and other bacteria. Three dogs were identified for this project, Jody, Pru and Oakley (figure 6.1), and it was agreed that a sustained period of training would be needed prior to testing to account for these project differences and time lag.

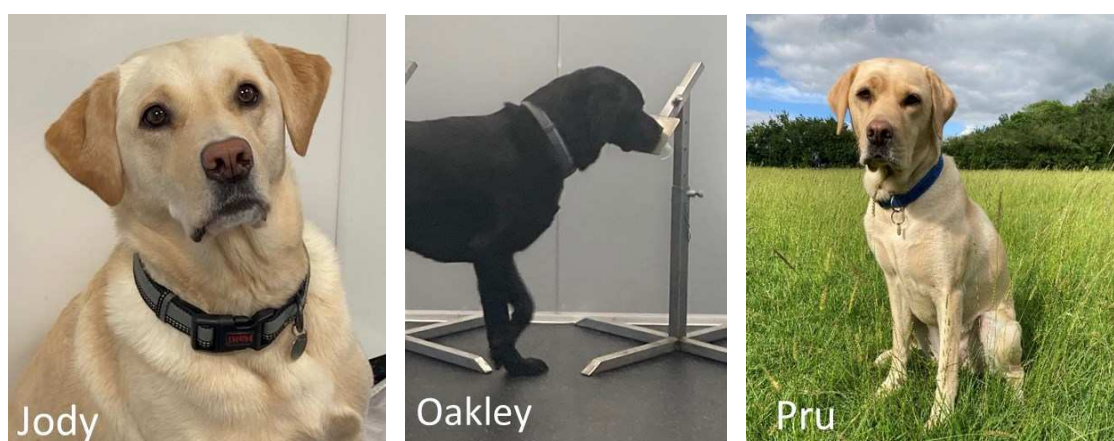


Figure 6.1: The dogs trained and tested in the Pa detection project

Jody, Oakley (who does not like having his picture taken) and Pru were selected for the Pa detection projection from a group of dogs at the MDDs charity centre in Milton Keynes. They are all labradors, one of two breeds along with spaniels that are typically chosen for medical detection purposes. None of the dogs had previously been trained or tested on Pa detection work within our lab group or any other project. Oakley's picture illustrates the metal testing rigs used for sample presentation.

Samples for initial training were produced in similar fashion to the huff tube lab model (methods, sections 2.2.2.). Bacterial isolates were revived from Microbank beads (Methods, section 2.1.3) in LB broth media and incubated overnight at 37°C on shaking platform at 200rpm. Samples were visually inspected for cloudy appearance to confirm high concentration of bacterial growth. Initially, 20µl aliquots of these cultured bacterial isolates, at neat concentration, were inoculated onto sterile

gauze by pipette under class II microbiological safety hood. The gauze was then submerged into 10ml of ceftrimide broth in 60ml sterile pots. The sample was vortexed briefly and incubated overnight at 37°C on shaking platform at 200rpm. The next day, a 200µl of each broth aliquot was taken using sterile pipette and OD₆₀₀ measured, as a proxy measure for bacterial concentration within the sample, and for reference in ad-hoc analysis of ongoing training. Due to the quantity of samples produced, colony counting for each training sample was not possible within the capacity of the workflow. The rest of the sample, still containing the piece of gauze in the 60ml pot, was then sealed and frozen at -80°C, and stored until ready for transport to the MDDs centre. Transport occurred in sealed insulated cool boxes, according to agreed protocol (appendix 7) and samples were stored again in a -80°C freezer. Samples were fully defrosted, according to agreed protocol, prior to use.

Training occurred between June and July of 2021, over four weeks. Each week, three sets of 25 different 10ml samples were produced in triplicate, consisting of 10 Pa isolates (labelled positives), 5-10 non-Pa isolates (a range of non-Pa bacterial isolates from pwCF, labelled negatives) and 5-10 of sterile ceftrimide broth (labelled blanks). Non-Pa isolates consisted of a selection of Sa, Bcc, Smt, Sma and Ax. Initially, samples were all provided at high starting concentrations (expected OD₆₀₀ 0.5 to 1.0) but over the four weeks, sample complexity was increased with reducing starting concentrations (produced by serial dilution, as per methods 2.1.4.) being inoculated onto the gauze. 225 samples were produced per week.

Training involved presenting four samples at a time, each mounted in a metal presenting rig with the sample at head height, covered by a metal grid, for the dog to sniff. Within the four samples presented there was one positive (Pa containing) target alongside blanks and then, over the training period, complexity was increased as samples containing non-Pa bacteria were introduced. A handler, familiar to the dogs, was present during training. They led the dog to the test rigs and then stood behind one-way glass to be out of view but able to observe the dog's response. The dog was free to

sniff the samples in their own time and declared their choice by either sitting or standing still by the sample. It was the role of the handler to determine when the dog had made their decision, **and so identification is reliant on both the dog and the handler's interpretation of the dog's actions.**

Correct identification of a Pa containing target was rewarded with an auditory click and a low-calorie treat.

In August 2021, the MDDs in-house statistician had performed independent preliminary analysis (not available) and felt that further training samples, with precise concentration gradients, going to lower bacterial concentrations, would help improve sensitivity training. Once further internal analysis (not available) confirmed improved sensitivity of performance in training sessions, it was agreed to proceed to formal testing.

Testing dogs with the huff tube lab model

Testing samples were produced in similar fashion to training samples, above, with clinical bacterial isolates revived from Microbank beads in LB broth and incubated overnight. However, this time, prior to inoculating gauze, OD₆₀₀ of each broth cultured isolate was measured. Prior work (methods, section 2.1.5) showed that an OD₆₀₀ of 1.0 equated approximately to a concentration of 10⁹ CFU/ml. Using the OD₆₀₀ values, aliquots of the broth culture were adjusted to produce high (10⁸ CFU/ml), medium (10⁵ CFU/ml) and low (10³ to 10⁴ CFU/ml) concentrations. This meant that inoculating volumes of 20µl pipetted onto gauze would contain high (~10⁶ CFU), medium (~10³ CFU) or low (~10-100 CFU) quantity of bacteria. Gauze was again submerged in 10ml of sterile cetrимide in 60ml sterile pots, sealed and vortexed briefly before incubation overnight, after which a further 200µl aliquot was taken for measurement of OD₆₀₀, and pots sealed, frozen, transported and stored in the same fashion as training samples.

A power calculation was performed by MDD in-house statistician and determined there was a minimal gain in observed sensitivity and specificity when testing sample size per dog increased beyond n=90. On this basis, the testing strategy would consist of 10 different clinical Pa isolates,

each produced in triplicate at high, medium and low inoculating quantities, providing 90 positive samples in total. 30 non-Pa clinical isolates, consisting of Sa, Bcc, Smt, Sma and Ax and all at high concentration, produced in triplicate would make up the 90 negative samples and there would be 45 sterile cetrimide broth samples. Samples were labelled with a randomly generated code.

To mimic any future real-world testing scenario, it was agreed that each sample could be presented to more than one dog, accepting a risk of scent contamination from contact with a previous dog's nose. The testing protocol was double-blinded; during each test run, the dog, handler and database coordinator did not know which sample was the positive target. A coded test sequence database was devised independently of the testing handlers, as per established methods of the MDD team. A dog was presented with four samples at a time, in the same test rigs as used in training (figure 6.1). Again, during the test, only one sample would be a positive target, and the others either Pa negative or sterile. The dog would again indicate its choice of sample by sitting or standing still by the sample in the test rig. The dog handler then confirmed the choice made by the dog to the database handler, the outcome entered into the database, and only then would the database be automatically unblinded to disclose immediately if this was correct or not. Correct choices were again rewarded with an auditory click and low-calorie treat. Incorrect choices were ignored, with no response to the dog given. Correct response rates were calculated by inoculating concentration going on to the gauze (high, medium or low) and each dog's individual as well as combined, 'all dogs' performance (mean and 95%CI calculated by the MDDs in-house statistician). Normal distribution of data was confirmed by assessed using Shapiro-Wilk and Kolmogorov-Smirnov tests. Statistical comparison of the proportion of samples determined by dogs (combined, all dogs) to be Pa and comparison of intra-dog performance was performed using two-way ANOVA with Tukey's multiple comparison test.

6.3. Results – training and testing the huff tube lab model with sniffer dogs

Dogs can detect Pa in samples produced in the huff tube lab model with high sensitivity and specificity

Initial training sample production and presentation of training samples to the dogs took place over four weeks. The decision to extend training with increasing dilute samples to improve sensitivity, warranted a further four weeks of sample production and presentation to the dogs. After this, testing of the huff tube lab model using cetrimide broth with the dogs was completed successfully, with test sample production over 2 weeks and testing protocol completed over 4 days, in which time three dogs were each presented with 225 samples (90 positives, 90 negatives and 45 sterile controls).

Overall, 270 positive target samples were encountered, with 235 correctly identified giving overall sensitivity (mean[95%CI]) of 87.0[82.5,90.5]% across the whole sample set. At high inoculating concentrations overall sensitivity was 100[95.9,100]%, medium was 97.8[92.3,99.4]% and at low was 63.3[53.0,72.6]% (Figure 6.2). This suggests that the dogs can successfully identify over 97% of Pa positive samples in the model when as few as 1000 CFU are applied to the gauze, and 63.0% of Pa positive samples when just 10-100 CFU are applied. Non-Pa containing samples were correctly dismissed with 90.0[85.8,93.0]% accuracy. Blank targets (sterile cetrimide broth) were dismissed correctly with 99.3[95.9,100]% accuracy.

Analysis of combined performance showed no difference between the proportion of samples correctly determined to be Pa at high versus medium inoculating concentrations ($p>0.05$).

Significantly more samples were correctly called as Pa at medium compared to low inoculating concentration ($p<0.0001$). Significantly more samples were correctly called as Pa at low inoculating concentration compared to incorrectly called non-Pa controls (10.0[7.0,14.2]%, $p<0.0001$).

Significantly more samples were incorrectly called Pa as non-Pa controls compared to sterile cetrimide broth (0.7[0.0,4.1]%, $p<0.05$).

All dogs performed statistically similarly ($p>0.05$) to each other and combined 'all dogs' performance at each starting concentration and in both non-Pa and sterile controls. Performance analysis by specific non-Pa bacteria species was not available.

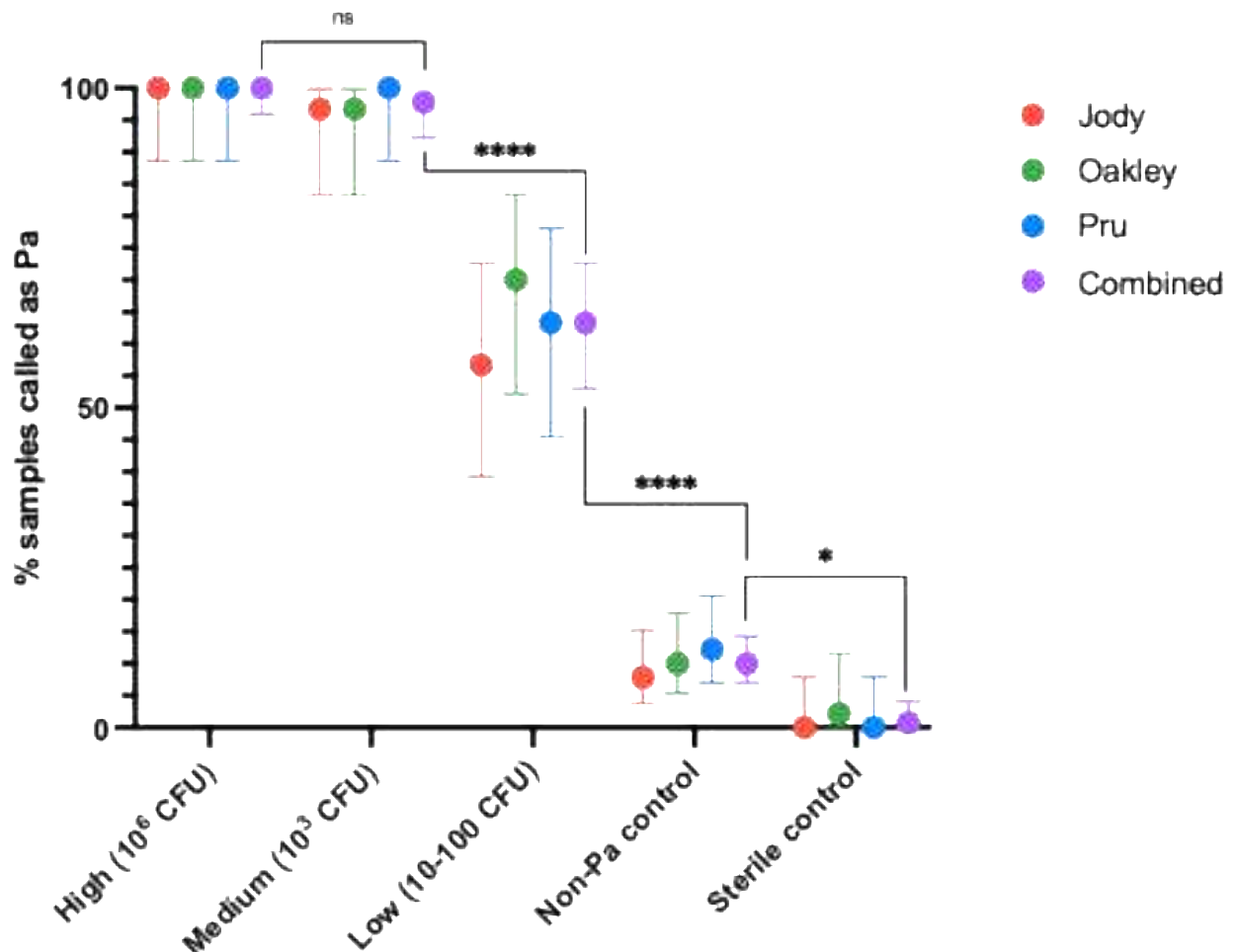


Figure 6.2: Samples identified by sniffer dogs as Pa from the huff tube model

Percentage of samples determined to be Pa by each sniffer dog (named Jody, Oakley and Pru) and pooled for all dogs (combined), with error bars indicating 95% CI. Results are compared by percentage of samples correctly called as Pa positive, divided into starting inoculation quantity of Pa on to gauze at high ($\sim 10^6$ CFU), medium ($\sim 10^3$ CFU) and low ($\sim 10-100$ CFU) quantities, and percentage of incorrectly called non-Pa controls (containing non-Pa bacteria) and sterile controls. Comparison by two-way ANOVA and Tukey's multiple comparison test with significance presented for combined results only. Pa = *Pseudomonas aeruginosa*, ns = non-significant, * = $p<0.05$, **** = $p<0.0001$.

6.4. Methods – Clinical pilot of huff tubes with sniffer dogs

The next step was to test the ability of dogs to detect Pa in huff tube samples collected from pwCF in a pilot study. 20 patients were identified from adult CF clinic at RBH who could all reliably still expectorate sputum (irrespective of CFTRm use). Ten were chronically infected with Pa (Leeds defined) and 10 had no chronic infection, or were chronically infected with other, non-Pa bacteria. People who commonly grew other, non-*aeruginosa*, *Pseudomonas* species, were excluded. All patients were recruited by email and consented by video call over the course of 2 weeks. Sample collection occurred under prior SRC-PAPA project ethics approval (Project ID 17/C4213/1). As discussed in methods (section 2.3.), amendments were required and approved to allow specifically huff tube sample collection, and to allow remote sample collection at a person's home and sample return by post (a method of sample collection that became commonplace during the covid-19 pandemic), along with updated and approved patient information sheets for all age groups (IRAS 233578 SA2).

Home testing kits were produced to Department for Transport UN3373 standards and labelling, with kits couriered to participants homes and pre-paid return envelopes included. Kits contained pre-assembled huff tubes, consisting of sterile gauze loaded into sterile mouthpiece adapted from a standard peak-flow metre mouthpiece, contained within a sterile sealed bag, assembled within a clean hood environment. Instructions with diagrams were provided on how to produce a huff into the huff tube (figure 6.3). Once the huff was completed, the included pot of sterile 10ml cefrimide broth was opened and the mouthpiece inverted and held over the top. A sterile swab was provided to allow the participant to push the piece of gauze out into the cefrimide and then the lid was closed, the sample swirled to submerge the gauze in broth and the pot then sealed with parafilm wrap and placed in the sample bag. A separate pot and sample bag was provided for sputum, to use at some point during the first week and again in week 4. A sample return sheet was completed, allowing documentation of current antibiotic treatments (oral, inhaled or intravenous), and samples returned the same day by post (figure 6.4).

PLEASE READ CAREFULLY:

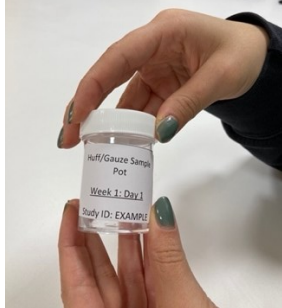
Instructions for huff/gauze sample collection and return of samples by post

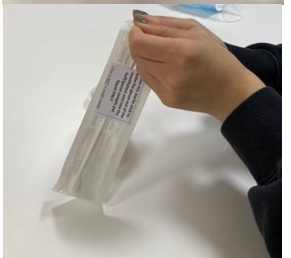
General points:

1. In the envelope there are three packs for testing across three separate days, approximately 48hrs apart – labelled Day 1, Day 3 and Day 5.
2. Please check each box and its contents are intact from the start. If there are any damaged items, please contact me and I will post you a replacement.
3. You can contact me on: j.king2@rbht.nhs.uk, or text/whatsapp/call me on 07841515548 if you have any issues or questions
4. **Please do your huff/gauze samples before your physio** (and therefore **before** any antibiotic nebs/inhalers you are due to take that day, but ideally **after** any mucolytics, e.g. DNase/Hypertonic saline)
5. One sputum sample is required at some point during the week and can be returned in any one of the three return boxes, alongside the huff/gauze sample
6. Please try to post samples within 12hrs of taking them using the pre-paid envelope included. You will likely need to drop them at the post office in view of the size of the pots/box
7. Please follow packing instructions precisely, as we must follow specific guidelines for posting patient samples

Inside this envelope, you will find:

	3 x huff/gauze test kit boxes: Day 1, 3 & 5
	1x sterile sputum pot inside sputum pot bag
	3 x pre-paid return envelopes for sample boxes to be returned in
	1 x instruction sheet
Inside each huff/gauze test kit box, you will find:	
	1x sterile mouthpiece loaded with sterile gauze in sealed sterile bag – <u>DO NOT OPEN THIS BAG UNTIL JUST BEFORE USE</u> in order to keep sterile

		1x huff/gauze sample pot (contains 10ml liquid) inside a huff/gauze sample bag with absorbent material (to be kept for return of sample)
		1 x sterile swab stick (not for sampling, just used to poke the gauze out of the mouthpiece and into the pot)
		1x return slip – to complete and include in the box before sending back
		1x security seal sticker – to seal the box before putting in the envelope
Instructions for taking a huff/gauze sample:		
		1. Wash your hands thoroughly 2. Open the box for the correct day of sampling and check no equipment is broken or has leaked in transit (if it has then please inform me)
		3. Remove the huff/gauze pot (contains some liquid) from the huff gauze sample bag and set on to a tabletop surface. Keep the bag, with the absorbent pads inside, to one side for returning the sample.
		4. Open the sterile bag, containing the mouthpiece loaded with gauze, by unfolding the wire fasteners and unrolling the top of the bag
		5. Remove the mouthpiece with your thumb and index finger (try to not touch the ends/inside of the tube).

	6. Take a deep breath in, place the cardboard end of the tube between your lips and make tight seal, and perform a ‘huff’ by breathing out, like you would in clinic/during physio. If you think you can do a second, bigger huff, then have another go before proceeding
 	7. Set the mouthpiece to the side, by standing it on its plastic end, open the huff/gauze pot by removing the film around the lid and setting that to one side. Remove the pot lid and set the lid down on its top. Open the sterile swab packet.
	8. Turn the mouthpiece upside down and hold the cardboard end directly over the opening to the huff/gauze pot. Use the sterile swab stick to poke through the gaps in the yellow plastic end and push the gauze out into the huff/gauze pot. This can take several ‘pokes.’
	9. Discard the mouthpiece and swab stick into the bin. Close the lid on the pot tightly, ensuring it has thread correctly and is flat, to ensure no leaks. You can re-use the film seal around the lid too, as extra protection, if it sticks back on.
	10. Swirl the pot to submerge the gauze in the liquid 11. Wrap the huff/gauze pot in the absorbent pad(s) from the huff/gauze sample bag and place inside the bag. Squeeze out any air in the bag and seal the bag, rolling up the excess around the pot.

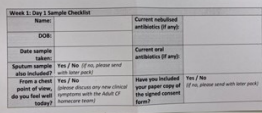
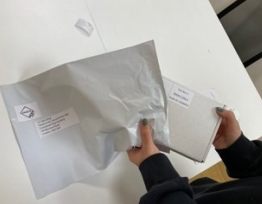
	12. Complete the return slip from the pot and remove the security seal sticker
	13. Place the huff/gauze sample bag inside the box with the completed return slip
	14. You only need to return one sputum sample in the first week of the study. If you are returning a sputum sample with this test then use the empty labelled sputum pot provided in the large envelope to collect a sample as you would in clinic. Then seal it inside the sputum sample bag (no absorbent material needed in this one) and place it alongside the huff/gauze sample in the box.
	15. Close the box and seal over the opening with the security seal bag
	16. Place the entire box inside one of the pre-paid polythene envelopes and fold the envelope flap over to seal tightly. Make sure the address, postage and UN3373 sticker are all clearly visible on the front and your 'sender address label' is visible on the back too.
	17. Return the package by post via your post box (if it will fit) or your local post office, ideally within 12 hours of the sample being produced. If you can send me a text/email when you have posted each sample box, that would be really helpful!
	18. Complete the 'day 3' and 'day 5' samples at approximately 48hr intervals.
THANK YOU SO MUCH FOR TAKING PART IN THE STUDY - PLEASE DO CONTACT ME IF YOU HAVE ANY ISSUES ALONG THE WAY OR IF ANY INSTRUCTIONS REQUIRE CLARIFICATION	

Figure 6.3: Instructions for home huff tube sampling for pilot study

This figure was printed as a two page double sided patient information sheet providing details of the contents of the home sampling kits, instructions on how and when to perform a home huff tube sample and sputum sample, and how to return by post.

Week 1: Day 1 Sample Checklist			
Name:		Current nebulised antibiotics (if any):	
DOB:			
Date sample taken:		Current oral antibiotics (if any):	
Sputum sample also included?	Yes / No (if no, please send with later pack)		
From a chest point of view, do you feel well today?	Yes / No (please discuss any new clinical symptoms with the Adult CF homecare team)	Have you included your paper copy of the signed consent form?	Yes / No (if no, please send with later pack)

Figure 6.4: Sample return slip for clinical pilot of huff tubes

An example of the sample return slip included in the huff tube home sampling kits. The slip provided prompts to ensure a sputum sample was returned at some point during the first week of sampling and identified whether the patient was feeling unwell or taking additional antibiotics, possibly indicating signs of an exacerbation, at the time of sample collection, to help with interpretation of expected results. As video consent was collected, a prompt to include the paper copy of the signed consent form was also included.

Three huff tube samples were collected, during a morning physiotherapy session, 48 hours apart over the course of one week (Day 1, 3 and 5), and one corresponding sputum sample provided. One further huff and sputum sample was to be provided at 4 weeks to assess medium term reproducibility. This would produce 80 huff samples in total. If applicable, people continued anti-Pa inhaled therapies; there were no changes to routine clinical care.

Samples were received at the research laboratory. Sputum samples were transferred to RBH for culture by standard microbiology laboratory methods, and results reviewed as gold standard comparator for the huff tube samples, plus clinically relevant results communicated to the patient's clinical team. Huff tube broth samples were incubated for 24 hours at 37°C at 200rpm. To look for

bacterial growth in the huff tube broth samples following incubation, three x 20µl spot of each sample was spotted onto LB agar plates using sterile pipette and allowed to dry. The plates were incubated overnight at 37°C. A 1ml aliquot of huff tube broth was saved at this point, to be used for colony counting by serial dilution (methods 2.1.4) of samples that demonstrated growth from the spots on agar. Huff tube broth sample pots, still containing the gauze, were labelled with a code, frozen at -80°C, and stored prior to transport and batch testing with the dogs. Testing methodology with the dogs was planned to occur in the same fashion as with the lab model (outlined in section 6.2).

6.5. Results – Clinical pilot of huff tubes tested with sniffer dogs

A clinical pilot huff tubes failed to capture Pa on gauze from a patient's huff

All 20 packs, containing 3 test kits with separate return post envelopes, were received by study participants. 6 (10%) packs contained one missing component due to human error; all 54 complete packs were returned, 6 (11%) had leakage of media in transit and were discarded. This was due to parafilm wrap, which had been wrapped around the lids to prevent leakage, and in hindsight obstructed proper closure after the sample was produced. All leaks were contained within the UN3373 biological sample standard packaging, which includes an absorbent pad, and did not risk local contamination.

Of the 10 chronic Pa patients included, 7 isolated Pa in the sputum provided, whilst none of the non-Pa patients isolated Pa. 48 huff tube samples were processed (24 from chronic Pa patients) of which only 2 (4.2%) had positive bacterial growth from the spots of huff sample broth spotted onto agar. Confirmatory identification of these growths was not performed, as paired sputum culture was the gold standard comparator and assumed to represent the same bacterial growth. As both samples

came from patients with chronic Pa infection, and both grew Pa on their paired sputum sample, this huff sample broth growth was assumed to Pa.

These results demonstrate failure to capture or culture Pa from a patient's huff in the huff-tube/cetrimide model. Possible reasons for this failure could be that there was:

- (i) insufficient deposit of bacteria on gauze from a single huff
- (ii) cetrimide broth might have been insufficiently supportive of growth outside an incubator and/or
- (iii) time spent in the post, at non-favourable incubation temperatures may have caused bacterial death within the sample; finally,
- (iv) 9 of these 10 patients were taking regular anti-Pa nebulised medication, which if carried in the breath may inhibit growth of small bacterial quantities.

Given the low likelihood of success with samples containing so little (or no) Pa that culture was negative, proceeding to testing with the dogs did not go ahead as planned.

Examining clinical pilot failure suggests LB broth may be favourable media over cetrimide in capturing bacteria in the huff tube model

To further examine the failure of this pilot, a small, in person, study during adult CF clinic made head-to-head comparisons of:

- (i) cough swabs versus single huffs versus multiple huffs onto gauze,
- (ii) cetrimide broth versus LB, a broth that likely provides more favourable growth condition for Pa but is non-selective, and,
- (iii) immediate versus delayed (48hr) incubation of samples, to represent the effect of transit in the post of home collected samples.

Five chronically Pa infected patients who could reliably produce sputum were recruited from adult CF clinic at RBH. In addition to a standard clinic sputum sample (or cough swab if sputum not possible on the day), each provided:

- A traditionally performed cough swab, the tip cut with sterile scissors and deposited into cetrimide broth incubated immediately
- A single huff on gauze in a huff tube, deposited into cetrimide broth, incubated immediately
- Five successive huffs on a single piece of gauze in a single huff tube, deposited into cetrimide broth, incubated immediately
- A traditionally performed cough swab, the tip cut with sterile scissors and deposited into LB broth, incubated immediately
- A single huff on gauze in a huff tube, deposited into LB broth, incubated immediately
- A single huff on gauze in a huff tube, deposited into cetrimide broth, held at room temperature for 48 hours before incubation (mimicking delayed sample processing due to transit time in the post)
- A single huff on gauze in a huff tube, deposited into LB broth, held at room temperature for 48 hours before incubation (mimicking delayed sample processing due to transit time in the post)

All of which were incubated at 37°C overnight on platform shaker at 200rpm. At the end of the incubation, three x 20µl of cultured broth media for each sample type was spotted onto LB agar using sterile pipette, plates allowed to dry and then incubated overnight to check for growth of any bacteria. If bacterial growth within the spots occurred, it was graded as **heavy** if individual colonies were not defined (TMTC, too many to count), **light** if individual colonies visible (CFU/ml estimated from the mean number of colonies present in the three x 20µl spots), and **no growth** if there were no visible colonies. Routine sputum or cough swab samples provided in clinic at the same time of research sample collection were sent to RBH laboratory for standard culture for comparison.

Four of five (4/5, 80%) participants produced a sputum sample, and one provided a cough swab due to absence of sputum at time of collection in clinic (table 6.1). All five (5/5, 100%) standard comparator samples cultured in RBH laboratory were Pa positive, though two (2/5, 40%) were also positive for Sa.

No huff tube gauze samples incubated in cetrimide, whether 1x huff (0/5, 0%) or 5x huffs (0/5, 0%) were performed, produced bacterial growth on agar. This suggested that cetrimide was insufficiently supportive of growth of the presumed very low bacterial numbers produced in this model. Two of five (2/5, 40%) traditionally sampled cough swabs grew heavy growth of bacteria when the swab tips were incubated in cetrimide broth, suggesting cough swabs sampled the airway more successfully than huff tubes in this model, with cetrimide.

All five (5/5, 100%) of 1x huff performed and incubated in LB showed bacterial growth on agar, four of five (4/5, 80%) were classed heavy growth and one of five (1/5, 20%) was light growth. Four of five (4/5, 80%) traditionally sampled cough swabs grew light growth of bacteria when the swab tips were incubated in LB broth, where one (1/5, 20%) showed no growth. This suggested that huff tubes sample the airway more successfully than cough swabs in this model, with LB. The identity of bacteria grown in LB broth was assumed to be the same as the comparator sputum, so that three (3/5, 60%) were assumed to be Pa, but two (2/5, 40%) could represent either Pa or Sa. This highlighted that future designs of this experiment would require an additional confirmatory process, such as PCR or MALDI-TOF, to identify bacteria grown in LB broth from huff tube samples.

Samples that received delayed incubation after 48hrs at room temperature showed the same pattern of growth as those that were incubated immediately, suggesting no impact from sample postal transit time.

Patient ID	Comparator sample type	Comparator sample culture result	Cough swab in cetrimide	1x huff in cetrimide	5x huff in cetrimide	1x huff in cetrimide + 48hrs	Cough swab in LB	1x huff in LB	1x huff in LB + 48hrs
021	Sputum	Pa and Sa					10-100	TMTC	TMTC
022	Cough swab	Pa from enrichment	TMTC				10-100	10-100	10-20
023	Sputum	Pa	TMTC					TMTC	TMTC
024	Sputum	Pa					10-50	TMTC	TMTC
025	Sputum	Pa and Sa					10-20	TMTC	TMTC

Key			
	Heavy growth	TMTC	Too many to count
	Light growth	10-100	Indicates approx. CFU/ml
	No growth		

Table 6.1: Clinic comparison of cetrimide and LB broth, sample types and timing of incubation of samples to evaluate failure of the first clinical pilot of huff tubes

Results of head-to-head comparison of cough swabs, single huffs, multiple huffs in cetrimide and Luria broth (LB), and the impact of delayed incubation to represent time in transit of postal samples. Samples were compared to clinic sputum (or cough swab if sputum unavailable) culture results from RBH laboratory, taken at the same clinic visit. Following incubation of different sample types in cetrimide or LB broth, samples were spotted onto agar to look for bacterial growth by growth of colonies. Key indicates degrees of growth of colonies, where 'heavy' showed too many colonies to count (TMTC) and 'light' showed distinct colonies that could be counted, and mean number across the three 20µl spots was calculated to estimate concentration (CFU/ml). Pa = Pseudomonas aeruginosa, Sa = Staphylococcus aureus, LB = Luria Bertani broth.

Redesigning the huff-tube model to use LB broth instead of cetrimide did not improve ability to capture Pa from a patient's huff

These results led to a redesign of the huff tube lab model and clinical pilot with the dogs to use LB broth instead of cetrimide. However, using a more general broth would allow growth of most bacteria (possibly including oral commensals) from a patient sample and could introduce a potentially more subtle/complex signal for discrimination by the dogs. A short test run performed using samples prepared similarly to the previous training scenario (section 6.2), but replacing cetrimide for LB, with Pa again inoculated onto gauze again at a range of low (10-100CFU), medium (10^3 CFU) and high (10^6 CFU) concentrations, and non-Pa isolates at high concentration, to produce:

- A total of 20 positive targets; 10 containing only Pa and 10 containing mixed Pa and non-Pa isolates
- 10 negative non-Pa containing samples.
- 10 blank controls of sterile LB.

It was quickly demonstrated that there was sufficient persistence of overall mean sensitivity (87.1%) and specificity (92.7%) for Pa containing samples (full results not available). On this basis, it was agreed that complete retraining with samples prepared in LB was not required and a repeat clinical pilot was started.

The same 20 participants underwent sample collection in the same manner as before, using collection kits that were prepared as before, except with LB in the huff tube collection pots instead of cetrimide. Once samples were returned, and because LB is a general medium which would allow any bacteria to grow, PCR was used a confirmatory step to identify the bacteria growing in any returned huff sample. Following incubation and prior to sample freezing, 1ml aliquots were taken from the gauze-broth mixtures by sterile pipette for colony counting and those with sufficient growth, assessed using PCR (as developed in chapter 5, section 5.3.1 and 5.32)

In this second pilot, 20 (100%) participants returned a sputum sample from week 1. Eight of 10 (80%) participants with chronic Pa infection grew Pa on their sputum sample, with one other growing Sa and one having no significant growth (table 6.2). None of the 10 non-Pa chronic infection participants grew Pa; one grew Smt and Sa and the rest had either no significant growth or grew upper respiratory tract flora.

Twenty (100%) returned the day 1 huff sample. Two participants failed to return the day 3 and day 5 huff samples. One participant's day 5 huff sample was lost in the return post. A total of 55 (92%) huff samples were returned in week 1. Twenty-five (45.5%) huff samples demonstrated growth on agar after incubation, eight on the first sample, nine on the second sample and eight on the third sample.

The same 8 participants showed bacterial growth on all three huff samples, with the addition of one further participant (ID:004) showing growth solely on their second sample. Seven of 25 (28%) produced more than one bacterial colony type on agar, indicating multiple bacteria in the huff sample broth post-incubation. Colony counts suggested post-incubation growth of bacteria from huff samples of $>10^6$ CFU/ml across all patients with bacterial growth.

Of the 10 chronic Pa participants, 4 (40%) had growth on agar from their post-incubation huff sample, 3 (30%) of which had corresponding Pa positive sputum. Prior to PCR confirmation, this would, at most, suggest a maximum of 30% success rate for capturing Pa in a huff tube.

Participant ID	Sputum culture Pa positive?	Growth from broth spotted onto agar (post-incubation)		
		Day 1	Day 3	Day 5
001	-	-	-	NR
002	-	-	-	-
003	-	+	+	+
004	+	-	+	-
005	+	-	-	-
006	-	+	+	+
007	+	-	-	-
008	-	-	-	-
009	+	+	+	+
010	-	-	NR	NR
011	+	-	-	-
013	-	-	-	-
014	-	+	+	+
015	-	+	+	+
016	+	+	+	+
017	+	-	-	-
018	-	-	-	-
019	-	+	+	+
020	-	+	+	+
025	+	-	NR	NR
	Total	8 +	9 +	8 +
	Grand total	25 +		

Key

000	Chronic Pa
000	No chronic Pa
+	Growth of Pa
+	Growth of bacteria
-	No bacterial growth
NR	Not returned/received

Table 6.2: Results of clinical pilot of huff tubes using LB broth media

20 patients (10 with and 10 without chronic Pa infection at baseline) participated in the second pilot, collecting huff samples at home and returning them by post. In a change to the original design, culture media in the huff sample collection pots was LB instead of ceftrimide. Three huff samples were collected, 48 hours apart across one week (Day 1, 3 and 5) and a single sputum sample returned at some point in the same week, for standard culture at RBH clinical laboratory. Participants with chronic Pa infection are coloured green and Pa positivity of corresponding sputum sample indicated as per the Key. ID 013 and 014 did not grow Pa on their sputum sample, despite being chronically infected with Pa.

All 20 participants returned the day 1 huff samples, so these were prioritised to be tested, following incubation, by PCR and compared to cultured Pa14 as positive control and sterile LB and PCR mastermix as negative controls (figure 6.5). This was repeated twice for confirmation. On both occasions, no huff samples were positive for Pa DNA on gel electrophoresis, suggesting that the huff tube model does not capture Pa effectively and bacteria seen, where there is bacterial growth, are likely oral commensals or other organisms. These samples were therefore not progressed to testing with the dogs. Due to project time constraints, further development of the huff tube model was not possible and the project put on hold for a future work within the group. .

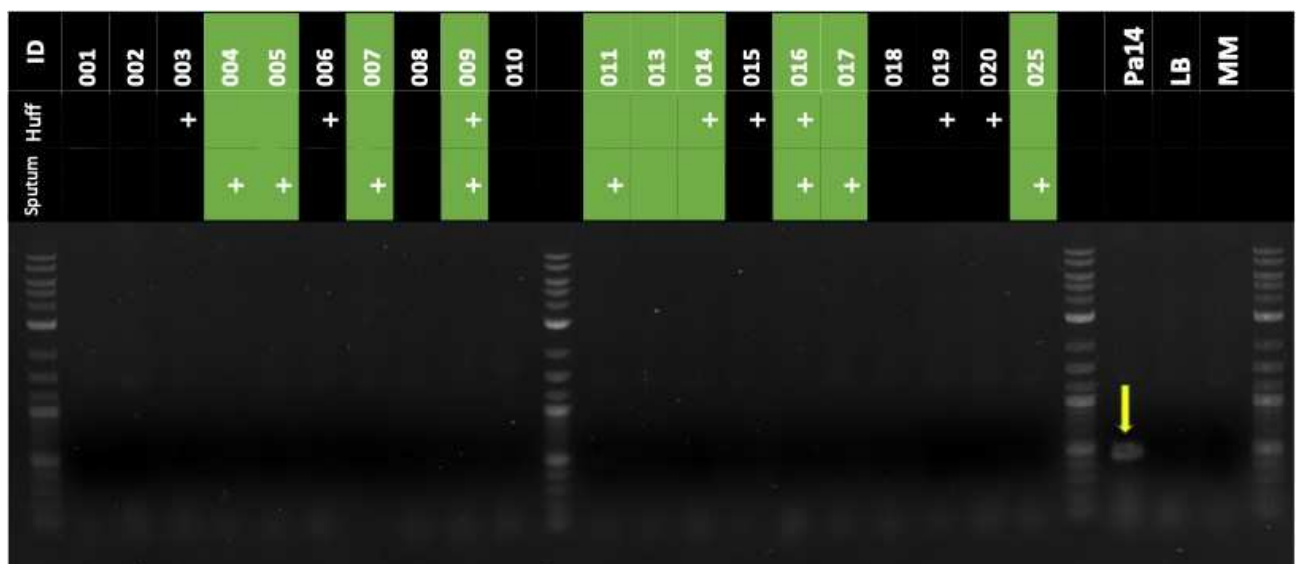


Figure 6.5: Confirmatory PCR results of huff samples collected in LB in clinical pilot

*Gel electrophoresis of PCR comparing post-incubation huff tube broth for the day 1 sample returned for each participant of the clinical pilot, compared with cultured Pa positive control, and sterile Luria Bertani (LB) negative control and mastermix (MM) negative control. Samples are indicated by participant number (ID row) above the results for their huff sample (huff row), sputum culture (sputum row) and gel electrophoresis results. Of the 20 participants, 10 were deemed chronically infected with Pa (indicated in green). Growth of bacteria from incubated huff samples on agar is indicated with a + in the **Huff row** and growth of Pa from comparator sputum samples, cultured in RBH laboratory, is indicated with a + in the **Sputum row**. The only positive PCR band is seen in Pa14*

positive control (yellow arrow), suggesting huff tube sampling failed to capture any Pa from a patient's huff and bacteria grown from huff samples on agar are not the same as Pa grown from comparator sputum samples.

6.6. Discussion

Through a period of training, dogs have already shown to be able to discriminate between Pa and other CF pathogens they have been trained with, and some which are new to them, by sniffing the headspace of filtered supernatant of broth cultures [130]. The same work also showed they could reliably detect Pa at concentrations as low as 10^5 CFU/ml. Work in this aim has expanded the limits of Pa detection with sniffer dogs, demonstrating their use within a realistic model for airway sampling. The huff tube model was designed to work similarly to the premise of a cough swab, capturing bacteria expelled from the lower airway as it is expelled during a forced expiratory manoeuvre, like a huff or cough, with the expectation that a larger surface area may capture more bacteria. In practice, the inoculating concentration of bacteria is unmeasurable, but using a model that allows bacterial expansion in broth can increase bacteria density, as shown in methodology development (methods, section 2.2.2).

By testing the dogs within this model, it was possible to demonstrate that Pa could be detected with 97.8% sensitivity when the inoculating bacterial load was 1000 CFU or more. Dropping the inoculating load to just 10-100 CFU, sensitivity was 63.3%, which is still comparable to standard culture of a cough swab [106,108]. The original design of the model used Pa selective broth media (cetrimide) to enhance specificity. The testing demonstrated that addition of the scent of cetrimide and gauze material, compared to use of filtered supernatant from bacterial culture in the previous study, did not impact the dog's ability to detect Pa [130]. This means any future use of sniffer dogs in Pa detection research, or in clinical practice, may be able to remove sample processing steps and

test directly from samples cultured simply overnight after collection. Challenges with the clinical pilot highlighted a need to re-examine the model with more general broth (LB), which would allow growth of any bacteria. Despite this, the dogs maintained sensitivity and specificity for Pa and did not require significant re-training for this change to the model.

Considering how dogs may be utilised in clinical practice, the model tested in this aim has demonstrated the ability of dogs to rapidly screen large quantities of samples with high diagnostic accuracy. A real world setup may use trained dogs to sniff samples in batches each day, with multiple trained dogs at a time, to provide repeat tests. Batches of samples from across a region could be taken in clinics and cultured in a bench top incubator overnight before sending for batch testing. The speed of throughput means hundreds of samples could be screened for Pa scent each week. Those screening positive could then be alerted to consider further diagnostic testing with sputum induction for example. The speed, simplicity of processing steps and resources required could be comparable or better than DNA based approaches, for example. An alternative approach could see the use of dogs in the clinic setting, sniffing samples, or other substrates directly at the point of care, although this has not been examined in this model and efficacy cannot be extrapolated from these results.

No other groups have published work on use of sniffer dogs to screen for the scent of cultured Pa isolates from pwCF, let alone use in clinical practice. Yet the simplicity of such a model which has demonstrated high specificity and sensitivity for Pa detection could be considered a highly attractive opportunity. Comparable work exists from the use of dogs to detect covid-19 infection [189].

Working on the hypothesis that sweat odour from people with covid-19 infection is different to those without, it has been shown that dogs could be trained within one to four hours to identify sweat from people with covid-19. Testing of 198 samples (101 covid-19 positive) suggested a frequency of correct identification by each of 8 trained dogs ranging from 83 to 100%. Use of trained sniffer dogs to screen for absence of covid-19 infection in the airport setting, in a triple blind

randomised control trial, showed similar concordance with RT-PCR results in ruling out covid-19, however, airport positivity rate was low (<1%) and so calculating sensitivity was not possible [190]. A further study, based in Rwanda, looked at the cost savings of using detection dogs compared with rapid antigen testing [191]. They demonstrated, across 5253 sweat samples collected by wiping a cotton pad in the axilla of people undertaking matched RT-PCR throat swabs, a sensitivity of 83.9% and specificity of 94.9% for detecting covid-19. A saving of \$14,000 (USD) across all tests was made compared with if rapid antigen tests had been employed instead. Work within the MDDs charity has also shown ability to identify malaria from the foot odour of Gambian school children, by collecting odour in a pair of ankle length nylon socks worn overnight [188]. The utilisation of sweat odour as a substrate for testing for infection status has yet to be explored in the context of Pa airway infection.

The clinical pilot of huff tubes demonstrated a failure to capture Pa in the huff of pwCF with chronic Pa infection. Exploration using head-to-head comparison of different culture media suggested initially that the issue related to use of a Pa selective media, perhaps providing insufficiently supportive growth conditions for the very small quantities Pa bacteria expelled during the huff. However, repeating the pilot with LB, a non-selective broth, and using the PCR methodology developed in parallel (chapter 5, section 5.3.1 and 5.3.2) showed that bacterial growth cultured from huff sample broth was not Pa. This was despite sputum samples taken at the same time indicating presence of Pa in the majority of those expected to be chronically infected and suggests the growth from huff sample broth were not indicative of bacteria grown in paired sputum samples from the lower airway. Interestingly, presence or absence of bacteria on huff samples culture in LB appeared to persist across the three samples taken for each participant. Assuming that what is being grown is oral commensal bacteria, this may represent difference in oral hygiene maintenance. Reasons for failing to capture lower airway bacteria, however, remain unclear. What is clear is that having no robust assessment of the collection methodology in parallel with, or prior to, developing the dogs training and testing, was a significant limitation of the design of this project. An assumption was made that this model would be equivalent or superior to cough swab collection. A secondary

confirmatory test, other than parallel sputum culture, was needed from the outset, to confirm or refute the presence of Pa in broth following huff capture on gauze.

Interestingly, the small in-clinic comparison study of cetrimide versus LB, huff(s) versus cough swab sampling, and immediate versus delayed sample processing, showed that when incubated in cetrimide broth, cough swabs tips grew bacteria in two of five samples, whilst no huff samples, irrespective of number of huffs, achieved growth. As cetrimide is Pa selective, it is probable that growth from the two cough swab tips was Pa. Whilst this is extremely limited data, it might suggest that placement of the cough swab tip in the oropharynx and above the airway more successfully captured lower airway bacteria. In a huff tube, despite the larger surface area of the gauze to capture airway sample on, placement of the tube after breath is expelled through mouth, may mean oral commensal bacteria are all that can be captured.

Failure of the huff tube model does not mean failure of this model as a whole. Firstly, the study pilot showed relative success in performing home collection of samples and successful return by post, although in future trials, collecting data on the time taken to receive samples from postage would be useful to know. Secondly, principles of airway secretion collection, and amplification of bacterial abundance in overnight culture will likely still apply to any other types of airway secretion collection methods considered and could then be examined with sniffer digs. Other methods of collection might include salivary samples, cough plates, oral rinses but also non-respiratory substrates such as sweat or urine could be considered and explored. Work performed in this project has formed part of the development of a major project, the PRECISION-CF innovation hub, that is currently underway within this research group. One of the three workstreams in this multi-centre hub project will be examining use of cough, throat, breath and saliva and also looking at skin secretions and urine, to develop novel ways to detect CF infections in an era of CFTRm where sputum availability is no longer guaranteed. This is discussed further in the next chapter.

Chapter 7 – Discussion

7.1. Summary of findings

In order to assess the impact of CFTRm on Pa in pwCF, two hypotheses were generated. Firstly, considering the environmental pressures on Pa in the CF airway from ASL and mucus dehydration, impaired mucociliary clearance, low environmental pH and oxygen state, and working on the assumption that CFTRm rescue of CFTR function leads to improvement in these pressures, it was hypothesised that treatment with CFTRm will lead to a reduction in sputum Pa culture frequency, density and shifts in bacterial virulence/persistence behaviours. Secondly, where spontaneous sputum expectoration was expected to reduce with use of CFTRm, alternative, non-invasive and operationally practical infection surveillance approaches could be developed to detect Pa in the airway and continue to guide treatment. Three aims were established to address these hypotheses.

Aim 1 was designed to determine the clinical impacts of CFTRm on lower airway Pa isolation frequency and abundance for up to 5 years pre- and 2 years post-ETI initiation in patients who were deemed chronically infected with Pa in at least one of the two years prior to starting. Paired analysis of the data of 148 pwCF meeting inclusion criteria was completed successfully. It confirmed an expectation that provision of sputum samples significantly and persistently - beyond return to standard clinical care after the covid-19 pandemic - reduced following introduction of ETI. There was no increase in the number of alternative sampling methods like cough swabs; in turn the total annual sample number per person reduced so much that the majority were no longer meeting Standards of Care, with more than 60% providing fewer than 4 samples annually [77]. Examining comparison of baseline characteristics at year -2 pre-ETI compared to years +1 and +2 post-ETI allowed the pandemic year (predominantly year -1) to be mitigated. Adjusting for the number of samples provided per person per year (pppy), a sustained reduction in the proportions of Pa positive samples, of samples exhibiting mucoid Pa phenotype, and median mucoid and non-mucoid Pa

abundance was observed. This compares well to the findings of other large studies from registries and real-world data [144,192]. Comparisons to the wider current literature are presented in the discussion of results chapter 3. Where this research goes further is in identifying these trends in pwCF who **already have chronic Pa infection** at baseline and shows that even in people exhibiting recurrent Pa positive airway sampling and persistent mucoid Pa phenotype, a hallmark of chronic Pa infection, for many years before ETI, it is possible to reduce or stop isolating Pa, or reduce abundance of Pa growths. This is not an artefact of increased sampling with less sensitive cough swabs either, as the number of cough swabs taken did not change. This research also highlights the relevance of classifying chronic Pa infection, post-CFTRm, by classical definitions like Leeds criteria, if adequate sampling frequency and quality cannot be maintained.

Aim 2 was designed to determine the phenotypic response of Pa to CFTRm by virulence and persistence assay of both Pa samples collected from pwCF pre- and post-CFTRm initiation and Pa subjected to *in vitro* environments that mimic some of the *in vivo* airway changes expected following CFTRm initiation. Firstly, Pa behaviours of stored isolates from the same cohort of patients from Aim 1, before and after CFTRm initiation, were evaluated using a basket of phenotypic assays that assessed growth rate, virulence (pyoverdine production, measured by RFI) and persistence behaviours (mucoidy, subjectively scored on congo red agar). No significant difference by any assay was seen in isolates collected before and after CFTRm initiation. However, the number of patients and their isolates meeting inclusion criteria was severely limited by timings of CFTRm initiation, with only 8 pwCF being eligible for evaluation. This is because for some, IVA started at a similar time to the first isolate storage in the SRC-PAPA repository, meaning few people had enough pre-IVA isolates for comparison and for the rest, ETI started during the pandemic, when isolate storage had to be suspended temporarily due to closure of academic labs, reducing the number of applicable post-ETI isolates available.

Secondly, in anticipation that examining Pa phenotypes created *in vivo* by CFTRm use would be challenging due to the number of stored isolates available, an experimental model was developed using *in vitro* broth based culture that sought to represent some of the environmental changes expected from CFTRm use, at three physiological pH levels (pH 6.5, 7.4 and 8.0) and two oxygen states (21% and 6% ambient) for serial passage of 11 clinical Pa isolates and one reference strain (Pa14). The same assays, MIC to tobramycin were then assessed and results analysed relative to normoxic pH 7.4 conditions. Growth measured by OD₆₀₀ was slower only in hypoxic conditions, but not different by pH. These results were not replicated when growth was assessed by colony counting. Pyoverdine production was lower in the presence of hypoxia at both pH 6.5 and 7.4. Subjectively scored mucoidy trended higher in hypoxic conditions compared to normoxic conditions. MIC to tobramycin was no different in any condition. These results might suggest that acute virulence, represented by pyoverdine production, is upregulated and persistence, represented by mucoidy, is downregulated as environmental conditions shift from low oxygen, low pH to normoxic and more neutral/higher pH, as might be expected with CFTRm use. These experiments were intended to be exploratory, with positive findings being the springboard for future work within the lab. Generalising these limited findings without more work is difficult, but they have been reviewed in the context of the little comparative literature available within the discussion of results chapter 4.

Aim 3 sought to address development, optimisation and then comparison of alternative, non-culture based and low complexity Pa surveillance methods, applied to a novel method of non-sputum based airway sampling termed a 'huff tube.' To do this, a laboratory model for growth of Pa clinical isolates on gauze in Pa selective media was developed, mimicking a novel huff tube device where patients perform a forced expiratory manoeuvre (a huff) onto a piece of gauze. The model demonstrated growth from low to high concentrations in <24 hours. Methods of rapid detection of Pa, with minimal processing direct from gauze incubated broth sample, using PCR, LAMP, MALDI-TOF MS and sniffer dogs were developed and compared and a clinical pilot of huff tubes performed. PCR was feasible direct from inoculated gauze cultured in the huff tube model with 100% specificity, but

sensitivity was limited to 10^4 to 10^5 CFU/ml, whilst LAMP proved too at risk of environmental contamination or subject to primer-dimer interaction to be useful. MALDI-TOF was insufficiently sensitive in this model, limited to $>10^8$ CFU/ml. The dogs showed significant promise as a high sensitivity screening tool for Pa, demonstrating sensitivity of 63% when just 10-100 CFU were inoculated onto gauze, and >97.8% when >1000 CFU applied. Non-Pa bacteria were correctly dismissed 90.0% of the time. Unfortunately, though, clinical pilots demonstrated a failure to sufficiently capture Pa on gauze from a person's huff. Again, these findings are reviewed in the context of up to date literature in the discussion sections of results chapters 5 and 6. Ultimately these results highlighted the need for finding alternative substrates, other than sputum, for already well developed tests like PCR and MALDI-TOF, or promising mass scale screening with dogs.

Below, the relevant literature published since the start of the project in 2020 is discussed in more detail, specifically considering the most recent developments in knowledge relating to the context of this project and development of its hypotheses. This includes more recent assessment of the impact of CFTRm for pwCF in terms of general clinical status, sputum production, Pa infection. It also looks at studies examining gaps in previous knowledge, like the impact of CFTRm on the CF lung microenvironment, Pa virulence and persistence mechanisms and progress in finding alternative substrates and tests for screening the CF airway for Pa in the absence of readily available sputum.

7.2. Post-approval studies for ETI

Since the start of this project, ETI has become available in the UK for pwCF from the age of 2 years and above. The majority of pwCF in the UK, approximately 85 to 90% based on genotype, now have access to effective CFTR modulation. A number of post-trial studies have examined the clinical effectiveness in the real world setting and have confirmed the findings from phase 3 trial data [142,193]. In a US CF registry based study of 16116 pwCF mean ppFEV₁ increased by 8.2% from baseline [142]. PEx rate reduced from 0.86 exacerbation pppy, to 0.18 (79% reduction), positive

bacterial culture rate declined, and annualised rate of death and lung transplantation significantly decreased. No new safety concerns were flagged beyond the known side effects from trial data. In a German CF registry study of 2645 pwCF, a mean ppFEV₁ increase of 11.3% was seen along with a 75.9% reduction in rate of pulmonary exacerbation and mean reduction in sweat chloride of 50.9mmol/L [193].

Respiratory outcome measures beyond the scope of the trials are also being examined. RECOVER (Real World Clinical Outcomes with Novel Modulator Therapy Combinations in people with CF) is a multi-centre study based across sites in the Republic of Ireland, Belfast and London that is measuring real-world outcomes in children and adults commencing ETI since Autumn 2020. Outcome measures include MBW and CT chest with standardised scoring (PRAGMA-CF scoring). 117 pwCF aged 12 and over were recruited with mean change in LCI of -2.5 and significant reduction in CT scores for air trapping, mucus plugging and bronchial wall thickening following ETI [194]. Bronchiectasis scoring however, did not change. This was contrary to a French study suggesting 5% of adults with CF saw reversal of specifically cylindrical type bronchiectasis in follow up CT imaging. These findings suggest a potential of ETI to reverse degrees of structural lung disease too.

ETI is not tolerated by all who are eligible and adverse reactions reported include mood deterioration, poor cognition, anxiety and sleep disturbance; there is poor understanding of the biological mechanisms for these or how they can best be managed [195]. For a small proportion, side effects outweigh perceived clinical benefit, and they have stopped taking ETI. Evidence of a variable response to ETI is also apparent with a wide range of sweat chloride reduction seen in the original trial data [91,92]. In a prospective single centre study, heterogeneity of response was examined, with 10% of patients having a sweat chloride reduction of <27mmol/L, increases of ppFEV₁ of <2.5 points, reduction in BMI of up to 0.13 and no change in CFQ-R scores [196]. Combining those who are intolerant or poor responders, one US centre estimates that 6% of their CF treatment population could be classed as having 'modulator refractory CF' [197]. Understanding the

impact of CFTRm on Pa infection and CF pathogenesis needs to be examined and interpreted in the context of a likely heterogenous drug response.

7.3. Impact of ETI on sputum production and Pa infection

This project has shown a significant reduction in the proportion of pwCF that can reliably expectorate sputum. As discussed previously, this is supported in several large registry and real-world studies [144,147]. As well as detecting and eradicating harmful pathogens like Pa, chronic infection definitions are dependent on regular and reliable airway sampling, and traditionally sputum expectoration has been an issue for children and young adults with well-preserved lung function [77]. Despite awareness of reduced sputum availability secondary to CFTRm use for many years, both anecdotally in clinical practice and through experience with IVA in those with gating mutations [105], there is no recently published evidence addressing the shortfall in sufficient sampling substrate, alternative sampling methods or the impact on our ability to reliably define chronic infection.

This project has identified a need to question the relevance of current accepted chronic infection definitions, like Leeds criteria, in the absence of sufficient sampling quantity and quality. If current definitions of chronic infection are no longer valid, treatment decisions regarding termination of inhaled antibiotic therapies for example, may be made incorrectly from misleading clinical evidence. The CF Foundation position paper on models of care in the era of effective CFTRm use for the majority of pwCF suggested that in the absence of reliable sputum testing, cough swabs should be used more frequently to rule out the presence of Pa and use of imaging may be used to help to target those that require more invasive infection surveillance, like BAL [152]. Additionally, they advised that more research is needed to consider suitability of more frequent sputum induction. A study is underway to evaluate the use of self-directed sputum induction at home in children [166]. Furthermore, all guidance on managing chronic Pa infection came from evidence that predates ETI;

the relevance of chronic Pa in a modulated airway also needs to be understood to safely make changes to treatment.

Multiple published studies, including the results reported in this thesis (chapter 3, accepted for publication in Annals ATS, Winter 2025), confirm that the frequency and abundance of Pa isolation have decreased since the introduction of ETI [144,146–148]. In the study population examined in this thesis, it was possible that Pa could be cleared even in those who were long deemed chronically infected, sometimes for up to 5 years prior to ETI initiation. Changes were as evident in people consistently growing clinically reported mucoid Pa as non-mucoid Pa, suggesting CFTR^m driven changes to the expression of persistence factors like mucoidy are occurring to Pa in the CF airway. Mechanisms for these observed and accepted changes to Pa in clinical practice warrant further investigation. This argument is made even more clear by the fact that Pa does not clear in everyone, and new Pa infection can still be acquired. If infection persists in some but not others, understanding the mechanisms of CFTR^m facilitated clearance may provide insight into better eradication in others.

7.4. Impact of ETI on CF lung microenvironment and CF pathogenesis

ETI was available for use in pwCF in the UK from December 2019 on managed access programmes only; general clinical availability for those aged 12 years and over came in once NHS reimbursement was agreed from September 2020, coinciding with the start of this project. At that time, no data was available to suggest the impact of ETI on CF lung microenvironment and CF pathogenesis; this was not examined in clinical trials.

As outlined in the introduction, studies relating to the impact of IVA, used in those with gating mutations from 2012, on the CF lung microenvironment were limited and presented mixed results. It appears likely that IVA improves measures of CF mucus elasticity and viscosity and mucociliary clearance towards normal, and evidence suggests reduced infiltration of the airway from

macrophages and reduced inflammatory response measured by NE activity, and presence of the pro-inflammatory interleukins, IL-1 β and IL-8 [94–96]. Prior to ETI roll out, no studies have explored the impact of IVA on airway pH and relative hypoxia within mucus plugs, and no additional data has been published since.

As a result, when this project commenced, the impact of ETI on the CF lung microenvironment could only be hypothesised from knowledge that ETI restored chloride transport through CFTR [158]. Here it was hypothesised that restoration of chloride, and bicarbonate, would lead to restoration of ASL hydration and pH, and in turn normalisation of mucus properties, including oxygen state with mucus plugs.

Since the start of this project a number of studies have attempted clarify the impact of CFTRm on the CF airway microenvironment. Use of a novel cell line of Human Bronchial Epithelial (HBE) cells from pwCF with either *G551D* or *F508del* homozygous mutations, showed that loss of function of CFTR in both genotypes lead to reduced ASL pH and evidence of mucus dehydration [198]. Applying IVA and ETI to the *G551D* and *F508del* cell line models respectively demonstrated no change in pH or mucin RNA expression, but mucus phenotype changed, showing relaxation of mucin ultrastructure and improved mucus transport. A similarly conducted study applying ETI to cultured CF HBE cell lines also showed improved PCL hydration and reduced mucus viscosity but also showed neutralisation of ASL pH [199]. Improved airway hydration and mucus viscoelastic properties are also demonstrated by improved mucociliary clearance in pwCF taking ETI in clinical practice compared to baseline [157]. Radioisotope labelling of airway mucus to measure real-time mucociliary clearance showed significant improvement in clearance time following ETI initiation in 44 pwCF. The same study also analysed sputum (by induction because 23 people could longer spontaneously expectorate sputum), and showed significantly reduced sputum solids compared to baseline, but no change in measures of elasticity and viscosity. This is contrary to other studies of sputum suggesting elastic and viscous modulus measurements are improved at both 3 and 12 months post-ETI initiation [146]. No change

was seen in the pH of exhaled breath condensate. No published literature to date has examined the effect of ETI on oxygen concentration in mucus plugs. Changes to mucus oxygen state considered in our hypothesis remain unproven.

Other factors in the CF microenvironment, not tested in the proposed hypotheses of this project, have demonstrated change since ETI. Examining the sputum of 79 pwCF, ETI use was associated with reduced levels of proinflammatory IL-8 and free NE, and proteome analysis shifted toward that of healthy controls [146]. Another study looking at proteomic analysis of pre- and post-ETI sputum samples from pwCF using liquid MS showed an increase in anti-inflammatory proteins [200]. However, principal component analysis this time showed that protein composition changed to a state that was distinct from that of sputum from both pwCF before ETI exposure and from healthy controls. There appeared to be evidence of incomplete resolution of neutrophilic inflammation and suggested risk of ongoing airway damage. Evidence of reduced systemic inflammation, with reduced circulating serum IgG and IgA is emerging [201]. Mucosal-associated Invariant T (MAIT) cells have also been examined; MAIT cells recognise bacterial metabolic compounds, play a role in antimicrobial action, accumulate in BAL from pwCF and can produce a range of cytokines [202]. Examining serum inflammatory cytokine and MAIT profiles in children with CF before and after ETI initiation, it was found that pre-ETI, MAIT cells exhibited an anergic/exhausted phenotype, effectively not functioning. Post-ETI there was restoration of number and function of these cells to levels similar of controls. Post-ETI inflammation profiling is so far limited, but evidence suggests local airway and systemic effects of ETI on inflammation.

Understanding of the impact of ETI on the CF lung microenvironment and the wider aspect of CF pathogenesis, beyond improvements in ASL hydration and mucociliary clearance, is in its infancy. Airway pH restoration seems probable, if not consistently demonstrated, whilst oxygen state in mucus plugs remains unclear. Markers of inflammation are improved but perhaps not to levels of

healthy controls and this is important to understand risk of residual lung disease and progression over time in pwCF post-ETI.

7.5. Impact of ETI on Pa virulence and persistence

Evidence from real world studies of IVA and ETI have shown that Pa isolation frequency and abundance is reduced, and this project has demonstrated that this occurs even in those deemed chronically infected with Pa before starting [96,144,147]. However, Pa is not eradicated in all with mucoid and non-mucoid phenotypes persisting, despite evidence of improved mucus properties and mucociliary clearance. Mitigating the ability to sample the airway with spontaneous sputum is likely to have impaired ability to study Pa phenotypic behaviours following ETI starting.

A recent study examined BAL samples from the same 5 lung regions pre- and 1.5 years post-ETI initiation and correlated these lung regions with evidence of structural lung disease, like bronchiectasis, from CT scans [203]. Their understandable hypothesis - that areas of lung with more severe lung disease would more likely have persisting Pa infection - was not proven. Surprisingly they found that if Pa cleared from one region, it cleared from all regions. If it persisted in one region, it persisted in all regions, irrespective of degree of structural lung disease on CT. Neutrophilic inflammation, measured by NE activity, persisted if Pa infection persisted, and cleared if Pa infection cleared. This suggests that important host and drug-response factors exist and determine Pa clearance, and that it is possible to achieve Pa clearance even in the presence of significant lung disease. Use of 16s RNA analysis of sinus, throat and sputum samples from pwCF, showed that Pa persisted in the lower and upper airway, albeit in lower quantities, and that in the majority these strains were the same as the ones pre-ETI [204]. Using phylogenomic analysis, they could see that these strains continued to evolve genetically following ETI, suggesting that Pa is trying to adapt to the new selective pressures of a CFTRm treated airway environment.

Phenotypic analysis of Pa isolates from 11 pwCF who were chronically infected with Pa at baseline and up to 21 months post-ETI starting offers further insight [163]. 105 different isolates were assessed for colonial lineage and again strain persistence existed, with no displacement by new strains following ETI. Mucoid phenotype, measured by expression of O-antigen, was sustained. There was also no change in susceptibility to a range of anti-Pa antibiotics, as measured by disc diffusion.

Modelling CF artificial sputum media (ASM) to represent changes in mucus hydration and biochemistry following ETI, CF Pa isolates, in a complex lung infection model in both normoxic and hypoxic conditions, were examined to assess their response to this new environment [205]. Oxygen state rather than biochemical sputum composition was found to have greater impact on microbial community as measured by scores of diversity and richness. They noticed that when reduced amino acids were present in CF ASM, modelling an anticipated effect of ETI, Pa production of rhamnolipids and quinolones both reduced. The authors highlight that these molecules are linked to Pa quorum sensing and may suggest reduced virulence and impaired killing of other bacteria in the CF lung environment. This is the only paper directly looking at aspects of Pa virulence in the context of expected CF lung environment, and no research looking at impact of specifically pH can be found. No data relating to other aspects of Pa virulence and persistence, such as motility, toxin and pyoverdine production and biofilm formation, appears to have been investigated yet.

This area of research is still in its infancy and hampered partly by lack of complete understanding of the impact of CFTRm on the CF airway and CF pathogenesis still. What is apparent from most recent research is that, despite use of CFTRm, Pa can persist in the airway irrespective of the degree of underlying structural lung disease, mucoid expression can continue suggesting persistence is not overcome and that clearance of Pa could be linked with reduced airway inflammation.

7.6. Advances and ongoing challenges in Pa detection

Prior to this research, non-culture based methods of Pa detection such as PCR and MALDI-TOF had already been proven to show high specificity and, with the optimum sample substrate (sputum or BAL) and established sample processing methods, high sensitivity to detect Pa airway infection in pwCF [119–123,125,141,206]. Attempts to adapt these methods to the huff tube model and to detect the presence of Pa in low quantity, in a non-sputum based sample were challenging. Starting quantity of bacteria probably still relies on definitive lower airway sampling for these tests to be used in their current design.

Examining the advances in PCR detection research, a number of recent reports have shown that PCR can be more sensitive than standard culture methods, demonstrating detectable but non-culturable Pa in respiratory secretions [175,207]. These findings significantly affected classification of pwCF by accepted Leeds chronic infection groups, which has implications for ongoing treatment. Quantitative PCR has even detected presence of Pa DNA in samples collected up to a year before becoming apparent by sputum culture, in children undergoing Pa eradication treatment [208]. Methodology adaptations are reported to assess cough swabs directly by PCR for Pa, without intermediary culture steps, though crucially, comparison of sensitivity compared to culture and PCR of sputum is not reported [175].

The challenges identified in this project, of trying to adapt PCR for use in non-sputum based analysis, have been explored by another group as well. Using paired cough swab and sputum samples, comparison of next generation sequencing methodology, as a culture independent method, to standard culture methods, has shown poor concordance for cough swabs, but good concordance for sputum samples [209]. Another study examined respiratory microbiome by PCR from throat swabs and cough swabs in a broad range of chronic lung diseases and was compared to simultaneous BAL PCR [210]. Throat swab respiratory microbiota correlated well with lower airway samples, and it was possible to distinguish between different chronic lung diseases on this basis. However, wide inter-

subject variation in respiratory microbiome meant these could not be used for clinical decision making and interestingly cough swab samples did not sequence sufficiently for analysis. These findings support the notion that alternative substrates for testing need to be explored in the absence of sputum in clinical practice.

Cough plates have been explored as alternatives to sputum and cough swabs. Cough plates use a sterile general agar plate with lid which is opened at the time of sampling and the patient coughs directly on the surface of the agar. Variable sensitivities and specificities for infection detections have been reported, when compared alongside cough swabs in clinical use, with some studies suggesting better and some suggesting worse results [211,212]. These methods were evaluated prior to use of CFTRm, and in general clinical practice have not replaced the use of cough swabs in those that cannot expectorate. However, as a simple method of non-invasively capturing bacteria, they have better proven success than the huff tube model and could be part of future work exploring alternative non-sputum based substrates for testing.

Testing for products of *Pa* metabolism, rather than directly culturing or detecting *Pa* DNA, offers an alternative method for screening of *Pa* in the airway. 2-alkyl-4-quinolones (AQs) are molecules produced and pumped into the local environment by *Pa* as part of its quorum sensing virulence control mechanisms and found in sputum, urine and serum from pwCF with *Pa* infection [213]. Serum levels of AQs correlate with clinical status and exacerbation as well [214]. More recently, in the absence of sputum, the presence of AQs in saliva from pwCF has been examined and shows 50% sensitivity and 100% specificity for detecting *Pa* infection when compared to standard culture of sputum samples [215]. Whilst sensitivity is low, as an adjunct for screening of *Pa* in the lower airway, this method offers promise. Exploring other by-products of *Pa* metabolism as biomarkers for lower airway infection could offer other alternative routes for screening.

No further work has been published examining the use of dogs to detect *Pa* in pwCF; to our knowledge, no other groups are currently exploring this. Use of dogs to screen for covid-19

infections offers a good example of how it can be used in practice, as discussed within the results of chapter 6 [189–191].

VOCs and breath analysis have been examined further and remain a strong contender as a culture independent method of Pa detection. We have seen clearly that VOC analysis from exhaled breath can discriminate between presence and absence of Pa infection in children with CF [181]. More recent work has shown the application of specific electronic VOC sensor panels within ‘electronic nose’ machines (eNose), as discussed in chapter 6. These have since demonstrated to be able to distinguish between pwCF with chronic Sa and Pa infection [184], and detect clearance of Sa from breath following eradication treatment [216]. A number of projects relating to VOCs are ongoing.

Recently published work looked at a device called ‘PneumoniaCheck’ which is designed for non-CF infections and takes sampling onto a filter pad from a patients cough at the time of respiratory illness. Using this device in a very small pilot study of 10 pwCF hospitalised with PEx, they collected 10 cough samples in the PneumoniaCheck device, though only 2 patients provided comparator sputum samples [217]. In an interesting design step, the collection filter pads were analysed with ELISA to look for the presence of amylase, as a marker of upper airway sample, and surfactant protein A, as a marker of lower airway sample. 9 of the 10 collected samples were deemed to be from lower airway samples, and pathogenic bacteria were detected in 7 samples, though there was insufficient comparator sputum to understand true sensitivity. Clearly such methods require much more evaluation, but whilst it is apparent that culture-independent and sputum independent testing is its infancy in the era of effective CFTRm for pwCF, all options should now be considered to find reliable alternatives.

7.7. Strengths and limitations

A major strength of this project is the completion of aim 1, providing assessment of a large cohort of pwCF, and adding further to the, now gathering, evidence that following introduction of ETI, there is a reduction in the frequency and abundance with which Pa grows from the CF airway. This work extends knowledge in the field by providing analysis of the impact in those who have long been deemed to be chronically infected with Pa and showing mucoid phenotype. Whilst identifying that it is possible to reduce or even eradicate Pa burden in those previously deemed chronically infected, it is equally as important to know that Pa persists in many, despite improvement in other clinical parameters. No clinical factors, such as ppFEV₁ response or genotype, could be linked to Pa response either. A more thorough assessment of drug response and tolerance would have been helpful, such as sweat chloride change and PEx frequency, though this was not available. Knowledge of underlying structural lung disease at baseline could also have provided useful context. The study also highlighted failure to continue to meet Standards of Care following CFTRm and, for the first time in published literature, shows the difficulty with interpreting previously accepted chronic infection definitions. Limitations have been discussed within the results chapter but reiterating here, as a single CF centre study in the UK, generalisability of results to other centres needs to be considered.

When considering the impact of CFTRm on Pa virulence and persistence phenotype in Aim 2, examining changes in isolates based on their *in vivo* exposure to CFTRm was severely hampered by lack of sufficient suitable stored specimens, predominantly due to the timing of IVA and ETI initiation relative, respectively, to the first start date and then later pause of sample collection secondary to the covid-19 pandemic. Had more appropriate samples been available, this would have been a better way to examine phenotypic change over time, as we recognise strain persistence in those that continue to grow Pa after starting CFTRm, and would demonstrate full *in vivo* effect of CFTRm [96]. Better still, seeing as we do not know the impact on phenotype from freezing and thawing the isolate, would be to have a design whereby samples were prospectively collected and assessed without needed for processing and storage.

Experiments instead then had to model expected changes, however a major limitation from the outset was the limited data available on the effect of any CFTRm on the CF airway microenvironment. The premise for the hypothesis and in turn experimental design had to be based on assumptions about the impact of CFTRm based on knowledge of the various roles CFTR plays in maintaining normal airway environment. Given the complexity of CFTR function, beyond ASL hydration and including pH balance and inflammation, as well secondary effects of localised hypoxia, high eDNA and complex changes to mucin and mucus architecture, this meant that a simple broth-based model was not able to replicate true environmental complexity. The model has however identified some possible differences in mucoidy and pyoverdine production at conditions that may reflect the effect of CFTRm, and these are worth exploring further, with greater array of assays, and should be considered in a more complex lung environment model, perhaps such as Air-Liquid Interface (ALI) cell culture models, or CF animal models. The importance of understanding the impact of CFTRm on *Pa* virulence and persistence phenotype is all the more important knowing that not all people clear *Pa* similarly following CFTRm. Understanding these mechanisms may lead to opportunity to clear infection for everyone.

In aim 3, alternative, non-culture based, detection methods were considered. The major strength of this aim was contributing to evidence that dogs can detect *Pa* from low inoculating concentrations with very high sensitivity and specificity, after only a brief period of culture in broth. This supports their use in future models of rapid detection or screening of *Pa* from similar or alternative substrates. Examining PCR and MS proved more challenging. The main reason for poor success was committing from the outset to testing PCR and MS on the output of the huff tube lab model, and the untested components of this design. Failure to clinically evaluate capture of bacteria on the huff tube gauze prior to examining PCR and MS, has meant generalisability of results to future collection methods may be difficult. Similarly, whilst aiming to test samples with PCR with as few processing steps as possible, use of standardised DNA extraction methods could have been performed to confirm that *Pa* DNA was detectable at similar sensitivity to the published literature [121,125].

It is unclear why the huff model failed to capture bacteria from a patient's huff in the clinical pilot, but ultimately a more robust assessment of this design prior to training and examining their effectiveness with dogs was needed as well. This work assumed that a huff tube would be equal or superior to a cough swab in capturing bacteria. Use of additional confirmatory testing, aside from lab standardised culture of sputum sample collected in parallel, could have helped determine the presence of *Pa* in broth from a huff sample sooner and more efficiently.

7.8. Future directions

Now the clinical impact of CFTRm on *Pa* infection in the CF airway is better understood, from this and other research published since the start of this project, future work should focus on the differences between those who clear infection and those for whom it persists. Understanding mechanisms of impact of CFTRm on the CF airway microenvironment and *Pa* virulence and persistence factors will be essential and may lead to developing opportunities to clear *Pa* infection in everyone. If *Pa* infection cannot be cleared in everyone, then parallel research into risk of morbidity and mortality in continued chronic *Pa* infection in a modulated airway environment is required, to assess how it differs from previous understanding of chronic *Pa* infection. It is interesting to note experiments demonstrating that presence of *Pa* can actually reduce chloride secretion in wild-type and LUM/IVA rescued *F508del*-CFTR, so the persistence of *Pa* in a modulated airway may directly reduce the effectiveness of CFTRm [218].

As discussed, it is apparent that there is a variable biochemical and clinical response to CFTRm and understanding this may also help determine difference in *Pa* infection clearance.

Vanzacaftor/Tezacaftor/Deutivacaftor (Alyftrek), is the next generation of CFTRm and has recently been licenced. It has been found non-inferior to ETI in terms of clinical benefits but may show increased biochemical response as measured by sweat chloride reduction [219]. The relationship of infection status with sweat chloride reduction is not known, but if considered as a measure of CFTR

function, then assessing the impact of those who have not yet cleared Pa, as they switch to Alyftrek, could be insightful. The medication is also once a day and may improve issues of medication adherence, which has not been assessed in any studies looking at Pa infection, but could play a role in variable response to CFTRm treatment.

Sputum provision, with current or new CFTRm is going to remain an issue, and this is severely hampering our ability to understand the lower airway infection status of pwCF now. This work, and the review of relevant literature illustrates a paucity of research to find alternative substrates and methods for infection detection that might be acceptable to do with regularity in the clinic and home setting. This urgently needs addressing if patient priorities are to be addressed, such as safely reducing treatment burden with inhaled antibiotics therapies [220], and before clinical practice of routine sampling diminishes completely and indolent infection may be missed. As addressed in the discussion this will impact use of current chronic infection definitions, and future work should examine their suitability and alternatives in the CFTRm era.

One project underway to address some of these issues is the Precision-CF innovation hub, of which I am a co-applicant. Working across three workstreams, this multi-centre hub looks at clinical and bench top research and aims to improve the detection, diagnosis and treatment plans of lung infections in people with CF. Three workstreams are planned. The first will examine interactions between common CF pathogens including Pa and Sa, looking at their survival and competition in the CF lung together. The second will explore the CF host inflammatory defence response to CF pathogens. and consider novel biomarkers to detect infection. The third will be directly exploring alternatives to sputum for detection of common pathogens in the CF lung and testing methods that deem suitable for pwCF. This final work stream will build on some of the preliminary work in this MD(Res) project and coordinate sample collection for multiple purposes, under single ethics approval, across many clinical sites in the UK. A keen focus on the issue of finding alternative substrates for Pa detection is planned, with sample collection including sputum and induced sputum

(as standard culture comparators), cough and throat swabs, saliva and breath from the airway and non-airway samples from urine and skin secretions. Analysis of samples will build on work with non-culture based techniques including MS, PCR and DNA based methods, and again on promising experience with sniffer dogs. Precision-CF will therefore address a number of key priorities for pwCF in the era of CFTRm.

References

- [1] Foundation CF. Cystic Fibrosis Foundation Patient Registry, 2016 Annual Data. Cystic Fibrosis Foundation; 2017.
- [2] Elborn JS. Cystic fibrosis. *Lancet* 2016;388:2519–31. [https://doi.org/10.1016/S0140-6736\(16\)00576-6](https://doi.org/10.1016/S0140-6736(16)00576-6).
- [3] Registry CFT. CF Trust Registry Annual Report Data 2020. 2020.
- [4] ANDERSEN DH. CYSTIC FIBROSIS OF THE PANCREAS AND ITS RELATION TO CELIAC DISEASE. *American Journal of Diseases of Children* 1938;56:344. <https://doi.org/10.1001/archpedi.1938.01980140114013>.
- [5] DI SANT'AGNESE PA, DARLING RC, PERERA GA, SHEA E. Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas; clinical significance and relationship to the disease. *Pediatrics* 1953;12:549–63.
- [6] Tsui LC, Buchwald M, Barker D, Braman JC, Knowlton R, Schumm JW, et al. Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. *Science* 1985;230:1054–7. <https://doi.org/10.1126/science.2997931>.
- [7] Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 1989;245:1059–65. <https://doi.org/10.1126/science.2772657>.
- [8] Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, et al. Identification of the cystic fibrosis gene: genetic analysis. *Science* 1989;245:1073–80. <https://doi.org/10.1126/science.2570460>.
- [9] Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, et al. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 1989;245:1066–73. <https://doi.org/10.1126/science.2475911>.
- [10] Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. *N Engl J Med* 2015;372:351–62. <https://doi.org/10.1056/NEJMra1300109>.
- [11] Dechecchi MC, Tamanini A, Cabrini G. Molecular basis of cystic fibrosis: from bench to bedside. *Ann Transl Med* 2018;6:334. <https://doi.org/10.21037/atm.2018.06.48>.
- [12] Boyle MP, De Boeck K. A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect. *Lancet Respir Med* 2013;1:158–63. [https://doi.org/10.1016/s2213-2600\(12\)70057-7](https://doi.org/10.1016/s2213-2600(12)70057-7).
- [13] Wilschanski M, Dupuis A, Ellis L, Jarvi K, Zielenski J, Tullis E, et al. Mutations in the Cystic Fibrosis Transmembrane Regulator Gene and *In Vivo* Transepithelial Potentials. *Am J Respir Crit Care Med* 2006;174:787–94. <https://doi.org/10.1164/rccm.200509-1377OC>.

- [14] McKone EF, Velentgas P, Swenson AJ, Goss CH. Association of sweat chloride concentration at time of diagnosis and CFTR genotype with mortality and cystic fibrosis phenotype. *Journal of Cystic Fibrosis* 2015;14:580–6. <https://doi.org/10.1016/j.jcf.2015.01.005>.
- [15] Davies JC, Griesenbach U, Alton E. Modifier genes in cystic fibrosis. *Pediatr Pulmonol* 2005;39:383–91. <https://doi.org/10.1002/ppul.20198>.
- [16] de Gracia J. Genotype-phenotype correlation for pulmonary function in cystic fibrosis. *Thorax* 2005;60:558–63. <https://doi.org/10.1136/thx.2004.031153>.
- [17] Sliker MG, Sanders EAM, Rijkers GT, Ruven HJT, van der Ent CK. Disease modifying genes in cystic fibrosis. *Journal of Cystic Fibrosis* 2005;4:7–13. <https://doi.org/10.1016/j.jcf.2005.05.006>.
- [18] Boucher RC. Evidence for airway surface dehydration as the initiating event in CF airway disease. *J Intern Med* 2007;261:5–16. <https://doi.org/10.1111/j.1365-2796.2006.01744.x>.
- [19] Boucher RC. An overview of the pathogenesis of cystic fibrosis lung disease. *Adv Drug Deliv Rev* 2002;54:1359–71. [https://doi.org/10.1016/S0169-409X\(02\)00144-8](https://doi.org/10.1016/S0169-409X(02)00144-8).
- [20] Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 2003;168:918–51. <https://doi.org/10.1164/rccm.200304-505SO>.
- [21] Flume PA, Mogayzel PJ, Robinson KA, Goss CH, Rosenblatt RL, Kuhn RJ, et al. Cystic Fibrosis Pulmonary Guidelines. *Am J Respir Crit Care Med* 2009;180:802–8. <https://doi.org/10.1164/rccm.200812-1845PP>.
- [22] Antus B, Barta I, Csiszer E, Kelemen K. Exhaled breath condensate pH in patients with cystic fibrosis. *Inflamm Res* 2012;61:1141–7. <https://doi.org/10.1007/s00011-012-0508-9>.
- [23] Vaughan J, Ngamtrakulpanit L, Pajewski TN, Turner R, Nguyen T-A, Smith A, et al. Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. *European Respiratory Journal* 2003;22:889–94. <https://doi.org/10.1183/09031936.03.00038803>.
- [24] Shoemark A, Wilson R. Exhaled Breath Condensate pH as a Non-invasive Measure of Inflammation in Non-CF Bronchiectasis. *ISRN Pulmonol* 2011;2011:1–6. <https://doi.org/10.5402/2011/169080>.
- [25] Tate S. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 2002;57:926–9. <https://doi.org/10.1136/thorax.57.11.926>.
- [26] Paget-Brown AO, Ngamtrakulpanit L, Smith A, Bunyan D, Hom S, Nguyen A, et al. Normative Data for pH of Exhaled Breath Condensate. *Chest* 2006;129:426–30. <https://doi.org/10.1378/chest.129.2.426>.

- [27] Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. *Nature* 2012;487:109–13. <https://doi.org/10.1038/nature11130>.
- [28] Kyle H, Ward JP, Widdicombe JG. Control of pH of airway surface liquid of the ferret trachea in vitro. *J Appl Physiol* (1985) 1990;68:135–40. <https://doi.org/10.1152/jappl.1990.68.1.135>.
- [29] Coakley RD, Boucher RC. Regulation and functional significance of airway surface liquid pH. *JOP* 2001;2:294–300.
- [30] Quinton PM. Cystic fibrosis: impaired bicarbonate secretion and mucoviscidosis. *Lancet* 2008;372:415–7. [https://doi.org/10.1016/S0140-6736\(08\)61162-9](https://doi.org/10.1016/S0140-6736(08)61162-9).
- [31] Quinton PM. Role of epithelial HCO₃⁻ transport in mucin secretion: lessons from cystic fibrosis. *Am J Physiol Cell Physiol* 2010;299:C1222–33. <https://doi.org/10.1152/ajpcell.00362.2010>.
- [32] Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. *Journal of Clinical Investigation* 2002;109:317–25. <https://doi.org/10.1172/JCI13870>.
- [33] Montgomery ST, Mall MA, Kicic A, Stick SM, Arest CF. Hypoxia and sterile inflammation in cystic fibrosis airways: mechanisms and potential therapies. *Eur Respir J* 2017;49. <https://doi.org/10.1183/13993003.00903-2016>.
- [34] Lin Q, Pilewski JM, Di YP. Acidic Microenvironment Determines Antibiotic Susceptibility and Biofilm Formation of Pseudomonas aeruginosa. *Front Microbiol* 2021;12. <https://doi.org/10.3389/fmicb.2021.747834>.
- [35] Piva TC, Luft C, Antunes KH, Marostica PJC, Pinto LA, Donadio MVF. Extracellular DNA in sputum is associated with pulmonary function and hospitalization in patients with cystic fibrosis. *Respir Med* 2020;172. <https://doi.org/10.1016/j.rmed.2020.106144>.
- [36] Morrison CB, Markovetz MR, Ehre C. Mucus, mucins, and cystic fibrosis. *Pediatr Pulmonol* 2019;54:S84–96. <https://doi.org/10.1002/ppul.24530>.
- [37] McElvaney OJ, Wade P, Murphy M, Reeves EP, McElvaney NG. Targeting airway inflammation in cystic fibrosis. *Expert Rev Respir Med* 2019;13:1041–55. <https://doi.org/10.1080/17476348.2019.1666715>.
- [38] Sagel SD, Gibson RL, Emerson J, McNamara S, Burns JL, Wagener JS, et al. Impact of Pseudomonas and Staphylococcus Infection on Inflammation and Clinical Status in Young Children with Cystic Fibrosis. *J Pediatr* 2009;154:183–188.e3. <https://doi.org/10.1016/j.jpeds.2008.08.001>.

- [39] ARMSTRONG DS, GRIMWOOD K, CARLIN JB, CARZINO R, GUTIÉRREZ JP, HULL J, et al. Lower Airway Inflammation in Infants and Young Children with Cystic Fibrosis. *Am J Respir Crit Care Med* 1997;156:1197–204. <https://doi.org/10.1164/ajrccm.156.4.96-11058>.
- [40] Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DWH. Early Pulmonary Inflammation in Infants with Cystic Fibrosis. *Am J Respir Crit Care Med* 1995;151:1075–82. <https://doi.org/10.1164/ajrccm/151.4.1075>.
- [41] Bruscia EM, Bonfield TL. Cystic Fibrosis Lung Immunity: The Role of the Macrophage. *J Innate Immun* 2016;8:550–63. <https://doi.org/10.1159/000446825>.
- [42] Koch C. Early infection and progression of cystic fibrosis lung disease. *Pediatr Pulmonol* 2002;34:232–6. <https://doi.org/10.1002/ppul.10135>.
- [43] Lee TWR, Brownlee KG, Conway SP, Denton M, Littlewood JM. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Journal of Cystic Fibrosis* 2003;2:29–34. [https://doi.org/10.1016/S1569-1993\(02\)00141-8](https://doi.org/10.1016/S1569-1993(02)00141-8).
- [44] Bhagirath AY, Li Y, Somayajula D, Dadashi M, Badr S, Duan K. Cystic fibrosis lung environment and *Pseudomonas aeruginosa* infection. *BMC Pulm Med* 2016;16:174. <https://doi.org/10.1186/s12890-016-0339-5>.
- [45] Courtney JM, Bradley J, McCaughan J, O'Connor TM, Shortt C, Bredin CP, et al. Predictors of mortality in adults with cystic fibrosis. *Pediatr Pulmonol* 2007;42:525–32. <https://doi.org/10.1002/ppul.20619>.
- [46] Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol* 2002;34:91–100. <https://doi.org/10.1002/ppul.10127>.
- [47] Kosorok MR, Zeng L, West SE, Rock MJ, Splaingard ML, Laxova A, et al. Acceleration of lung disease in children with cystic fibrosis after *Pseudomonas aeruginosa* acquisition. *Pediatr Pulmonol* 2001;32:277–87. <https://doi.org/10.1002/ppul.2009.abs>.
- [48] Nixon GM, Armstrong DS, Carzino R, Carlin JB, Olinsky A, Robertson CF, et al. Clinical outcome after early *Pseudomonas aeruginosa* infection in cystic fibrosis. *J Pediatr* 2001;138:699–704. <https://doi.org/10.1067/mpd.2001.112897>.
- [49] Smith WD, Bardin E, Cameron L, Edmondson CL, Farrant K V, Martin I, et al. Current and future therapies for *Pseudomonas aeruginosa* infection in patients with cystic fibrosis. *FEMS Microbiol Lett* 2017;364. <https://doi.org/10.1093/femsle/fnx121>.
- [50] Winstanley C, O'Brien S, Brockhurst MA. *Pseudomonas aeruginosa* Evolutionary Adaptation and Diversification in Cystic Fibrosis Chronic Lung Infections. *Trends Microbiol* 2016;24:327–37. <https://doi.org/10.1016/j.tim.2016.01.008>.

- [51] Pritt B, O'Brien L, Winn W. Mucoid *Pseudomonas* in cystic fibrosis. *Am J Clin Pathol* 2007;128:32–4. <https://doi.org/10.1309/kjrpc7dd5tr9ntdm>.
- [52] Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, Green CG, et al. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *JAMA* 2005;293:581–8. <https://doi.org/10.1001/jama.293.5.581>.
- [53] de Kerchove AJ, Elimelech M. Bacterial swimming motility enhances cell deposition and surface coverage. *Environ Sci Technol* 2008;42:4371–7. <https://doi.org/10.1021/es703028u>.
- [54] Haiko J, Westerlund-Wikstrom B. The role of the bacterial flagellum in adhesion and virulence. *Biology (Basel)* 2013;2:1242–67. <https://doi.org/10.3390/biology2041242>.
- [55] Feldman M, Bryan R, Rajan S, Scheffler L, Brunnert S, Tang H, et al. Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* 1998;66:43–51. <https://doi.org/10.1128/IAI.66.1.43-51.1998>.
- [56] Moradali MF, Ghods S, Rehm BH. *Pseudomonas aeruginosa* Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. *Front Cell Infect Microbiol* 2017;7:39. <https://doi.org/10.3389/fcimb.2017.00039>.
- [57] Nair C, Shoemark A, Chan M, Ollosson S, Dixon M, Hogg C, et al. Cyanide levels found in infected cystic fibrosis sputum inhibit airway ciliary function. *Eur Respir J* 2014;44:1253–61. <https://doi.org/10.1183/09031936.00097014>.
- [58] Lamont IL, Beare PA, Ochsner U, Vasil AI, Vasil ML. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences* 2002;99:7072–7. <https://doi.org/10.1073/pnas.092016999>.
- [59] Kang D, Revtovich A V., Chen Q, Shah KN, Cannon CL, Kirienko N V. Pyoverdine-Dependent Virulence of *Pseudomonas aeruginosa* Isolates From Cystic Fibrosis Patients. *Front Microbiol* 2019;10. <https://doi.org/10.3389/fmicb.2019.02048>.
- [60] Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* 2009;7:654–65. <https://doi.org/10.1038/nrmicro2199>.
- [61] Ding F, Oinuma KI, Smalley NE, Schaefer AL, Hamwy O, Greenberg EP, et al. The *Pseudomonas aeruginosa* Orphan Quorum Sensing Signal Receptor QscR Regulates Global Quorum Sensing Gene Expression by Activating a Single Linked Operon. *MBio* 2018;9. <https://doi.org/10.1128/mBio.01274-18>.
- [62] Schaible B, Taylor CT, Schaffer K. Hypoxia increases antibiotic resistance in *Pseudomonas aeruginosa* through altering the composition of multidrug efflux pumps. *Antimicrob Agents Chemother* 2012;56:2114–8. <https://doi.org/10.1128/AAC.05574-11>.

- [63] Hogardt M, Heesemann J. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. *Int J Med Microbiol* 2010;300:557–62.
<https://doi.org/10.1016/j.ijmm.2010.08.008>.
- [64] Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, et al. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. *J Clin Invest* 2002;109:317–25. <https://doi.org/10.1172/JCI13870>.
- [65] Valentini M, Filloux A. Biofilms and Cyclic di-GMP (c-di-GMP) Signaling: Lessons from *Pseudomonas aeruginosa* and Other Bacteria. *J Biol Chem* 2016;291:12547–55.
<https://doi.org/10.1074/jbc.R115.711507>.
- [66] Høiby N, Ciofu O, Bjarnsholt T. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol* 2010;5:1663–74. <https://doi.org/10.2217/fmb.10.125>.
- [67] Pedersen SS, Møller H, Espersen F, Sørensen CH, Jensen T, Høiby N. Mucosal immunity to *Pseudomonas aeruginosa* alginate in cystic fibrosis. *APMIS* 1992;100:326–34.
- [68] Goldstein W, Döring G. Lysosomal enzymes from polymorphonuclear leukocytes and proteinase inhibitors in patients with cystic fibrosis. *Am Rev Respir Dis* 1986;134:49–56.
<https://doi.org/10.1164/arrd.1986.134.1.49>.
- [69] Bruce MC, Poncz L, Klinger JD, Stern RC, Tomashefski JF, Dearborn DG. Biochemical and pathologic evidence for proteolytic destruction of lung connective tissue in cystic fibrosis. *Am Rev Respir Dis* 1985;132:529–35. <https://doi.org/10.1164/arrd.1985.132.3.529>.
- [70] Jensen PØ, Bjarnsholt T, Phipps R, Rasmussen TB, Calum H, Christoffersen L, et al. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology (Reading)* 2007;153:1329–38. <https://doi.org/10.1099/mic.0.2006/003863-0>.
- [71] Lin Q, Pilewski JM, Di YP. Acidic Microenvironment Determines Antibiotic Susceptibility and Biofilm Formation of *Pseudomonas aeruginosa*. *Front Microbiol* 2021;12:747834.
<https://doi.org/10.3389/fmicb.2021.747834>.
- [72] Mahenthiralingam E, Campbell ME, Speert DP. Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* 1994;62:596–605. <https://doi.org/10.1128/IAI.62.2.596-605.1994>.
- [73] Hogardt M, Hoboth C, Schmoldt S, Henke C, Bader L, Heesemann J. Stage-specific adaptation of hypermutable *Pseudomonas aeruginosa* isolates during chronic pulmonary infection in patients with cystic fibrosis. *J Infect Dis* 2007;195:70–80. <https://doi.org/10.1086/509821>.

- [74] Amiel E, Lovewell RR, O'Toole GA, Hogan DA, Berwin B. *Pseudomonas aeruginosa* Evasion of Phagocytosis Is Mediated by Loss of Swimming Motility and Is Independent of Flagellum Expression. *Infect Immun* 2010;78:2937–45. <https://doi.org/10.1128/IAI.00144-10>.
- [75] Visca P, Imperi F, Lamont IL. Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* 2007;15:22–30. <https://doi.org/10.1016/j.tim.2006.11.004>.
- [76] Bragonzi A, Paroni M, Nonis A, Cramer N, Montanari S, Rejman J, et al. *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. *Am J Respir Crit Care Med* 2009;180:138–45. <https://doi.org/10.1164/rccm.200812-1943OC>.
- [77] Southern KW, Burgel P-R, Castellani C, De Boeck K, Davies JC, Dunlevy F, et al. Standards for the care of people with cystic fibrosis (CF). *Journal of Cystic Fibrosis* 2023;22:961–2. <https://doi.org/10.1016/j.jcf.2023.09.009>.
- [78] Horsley AR, Belcher J, Bayfield K, Bianco B, Cunningham S, Fullwood C, et al. Longitudinal assessment of lung clearance index to monitor disease progression in children and adults with cystic fibrosis. *Thorax* 2022;77:357–63. <https://doi.org/10.1136/thoraxjnl-2021-216928>.
- [79] Rybacka A, Karmelita-Katulska K. The Role of Computed Tomography in Monitoring Patients with Cystic Fibrosis. *Pol J Radiol* 2016;81:141–5. <https://doi.org/10.12659/PJR.896051>.
- [80] Harman K, Dobra R, Davies JC. Disease-modifying drug therapy in cystic fibrosis. *Paediatr Respir Rev* 2018;26:7–9. <https://doi.org/10.1016/j.prrv.2017.03.008>.
- [81] Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Drevinek P, et al. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* 2011;365:1663–72. <https://doi.org/10.1056/NEJMoa1105185>.
- [82] Davies JC, Cunningham S, Harris WT, Lapey A, Regelman WE, Sawicki GS, et al. Safety, pharmacokinetics, and pharmacodynamics of ivacaftor in patients aged 2-5 years with cystic fibrosis and a CFTR gating mutation (KIWI): an open-label, single-arm study. *Lancet Respir Med* 2016;4:107–15. [https://doi.org/10.1016/S2213-2600\(15\)00545-7](https://doi.org/10.1016/S2213-2600(15)00545-7).
- [83] Rosenfeld M, Wainwright CE, Higgins M, Wang LT, McKee C, Campbell D, et al. Ivacaftor treatment of cystic fibrosis in children aged 12 to <24 months and with a CFTR gating mutation (ARRIVAL): a phase 3 single-arm study. *Lancet Respir Med* 2018;6:545–53. [https://doi.org/10.1016/S2213-2600\(18\)30202-9](https://doi.org/10.1016/S2213-2600(18)30202-9).
- [84] Rosenfeld M, Cunningham S, Harris WT, Lapey A, Regelman WE, Sawicki GS, et al. An open-label extension study of ivacaftor in children with CF and a CFTR gating mutation initiating treatment at age 2-5 years (KLIMB). *J Cyst Fibros* 2019;18:838–43. <https://doi.org/10.1016/j.jcf.2019.03.009>.

- [85] Volkova N, Moy K, Evans J, Campbell D, Tian S, Simard C, et al. Disease progression in patients with cystic fibrosis treated with ivacaftor: Data from national US and UK registries. *J Cyst Fibros* 2020;19:68–79. <https://doi.org/10.1016/j.jcf.2019.05.015>.
- [86] Bessonova L, Volkova N, Higgins M, Bengtsson L, Tian S, Simard C, et al. Data from the US and UK cystic fibrosis registries support disease modification by CFTR modulation with ivacaftor. *Thorax* 2018;73:731–40. <https://doi.org/10.1136/thoraxjnl-2017-210394>.
- [87] Wainwright CE, Elborn JS, Ramsey BW. Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med* 2015;373:1783–4. <https://doi.org/10.1056/NEJMc1510466>.
- [88] Taylor-Cousar JL, Munck A, McKone EF, van der Ent CK, Moeller A, Simard C, et al. Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del. *N Engl J Med* 2017;377:2013–23. <https://doi.org/10.1056/NEJMoa1709846>.
- [89] Taylor-Cousar JL, Mall MA, Ramsey BW, McKone EF, Tullis E, Marigowda G, et al. Clinical development of triple-combination CFTR modulators for cystic fibrosis patients with one or two F508del alleles. *ERJ Open Res* 2019;5. <https://doi.org/10.1183/23120541.00082-2019>.
- [90] Davies JC, Moskowitz SM, Brown C, Horsley A, Mall MA, McKone EF, et al. VX-659-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. *N Engl J Med* 2018;379:1599–611. <https://doi.org/10.1056/NEJMoa1807119>.
- [91] Heijerman HGM, McKone EF, Downey DG, Van Braeckel E, Rowe SM, Tullis E, et al. Efficacy and safety of the elexacaftor plus tezacaftor plus ivacaftor combination regimen in people with cystic fibrosis homozygous for the F508del mutation: a double-blind, randomised, phase 3 trial. *Lancet* 2019;394:1940–8. [https://doi.org/10.1016/S0140-6736\(19\)32597-8](https://doi.org/10.1016/S0140-6736(19)32597-8).
- [92] Middleton PG, Mall MA, Drevinek P, Lands LC, McKone EF, Polineni D, et al. Elexacaftor-Tezacaftor-Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. *N Engl J Med* 2019;381:1809–19. <https://doi.org/10.1056/NEJMoa1908639>.
- [93] Mall MA, Brugha R, Gartner S, Legg J, Moeller A, Mondejar-Lopez P, et al. Efficacy and Safety of Elexacaftor/Tezacaftor/Ivacaftor in Children 6 Through 11 Years of Age with Cystic Fibrosis Heterozygous for F508del and a Minimal Function Mutation: A Phase 3b, Randomized, Placebo-controlled Study. *Am J Respir Crit Care Med* 2022;206:1361–9. <https://doi.org/10.1164/rccm.202202-0392OC>.
- [94] Birket SE, Davis JM, Fernandez-Petty CM, Henderson AG, Oden AM, Tang L, et al. Ivacaftor Reverses Airway Mucus Abnormalities in a Rat Model Harboring a Humanized G551D-CFTR. *Am J Respir Crit Care Med* 2020;202:1271–82. <https://doi.org/10.1164/rccm.202002-0369OC>.

- [95] Harwood KH, McQuade RM, Jarnicki A, Schneider-Futschik EK. Ivacaftor Alters Macrophage and Lymphocyte Infiltration in the Lungs Following Lipopolysaccharide Exposure. *ACS Pharmacol Transl Sci* 2022;5:419–28. <https://doi.org/10.1021/acsptsci.2c00007>.
- [96] Hisert KB, Heltshe SL, Pope C, Jorth P, Wu X, Edwards RM, et al. Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. *Am J Respir Crit Care Med* 2017;195:1617–28. <https://doi.org/10.1164/rccm.201609-1954OC>.
- [97] Harris JK, Wagner BD, Zemanick ET, Robertson CE, Stevens MJ, Heltshe SL, et al. Changes in airway microbiome and inflammation with ivacaftor treatment in patients with cystic fibrosis and the G551D mutation. *Ann Am Thorac Soc* 2020;17:212–20. <https://doi.org/10.1513/AnnalsATS.201907-493OC>.
- [98] Bernarde C, Keravec M, Mounier J, Gouriou S, Rault G, Ferec C, et al. Impact of the CFTR-potentiator ivacaftor on airway microbiota in cystic fibrosis patients carrying a G551D mutation. *PLoS One* 2015;10:e0124124. <https://doi.org/10.1371/journal.pone.0124124>.
- [99] Rowe SM, Heltshe SL, Gonska T, Donaldson SH, Borowitz D, Gelfond D, et al. Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *Am J Respir Crit Care Med* 2014;190:175–84. <https://doi.org/10.1164/rccm.201404-0703OC>.
- [100] Heltshe SL, Rowe SM, Skalland M, Baines A, Jain M, Network GI of the CFFTD. Ivacaftor-treated Patients with Cystic Fibrosis Derive Long-Term Benefit Despite No Short-Term Clinical Improvement. *Am J Respir Crit Care Med* 2018;197:1483–6. <https://doi.org/10.1164/rccm.201710-2046LE>.
- [101] Millar BC, McCaughan J, Rendall JC, Downey DG, Moore JE. *Pseudomonas aeruginosa* in cystic fibrosis patients with c.1652GA (G551D)-CFTR treated with ivacaftor-Changes in microbiological parameters. *J Clin Pharm Ther* 2018;43:92–100. <https://doi.org/10.1111/jcpt.12616>.
- [102] Neerinx AH, Whiteson K, Phan JL, Brinkman P, Abdel-Aziz MI, Weersink EJM, et al. Lumacaftor/ivacaftor changes the lung microbiome and metabolome in cystic fibrosis patients. *ERJ Open Res* 2021;7:31–2020. <https://doi.org/10.1183/23120541.00731-2020>.
- [103] Hazlett HF, Hampton TH, Aridgides DS, Armstrong DA, Dessaint JA, Mellinger DL, et al. Altered iron metabolism in cystic fibrosis macrophages: the impact of CFTR modulators and implications for *Pseudomonas aeruginosa* survival. *Sci Rep* 2020;10. <https://doi.org/10.1038/s41598-020-67729-5>.

- [104] Guimbellot JS, Baines A, Paynter A, Heltshe SL, VanDalfsen J, Jain M, et al. Long term clinical effectiveness of ivacaftor in people with the G551D CFTR mutation. *J Cyst Fibros* 2021;20:213–9. <https://doi.org/10.1016/j.jcf.2020.11.008>.
- [105] Heltshe SL, Mayer-Hamblett N, Burns JL, Khan U, Baines A, Ramsey BW, et al. *Pseudomonas aeruginosa* in cystic fibrosis patients with G551D-CFTR treated with ivacaftor. *Clin Infect Dis* 2015;60:703–12. <https://doi.org/10.1093/cid/ciu944>.
- [106] Ahmed B, Bush A, Davies JC. How to use: bacterial cultures in diagnosing lower respiratory tract infections in cystic fibrosis. *Arch Dis Child Educ Pract Ed* 2014;99:181–7. <https://doi.org/10.1136/archdischild-2012-303408>.
- [107] Armstrong DS, Grimwood K, Carlin JB, Carzino R, Olinsky A, Phelan PD. Bronchoalveolar lavage or oropharyngeal cultures to identify lower respiratory pathogens in infants with cystic fibrosis. *Pediatr Pulmonol* 1996;21:267–75. [https://doi.org/10.1002/\(SICI\)1099-0496\(199605\)21:5<267::AID-PPUL1>3.0.CO;2-K](https://doi.org/10.1002/(SICI)1099-0496(199605)21:5<267::AID-PPUL1>3.0.CO;2-K).
- [108] Equi AC, Pike SE, Davies J, Bush A. Use of cough swabs in a cystic fibrosis clinic. *Arch Dis Child* 2001;85:438–9. <https://doi.org/10.1136/ad.85.5.438>.
- [109] Ramsey BW, Wentz KR, Smith AL, Richardson M, Williams-Warren J, Hedges DL, et al. Predictive value of oropharyngeal cultures for identifying lower airway bacteria in cystic fibrosis patients. *Am Rev Respir Dis* 1991;144:331–7. <https://doi.org/10.1164/ajrccm/144.2.331>.
- [110] Rosenfeld M, Emerson J, Accurso F, Armstrong D, Castile R, Grimwood K, et al. Diagnostic accuracy of oropharyngeal cultures in infants and young children with cystic fibrosis. *Pediatr Pulmonol* 1999;28:321–8. [https://doi.org/10.1002/\(sici\)1099-0496\(199911\)28:5<321::aid-ppul3>3.0.co;2-v](https://doi.org/10.1002/(sici)1099-0496(199911)28:5<321::aid-ppul3>3.0.co;2-v).
- [111] CF Trust. Laboratory Standards for Processing Samples from People with Cystic Fibrosis . 2010.
- [112] Jain K, Wainwright C, Smyth AR. Bronchoscopy-guided antimicrobial therapy for cystic fibrosis. *Cochrane Database of Systematic Reviews* 2018;2018. <https://doi.org/10.1002/14651858.CD009530.pub4>.
- [113] Ronchetti K, Tame J-D, Paisey C, Thia LP, Doull I, Howe R, et al. The CF-Sputum Induction Trial (CF-SpIT) to assess lower airway bacterial sampling in young children with cystic fibrosis: a prospective internally controlled interventional trial. *Lancet Respir Med* 2018;6:461–71. [https://doi.org/10.1016/S2213-2600\(18\)30171-1](https://doi.org/10.1016/S2213-2600(18)30171-1).

- [114] Ratjen F, Walter H, Haug M, Meisner C, Grasemann H, Doring G. Diagnostic value of serum antibodies in early *Pseudomonas aeruginosa* infection in cystic fibrosis patients. *Pediatr Pulmonol* 2007;42:249–55. <https://doi.org/10.1002/ppul.20562>.
- [115] Hayes Jr. D, Farrell PM, Li Z, West SE. *Pseudomonas aeruginosa* serological analysis in young children with cystic fibrosis diagnosed through newborn screening. *Pediatr Pulmonol* 2010;45:55–61. <https://doi.org/10.1002/ppul.21083>.
- [116] Douglas TA, Brennan S, Berry L, Winfield K, Wainwright CE, Grimwood K, et al. Value of serology in predicting *Pseudomonas aeruginosa* infection in young children with cystic fibrosis. *Thorax* 2010;65:985–90. <https://doi.org/10.1136/thx.2009.132845>.
- [117] Davies J. The coronavirus pandemic has forced rapid changes in care protocols for cystic fibrosis. *Nature* 2020;583:S15. <https://doi.org/10.1038/d41586-020-02112-y>.
- [118] Baillie S, Ireland K, Warwick S, Wareham D, Wilks M. Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry: rapid identification of bacteria isolated from patients with cystic fibrosis. *Br J Biomed Sci* 2013;70:144–8. <https://doi.org/10.1080/09674845.2013.11669948>.
- [119] Fernández-Olmos A, García-Castillo M, Morosini M-I, Lamas A, Máiz L, Cantón R. MALDI-TOF MS improves routine identification of non-fermenting Gram negative isolates from cystic fibrosis patients. *Journal of Cystic Fibrosis* 2012;11:59–62. <https://doi.org/10.1016/j.jcf.2011.09.001>.
- [120] Fothergill JL, Ledson MJ, Walshaw MJ, McNamara PS, Southern KW, Winstanley C. Comparison of real time diagnostic chemistries to detect *Pseudomonas aeruginosa* in respiratory samples from cystic fibrosis patients. *J Cyst Fibros* 2013;12:675–81. <https://doi.org/10.1016/j.jcf.2013.04.007>.
- [121] Deschaght P, Van daele S, De Baets F, Vaneechoutte M. PCR and the detection of *Pseudomonas aeruginosa* in respiratory samples of CF patients. A literature review. *Journal of Cystic Fibrosis* 2011;10:293–7. <https://doi.org/10.1016/j.jcf.2011.05.004>.
- [122] De Vos D, Lim Jr. A, Pirnay JP, Struelens M, Vandenvelde C, Duinslaeger L, et al. Direct detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *oprI* and *oprL*. *J Clin Microbiol* 1997;35:1295–9. <https://doi.org/10.1128/JCM.35.6.1295-1299.1997>.
- [123] Hou T-Y, Chiang-Ni C, Teng S-H. Current status of MALDI-TOF mass spectrometry in clinical microbiology. *J Food Drug Anal* 2019;27:404–14. <https://doi.org/10.1016/j.jfda.2019.01.001>.

- [124] Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev 2013;26:547–603. <https://doi.org/10.1128/CMR.00072-12>.
- [125] Deschaght P, De Baere T, Van Simaey L, Van daele S, De Baets F, De Vos D, et al. Comparison of the sensitivity of culture, PCR and quantitative real-time PCR for the detection of *Pseudomonas aeruginosa* sputum of cystic fibrosis patients. BMC Microbiol 2009;9:244. <https://doi.org/10.1186/1471-2180-9-244>.
- [126] Brendish NJ, Schiff HF, Clark TW. Point-of-care testing for respiratory viruses in adults: The current landscape and future potential. J Infect 2015;71:501–10. <https://doi.org/10.1016/j.jinf.2015.07.008>.
- [127] Rogers GB, Shaw D, Marsh RL, Carroll MP, Serisier DJ, Bruce KD. Respiratory microbiota: addressing clinical questions, informing clinical practice. Thorax 2015;70:74–81. <https://doi.org/10.1136/thoraxjnl-2014-205826>.
- [128] Rogers GB, Hoffman LR, Carroll MP, Bruce KD. Interpreting infective microbiota: the importance of an ecological perspective. Trends Microbiol 2013;21:271–6. <https://doi.org/10.1016/j.tim.2013.03.004>.
- [129] Cuthbertson L, Walker AW, Oliver AE, Rogers GB, Rivett DW, Hampton TH, et al. Lung function and microbiota diversity in cystic fibrosis. Microbiome 2020;8:45. <https://doi.org/10.1186/s40168-020-00810-3>.
- [130] Davies JC, Alton E, Simbo A, Murphy R, Seth I, Williams K, et al. Training dogs to differentiate *Pseudomonas aeruginosa* from other cystic fibrosis bacterial pathogens: not to be sniffed at? Eur Respir J 2019;54. <https://doi.org/10.1183/13993003.00970-2019>.
- [131] Savelev SU, Perry JD, Bourke SJ, Jary H, Taylor R, Fisher AJ, et al. Volatile biomarkers of *Pseudomonas aeruginosa* in cystic fibrosis and noncystic fibrosis bronchiectasis. Lett Appl Microbiol 2011;52:610–3. <https://doi.org/10.1111/j.1472-765X.2011.03049.x>.
- [132] Dryahina K, Sovová K, Nemec A, Španěl P. Differentiation of pulmonary bacterial pathogens in cystic fibrosis by volatile metabolites emitted by their *in vitro* cultures: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and the *Burkholderia cepacia* complex. J Breath Res 2016;10:037102. <https://doi.org/10.1088/1752-7155/10/3/037102>.
- [133] Nasir M, Bean HD, Smolinska A, Rees CA, Zemanick ET, Hill JE. Volatile molecules from bronchoalveolar lavage fluid can ‘rule-in’ *Pseudomonas aeruginosa* and ‘rule-out’ *Staphylococcus aureus* infections in cystic fibrosis patients. Sci Rep 2018;8:826. <https://doi.org/10.1038/s41598-017-18491-8>.

- [134] Pabary R, Huang J, Kumar S, Alton EFWF, Bush A, Hanna GB, et al. Does mass spectrometric breath analysis detect *Pseudomonas aeruginosa* in cystic fibrosis? *European Respiratory Journal* 2016;47:994–7. <https://doi.org/10.1183/13993003.00944-2015>.
- [135] Lammers M, Kos R, De Vries R, Brinkman P, Dagelet JWF, Rutjes NW, et al. Detection of *Pseudomonas aeruginosa* infection in cystic fibrosis patients by eNose technology. *European Respiratory Journal* 2018;52:PA3412. <https://doi.org/10.1183/13993003.congress-2018.PA3412>.
- [136] Andrews JM. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy* 2001;48:5–16. https://doi.org/10.1093/jac/48.suppl_1.5.
- [137] Murphy RA, Coates M, Thrane S, Sabnis A, Harrison J, Schelenz S, et al. Synergistic Activity of Repurposed Peptide Drug Glatiramer Acetate with Tobramycin against Cystic Fibrosis *Pseudomonas aeruginosa*. *Microbiol Spectr* 2022;10:e0081322. <https://doi.org/10.1128/spectrum.00813-22>.
- [138] Bernardy EE, Petit RA, Raghuram V, Alexander AM, Read TD, Goldberg JB. Genotypic and Phenotypic Diversity of *Staphylococcus aureus* Isolates from Cystic Fibrosis Patient Lung Infections and Their Interactions with *Pseudomonas aeruginosa*. *MBio* 2020;11. <https://doi.org/10.1128/mBio.00735-20>.
- [139] Krishna PS, Woodcock SD, Pfeilmeier S, Bornemann S, Zipfel C, Malone JG. *Pseudomonas syringae* addresses distinct environmental challenges during plant infection through the coordinated deployment of polysaccharides 2021. <https://doi.org/10.1101/2021.06.18.449010>.
- [140] Hart A, Kite PE. Comparison of four selective agars for the isolation of pseudomonads. *Appl Environ Microbiol* 1977;33:1209–14. <https://doi.org/10.1128/AEM.33.5.1209-1214.1977>.
- [141] Héry-Arnaud G, Nowak E, Caillon J, David V, Dirou A, Revert K, et al. Evaluation of quantitative PCR for early diagnosis of *Pseudomonas aeruginosa* infection in cystic fibrosis: a prospective cohort study. *Clinical Microbiology and Infection* 2017;23:203–7. <https://doi.org/10.1016/j.cmi.2016.11.016>.
- [142] Bower JK, Volkova N, Ahluwalia N, Sahota G, Xuan F, Chin A, et al. Real-world safety and effectiveness of elexacaftor/tezacaftor/ivacaftor in people with cystic fibrosis: Interim results of a long-term registry-based study. *Journal of Cystic Fibrosis* 2023;22:730–7. <https://doi.org/10.1016/j.jcf.2023.03.002>.
- [143] Gj C, S M, Tc L, N S, Ss S, T M, et al. Real-world impact of Elexacaftor-Tezacaftor-Ivacaftor treatment in young people with Cystic Fibrosis: A longitudinal study. *Respir Med* 2025;236:107882. <https://doi.org/10.1016/j.rmed.2024.107882>.

- [144] Dittrich A-M, Sieber S, Naehrlich L, Burkhart M, Hafkemeyer S, Tümmler B. Use of elexacaftor/tezacaftor/ivacaftor leads to changes in detection frequencies of *Staphylococcus aureus* and *Pseudomonas aeruginosa* dependent on age and lung function in people with cystic fibrosis. *International Journal of Infectious Diseases* 2024;139:124–31. <https://doi.org/10.1016/j.ijid.2023.11.013>.
- [145] Sheikh S, Britt RD, Ryan-Wenger NA, Khan AQ, Lewis BW, Gushue C, et al. Impact of elexacaftor–tezacaftor–ivacaftor on bacterial colonization and inflammatory responses in cystic fibrosis. *Pediatr Pulmonol* 2023;58:825–33. <https://doi.org/10.1002/ppul.26261>.
- [146] Schaupp L, Addante A, Völler M, Fentker K, Kuppe A, Bardua M, et al. Longitudinal effects of elexacaftor/tezacaftor/ivacaftor on sputum viscoelastic properties, airway infection and inflammation in patients with cystic fibrosis. *European Respiratory Journal* 2023;62:2202153. <https://doi.org/10.1183/13993003.02153-2022>.
- [147] Nichols DP, Morgan SJ, Skalland M, Vo AT, Van Dalfsen JM, Singh SBP, et al. Pharmacologic improvement of CFTR function rapidly decreases sputum pathogen density, but lung infections generally persist. *Journal of Clinical Investigation* 2023;133. <https://doi.org/10.1172/JCI167957>.
- [148] Schnell A, Hober H, Kaiser N, Ruppel R, Geppert A, Tremel C, et al. Ellexacaftor – Tezacaftor – Ivacaftor treatment improves systemic infection parameters and *Pseudomonas aeruginosa* colonization rate in patients with cystic fibrosis a monocentric observational study. *Heliyon* 2023;9:e15756. <https://doi.org/10.1016/j.heliyon.2023.e15756>.
- [149] Deasy KF, Ibrahim H, Mc Carthy M, Fleming C, Dorgan J, Mc Carthy Y, et al. Real-world Ellexacaftor/Tezacaftor/Ivacaftor(ETI) changes prospective sputum collection and microbiological reporting in a single centre pilot CF cohort. *Adult Cystic Fibrosis, European Respiratory Society*; 2023, p. 15. <https://doi.org/10.1183/23120541.ACF-2023.15>.
- [150] Mossop M, Robinson L, Jiang J-H, Peleg AY, Blakeway L V., Macesic N, et al. Characterisation of key genotypic and phenotypic traits of clinical cystic fibrosis *Staphylococcus aureus* isolates. *J Med Microbiol* 2023;72. <https://doi.org/10.1099/jmm.0.001703>.
- [151] Campbell MJ, Gardner MJ. Statistics in Medicine: Calculating confidence intervals for some non-parametric analyses. *BMJ* 1988;296:1454–6. <https://doi.org/10.1136/bmj.296.6634.1454>.
- [152] Goetz DM, Brown RF, Filigno SS, Bichl SL, Nelson AL, Merlo CA, et al. Cystic fibrosis foundation position paper: Redefining the CF care model. *Journal of Cystic Fibrosis* 2024;23:1055–65. <https://doi.org/10.1016/j.jcf.2024.08.007>.

- [153] Beck MR, Hornick DB, Pena TA, Singh SB, Wright BA. Impact of elexacaftor/tezacaftor/ivacaftor on bacterial cultures from people with cystic fibrosis. *Pediatr Pulmonol* 2023;58:1569–73. <https://doi.org/10.1002/ppul.26362>.
- [154] Mianowski L, Doléans-Jordheim A, Barraud L, Rabilloud M, Richard M, Josserand RN, et al. One year of ETI reduces lung bacterial colonisation in adults with cystic fibrosis. *Sci Rep* 2024;14:29298. <https://doi.org/10.1038/s41598-024-77246-4>.
- [155] Hatfield L, Bianco B, Gavillet H, Burns P, Rivett D, Smith M, et al. Effects of postage on recovery of pathogens from cystic fibrosis sputum samples. *Journal of Cystic Fibrosis* 2023;22:816–22. <https://doi.org/10.1016/j.jcf.2023.03.008>.
- [156] Robey RC, Crozier O, Frame A, Martin K, Ashcroft P, Iqbal N, et al. The benefits of COVID lockdowns on respiratory health: What factors may have contributed to the decline in hospital admissions for cystic fibrosis? *Respir Med Res* 2023;84:101061. <https://doi.org/10.1016/j.resmer.2023.101061>.
- [157] Donaldson SH, Corcoran TE, Pilewski JM, Mogayzel P, Laube BL, Boitet ER, et al. Effect of elexacaftor/tezacaftor/ivacaftor on mucus and mucociliary clearance in cystic fibrosis. *Journal of Cystic Fibrosis* 2024;23:155–60. <https://doi.org/10.1016/j.jcf.2023.10.010>.
- [158] Bijvelds MJC, Roos FJM, Meijssen KF, Roest HP, Verstegen MMA, Janssens HM, et al. Rescue of chloride and bicarbonate transport by elexacaftor-ivacaftor-tezacaftor in organoid-derived CF intestinal and cholangiocyte monolayers. *Journal of Cystic Fibrosis* 2022;21:537–43. <https://doi.org/10.1016/j.jcf.2021.12.006>.
- [159] Guillaume O, Butnarusu C, Visentin S, Reimhult E. Interplay between biofilm microenvironment and pathogenicity of *Pseudomonas aeruginosa* in cystic fibrosis lung chronic infection. *Biofilm* 2022;4:100089. <https://doi.org/10.1016/j.bioflm.2022.100089>.
- [160] Hauser AR. *Pseudomonas aeruginosa*: so many virulence factors, so little time. *Crit Care Med* 2011;39:2193–4. <https://doi.org/10.1097/CCM.0b013e318221742d>.
- [161] Murphy RA, Simbo A, Pallant L, Bardin G, Davies JC. 196 The impact of pH on antibiotic susceptibility of clinical *Pseudomonas aeruginosa* strains. *Journal of Cystic Fibrosis* 2017;16:S117. [https://doi.org/10.1016/S1569-1993\(17\)30560-X](https://doi.org/10.1016/S1569-1993(17)30560-X).
- [162] Rehm K, Vollenweider V, Kümmerli R, Bigler L. Rapid identification of pyoverdines of fluorescent *Pseudomonas* spp. by UHPLC-IM-MS. *Biometals* 2023;36:19–34. <https://doi.org/10.1007/s10534-022-00454-w>.
- [163] Ledger EL, Smith DJ, Teh JJ, Wood ME, Whibley PE, Morrison M, et al. Impact of CFTR Modulation on *Pseudomonas aeruginosa* Infection in People With Cystic Fibrosis. *J Infect Dis* 2024;230:e536–47. <https://doi.org/10.1093/infdis/jiae051>.

- [164] Bryant RE, Fox K, Oh G, Morthland VH. -Lactam Enhancement of Aminoglycoside Activity under Conditions of Reduced pH and Oxygen Tension That May Exist in Infected Tissues. *Journal of Infectious Diseases* 1992;165:676–82. <https://doi.org/10.1093/infdis/165.4.676>.
- [165] Casey M, Gabillard-Lefort C, McElvaney OF, McElvaney OJ, Carroll T, Heeney RC, et al. Effect of elexacaftor/tezacaftor/ivacaftor on airway and systemic inflammation in cystic fibrosis. *Thorax* 2023;78:835–9. <https://doi.org/10.1136/thorax-2022-219943>.
- [166] Ronchetti K, Tame J-D, Thia L, Aseeri A, Weiser R, Mahenthiralingam E, et al. WS13.01 The cystic fibrosis Home Sputum-Induction Trial (CF-HomeSpIT - ISRCTN86523335) to evaluate home sputum-induction and early morning saliva sampling in children with cystic fibrosis. *Journal of Cystic Fibrosis* 2023;22:S25–6. [https://doi.org/10.1016/S1569-1993\(23\)00259-X](https://doi.org/10.1016/S1569-1993(23)00259-X).
- [167] Soroka M, Wasowicz B, Rymaszewska A. Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? *Cells* 2021;10:1931. <https://doi.org/10.3390/cells10081931>.
- [168] Wong YP, Othman S, Lau YL, Radu S, Chee HY. Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *J Appl Microbiol* 2018;124:626–43. <https://doi.org/10.1111/jam.13647>.
- [169] Li C, Shi Y, Yang G, Xia XS, Mao X, Fang Y, et al. Establishment of loop-mediated isothermal amplification for rapid detection of *Pseudomonas aeruginosa*. *Exp Ther Med* 2019;17:131–6. <https://doi.org/10.3892/etm.2018.6910>.
- [170] Zhang S, Xu X, Wu Q, Zhang J. Rapid and sensitive detection of *Pseudomonas aeruginosa* in bottled water by loop-mediated isothermal amplification. *European Food Research and Technology* 2013;236:209–15. <https://doi.org/10.1007/s00217-012-1876-7>.
- [171] Landaverde L, Wong W, Hernandez G, Fan A, Klapperich C. Method for the elucidation of LAMP products captured on lateral flow strips in a point of care test for HPV 16. *Anal Bioanal Chem* 2020;412:6199–209. <https://doi.org/10.1007/s00216-020-02702-9>.
- [172] Lorenz TC. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *J Vis Exp* 2012:e3998. <https://doi.org/10.3791/3998>.
- [173] Meagher RJ, Priye A, Light YK, Huang C, Wang E. Impact of primer dimers and self-amplifying hairpins on reverse transcription loop-mediated isothermal amplification detection of viral RNA. *Analyst* 2018;143:1924–33. <https://doi.org/10.1039/c7an01897e>.
- [174] Sidstedt M, Rådström P, Hedman J. PCR inhibition in qPCR, dPCR and MPS-mechanisms and solutions. *Anal Bioanal Chem* 2020;412:2009–23. <https://doi.org/10.1007/s00216-020-02490-2>.

- [175] Gavillet H, Hatfield L, Rivett D, Jones A, Maitra A, Horsley A, et al. Bacterial Culture Underestimates Lung Pathogen Detection and Infection Status in Cystic Fibrosis. *Microbiol Spectr* 2022;10. <https://doi.org/10.1128/spectrum.00419-22>.
- [176] Qiu X, Liu X, Wang R, Ma X, Han L, Yao J, et al. Accurate, Sensitive, and Rapid Detection of *Pseudomonas aeruginosa* Based on CRISPR/Cas12b with One Fluid-Handling Step. *Microbiol Spectr* 2023;11. <https://doi.org/10.1128/spectrum.03523-22>.
- [177] Larrouy-Maumus G, Clements A, Filloux A, McCarthy RR, Mostowy S. Direct detection of lipid A on intact Gram-negative bacteria by MALDI-TOF mass spectrometry. *J Microbiol Methods* 2016;120:68–71. <https://doi.org/10.1016/j.mimet.2015.12.004>.
- [178] Krenkel H, Brown J, Morris M, Cramer R. The use of salts, buffers and surfactants in LAP-MALDI MS. *Int J Mass Spectrom* 2023;493:117134. <https://doi.org/10.1016/j.ijms.2023.117134>.
- [179] Labarca JA, Pegues DA, Wagar EA, Hindler JA, Bruckner DA. Something's Rotten: A Nosocomial Outbreak of Malodorous *Pseudomonas aeruginosa*. *Clinical Infectious Diseases* 1998;26:1440–5. <https://doi.org/10.1086/516370>.
- [180] Davis TJ, Karanjia A V., Bhebhe CN, West SB, Richardson M, Bean HD. *Pseudomonas aeruginosa* Volatilome Characteristics and Adaptations in Chronic Cystic Fibrosis Lung Infections. *MSphere* 2020;5. <https://doi.org/10.1128/mSphere.00843-20>.
- [181] Kos R, Brinkman P, Neerincx AH, Paff T, Gerritsen MG, Lammers A, et al. Targeted exhaled breath analysis for detection of *Pseudomonas aeruginosa* in cystic fibrosis patients. *Journal of Cystic Fibrosis* 2022;21:e28–34. <https://doi.org/10.1016/j.jcf.2021.04.015>.
- [182] Dryahina K, Sovová K, Nemec A, Španěl P. Differentiation of pulmonary bacterial pathogens in cystic fibrosis by volatile metabolites emitted by their *in vitro* cultures: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia* and the *Burkholderia cepacia* complex. *J Breath Res* 2016;10:037102. <https://doi.org/10.1088/1752-7155/10/3/037102>.
- [183] Goeminne PC, Vandendriessche T, Van Eldere J, Nicolai BM, Hertog ML, Dupont LJ. Detection of *Pseudomonas aeruginosa* in sputum headspace through volatile organic compound analysis. *Respir Res* 2012;13:87. <https://doi.org/10.1186/1465-9921-13-87>.
- [184] Licht J-C, Seidl E, Slingers G, Waters V, de Vries R, Post M, et al. Exhaled breath profiles to detect lung infection with *Staphylococcus aureus* in children with cystic fibrosis. *Journal of Cystic Fibrosis* 2023;22:888–93. <https://doi.org/10.1016/j.jcf.2023.02.010>.

- [185] Willis CM, Britton LE, Harris R, Wallace J, Guest CM. Volatile organic compounds as biomarkers of bladder cancer: Sensitivity and specificity using trained sniffer dogs. *Cancer Biomarkers* 2011;8:145–53. <https://doi.org/10.3233/CBM-2011-0208>.
- [186] Martos Martinez-Caja A, De Herdt V, Boon P, Brandl U, Cock H, Parra J, et al. Seizure-alerting behavior in dogs owned by people experiencing seizures. *Epilepsy & Behavior* 2019;94:104–11. <https://doi.org/10.1016/j.yebeh.2019.02.001>.
- [187] Rooney NJ, Guest CM, Swanson LCM, Morant S V. How effective are trained dogs at alerting their owners to changes in blood glycaemic levels?: Variations in performance of glycaemia alert dogs. *PLoS One* 2019;14:e0210092. <https://doi.org/10.1371/journal.pone.0210092>.
- [188] Guest C, Pinder M, Doggett M, Squires C, Affara M, Kandeh B, et al. Trained dogs identify people with malaria parasites by their odour. *Lancet Infect Dis* 2019;19:578–80. [https://doi.org/10.1016/S1473-3099\(19\)30220-8](https://doi.org/10.1016/S1473-3099(19)30220-8).
- [189] Grandjean D, Sarkis R, Lecoq-Julien C, Benard A, Roger V, Levesque E, et al. Can the detection dog alert on COVID-19 positive persons by sniffing axillary sweat samples? A proof-of-concept study. *PLoS One* 2020;15:e0243122. <https://doi.org/10.1371/journal.pone.0243122>.
- [190] Kantele A, Paajanen J, Turunen S, Pakkanen SH, Patjas A, Itkonen L, et al. Scent dogs in detection of COVID-19: triple-blinded randomised trial and operational real-life screening in airport setting. *BMJ Glob Health* 2022;7:e008024. <https://doi.org/10.1136/bmjgh-2021-008024>.
- [191] Mutesa L, Misbah G, Remera E, Ebberts H, Schalke E, Tuyisenge P, et al. Use of trained scent dogs for detection of COVID-19 and evidence of cost-saving. *Front Med (Lausanne)* 2022;9. <https://doi.org/10.3389/fmed.2022.1006315>.
- [192] Nichols DP, Morgan SJ, Skalland M, Vo AT, Van Daltsen JM, Singh SBP, et al. Pharmacologic improvement of CFTR function rapidly decreases sputum pathogen density, but lung infections generally persist. *Journal of Clinical Investigation* 2023;133. <https://doi.org/10.1172/JCI167957>.
- [193] Sutharsan S, Dillenhoefer S, Welsner M, Stehling F, Brinkmann F, Burkhart M, et al. Impact of elxacaftor/tezacaftor/ivacaftor on lung function, nutritional status, pulmonary exacerbation frequency and sweat chloride in people with cystic fibrosis: real-world evidence from the German CF Registry. 2023.
- [194] McNally P, Lester K, Stone G, Elnazir B, Williamson M, Cox D, et al. Improvement in Lung Clearance Index and Chest Computed Tomography Scores with Elxacaftor/Tezacaftor/Ivacaftor Treatment in People with Cystic Fibrosis Aged 12 Years and

- Older – The RECOVER Trial. *Am J Respir Crit Care Med* 2023;208:917–29.
<https://doi.org/10.1164/rccm.202308-1317OC>.
- [195] VanElzakker MB, Tillman EM, Yonker LM, Ratai E-M, Georgiopoulos AM. Neuropsychiatric adverse effects from CFTR modulators deserve a serious research effort. *Curr Opin Pulm Med* 2023;29:603–9. <https://doi.org/10.1097/MCP.0000000000001014>.
- [196] Alicandro G, Gramegna A, Bellino F, Sciarra SC, Lanfranchi C, Contarini M, et al. Heterogeneity in response to Elexacaftor/Tezacaftor/Ivacaftor in people with cystic fibrosis. *Journal of Cystic Fibrosis* 2024;23:1072–9. <https://doi.org/10.1016/j.jcf.2024.04.013>.
- [197] Somerville L, Borish L, Noth I, Albon D. Modulator-refractory cystic fibrosis: Defining the scope and challenges of an emerging at-risk population. *Ther Adv Respir Dis* 2024;18. <https://doi.org/10.1177/17534666241297877>.
- [198] Morrison CB, Shaffer KM, Araba KC, Markovetz MR, Wykoff JA, Quinney NL, et al. Treatment of Cystic Fibrosis Airway Cells with CFTR Modulators Reverses Aberrant Mucus Properties via Hydration. *European Respiratory Journal* 2022;59. <https://doi.org/10.1183/13993003.00185-2021>.
- [199] Ludovico A, Moran O, Baroni D. Modulator Combination Improves In Vitro the Microrheological Properties of the Airway Surface Liquid of Cystic Fibrosis Airway Epithelia. *Int J Mol Sci* 2022;23. <https://doi.org/10.3390/ijms231911396>.
- [200] Maher RE, Barry PJ, Emmott E, Jones AM, Lin L, McNamara PS, et al. Influence of highly effective modulator therapy on the sputum proteome in cystic fibrosis. *Journal of Cystic Fibrosis* 2024;23:269–77. <https://doi.org/10.1016/j.jcf.2023.10.019>.
- [201] Pepe A, Fevola C, Dolce D, Campana S, Ravenni N, Taccetti G, et al. Elexacaftor/tezacaftor/ivacaftor and inflammation in children and adolescents with cystic fibrosis: a retrospective dual-center cohort study. *Ther Adv Respir Dis* 2025;19. <https://doi.org/10.1177/17534666251314706>.
- [202] Bardin E, Hunzinger N, Lamy E, Roquencourt C, Zhou B, Tabache Y, et al. Short-term modification of breathprint by Elexacaftor/Tezacaftor/Ivacaftor in a paediatric cohort. *Journal of Cystic Fibrosis* 2025;24:606–12. <https://doi.org/10.1016/j.jcf.2025.01.004>.
- [203] Durfey SL, Kapnadak SG, Pena T, Willmering MM, Godwin JD, Teresi ME, et al. *Pseudomonas* infections persisting after CFTR modulators are widespread throughout the lungs and drive lung inflammation. *Cell Host Microbe* 2025. <https://doi.org/10.1016/j.chom.2025.07.009>.
- [204] Armbruster CR, Hilliam YK, Zemke AC, Atteih S, Marshall CW, Moore J, et al. Persistence and evolution of *Pseudomonas aeruginosa* following initiation of highly effective modulator therapy in cystic fibrosis. *MBio* 2024;15. <https://doi.org/2>.

- [205] González CT, Martin C, Crane M, Gutierrez K, Thomas J, Okros M, et al. Microenvironmental Effects of a Non-Antibiotic Therapy for a Chronic Polymicrobial Infection Alter 2 Microbial Physiology, Competition, and Virulence 3 Running title 4 Mimicking CFTR Modulators on Lung Microbiome 5 n.d. <https://doi.org/10.1093/ismejo/wraf125/8162675>.
- [206] Singhal N, Kumar M, Kanaujia PK, Viridi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front Microbiol* 2015;6:791. <https://doi.org/10.3389/fmicb.2015.00791>.
- [207] Mangiaterra G, Amiri M, Di Cesare A, Pasquaroli S, Manso E, Cirilli N, et al. Detection of viable but non-culturable *Pseudomonas aeruginosa* in cystic fibrosis by qPCR: a validation study. *BMC Infect Dis* 2018;18:701. <https://doi.org/10.1186/s12879-018-3612-9>.
- [208] Blanchard AC, Rooney AM, Yau Y, Zhang Y, Stapleton PJ, Horton E, et al. Early detection using qPCR of *Pseudomonas aeruginosa* infection in children with cystic fibrosis undergoing eradication treatment. *Journal of Cystic Fibrosis* 2018;17:723–8. <https://doi.org/10.1016/j.jcf.2018.02.008>.
- [209] Fenn D, Abdel-Aziz MI, Brinkman P, Kos R, Neerincx AH, Altenburg J, et al. Comparison of microbial composition of cough swabs and sputum for pathogen detection in patients with cystic fibrosis. *Journal of Cystic Fibrosis* 2022;21:52–60. <https://doi.org/10.1016/j.jcf.2021.08.031>.
- [210] Ahmed B, Cox MJ, Cuthbertson L, James PL, Cookson WOC, Davies JC, et al. Comparison of the upper and lower airway microbiota in children with chronic lung diseases. *PLoS One* 2018;13:e0201156. <https://doi.org/10.1371/journal.pone.0201156>.
- [211] Maiya S. Cough plate versus cough swab in patients with cystic fibrosis; a pilot study. *Arch Dis Child* 2004;89:577–9. <https://doi.org/10.1136/adc.2003.037481>.
- [212] Chavasse RJ, Cordle R, Petkar H. Cough plates for microbiological surveillance in cystic fibrosis. *Arch Dis Child* 2007;92:279. <https://doi.org/10.1136/adc.2006.110353>.
- [213] Barr HL, Halliday N, Barrett DA, Williams P, Forrester DL, Peckham D, et al. Diagnostic and prognostic significance of systemic alkyl quinolones for *P. aeruginosa* in cystic fibrosis: A longitudinal study. *Journal of Cystic Fibrosis* 2017;16:230–8. <https://doi.org/10.1016/j.jcf.2016.10.005>.
- [214] Barr HL, Halliday N, Cámara M, Barrett DA, Williams P, Forrester DL, et al. *Pseudomonas aeruginosa* quorum sensing molecules correlate with clinical status in cystic fibrosis. *European Respiratory Journal* 2015;46:1046–54. <https://doi.org/10.1183/09031936.00225214>.

- [215] Webb K, Cámara M, Zain NMM, Halliday N, Bruce KD, Nash EF, et al. Novel detection of specific bacterial quorum sensing molecules in saliva: Potential non-invasive biomarkers for pulmonary *Pseudomonas aeruginosa* in cystic fibrosis. *Journal of Cystic Fibrosis* 2022;21:626–9. <https://doi.org/10.1016/j.jcf.2021.08.030>.
- [216] Seidl E, Licht J-C, de Vries R, Ratjen F, Grasemann H. Exhaled Breath Analysis Detects the Clearance of *Staphylococcus aureus* from the Airways of Children with Cystic Fibrosis. *Biomedicine* 2024;12:431. <https://doi.org/10.3390/biomedicine12020431>.
- [217] Tolle JJ, Jadhaio S, Patel B, Sun H, Eastman S, Hartert T, et al. Detection of pathogenic bacteria and biomarkers in lung specimens from cystic fibrosis patients. *J Breath Res* 2024;18:046004. <https://doi.org/10.1088/1752-7163/ad56bc>.
- [218] Stanton BA. Effects of *Pseudomonas aeruginosa* on CFTR chloride secretion and the host immune response. *Am J Physiol Cell Physiol* 2017;312:C357–66. <https://doi.org/10.1152/ajpcell.00373.2016>.
- [219] Keating C, Yonker LM, Vermeulen F, Prais D, Linnemann RW, Trimble A, et al. Vanzacaftor-tezacaftor-deutivacaftor versus elexacaftor-tezacaftor-ivacaftor in individuals with cystic fibrosis aged 12 years and older (SKYLINE Trials VX20-121-102 and VX20-121-103): results from two randomised, active-controlled, phase 3 trials. *Lancet Respir Med* 2025;13:256–71. [https://doi.org/10.1016/S2213-2600\(24\)00411-9](https://doi.org/10.1016/S2213-2600(24)00411-9).
- [220] Rowbotham NJ, Smith S, Elliott ZC, Cupid B, Allen LJ, Cowan K, et al. A refresh of the top 10 research priorities in cystic fibrosis. *Thorax* 2023;78:840–3. <https://doi.org/10.1136/thorax-2023-220100>.

Appendices

Appendix 1: SRC-PAPA repository collection and storage SOP

Pseudomonas aeruginosa clinical isolate collection
SOP Reference: CFGT/SOP/215
Version Number: Final version 1; 03/JUL/2015
Effective Date: 03/JUL/2015
Procedure Authors: A Turnbull and Michelle Manvell

OUTLINE OF PROCEDURE

Collection and storage of *Pseudomonas aeruginosa* (Pa) clinical isolates, obtained from the microbiology department of the Royal Brompton Hospital.

Relevant health and safety reference documents:

Bio1 risk
assessment
COSHH
assessment
Code of Practice for working with PA and unscreened human
tissue Local rules Gene Therapy
NHLI H&S Policy

Personal Protective Equipment:

Fastened, Howie Lab coat.
Nitrile gloves, required when handling of bacteria and chemicals
Safety glasses, when there is a risk of splashes as indicated in the protocol.

Equipment needed:

Sterile inoculation loops
Microbank microbial storage
vials
Class II microbiological safety cabinet
Screw-top container within sample transport containers (leakproof, sealed;
screwtop biohazard transport jar); must be UN 3373 approved for transport
between sites see
<http://www3.imperial.ac.uk/safety/subjects/dangerousgoods/diagnosticspecimen>
S
Paper towels
2% Virkon or 2% Distel

TRANSPORT OF SAMPLES

- The packaging (follow IATA PI-650 instructions), labelling and marking requirements for “Biological Substances, Category B” (UN3373) must be applied.

<http://www3.imperial.ac.uk/safety/subjects/dangerousgoods/diagnosticspecimens>

- The package must be concealed from public view eg by concealing in an anonymous outer package such as a shopping bag
- The package must never be left unattended at any stage
- The College employee must carry his / her ID card at all times

STORAGE OF SAMPLES

- Using a permanent marker, label a separate Microbank vial for each isolate to be stored. Each isolate will be labelled with a lab number in the form of the year, followed by the letter B, followed by a six-digit number (e.g. 14B012435). This whole lab number should be written on the Microbank vial, along with the Box number, and the place number within the box. The lab number, box number, and place number should then be entered into the sample lab book, and the box and place numbers should follow on chronologically from the previous numbers. Note that a single cetrimide agar plate will contain up to six isolates, which will each be individually labelled with a lab number. Where both a non-mucoid and a mucoid Pa strain have been cultured, these will also be labelled with as "Pa", and "PaM", respectively. If the "Pa" and "PaM" labelling is present, this should also be recorded in the sample lab book in the appropriate column.
- All of the following stages should be carried out in a class II microbiological safety cabinet
- Using aseptic technique, unscrew the Microbank vial cap.
- Using a sterile inoculating loop, pick off a scrape of bacteria from a pure culture isolate (aiming for a large quantity of bacteria to be on the loop) and place this into the cryopreservative. Gently agitate the loop among the beads to detach the bacteria from the loop. Discard the loop, first disinfecting in 1% Virkon or 1% Distel.
- Using aseptic technique, replace the cap on the microbank vial tightly, and invert it 4-5 times to emulsify the organism. DO NOT VORTEX.
- Let the microbank vial sit for 2 minutes to allow the isolate to bind to the beads.
- Remove the cap and use a sterile disposable pipette to remove as much of the cryopreservative as possible (there is approximately 850microlitres). The beads should be as free of liquid as possible.
- Close the Microbank vial finger tight only. Do not overtighten.
- Place the Microbank vials back into the freezer box (or the current freezer box if mid-way through a box) and freeze at -80.

RECOVERY OF BACTERIAL ISOLATES

- Remove the Microbank vial(s) from the -80 freezer and place in a polystyrene container containing dry ice.
- Transport the Microbank vial(s) in the container to the class II microbiological safety cabinet.
- Using aseptic technique, open the Microbank vial and, using a sterile needle or forceps remove one bead. Place this on agar and allow to thaw, or place directly into an appropriate liquid medium. Close the Microbank vial finger tight and return as soon as possible to the freezer, to avoid thawing of the remaining beads.
- The thawed bead can then be streaked directly onto agar or inoculated into an appropriate liquid medium.

SPILLAGE PROCEDURES

Check the bio1 form and code of practice for working with Pa

Any pathogen samples spilled before having been fixed must be rendered inactive using a 2% Virkon solution mixed 1:1 with waste to give a final concentration of 1%, and then waste can be disposed of via yellow clinical waste bins or can be put in GM waste bags for autoclaving.

WASTE DISPOSAL

Check the bio1 form and code of practice for working with PA

Any live pathogen waste that cannot be easily inactivated in Virkon must be disposed of in a clear autoclave waste bags (at all times) in a leak proof solid container; transported to the autoclave must be in the closed solid blue container and the container with the waste should be autoclaved. **The clear bag must not be sealed to allow steam to penetrate.**

PA waste should not be left unattended outside the laboratory. If there is a delay in waste inactivation, the blue lid should be sealed with a tag till the autoclave is available.

Any non-fixed samples containing both human cells and pathogens should be rendered inactive via autoclaving, where possible, and should be disposed of in yellow bins marked "Pathological Specimens for incineration"

Hospital waste disposal: use yellow bins marked "Pathological Specimens for incineration" and incinerated via routine hospital waste disposal routes.

Orange clinical waste bags (EKB); or Tiger Waste (RBH) can be used for any non-infective, general clinical waste, and when they are $\frac{3}{4}$ full, they should be tied with a waste tag, and marked with the department, room number and date. They should be left inside the lab until ready to be disposed of.

The boxes should be sealed during transport to the. The small grey crates can then be put directly in the autoclave.

Appendix 2: SRC-PAPA Revival of frozen stored repository samples SOP

Microorganism storage and revival from frozen
SOP Reference: CFGT/SOP/221
Version Number: Final version 2; 29/FEB/2016
Effective Date: 29/FEB/2016
Last Review Date: 29/FEB/2016
Procedure Authors: Wynne Smith

OUTLINE OF PROCEDURE

Collection and storage of *Pseudomonas aeruginosa* (Pa), *Haemophilus influenzae* (Hi), *Streptococcus pneumoniae* (Sp), *Moraxella catarrhalis* (Mc) and *Staphylococcus aureus* (Sa) from clinical isolates, obtained from the microbiology department of the Royal Brompton Hospital. Also PA01, PA14 laboratory strains, GM *Pseudomonas* strains and *Chromobacterium violaceum* (including CV026 strain). Reculturing onto agar and then into broth as needed. Quantification of bacteria

SECTION 1 Summary of health and safety

GENERAL The main risks involved in this protocol are:

- Exposure to Class II bacteria *Pseudomonas aeruginosa* (Pa) *Haemophilus influenzae* (Hi), *Streptococcus pneumoniae* (Sp), *Moraxella catarrhalis* (Mc) and *Staphylococcus aureus* (Sa)
- Exposure to *Chromobacterium violaceum* which doesn't have a hazard class, but will be treated as class II as this is a rare cause of disease in humans.
- Risk of needle-stick injury – **DO NOT RE-SHEATH** needle after use. Dispose in a sharps bin. (Note: blunt needles cannot be substituted as they do not work with the micro-beads).

PROTECTIVE EQUIPMENT

- Class II Biosafety Hood
- Laboratory coat
- Nitrile gloves
- Protective eyewear
- Biological safety box for transportation
- Biological safety screw-lidded tub for transportation
- Freezer gloves

EQUIPMENT

- Class II Biosafety Hood
- Centrifuge
- Incubator
- -80 °C Freezer
- Vortex
- Spectrophotometer

SPILLAGE PROCEDURES

- Any spillage of bacteria within rotors should be dealt with in a Class II microbiological safety cabinet – transport sealed rotor buckets to cabinet for opening. Clean up major liquid spillages with 2% vol/vol Distel/Anistel (to give a final concentration of 1% disinfectant). 1% disinfectant can be used for surface wipes of any residual contamination. Rinse rotor buckets thoroughly with water after disinfection and dry before placing back in centrifuge.
- Spillage outside of containment – the contaminated area will be evacuated to allow aerosols to settle for minimum of 1 hour, gross liquid will be adsorbed, and cleaned with 2% Virkon/Distel vol:vol (to give a 1% final concentration) for minimum contact time of 10 minutes and wiped clean. All gloves and towels will be autoclaved.

FIRST AID PROCEDURES

- Exposure to Class 2 pathogens- if on skin wash thoroughly with soap and water and if a needle-stick injury – encourage wound to bleed. For mouth or eyes splash, flush out with lots of water for 10 minutes – contact Safety Officer and Occupational Health for advice within same or next working day. For inhalation notify Health and Safety and Occupational Health.

WASTE DISPOSAL

- Used agar plates, inoculation loops, pipette tips, cuvettes and needles will be autoclaved in sharps containers, or biohazard boxes as appropriate
- Inoculation loops, used pipette tips and cuvettes will be sterilised using Distel or Virkon at a final concentration of 1% for 10 minutes prior to disposal
- All biological waste, contaminated gloves and towels will be autoclaved prior to disposal by incineration
- Large volumes of broth waste will either be autoclaved or mixed 1:1 volume with a solution of 2% Distel or Virkon at a final concentration of 1% for 60 minutes for sterilisation before disposal to sink

SECTION 2 Protocol

Sample collection, handling and subsequent transportation:

- Agar plates identified as culturing pure growth of the above organisms will be collected from RBHT microbiology twice weekly, or as necessary
- All transportation in double containment
- Transported into class II microbiological safety cabinet (MSC)
- All processing work will be undertaken in a class II MSC unless where clearly stated
- Hands should be washed after all laboratory work

Sample processing and freezing (within class II MSC):

- Each sample identifier is labelled onto microbank storage bead tubes (Prolab diagnostics) using a permanent marker. This information is also recorded into the laboratory written record. Both laboratory record and tube are also labelled with planned freezer location of tube (eg “box 2, position 24”).
- Using asepsis, microbank vial lid is removed

- Bacterial colonies are taken from the agar and inoculated into the microbank vial to achieve a 3-4 McFarland standard (if possible) using a sterile loop and the lid of the microbank vial is replaced
- The microbank vial is inverted 4-5 times, and left to sit for a minimum of 2 minutes
- Using a sterile disposable pipette, aspirate the microbank vial to dryness by removing a volume of approximately 900 microlitres. Replace the cap of the microbank vial
- The microbank vial is then placed in the freezer at the correct location (eg in box 2 at position 24) at a temperature of -70 - -80C
- Spent agar plates will have lids replaced and be securely wrapped in laboratory film before being added to sharps containers and then autoclaved
- Used inoculation loops, pipette tips and any waste microbank vial broth will be placed into a liquid waste receptacle containing 2% Distel solution (to achieve a final concentration of no less than 1% Distel). After 60 minutes exposure these are safe to be disposed of.

Reculturing onto agar when needed:

- Dry ice will be used in an appropriate, loose-lidded container
- Safety gloves will be worn when moving or handling dry ice or specimens preserved therein
- Microbank vials will be removed from the freezer and placed on dryice

Without allowing the microbank vial to thaw, a single bead is removed from the vial using a 19G 2 inch Microlance needle. The microbank vial with remaining beads is returned to the dry ice, and subsequently to the correct position in the freezer.

- The bead is transferred onto a selected agar plate, previously labelled on the base with corresponding details of culture, date, and user, with a permanent marker
- The bead is left to thaw on the agar plate for 2 to 5 minutes
- A sterile inoculation loop is used to inoculate the agar from the thawed bead
- Agar plates are cultured form 12-24 hours in the warm room (held at 30°C or 37°C depending on organism).
- Used inoculation loops will be placed into a liquid waste receptacle containing 2% Distel solution (to achieve a final concentration of no less than 1% Distel).
- Used Microlance needles will be placed into sharps containers before autoclaving. Do not re-sheathe used needles.

Reculturing from agar plates into broth:

- 30ml of prepared and sterilised Lennox Luria Bertani broth is added to sterile 50ml Erlenmeyer flasks with sterile cotton wool and foil lids and labels applied
- A sterile loop is used to remove a sweep of 10-20 colonies and transferred into the Lennox Luria Bertani broth and the cotton wool and foil lid reapplied afterwards.
- If needed, to mix the bacteria, these will be vortexed with the lids held firmly in place inside the microbiological safety cabinet
- Erlenmeyer flasks are contained in an old ice cream tub, six per tub so as to be secure. If fewer than 6 are needed, empty flasks should be used as space savers.

- Flasks and tubs are then slid into an air-tight zip-lock bag which is then fastened before removal from the class II MSC
- Broth is cultured at 30-37C, either static or agitated for 20-24 hours
- Broth cultures agitated on a shaker / rocker will be secured in place within the tubs using sticky tape and purpose-built attachment bars so that the cultures cannot tip up should the shaker / rocker speed change.

Quantification of bacteria

- Broth cultures are vortexed for a minimum of 10 seconds and speed of 1800-2200 rpm until there is no clumping of matter seen in the sample on visual inspection. This is performed with foil lids attached and with the class II MSC to protect staff.
- A 1 ml volume of broth culture will be pipetted into a single cuvette
- The cuvette is then transferred to a spectrophotometer within the class

Appendix 3: Miles and Misra Method of Colony Counting

Miles and Misra Method of Colony Counting	
SOP Reference: CFGT/SOP/xxx	
Version Number: 1	Final version x; DD/MON/YYYY
Effective Date:	
Last Review Date:	
Procedure Authors: Ronan Murphy	
Approved by London:	
Signature:	
Date: 28/11/16	

OUTLINE OF PROCEDURE

To determine the number of colony forming units in a bacterial suspension

SECTION 1 Summary of health and safety

GENERAL The main risks involved in this protocol are:

Exposure to Class II bacteria from microbiology culture collection

PROTECTIVE EQUIPMENT

Class II Biosafety Hood

Laboratory coat

Nitrile gloves Protective

eyewear

EQUIPMENT

Class II Biosafety Hood 37°C

Incubator

Vortex

Pipette

SPILLAGE PROCEDURES

Spillage outside of containment – the contaminated area will be evacuated to allow aerosols to settle for minimum of 1 hour, gross liquid will be adsorbed, and cleaned with 2% Virkon/Distel vol:vol (to give a 1% final concentration) for minimum contact time of 1 hour and wiped clean. All gloves and towels will be autoclaved.

WASTE DISPOSAL

Used agar plates, pipette tips and cuvettes will be autoclaved in sharps containers, or biohazard boxes as appropriate

Used pipette tips and cuvettes will be sterilised using Distel or Virkon at a final concentration of 1% for 1 hour prior to disposal

• **SECTION 2 Protocol**

The bacterial suspension is serially diluted by adding 1x of suspension to 9x of diluent (e.g. 100 µl bacteria in 900 µl PBS). When the quantity of bacteria is unknown, dilutions should be made to at least 10^{-8}

- The surfaces of the plates need to be sufficiently dry to allow a 20µl drop to be absorbed in 15–20 minutes

- Plates are divided into 4 equal sectors and each sector labelled with a dilution factor

In each sector, 5 x 20 µl (total 100 µl) of the appropriate dilution is dropped onto the surface of the agar and the drop allowed to spread naturally. It is important to avoid touching the surface of the agar with the pipette

- The plates are left upright on the bench to dry (may take up to 30mins) before inversion and incubation at 37 °C for 18 – 24 hours (or appropriate incubation conditions considering the organism and agar used)
- Each sector is observed for growth, high concentrations will give a confluent growth over the area of the drop, or a large number of small/merged colonies. Colonies are counted in the sector where the highest number of full-size discrete colonies can be seen (usually sectors containing between 2-20 colonies are counted)
- The following equation is used to calculate the number of colony forming units (CFU) per ml from the original aliquot / sample:

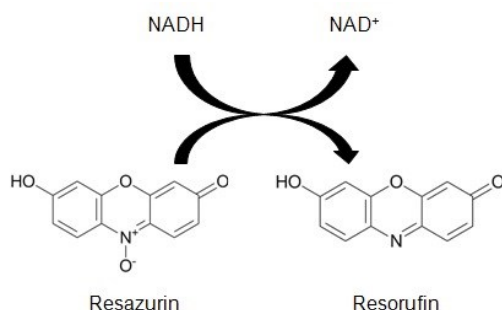
$$\text{CFU per ml} = \text{Average number of colonies for a dilution} \times 50 \times \text{dilution factor}$$

Appendix 4: Resazurin assay

Resazurin reduction assay

Viable cells with active metabolism will reduce resazurin to resorufin.

<https://www.ncbi.nlm.nih.gov/books/NBK144065/>



Resazurin = Dark blue, no fluorescence.

Resorufin = pink, fluorescent.

Protocol for measuring *Pseudomonas aeruginosa* viability.

Resazurin preparation

- Prepare 1% stock solution of resazurin – Dissolve 0.5g of resazurin sodium salt into 50ml of dH₂O.
- Prepare 0.02% solution of resazurin – dilute 1% stock at 1:50 in distilled water. Then sterile filter through 0.2 µm cellulose filter.

Protocol

- Add resazurin solution at 10% of total volume in the well. Therefore for 200µl of bacterial solution add 20µl of 0.02% resazurin. Final concentration = 0.002%.
- For negative control, add 20µl of the solution to wells containing no bacteria.
- Use wells with highest bacterial concentration to set the gain, by selecting the appropriate wells, setting gain to 95% of maximum and starting adjustment.
 - Take note of the gain value, and do not change this value between experiments!
- Either cover with foil and incubate at 37°C for 1-2 hours for endpoint measurement, or incubate in plate reader with readings every 5 minutes for 30-60 minutes
 - Remember to turn the incubator on in the plate reader.
- Measure fluorescence with excitation at 544nm and emission at 590nm (544/590).

Appendix 5: Huff tube lab model SOP

Equipment

- 15ml falcon flasks
- LB and cetrinide broth and agar
- Sterile green tip needles
- Sterile inoculation loops
- 96-well plates
- 2ml Eppendorf tubes
- Sterile Phosphate Buffer Solution (PBS)
- Sterile gauze
- 70% ethanol solution
- Scissors and forceps
- Petri dishes (square and round)
- 1000, 200 Pipettes and tips
- Disinfectant

Method – work under category 2 hood to avoid sample contamination

Preparation:

- Prepare sufficient supply of sterile cetrinide and LB broth according to SOPs
- Prepare sufficient supply of sterile LB agar plates (square and round)

Day 1:

- Choose relevant samples from the repository freezer
- Prepare 5ml LB broth in 15ml falcon flasks
- Use green needle to extract 2 beads from each freezer sample tube
 - 1 to be dropped into the LB broth and vortexed for 5 seconds
 - 1 onto LB agar round plate and streak out (see separate SOP), removing bead in to
- Incubate falcon tubes overnight at 37°C, 200RPM overnight, incubate LB plates at 37°C

Day 2:

- Check broth samples for growth by assessing for cloudiness/bacterial sediment
- Remove beads using inoculation loop and discard into disinfectant
- Centrifuge falcon tubes at 3500RPM, 15 mins, 22°C
- Discard supernatant into disinfectant
- Re-suspend in 5ml sterile PBS and vortex until pellet dissolved

- Prepare 8 x Eppendorf tubes with 900µl cetrimide broth
- Pipette 100µl of neat sample suspension into 1st tube, vortex, sample 100µl of that into the next Eppendorf and repeat for serial dilution to at log-8
- Using 96 well plate, pipette 200µl of each neat sample suspension into row 1sp, and then each corresponding dilution along the row along with neat PBs and neat cetrimide as blanks
- Perform Optical Density (OD 600nm) measurement using plate reader for starting OD for all neat sample suspensions and correct against PBS blank
- Remove sample from plate reader, seal plate with optically clear breathable membrane film, turn on plate reader incubator at 37°C and start 24hr growth curves (readings every 30 min)
- Using neat suspension and cetrimide serial dilutions, pipette 3x 20µl spots for each strain onto LB agar for colony counting (and incubate overnight at 37°C)
- Prepare 60ml sterile pots with lids with 10ml cetrimide broth under hood and label pots with strain/starting dilution
- Assemble sterile field using sterile large petri dishes. Sterilize forceps and scissors in 70% ethanol and allow to dry. Cut sterile gauze into quarters (5x5cm piece) and lay out in 2 ply pieces.
- Inoculate 20µl of each neat suspension and cetrimide dilution for each strain onto separate pieces of gauze. Drop gauze into the 60ml pots/10ml cetrimide with sterile forceps (starting with lowest starting dilution and working up towards neat to avoid cross contamination) and re-sterilise the forceps between strains). Close lids tight
- Vortex pots and incubate on shaker at 200RPM, 37°C overnight
- Finally read Day 1 LB plates to check for single colony growth

Day 3

- Complete overnight growth OD measurement and save data
- Remove gauze pots from incubator and inspect for broth cloudiness/bacterial debris
- Place 3 x 20µl spots of each starting dilution onto LB agar and incubate overnight – this will later identify the threshold of growth viability by starting concentration – read on day 4
- Identify samples to perform colony counting on – include most likely 10² starting concentration sample (as estimated by OD or from previous day's starting colony counts if ready)
- Perform serial dilution in 96 well plate using multichannel pipette (180µl PBS with 20µl sample) and spot out onto LB agar for colony counting day 4.
- Colony count previous days starting dilution samples.

WarmStart Colorimetric LAMP 2X Master Mix Typical LAMP Protocol (M1800)

Overview

Reaction Setup: For simplicity in setting up reactions, we recommend making stocks of the LAMP primers at a usable concentration. For example, we suggest a 10X Primer Mix containing all 6 LAMP primers.

A 10X LAMP Primer Mix contains:

PRIMER	10X CONCENTRATION (STOCK)	1X CONCENTRATION (FINAL)
FIP	16 μ M	1.6 μ M
BIP	16 μ M	1.6 μ M
F3	2 μ M	0.2 μ M
B3	2 μ M	0.2 μ M
LOOP F	4 μ M	0.4 μ M
LOOP B	4 μ M	0.4 μ M

Note: Make primer stock in molecular biology grade H₂O rather than TE or other buffer in order to avoid carryover of additional buffer to the LAMP reaction. Prepare primer stocks in nuclease free water and store at –20°C for up to 2 years.

1. Thaw all components to be used at room temperature and place on ice. Salt may appear in the bottom of the tube so vortex briefly or invert tubes several times to mix thoroughly. Centrifuge to collect material and place on ice.
2. Prepare reaction mix as described below using Colorimetric LAMP Master Mix, LAMP primers and nuclease free water. Volumes listed per 25 μ l LAMP reaction, but other volumes (10, 20, 50 μ l etc.) are all effective if desired, just adjust volumes accordingly. Sample is assumed here to be 1 μ l, but for higher sample volumes add as needed and reduce volume of H₂O to compensate. For non-template reactions add equivalent volume of H₂O or sample storage buffer.

	DNA TARGET	RNA TARGET DETECTION	NO- TEMPLATE CONTROL (NTC)
WarmStart Colorimetric LAMP 2X Master Mix	12.5 µl	12.5 µl	12.5 µl
LAMP Primer Mix (10X)	2.5 µl	2.5 µl	2.5 µl
Target DNA	1 µl	–	–
Target RNA	–	1 µl	–
dH ₂ O	9 µl	9 µl	10 µl
Total Volume	25 µl	25 µl	25 µl

3. Vortex reaction mix and centrifuge to collect material.

4. Pipet 24 µl per reaction into desired reaction vessels and add 1 µl of sample. Mix by vortexing or by pipetting if using a plate or similar vessel, centrifuge to collect if necessary. Check that reaction solutions have a bright pink color, which indicates initial high pH required for successful pH-LAMP reaction.

5. Seal reaction vessels.
6. Incubate at 65°C for 30 minutes.
7. Remove tubes or vessels from incubation and examine by eye. Positive reactions will have turned yellow while negative

controls should remain pink. If color change is not robust, e.g. an orange color is visible, return reactions to 65°C for an additional 10 minutes. Reactions can be examined earlier if desired, and high copy or input reactions can exhibit full color change in as little as 10–15 minutes. Color will be visible directly on removal from incubation temperature, but can be intensified by allowing reaction to cool to room temperature.

8. The result can be photographed or scanned to record the colorimetric results, or simply kept at room temperature in the reaction vessel.

Appendix 7: Medical Detection Dogs – SOP for transport, storage, handling and disposal of samples

Background

We are investigating whether *Pseudomonas aeruginosa* (Pa) can be detected by medical detection dogs (MDDs) when presented with samples of Pa that have been inoculated onto pieces of gauze and briefly incubated in cetrimide broth. This progresses from previous work where Pa scent was detected in filtered bacterial supernatant. Training and testing samples will be produced at the NHLI laboratory (EKB building) in South Kensington and transported for use at MDDs centre in Milton Keynes.

In difference to previous protocol, the dogs will be presented with samples containing potentially live bacteria. The following SOP outlines requirements for transport, storage, handling and disposal of samples.

Transport

Gauze/broth culture mixture will be contained within 60ml plastic sterilin pots (figure 1) with a secured screw top cap. During transport, these pots will be placed within a second container, plastic or polystyrene, with secured lid to provide two layers of containment in accordance with requirements for transport of UN3373 Category B Biological Substances. All outer packaging will be labelled indication this category status (figure 2).



Figure 1: 60ml Sterilin pots with screw top lids



Figure 2: Example sticker for packaging

Storage

After production, samples will be stored at -80°C and during any transport, ice packs placed around the pots will keep samples chilled prior to freezing at -80°C on arrival at MDD centre. If samples are being transported immediately after production then they will be transported on ice prior to freezing at -80°C on arrival at MDD centre, unless being used immediately for training/testing. Frozen samples should be removed from the freezer 2 hours prior to any training/testing scenario

and allowed to warm to room temperature before use. Samples will be visibly defrosted and liquid before use.

After use, sample pots can be closed using the original lid and re-frozen at -80°C. If required again they should be defrosted in the same manner as previous.

Handling

Samples should be handled with caution so as not spill contents. Wear appropriate personal protective equipment (disposable glove, white coat or plastic apron) when opening, closing or handling open samples, to protect sample handler and to reduce risk of contamination of the sample. Gloves should be changed between handling different samples to avoid cross-contamination. The pots fit directly into the testing rig and will be covered with a barrier plate. Any visible spillage of the sample (if the rig is knocked or sample comes loose) should be cleaned using appropriate disinfectant (for example 1:100 dilution Verkon/Distel disinfectant). Spilt sample pots should be closed with the lid and disposed of as a below, do not continue to use for testing in case some has spilt onto the outside of the pot.

Disposal

When a sample has been used and no longer required, re-seal with the lid and place inside an autoclave bag. Autoclave according to local protocol/settings and dispose autoclave waste according local site collection arrangements.