THE ROLE OF RESPIRATORY VIRUSES IN
EXACERBATIONS OF CYSTIC FIBROSIS IN ADULTS

A thesis submitted to The University of Manchester for the degree of
Doctor of Philosophy
in the Faculty of Medical and Human Sciences.

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WILLIAM GEORGE FLIGHT

SCHOOL OF MEDICINE
## Contents

List of Tables .................................................................................................................. 8
List of Figures .................................................................................................................. 10
List of Abbreviations ....................................................................................................... 12
Abstract ............................................................................................................................ 14
Declaration ......................................................................................................................... 15
Copyright Statement ......................................................................................................... 16
Dedication .......................................................................................................................... 17
Acknowledgements ............................................................................................................ 18
The Author ......................................................................................................................... 19
Preface ............................................................................................................................... 20

1.0 Cystic Fibrosis – An Overview .................................................................................... 24
  1.1 Introduction ................................................................................................................. 24
  1.2 Genetics & Pathophysiology ..................................................................................... 24
  1.3 Clinical Features of CF – Pulmonary ......................................................................... 25
  1.4 Clinical Features of CF – Gastrointestinal & Nutritional ........................................ 25
  1.5 Clinical Features of CF – Metabolic .......................................................................... 26
  1.6 Clinical Features of CF – Others .............................................................................. 27
  1.7 Microbiology of CF Lung Disease ............................................................................. 27
    1.7.1 Pseudomonas aeruginosa .................................................................................... 28
    1.7.2 Burkholderia cepacia complex (BCC) ............................................................... 29
    1.7.3 Methicillin-Resistant Staphylococcus aureus (MRSA) ....................................... 29
    1.7.4 Non-Tuberculous Mycobacteria (NTM) ............................................................ 29
    1.7.5 Emerging Bacterial Pathogens .......................................................................... 30
    1.7.6 Fungi .................................................................................................................... 30
  1.8 The CF Lung Microbiome ........................................................................................ 30
    1.8.1 Methods Used to Characterise the Lung Microbiome ....................................... 31
    1.8.2 Limitations of Culture-Independent Microbiology ............................................ 32
  1.9 Diagnosis of Cystic Fibrosis ...................................................................................... 33
  1.10 Treatment of Cystic Fibrosis .................................................................................... 33
    1.10.1 Maintenance Pulmonary Therapy ..................................................................... 33
    1.10.2 Treatment of Pulmonary Exacerbations (PEx) ................................................ 34
    1.10.3 Treatment of Extra-Pulmonary Manifestations of CF ...................................... 35
    1.10.4 Novel Therapies – Correcting the Underlying CFTR Defect .............................. 35
2.0 Viral Respiratory Infections – An Overview ..................................................37
2.1 Introduction .................................................................................................37
2.2 Diagnosis of Viral Respiratory Infection ................................................ 37
2.3 Epidemiology of Viral Respiratory Infections ....................................... 38
2.4 Specific Viral Respiratory Infections .........................................................39
  2.4.1 Rhinovirus ...............................................................................................39
  2.4.2 Respiratory Syncytial Virus (RSV) ..........................................................40
  2.4.3 Parainfluenza Virus .................................................................................40
  2.4.4 Influenza ..................................................................................................41
  2.4.5 Coronavirus ............................................................................................42
  2.4.6 Adenovirus .............................................................................................43
  2.4.7 Metapneumovirus ..................................................................................43
  2.4.8 Bocavirus ...............................................................................................43
2.5 Clinical Syndromes Caused by Respiratory Viruses .............................44
  2.5.1 The Common Cold ..................................................................................44
  2.5.2 Influenza ..................................................................................................44
  2.5.3 Croup .......................................................................................................44
  2.5.4 Bronchiolitis ......................................................................................... 45
  2.5.5 Community Acquired Pneumonia (CAP) ..................................................45
  2.5.6 Novel Coronavirus-Associated Acute Respiratory Syndromes ..........46
2.6 The Effect of Respiratory Viruses in Chronic Lung Disease .................47
2.8 Treatment of Viral Respiratory Infections ............................................48
3.0 Respiratory Viruses in Cystic Fibrosis – A Literature Review ............51
3.1 Introduction .................................................................................................51
3.2 Diagnosis of Viral Respiratory Infections in Cystic Fibrosis ...............51
3.3 Incidence of Viral Respiratory Infections in Cystic Fibrosis ................51
3.4 Clinical Features of Viral Respiratory Infections in CF .........................53
3.5 Viral Respiratory Infections and Progression of CF Lung Disease .......54
3.6 Virus-Bacteria Interaction in CF .................................................................55
3.7 Prevention of Viral Respiratory Infections in Cystic Fibrosis ...............56
3.8 Treatment of Viral Respiratory Infections in CF ....................................57
4.0 Study Aims and Research Questions .......................................................61
4.1 Aims .............................................................................................................61
4.2 Primary Research Questions ..................................................................61
4.3 Secondary Research Questions ...............................................................61
5.0 Methods .....................................................................................................63
5.1 Study Overview ..........................................................................................63
5.2 Participants ................................................................................................63
7.0 The Clinical Impact of Viral Respiratory Infection in Adults with Cystic Fibrosis

7.1 Abstract .................................................................................................................. 104
7.2 Introduction .............................................................................................................. 106
7.3 Methods.................................................................................................................... 106
  7.3.1 Study Overview .................................................................................................. 106
  7.3.2 Virological Analysis .......................................................................................... 107
  7.3.3 Clinical Outcomes .............................................................................................. 107
  7.3.4 Statistical Analysis ............................................................................................ 108
7.4 Results....................................................................................................................... 108
  7.4.1 Overview of Study Findings Reported in Chapter 6 ......................................... 108
  7.4.2 Acute Changes in FEV\textsubscript{1} in Relation to Virology Result .................... 109
  7.4.3 VRI and Failure of Intravenous Antibiotic Therapy ........................................... 109
  7.4.4 Impact of VRI and PEx on Long-term Decline in FEV\textsubscript{1} ............................. 109
  7.4.5 Effect of VRI on Inflammatory Markers ........................................................... 111
  7.4.6 Symptoms of VRI ............................................................................................. 111
  7.4.7 The Clinical Impact of Different Respiratory Viruses ....................................... 114
  7.4.8 Effect of PCR Cycle Threshold (CT) on Clinical Parameters ............................. 115
  7.4.9 Diagnostic Value of URTI Symptom Score ....................................................... 115
7.5 Discussion ................................................................................................................. 118

8.0 The Effect of Climate and Season on Viral Respiratory Infections and Pulmonary Exacerbations in Adults with Cystic Fibrosis

8.1 Abstract .................................................................................................................... 123
8.2 Introduction .............................................................................................................. 131
8.3 Methods.................................................................................................................... 126
  8.3.1 Study Design ..................................................................................................... 126
  8.3.2 Virological Analysis ......................................................................................... 127
  8.3.4 Meteorological Data ....................................................................................... 127
  8.3.5 Statistical Analysis ......................................................................................... 127
8.4 Results ....................................................................................................................... 128
  8.4.1 Patient Demographics ..................................................................................... 128
  8.4.2 Climate Data ................................................................................................... 129
  8.4.3 Seasonality of Viral Respiratory Infection ...................................................... 129
  8.4.4 Seasonality of PEx and Antibiotic Prescription ............................................ 131
  8.4.5 Climate and Viral Respiratory Infection ......................................................... 133
  8.4.6 Climate Variables and Incidence of Pulmonary Exacerbation ...................... 134
8.5 Discussion ................................................................................................................. 136
### 9.0 The Spectrum of Rhinovirus Species Affecting Adults with Cystic Fibrosis

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1 Abstract</td>
<td>142</td>
</tr>
<tr>
<td>9.2 Introduction</td>
<td>143</td>
</tr>
<tr>
<td>9.3 Methods</td>
<td>144</td>
</tr>
<tr>
<td>9.3.1 Patient Selection</td>
<td>144</td>
</tr>
<tr>
<td>9.3.2 Rhinovirus Sequencing</td>
<td>144</td>
</tr>
<tr>
<td>9.3.3 Correlation of Sequencing Results with Clinical Data</td>
<td>145</td>
</tr>
<tr>
<td>9.3.4 Statistical Analysis</td>
<td>146</td>
</tr>
<tr>
<td>9.4 Results</td>
<td>146</td>
</tr>
<tr>
<td>9.4.1 Patient and Sample Selection</td>
<td>146</td>
</tr>
<tr>
<td>9.4.2 Rhinovirus Sequencing Results</td>
<td>147</td>
</tr>
<tr>
<td>9.4.3 Evidence of the Potential for Chronic Rhinovirus Carriage in CF</td>
<td>147</td>
</tr>
<tr>
<td>9.4.4 Clinical Effects of Different Rhinovirus Species</td>
<td>151</td>
</tr>
<tr>
<td>9.5 Discussion</td>
<td>152</td>
</tr>
</tbody>
</table>

### 10.0 Development of Ribosomal Intergenic Spacer Analysis (RISA) as a Tool to Profile the Bacterial Diversity of Cystic Fibrosis Sputum

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1 Abstract</td>
<td>156</td>
</tr>
<tr>
<td>10.2 Introduction</td>
<td>158</td>
</tr>
<tr>
<td>10.3 Methods</td>
<td>159</td>
</tr>
<tr>
<td>10.3.1 Clinical Samples</td>
<td>159</td>
</tr>
<tr>
<td>10.3.2 Conventional Sputum Culture</td>
<td>159</td>
</tr>
<tr>
<td>10.3.3 Nucleic Acid Extraction</td>
<td>159</td>
</tr>
<tr>
<td>10.3.4 Ribosomal Intergenic Spacer Analysis (RISA)</td>
<td>160</td>
</tr>
<tr>
<td>10.3.5 16S rRNA Gene Pyrosequencing</td>
<td>160</td>
</tr>
<tr>
<td>10.3.6 Statistical Analysis</td>
<td>161</td>
</tr>
<tr>
<td>10.4 Results</td>
<td>161</td>
</tr>
<tr>
<td>10.4.1 Patient Demographics</td>
<td>161</td>
</tr>
<tr>
<td>10.4.2 RISA Analysis</td>
<td>162</td>
</tr>
<tr>
<td>10.4.3 Application of RISA to Pure Cultures of CF Pathogens</td>
<td>164</td>
</tr>
<tr>
<td>10.4.4 Reproducibility of RISA</td>
<td>166</td>
</tr>
<tr>
<td>10.4.5 Longitudinal Changes in RISA Profiles</td>
<td>166</td>
</tr>
<tr>
<td>10.4.6 Comparison of RISA with 16S rRNA Gene Pyrosequencing &amp; Standard Culture</td>
<td>167</td>
</tr>
<tr>
<td>10.4.7 Predictors of Bacterial Sputum Diversity</td>
<td>172</td>
</tr>
<tr>
<td>10.5 Discussion</td>
<td>173</td>
</tr>
</tbody>
</table>

### 11.0 The Impact of Viral Respiratory Infection on the Respiratory Microbiome of Adults with Cystic Fibrosis

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.1 Abstract</td>
<td>178</td>
</tr>
<tr>
<td>11.2 Introduction</td>
<td>180</td>
</tr>
<tr>
<td>11.3 Methods</td>
<td>181</td>
</tr>
<tr>
<td>11.3.1 Study Overview</td>
<td>181</td>
</tr>
</tbody>
</table>
11.3.2 Patient Selection ................................................................. 181
11.3.3 Classification of Study Visits ............................................... 181
11.3.4 Virological Analysis ............................................................ 182
11.3.5 Conventional Sputum Culture ............................................. 182
11.3.6 16S rRNA Gene Pyrosequencing ........................................ 182
11.3.7 Ribosomal Intergenic Spacer Analysis (RISA) ....................... 183
11.3.8 Statistical Analysis .............................................................. 183

11.4 Results .................................................................................. 184
11.4.1 Patient Characteristics ......................................................... 184
11.4.2 Study Visits ........................................................................ 184
11.4.3 Conventional Sputum Culture Results ................................. 185
11.4.4 16S rRNA Gene Pyrosequencing Results ............................ 186
11.4.5 Ribosomal Intergenic Spacer Analysis (RISA) Results .......... 189

11.5 Discussion ............................................................................. 190

12.0 Summary and Discussion ....................................................... 195

13.0 Appendix 1. Presence of Individual Symptom Score Components by Virus Type ................................................. 203

14.0 Appendix 2. Ribosomal Intergenic Spacer Analysis (RISA) Profiles of Selected Clinical Samples in Comparison with Pure Cultures of Reference Strains ................................................................. 205

15.0 Appendix 3. Sequential 16S rRNA Pyrosequencing Results for Individual Study Participants ......................................................... 207

16.0 Appendix 4. Sequential Ribosomal Intergenic Spacer Analysis (RISA) Profiles for Individual Study Participants ......................................................... 211

17.0 References ............................................................................. 215

Word Count: 47,634 words (excluding references)
<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Incidence of viral respiratory infections in CF – studies using</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>conventional virological techniques</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Incidence of viral respiratory infections in CF – studies using</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>polymerase chain reaction (PCR) techniques</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Primers and probes for respiratory virus polymerase chain</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>reaction assays</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Primers used in rhinovirus sequencing assays</td>
<td>70</td>
</tr>
<tr>
<td>6.1</td>
<td>Baseline demographics of study participants.</td>
<td>93</td>
</tr>
<tr>
<td>6.2</td>
<td>Combinations of viruses seen in dual viral infection</td>
<td>94</td>
</tr>
<tr>
<td>6.3</td>
<td>Incidence of viral respiratory infection in adults with CF</td>
<td>95</td>
</tr>
<tr>
<td>6.4</td>
<td>Demographic risk factors for viral respiratory infection</td>
<td>95</td>
</tr>
<tr>
<td>6.5</td>
<td>The effect of PCR cycle threshold on pulmonary</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>exacerbation status &amp; need for antibiotic therapy</td>
<td></td>
</tr>
<tr>
<td>6.6</td>
<td>Percentage of cases of each virus detected by sputum, nose-</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>and throat swabs</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>Risk factors for decline in FEV$_1$ over twelve months using a</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>multiple regression model</td>
<td></td>
</tr>
<tr>
<td>7.2</td>
<td>Inflammatory marker levels according to virology result</td>
<td>111</td>
</tr>
<tr>
<td>7.3</td>
<td>Odds of individual symptoms being present at virus-positive</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>compared with virus-negative visits</td>
<td></td>
</tr>
<tr>
<td>7.4</td>
<td>Clinical outcomes for rhinovirus-positive visits in</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>comparison with visits positive for other viruses</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>Effect of polymerase chain reaction cycle threshold (CT)</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>value on clinical outcomes amongst all virus-positive visits</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>Clinical outcomes of virus-positive study visits when</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>classified by positivity of sputum samples</td>
<td></td>
</tr>
<tr>
<td>7.7</td>
<td>Diagnostic value of the Johnston URTI score thresholds</td>
<td>117</td>
</tr>
<tr>
<td>8.1</td>
<td>Baseline demographics of patients in the climate study</td>
<td>128</td>
</tr>
<tr>
<td>8.2</td>
<td>Severity of pulmonary exacerbations by season of onset</td>
<td>133</td>
</tr>
<tr>
<td>8.3</td>
<td>The effect of ambient temperature and relative humidity on the</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td>incidence of viral respiratory infection (VRI) using a</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>8.4</td>
<td>The effect of ambient temperature and relative humidity on incidence of pulmonary exacerbation</td>
<td>136</td>
</tr>
<tr>
<td>9.1</td>
<td>Baseline demographics of patients providing rhinovirus samples for sequencing</td>
<td>146</td>
</tr>
<tr>
<td>9.2</td>
<td>Serial rhinovirus PCR results and clinical outcomes in a single patient with CF and chronic rhinovirus infection</td>
<td>149</td>
</tr>
<tr>
<td>9.3</td>
<td>Comparison of clinical outcomes between rhinovirus A and B infection</td>
<td>151</td>
</tr>
<tr>
<td>10.1</td>
<td>Baseline demographics of study participants</td>
<td>162</td>
</tr>
<tr>
<td>10.2</td>
<td>Demographic characteristics of patients by RISA sub-group</td>
<td>169</td>
</tr>
<tr>
<td>10.3</td>
<td>Comparison of measures of bacterial community diversity and composition by RISA sub-group</td>
<td>171</td>
</tr>
<tr>
<td>10.4</td>
<td>Relationship between demographic variables and sputum bacterial diversity assessed using multiple linear regression</td>
<td>172</td>
</tr>
<tr>
<td>11.1</td>
<td>Baseline characteristics of study participants</td>
<td>184</td>
</tr>
<tr>
<td>11.2</td>
<td>Breakdown of viruses identified during the study</td>
<td>185</td>
</tr>
<tr>
<td>11.3</td>
<td>Clinical characteristics of study visits by virological and exacerbation status</td>
<td>185</td>
</tr>
<tr>
<td>11.4</td>
<td>Comparison of bacterial diversity indices by exacerbation and virology visit status</td>
<td>187</td>
</tr>
</tbody>
</table>
**List of Figures**

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Legend</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Overview of the rhinovirus sequencing method</td>
<td>75</td>
</tr>
<tr>
<td>5.2</td>
<td>Overview of 16S rRNA gene pyrosequencing technique using the 454 FLX+ Genome Sequencer (Roche, Basel, Switzerland)</td>
<td>79</td>
</tr>
<tr>
<td>5.3</td>
<td>Overview of post-16S rRNA gene pyrosequencing bioinformatic analysis</td>
<td>80</td>
</tr>
<tr>
<td>6.1</td>
<td>Breakdown of all viruses identified during the study</td>
<td>94</td>
</tr>
<tr>
<td>6.2</td>
<td>Sensitivity of different combinations of sputum, nose- &amp; throat-swabs for the diagnosis of viral respiratory infection in adults with CF</td>
<td>98</td>
</tr>
<tr>
<td>6.3</td>
<td>Breakdown of viruses identified by each sample type at study visits where only one specimen was positive</td>
<td>98</td>
</tr>
<tr>
<td>7.1</td>
<td>Relative change in FEV₁ in relation to A) number of virus-positive visits and B) number of pulmonary exacerbations during study follow-up</td>
<td>110</td>
</tr>
<tr>
<td>7.2</td>
<td>Presence of upper respiratory tract infection symptom by virology result</td>
<td>113</td>
</tr>
<tr>
<td>7.3</td>
<td>Presence of pulmonary exacerbation symptoms by virology result</td>
<td>113</td>
</tr>
<tr>
<td>7.4</td>
<td>Receiver operating characteristic (ROC) curve for diagnosis of viral respiratory infection using the Johnston URTI score</td>
<td>117</td>
</tr>
<tr>
<td>8.1</td>
<td>Mean temperature and relative humidity during the study</td>
<td>130</td>
</tr>
<tr>
<td>8.2</td>
<td>Breakdown of viruses identified during the study</td>
<td>130</td>
</tr>
<tr>
<td>8.3</td>
<td>Monthly incidence of viral respiratory infection</td>
<td>131</td>
</tr>
<tr>
<td>8.4</td>
<td>Incidence of pulmonary exacerbation (PEx) and viral respiratory infection (VRI) by calendar month</td>
<td>132</td>
</tr>
<tr>
<td>8.5</td>
<td>Rate of antibiotic prescription by calendar month</td>
<td>132</td>
</tr>
<tr>
<td>9.1</td>
<td>Phylogenetic tree showing rhinoviruses detected in clinical samples from the Manchester CF Virology Study and reference rhinovirus strains reported in Lee <em>et al</em></td>
<td>148</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>Expanded phylogenetic tree to illustrate the persistence of a near-identical strain of rhinovirus A for over two years in an adult with CF</td>
<td></td>
</tr>
<tr>
<td>10.1</td>
<td>Ribosomal intergenic spacer analysis (RISA) profiles of the initial sputum samples provided by 93 study participants</td>
<td></td>
</tr>
<tr>
<td>10.2</td>
<td>RISA profiles of all sputum samples culture-positive for <em>Pseudomonas aeruginosa</em> in relation to profiles from pure cultures of three <em>P. aeruginosa</em> strains</td>
<td></td>
</tr>
<tr>
<td>10.3</td>
<td>Within-sample reproducibility of RISA when repeated on three separate occasions</td>
<td></td>
</tr>
<tr>
<td>10.4</td>
<td>Sequential RISA profiles from three patients known to be chronically infected with <em>Pseudomonas aeruginosa</em></td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>Comparison of sputum RISA profiles with conventional culture and 16S rRNA pyrosequencing</td>
<td></td>
</tr>
<tr>
<td>10.6</td>
<td>Effect of FEV₁ and age on sputum bacterial diversity</td>
<td></td>
</tr>
<tr>
<td>11.1</td>
<td>Sputum bacterial diversity by visit status as measured by A) Shannon Index and B) richness (i.e. total number of genera identified)</td>
<td></td>
</tr>
<tr>
<td>11.2</td>
<td>Longitudinal changes in the sputum 16S rRNA gene pyrosequencing results from four representative patients with CF</td>
<td></td>
</tr>
<tr>
<td>11.3</td>
<td>Multi-dimensional scaling plots to show the degree of similarity of bacterial populations detected by 16S rRNA gene pyrosequencing for sputum samples clustered by A) individual patients and B) visit status</td>
<td></td>
</tr>
<tr>
<td>11.4</td>
<td>Bacterial diversity within sequential sputum samples as detected by ribosomal intergenic spacer analysis (RISA)</td>
<td></td>
</tr>
</tbody>
</table>
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPA</td>
<td>Allergic bronchopulmonary aspergillosis</td>
</tr>
<tr>
<td>ACT</td>
<td>Airway clearance technique</td>
</tr>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCC</td>
<td><em>Burkholderia cepacia</em> complex</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone-mineral density</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAP</td>
<td>Community acquired pneumonia</td>
</tr>
<tr>
<td>CBAVD</td>
<td>Congenital bilateral absence of vas deferens</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFRD</td>
<td>Cystic fibrosis-related diabetes mellitus</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CMUH</td>
<td>Central Manchester University Hospitals NHS Foundation Trust</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold (of a polymerase chain reaction assay)</td>
</tr>
<tr>
<td>DIOS</td>
<td>Distal intestinal obstruction syndrome</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalised estimating equations</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>IQR</td>
<td>Inter-quartile range</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LRTI</td>
<td>Lower respiratory tract infection</td>
</tr>
<tr>
<td>MACFC</td>
<td>Manchester Adult Cystic Fibrosis Centre</td>
</tr>
<tr>
<td>MERS</td>
<td>Middle East Respiratory Syndrome</td>
</tr>
<tr>
<td>MPV</td>
<td>Metapneumovirus</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequence based amplification</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information (USA)</td>
</tr>
<tr>
<td>NHS</td>
<td>National Health Service</td>
</tr>
<tr>
<td>NTM</td>
<td>Non-tuberculous mycobacteria</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEx</td>
<td>Pulmonary exacerbation of cystic fibrosis</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>RISA</td>
<td>Ribosomal intergenic spacer analysis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Terminal restriction fragment length polymorphism profiling</td>
</tr>
<tr>
<td>UHSM</td>
<td>University Hospital of South Manchester NHS Foundation Trust</td>
</tr>
<tr>
<td>URTI</td>
<td>Upper respiratory tract infection</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region (relating to viral RNA)</td>
</tr>
<tr>
<td>VRI</td>
<td>Viral respiratory infection</td>
</tr>
</tbody>
</table>
Abstract
Viral respiratory infections (VRI) are common in children with cystic fibrosis (CF) and are associated with significant clinical deterioration. Little previous research has been conducted on VRI in adults with CF. This thesis describes a prospective study to determine the epidemiology and clinical impact of VRI among 100 adults with CF.

The incidence of identifiable VRI was 1.66 cases/patient-year. Rhinovirus accounted for 72.5% of viruses. Identifiable VRI was associated with increased risk of pulmonary exacerbation, increased respiratory symptoms and higher C-reactive protein levels.

Changes in the climate and seasons affected the incidence of identifiable VRI. Rhinovirus was most common in autumn and other viruses predominated during winter. Warmer ambient temperatures were associated with increased risk of rhinovirus infection while other viruses were more common in colder temperatures.

Genetic sequencing of a subset of 42 rhinoviruses identified during the study showed that rhinovirus A accounted for 69% of cases and was associated with more severe respiratory symptoms and higher C-reactive protein levels than rhinovirus B.

The impact of identifiable VRI on changes to bacterial communities within the lungs of patients with CF was investigated. Ribosomal intergenic spacer analysis (RISA) was developed as a tool to profile the bacterial diversity of CF sputum and was compared with standard culture and 16S rRNA gene pyrosequencing. No consistent effect of identifiable VRI on the microbial diversity of CF sputum was detected with any of these methods in longitudinal analysis of a subset of 18 patients.
Declaration

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In Memory of Chris Parker
1949 – 2013
Acknowledgements

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I am very grateful to Julie Morris for her expert advice on statistical analysis. Helen Flight’s immense knowledge of clinical research methodology and NHS ethics committee bureaucracy has been invaluable. She is also a wonderful wife and mother.

The research costs of the study were paid for by the Manchester Adult CF Centre’s charitable fund for which I am immensely grateful. I would also like to extend my thanks to the patients of the Manchester Adult CF Centre for agreeing to take part in the study and suffering the intrusion of quite so many nose and throat swabs.

Finally, I would like to thank and pay tribute to Christine Parker who sadly died shortly before submission of the final version of this thesis. Chris was a source of great support over the fifteen years I knew her and she provided invaluable advice on study design, statistics and preparation of this thesis. She will be missed hugely.
The Author

Dr Flight obtained his MBChB degree from the University of Sheffield in 2002. Following his pre-registration house officer year in Stoke-on-Trent he trained in general medicine in New Zealand, Lancaster and Manchester, obtaining the MRCP(UK) diploma in 2006. After further senior house officer posts in oncology and critical care he began specialist training in respiratory medicine in the North Western deanery in 2007. Between 2010 and 2013 he worked as clinical fellow to the Manchester Adult Cystic Fibrosis Centre during which time he conducted the research project leading to this thesis.
Preface
The main aim of the research project leading to this thesis was to determine the incidence and clinical impact of viral respiratory infection (VRI) among adults with cystic fibrosis (CF). The thesis also encompasses the epidemiology of VRI; the effect of the climate on viral infections; the genetic diversity of rhinoviruses affecting patients with CF and the impact of VRI on bacterial communities within the CF lung.

The thesis begins with an introduction to CF itself to set the context for the research, with an emphasis on the microbiology of CF lung disease. Chapter Two then discusses our current understanding of respiratory viruses and their relevance to other respiratory conditions such as asthma and chronic obstructive pulmonary disease. The final introductory chapter represents a review of the literature regarding previous studies on the role of VRI in patients with CF.

Chapter Four presents the aims of the overall study and the research questions it was designed to answer. The detailed methods of the study are reported in Chapter Five.

The results of the thesis are presented as a series of self-contained research papers in the style of journal articles. Each of the papers covers a distinct but related topic. There is inevitably some overlap with regard to the introduction and methods of each paper but this has been kept to a minimum wherever possible.

Chapter Six, the first results paper, documents the incidence of VRI and shows a clear association between VRI and pulmonary exacerbations (PEx) of CF. Chapter Seven centres on the effect of VRI on symptoms, lung function and other clinical parameters. Chapter Eight marries the virological data from the study with meteorological variables to examine the effect of the climate and seasons on the risk of VRI among adults with CF.

The fourth results paper, Chapter Nine, describes the genetic sequencing of a sub-set of rhinoviruses, the most common viral pathogens encountered in the study, to determine if particular sub-types were associated with more severe clinical outcomes. The final two chapters examine bacterial communities detected in the sputa of patients with CF. Firstly, in Chapter Ten, a technique known as ribosomal intergenic spacer
analysis (RISA) was evaluated as a novel means of profiling the diversity bacteria within the lungs of CF patients. In Chapter Eleven this method was then used alongside conventional culture and 16S rRNA gene pyrosequencing to investigate whether VRI led to changes in the bacteria within the CF lung.

Finally, a summary chapter reviews the key findings of the project and addresses both the strengths and weaknesses of the wider study. A number of key areas for future research are highlighted.

This research project has led to several publications, conference presentations and awards as listed below.

**Publications Arising from this Work**

Prizes and Awards for this Work

- North West Thoracic Society Specialist Registrar Abstract Prize, October 2012
- Semi-Finalist, Junior Investigator Competition, North American Cystic Fibrosis Conference, Orlando, October 2012
- Travel Award, European Cystic Fibrosis Society Conference, Lisbon, June 2013
Chapter One

Cystic Fibrosis – An Overview
1.0 Cystic Fibrosis – An Overview

1.1 Introduction

Cystic fibrosis (CF) is an autosomal recessive condition that leads to chronic endobronchial infection, respiratory failure and premature death.\(^1\) CF affects approximately 1 in 2500 live births in Caucasian populations making it the most common life-threatening inherited condition in the UK.\(^2\) Survival has steadily improved over the last three decades as a result of advances in supportive care and a specialist multi-disciplinary approach to patient management.\(^3\) It is estimated that babies with CF born after the year 2000 are likely to live into their 50s and beyond which makes a striking comparison with the median age of death of 16 years only 30 years ago.\(^4,5\) The introduction of newborn screening for CF and the promise of targeted therapies to correct the underlying pathophysiological defects in CF raise the hope of further improvements in survival for the future.\(^6-9\)

1.2 Genetics & Pathophysiology

CF is a genetic condition with an autosomal recessive mode of inheritance.\(^10\) The affected gene is located on the long arm of chromosome 7 and was first identified in 1989.\(^11-13\) The CF gene codes for a membrane protein named cystic fibrosis transmembrane conductance regulator (CFTR), the primary role of which is to transport chloride ions across the cell membrane.\(^14\)

More than 1800 different CFTR mutations have been identified.\(^14\) CFTR mutations are categorised into six groups: Class I – failed synthesis of CFTR in the nucleus; Class II – impaired maturation of CFTR protein; Class III – defective opening of the CFTR channel; Class IV – impaired chloride transport through the CFTR protein; Class V – reduced production of CFTR and Class VI – accelerated turnover at the cell surface.\(^15\) In the global CF population, the most common mutation is termed p.Phe508del (formerly F508del), a class II mutation involving a deletion of phenylalanine at position 508. The F508del mutation is found in 74.1% of UK CF patients, with 57.5% being F508del homozygous.\(^16\) Non-Caucasian patients have a lower prevalence of F508del. CFTR mutation status alone does not determine the clinical phenotype of individual patients with CF, however, and it is believed that
numerous other “modifier” genes and environmental factors interact with CFTR to determine disease severity.\textsuperscript{17}

In the healthy state, CFTR functions as an ion channel and allows the transfer of chloride and bicarbonate across the cell membrane.\textsuperscript{15} CFTR is expressed in many bodily organs including the lungs, liver, pancreas, gastrointestinal tract, reproductive system and sweat glands.\textsuperscript{15} This distribution corresponds to the typical clinical features of CF. The precise pathophysiology of CF has still not been fully elucidated, however. Proposed mechanisms of CF-related lung disease include: the “low-volume hypothesis” whereby impaired CFTR function leads to loss of sodium and water from the airway surface liquid;\textsuperscript{18} the contrasting “high-salt hypothesis” suggesting that excess sodium in airway surface liquid leads to impaired antibacterial defences\textsuperscript{19} and the “inflammation first” hypothesis suggesting that an underlying pro-inflammatory state sets up a cycle of infection and inflammation.\textsuperscript{20}

1.3 Clinical Features of CF – Pulmonary
CF typically leads to chronic endobronchial infection, bronchiectasis and colonisation with pathogenic microorganisms.\textsuperscript{14} Common symptoms include a productive cough, breathlessness, haemoptysis and pleuritic chest pain. Unfortunately, respiratory failure continues to be the ultimate cause of premature death in over 80\% of patients with CF. The usual pattern of CF lung disease reflects a progressive decline in lung function punctuated by intermittent exacerbations.\textsuperscript{14} There is no universally accepted definition of a pulmonary exacerbation (PEx) in CF but usual features include malaise, increased sputum production, anorexia, weight loss and a fall in lung function.\textsuperscript{21} PEx are increasingly recognised as harmful events and a direct link has been observed between exacerbations and impaired quality of life, accelerated lung function decline and increased mortality.\textsuperscript{22-24} In a large study using data from the US Cystic Fibrosis Foundation Patient Registry, pulmonary exacerbations were found to be more common during the winter months.\textsuperscript{25}

1.4 Clinical Features of CF – Gastrointestinal & Nutritional
CF was first described as a pancreatic disorder leading to malnutrition in infants.\textsuperscript{26} Exocrine pancreatic insufficiency affects up to 90\% of CF patients and presents with
steatorrhoea, failure to thrive or fat-soluble vitamin deficiencies.\textsuperscript{14} Pancreatitis most commonly affects those with preserved pancreatic function.\textsuperscript{27} Further gastrointestinal manifestations of CF include: liver cirrhosis and portal hypertension due to fatty infiltration of the bile ducts;\textsuperscript{28} meconium ileus, where the bowel is obstructed by tenacious meconium shortly after birth;\textsuperscript{29} distal intestinal obstruction syndrome (DIOS), effectively the adult equivalent of meconium ileus\textsuperscript{30} and an increased risk of gastrointestinal malignancy.\textsuperscript{31-33} Low body-mass index (BMI) as a consequence of malabsorption is common in CF and is associated with poor outcomes.\textsuperscript{34} High-calorie diets are routinely recommended alongside pancreatic enzyme replacement therapy for those with exocrine pancreatic insufficiency.\textsuperscript{35} Additional strategies for the optimisation of body weight include dietary supplements and enteral feeding through nasogastric or gastrostomy tubes.\textsuperscript{35,36}

1.5 Clinical Features of CF – Metabolic

CF-related diabetes mellitus (CFRD) affects an increasing proportion of CF patients as they age.\textsuperscript{1} Over a quarter of those over 25 years of age have CFRD and a further proportion have impaired glucose tolerance which often becomes clinically apparent during a pulmonary exacerbation.\textsuperscript{1} CFRD has features of both type I and type II diabetes mellitus but is distinct from both.\textsuperscript{37,38} CFRD is associated with decreased survival and deteriorating lung function, especially in young women.\textsuperscript{39} Annual oral glucose tolerance testing to screen for CFRD is recommended for all CF patients over the age of ten years.\textsuperscript{40} Guidelines on the management of CFRD have been published and centre on the use of insulin to control hyperglycaemia.\textsuperscript{38,40,41} Conventional wisdom suggests that CFRD patients are predominantly at risk of microvascular, but not macrovascular, complications of diabetes and yearly screening for diabetic retinopathy is recommended.\textsuperscript{42} As average life expectancy increases for patients with CF, however, it is likely that the macrovascular consequences of diabetes will become a more significant clinical problem.

Low bone mineral density (BMD) is common in patients with CF and is thought to be largely due to vitamin D deficiency and the effects of chronic inflammation.\textsuperscript{43} Low BMD becomes more prevalent with increased age and leads to an increased risk of bone fractures.\textsuperscript{44} A bone densitometry scan every 1 to 3 years and annual
measurement of vitamin D levels is recommended for adults with CF. Treatment options for low BMD include calcium/vitamin D supplementation and bisphosphonate therapy.

### 1.6 Clinical Features of CF – Others

Although much of the morbidity from CF relates to its pulmonary and gastrointestinal manifestations, it is a truly multi-system disorder. Cutaneous vasculitis and a CF-associated arthropathy are well recognised. Renal complications of CF include chronic kidney disease, often as a consequence of aminoglycoside therapy, and an increased incidence of renal calculi. Psychological disorders such as depression affect more than 1 in 5 patients and sub-optimal adherence with therapy is common. Genitourinary effects of CF include urinary stress incontinence and male infertility due to congenital bilateral absence of the vas deferens (CBAVD).

### 1.7 Microbiology of CF Lung Disease

Chronic endobronchial infection is a cardinal feature of CF lung disease. Conventional culture-based microbiology has evolved to identify a number of so-called “typical CF pathogens” which are believed to have the greatest clinical significance. Using this approach, *Staphylococcus aureus* and *Haemophilus influenzae* are the predominant pathogens seen in children with CF. The prevalence of *Pseudomonas aeruginosa* infection increases with age to become the most prevalent pathogen in adults with CF. A diverse group of other opportunistic bacteria, mycobacteria and fungi may also be recovered from the respiratory tract of patients with CF. Acquisition of certain pathogens, particularly *P. aeruginosa* and *Burkholderia* species, is associated with substantial clinical deterioration and impaired longevity. Many pathogenic organisms cultured from CF patients, including *P. aeruginosa*, *Burkholderia* spp, Methicillin-Resistant *S. aureus* (MRSA) and *Pandorea* spp, are capable of transmission between individuals demonstrating the need for rigorous infection control procedures within CF units. Identification of these organisms remains vitally important in CF care and the key pathogens are discussed in detail below. Increasingly, however, culture-independent analysis of CF respiratory samples has identified that the microbial communities within the CF lung are much more complex than is suggested by standard culture alone. The rapidly
evolving field of the CF lung microbiome is considered in Section 1.8. Viruses are discussed separately in Chapters 2 and 3.

1.7.1 *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is an aerobic, flagellate Gram-negative bacillus. Environmental sources of *P. aeruginosa* include soil, water and sanitary systems in hospitals. Data from the USA and Australia suggest that warmer ambient temperatures lead to higher rates of *P. aeruginosa* carriage amongst the CF population. In CF, more than 80% of adults over the age of 25 years are infected with *P. aeruginosa*. On initial infection, *P. aeruginosa* displays a non-mucoid phenotype which is often amenable to eradication with antibiotics. Once established, *P. aeruginosa* colonies form a biofilm within the airway through production of alginate and other chemicals. This is termed the mucoid phenotype and, once established, is regarded as impossible to eradicate. Alongside these phenotypic changes, repeated courses of antimicrobials lead to mutations conveying antibiotic resistance.

Numerous studies have shown that acquisition of *P. aeruginosa* in CF is associated with increased morbidity, deterioration of lung function and reduced survival. This has led to great interest in early eradication of *P. aeruginosa* in the hope of delaying established infection. Many different antibiotic regimens for *P. aeruginosa* eradication are effective but there is no agreed “gold-standard.” Common strategies include the use of oral ciprofloxacin alongside nebulised tobramycin or colistin. There is a pressing need for head-to-head comparisons of different eradication strategies to optimise current clinical practice.

Studies have clearly demonstrated that certain strains of *P. aeruginosa* are capable of transmission between individuals with CF. The identification of such cross-infection has led to the segregation of patients within many CF units and CF-specific infection control guidelines have been published in recent years. Patient segregation, infection control measures and early eradication of *P. aeruginosa* have led to lower levels of *P. aeruginosa* infection in individual CF units. Epidemic strains of *P. aeruginosa* appear to be associated with increased morbidity and
treatment requirements but are not associated with reduced survival when compared with sporadic strains.\textsuperscript{81-83}

1.7.2 \textit{Burkholderia cepacia complex (BCC)}

\textit{Burkholderia cepacia} complex, formerly known as \textit{Pseudomonas cepacia}, refers to a group of at least 17 related species of Gram-negative bacilli.\textsuperscript{52,84} \textit{Burkholderia} species are very common in the general environment and were first described as plant pathogens.\textsuperscript{85} \textit{BCC} is only rarely of medical importance in the non-CF population. \textit{Burkholderia} species colonise fewer than 4\% of CF patients\textsuperscript{70} but they are frequently associated with poor outcomes.\textsuperscript{86,87} The “cepacia syndrome” is the most dramatic manifestation of BCC infection and refers to a rapidly progressive pneumonitis associated with a \textit{Burkholderia} bacteraemia.\textsuperscript{88} Treatment of BCC infection is hindered by its inherently extensive antibiotic resistance profile.\textsuperscript{14} \textit{Burkholderia cenocepacia} has a particularly adverse prognosis and was responsible for a number of devastating outbreaks within CF units in the last century.\textsuperscript{87} The advent of patient segregation and rigorous infection control measures has led to a dramatic fall in cases of epidemic \textit{B. cenocepacia} in the UK CF population.\textsuperscript{89}

1.7.3 \textit{Methicillin-Resistant Staphylococcus aureus (MRSA)}

MRSA is a major hospital-acquired pathogen in the non-CF population.\textsuperscript{90} Over 30\% of CF patients in North America are positive for MRSA although the prevalence among UK patients remains below 3\%.\textsuperscript{1,91} Infection with MRSA has been shown to be associated with a significant decline in lung function and an increased risk of death.\textsuperscript{92,93} Eradication of MRSA has been achieved in over 80\% of affected patients in one large CF centre\textsuperscript{94} although the long-term clinical benefits of such a strategy remain unproven.

1.7.4 \textit{Non-Tuberculous Mycobacteria (NTM)}

The prevalence of chronic respiratory colonisation with NTM among CF patients in the UK is 3.9\%.\textsuperscript{91} \textit{M. avium} complex and \textit{M. abscessus} are the most common NTM species encountered in CF.\textsuperscript{1,95,96} Concern has been expressed in the scientific literature that the incidence of NTM infection in CF may be rising. Some investigators have hypothesised that the widespread use of prophylactic azithromycin
may be responsible for this through its inhibition of autophagy.\textsuperscript{97} A further recent finding related to NTM in CF has been the discovery that \textit{M. abscessus} is capable of transmission between patients.\textsuperscript{98} Further work on the scale of this problem in the UK is underway. Guidelines on the management of NTM infection in the general population have been published\textsuperscript{99,100} and a joint Cystic Fibrosis Foundation – European Cystic Fibrosis Society guideline on NTM in CF is currently in production. The clinical efficacy of anti-mycobacterial therapy for NTM infection in CF remains unclear.

1.7.5 Emerging Bacterial Pathogens
A range of other bacterial pathogens may infect the lungs of CF patients. Such organisms are typically Gram-negative bacilli and include \textit{Stenotrophomonas maltophilia}, \textit{Achromobacter xylosoxidans}, \textit{Ralstonia} species and \textit{Pandorea} species.\textsuperscript{52} The full clinical impact of these pathogens has yet to be established with certainty.

1.7.6 Fungi
\textit{Aspergillus} species are ubiquitous in the environment and are responsible for a spectrum of clinical diseases in CF. The most common clinical manifestation is allergic bronchopulmonary aspergillosis (ABPA). ABPA affects up to 15\% of CF patients\textsuperscript{101,102} and presents with wheeze, breathlessness, decline in lung function and new radiographic changes. Guidelines on the diagnosis of ABPA have been published\textsuperscript{101} and treatment centres on the use of corticosteroids and/or antifungals such as itraconazole. In the post-lung transplant setting, invasive aspergillosis is a potentially fatal complication.\textsuperscript{103} The role of other fungi in CF is less clear but sputum colonisation with \textit{Candida albicans} is associated with an increased risk of pulmonary exacerbations and an accelerated decline in lung function.\textsuperscript{104}

1.8 The CF Lung Microbiome
Standard microbiological practice has evolved over many decades to facilitate the growth \textit{in vitro} of specified organisms of interest using selective culture media. Standard culture may not provide a true representation of the microbial composition of the original sample since the species most adapted to survival in the chosen culture medium will prosper at the expense of others. One means of removing this bias is to
discard conventional culture all together. Over the last decade, culture-independent techniques have been developed to characterise entire microbial communities within clinical samples from sites such as the bowel, genitourinary system and respiratory tract. The term “microbiome” has been coined to describe the population of micro-organisms within a specific niche.

Culture-independent microbiological techniques rely on the identification of microbial nucleic acids within a sample and were first used to demonstrate the bacterial diversity of environmental samples such as soil and sea water. The most common culture-independent technique currently used in analysis of the respiratory microbiome is 16S ribosomal RNA gene pyrosequencing. 16S rRNA has an important role in the structure of the ribosome and is present in all bacterial species. The gene coding for 16S rRNA has sufficient similarity across species to capture virtually all bacteria whilst simultaneously being diverse enough to allow differentiation between species. Amplification of the 16S gene product using PCR allows a profile of all the bacteria within a sample to be generated based on the genetic sequences of individual nucleic acid fragments found within the specimen. 16S rRNA gene sequencing studies have demonstrated that the healthy human respiratory tract is not sterile but is colonised with bacterial flora. The respiratory microbiome has been found to be disrupted in asthma, chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis and healthy cigarette smokers.

1.8.1 Methods Used to Characterise the Lung Microbiome
The first application of culture-independent techniques in CF lung disease was reported in 2003. Rogers et al used Terminal Restriction Fragment Length Polymorphism (T-RFLP) profiling, a form of bacterial 16S rDNA sequencing, to analyse 14 sputum samples from five CF patients. This technique identified typical CF pathogens as well as a number of bands relating to other bacterial species not usually detected by standard culture. The authors subsequently refined the technique to include 16S rRNA sequencing which theoretically allows a distinction to be made between metabolically active and dead bacteria. Further important developments in the field were made by Tunney et al with their demonstration that
anaerobic bacteria such as *Veillonella* spp are highly prevalent in CF sputum and are largely undetected unless specific anaerobic cultures or molecular methods are used.\textsuperscript{120}

The CF microbiome is increasingly recognised as a highly variable entity, both between different patients and even within samples taken from the same individual.\textsuperscript{52} Willner *et al* took multiple samples from the lungs of two patients with CF, one obtained at post-mortem and the second explanted at transplantation.\textsuperscript{121} Microbial communities were found to differ significantly within a single patient suggesting that there are varying niches with their own ecology within the CF lung. Cox *et al* have also shown that the CF microbiome varies with age and lung function.\textsuperscript{122} In their cohort, increased age and a lower FEV\textsubscript{1} was linked with a marked reduction in bacterial diversity and richness. Similarly, Zhao *et al* found that the respiratory microbiome became significantly less diverse in three patients with progressive clinical deterioration while three stable patients maintained higher levels of bacterial diversity.\textsuperscript{123} Somewhat surprisingly, Tunney and colleagues found that sputum microbial diversity and abundance varies relatively little following treatment with antibiotics for a pulmonary exacerbation of CF.\textsuperscript{124} It is clear that the mechanisms behind changes in the CF microbiome require substantial further investigation before they are truly understood. The impact of respiratory viruses on bacterial communities within the lung has not been studied previously.

\textbf{1.8.2 Limitations of Culture-Independent Microbiology}

Although culture-independent bacteriology has made a number of important discoveries in respiratory medicine, these techniques do have a number of limitations. Sputum inevitably passes through the pharynx in the process of expectoration leading to the possibility of contamination by oral bacteria. Similarly, the bronchoscope must be passed through the upper airway in order to obtain bronchoalveolar lavage (BAL) samples. Notably, Charlson *et al* found that in healthy individuals the bacterial profile of the lung as sampled by BAL was indistinguishable from that of the upper respiratory tract.\textsuperscript{112} In the context of CF, Goddard *et al* performed bacterial 16S rRNA gene pyrosequencing of specimens taken directly from explanted CF lungs and compared them with results from oropharyngeal swabs and sputum collected
immediately prior to transplantation surgery.\textsuperscript{125} Samples from the explanted lungs were significantly less diverse than the other sample types suggesting possible contamination from the upper airway flora in these specimens. In contrast, Rogers \textit{et al} performed 16S rRNA sequencing on matched sputum and mouthwash samples from 19 patients with CF.\textsuperscript{126} The authors reported no evidence of significant contamination of sputum by bacteria in the oral cavity. The importance of upper airway contamination of lower respiratory samples remains controversial.

A second major limitation of molecular bacteriological techniques is that nucleic acids are amplified regardless of whether the organism that produced them is dead or alive. Emphasis on studying bacterial RNA helps reduce the impact of this as transcription requires the presence of viable bacteria.\textsuperscript{118} Allowing for these factors, culture-independent techniques present an exciting means of unravelling the microbial factors involved in CF lung disease.

1.9 Diagnosis of Cystic Fibrosis

The diagnosis of CF is made primarily on clinical grounds with supporting evidence obtained from sweat chloride measurements, genetic analysis, nasal/rectal potential difference studies or newborn screening trypsinogen tests. Guidelines on the diagnosis of CF in patients presenting with compatible symptoms are available.\textsuperscript{7,127,128} Newborn screening for CF is now routine practice throughout the UK and will lead to the earlier diagnosis of the condition in asymptomatic infants. It is to be hoped that the newborn screening program will allow improved management of CF in children and prevent the development of important complications of the disease.

1.10 Treatment of Cystic Fibrosis

1.10.1 Maintenance Pulmonary Therapy

A longstanding component of CF respiratory care has been the use of airway clearance techniques (ACT) to help expectorate sputum. Inherent methodological difficulties mean that there is little conclusive trial data demonstrating the effectiveness of ACT over no therapy.\textsuperscript{129} The UK CF Trust and the US CF Foundation recommend that ACT should be used by all CF patients but do not favour any particular method of chest clearance.\textsuperscript{130,131}
Several inhaled therapies are available to try and maintain lung health in CF.\textsuperscript{132} Nebulised recombinant human deoxyribonuclease and hypertonic saline both improve lung function and reduce the frequency of exacerbations.\textsuperscript{133,134} Regular nebulised antibiotics are used for the eradication of \textit{P. aeruginosa} or for suppression of respiratory infection in patients with chronic infection. Tobramycin has the best evidence base and has been shown to improve lung function, reduce the frequency of exacerbations and reduce the need for intravenous antibiotics in patients with chronic \textit{P. aeruginosa} infection.\textsuperscript{135-137} Aztreonam lysine is now available as an alternative nebulised antibiotic with equivalent efficacy to tobramycin in patients chronically infected with \textit{P. aeruginosa}.\textsuperscript{138,139} Many CF centres, especially in Europe, also have considerable experience with the use of nebulised colistin in this setting. In addition to these nebulised preparations, dry-powder formulations of tobramycin and colistin are now available for routine use in CF and appear to have non-inferiority with their nebulised counterparts.\textsuperscript{140-142}

Prophylactic antibiotics have long been used in CF. Maintenance azithromycin improves lung function and reduces the frequency of pulmonary exacerbations in both adults and children.\textsuperscript{143-147} Regular anti-staphylococcal antibiotics have been used extensively in the paediatric population but potential links with increased rates of \textit{P. aeruginosa} infection has led to the North American CF Foundation recommending against their use.\textsuperscript{132,148,149} This is an area of controversy, however, and the use of prophylactic flucloxacillin remains common in UK paediatric CF centres.

1.10.2 \hspace{1em} Treatment of Pulmonary Exacerbations (PEx)

The treatment of pulmonary exacerbations of CF requires a multi-faceted approach. Antibiotics are central to the treatment of PEx with mild cases requiring oral antibiotics and more severe cases needing intravenous therapy. When given intravenously, a combination of an aminoglycoside and a beta-lactam antibiotic is typically used.\textsuperscript{150} Additional aspects of the management of PEx may include bronchodilator therapy, oxygen administration, non-invasive ventilation, airway clearance techniques and correction of hyperglycaemia, haemoptysis or pneumothorax. Guidelines on the management of PEx and its complications have
been published.21,151 The lack of a standardised definition of PEx hinders a robust evidence-based approach to its management, however.

1.10.3 Treatment of Extra-Pulmonary Manifestations of CF
The treatment of the non-pulmonary features of CF is discussed in Sections 1.4 to 1.6.

1.10.4 Novel Therapies – Correcting the Underlying CFTR Defect
There has been considerable effort expended on the development of therapies to reverse the underlying cause of CF ever since the CFTR gene was discovered. Unfortunately, many of these prospective therapies have proven unsuccessful. Examples include denufosol, an epithelial sodium channel modifier,152 and ataluren which was designed to correct the CFTR defect in class I mutations associated with a premature stop codon.153 An alternative strategy which has been under investigation for many years relates to gene therapy.8 A phase IIB study of gene therapy for CF is currently in progress whereby patients will be treated with a nebulised liposomal preparation to deliver functional CFTR directly to the airways.154 Gene therapy for CF remains experimental and is some years away from possible translation into clinical practice.

Over the last two years, however, the first effective therapy to reverse the underlying CFTR defect causing CF has become available.155 Ivacaftor is a CFTR potentiator and is licensed for the treatment of CF patients carrying at least one copy of the G551D mutation.6 G551D is a class III “gating” mutation in which CFTR reaches the cell membrane but fails to open correctly to allow the passage of chloride ions. Ivacaftor acts to open the chloride channel and leads to a dramatic reduction in sweat chloride levels in patients with G551D taking the drug.6 Most importantly, ivacaftor is also associated with significant improvements in lung function, weight and quality of life in these patients.6,9,156 Unfortunately, G551D is found in only 5.6% of the UK CF population91 and ivacaftor has no tangible effect on CFTR function for patients with other classes of CFTR mutation such as F508del.157 Despite this, ivacaftor has shown beyond doubt that therapies correcting the root cause of CF have the potential to succeed and a number of other therapeutic compounds are currently under development.158-160
Chapter Two

Viral Respiratory Infections –
An Overview
2.0 Viral Respiratory Infections – An Overview

2.1 Introduction

The term “viral respiratory infection” (VRI) describes the group of clinical conditions caused by a spectrum of viruses infecting the respiratory tract. VRIs include the common cold, viral pharyngitis, conjunctivitis, bronchiolitis, croup and influenza. This group of conditions can be split further into upper respiratory tract infections (URTIs) such as the common cold and lower respiratory tract infections (LRTIs) such as influenza and bronchiolitis. Although broadly representative, this division is not always useful as influenza infection presents with varying degrees of severity and in some cases can be indistinguishable from the common cold. Asymptomatic VRI is well recognised with 35% of healthy children undergoing elective general anaesthesia proving positive for a respiratory virus in one recent study.

2.2 Diagnosis of Viral Respiratory Infection

Diagnosis of VRI has changed dramatically over the last two decades with the advent of molecular technologies. Conventional virology methods rely on cell culture, immunofluorescence or serological responses. All of these methods are limited by low sensitivity and long laboratory turn-around times which compromise their impact on the management of individual patients. There has been a recent resurgence of interest in antigen detection techniques such as immunofluorescence in the form of point-of-care testing kits. Such methods remain under investigation but raise the prospect of ultra-rapid viral diagnosis and may have a particularly important role in the setting of future viral pandemics.

Currently, real-time polymerase chain reaction (PCR) assays are the principal method used for the diagnosis of VRI allow rapid, same-day identification of viruses. PCR involves the extraction of nucleic acids from clinical samples and the amplification of target DNA within the specimen using the enzyme DNA polymerase, originally obtained from the heat-stable bacterium Thermophilus aquaticus. In comparison with conventional techniques, PCR has consistently demonstrated substantially higher levels of sensitivity. Kuypers et al, for instance, showed that a PCR panel for seven respiratory viruses had a sensitivity of over 99% when compared with fluorescent antibody assays. In a separate study, Templeton et al analysed 358
specimens for seven respiratory viruses and found that PCR detected all 67 culture-
positive samples as well as a further 20 cases which were negative by viral culture. The precise sensitivity and specificity of any given PCR assay depends on several factors including the viruses studied, the nucleic acid extraction technique, the PCR reagents and instruments and both the timing and quality of the specimen used. Samples collected some days after the onset of a respiratory illness, when viral shedding is likely to have fallen considerably, may have lower detection rates, for example. It must also be noted that although the sensitivity of viral culture is lower than PCR, culture retains a vital role in the investigation of novel viruses such as pandemic influenza strains.

Each of the virological techniques described above may be applied to a variety of different specimen types when attempting to diagnose VRI. Respiratory samples suitable for PCR analysis include nose and throat swabs, nasal washes, nasal secretions, sputum and bronchoalveolar lavage fluid. There is no universally agreed gold standard diagnostic strategy and the optimal combination of specimens may vary among different patient populations. Studies from children have shown that the sensitivity of nasal swabs is broadly equivalent to that achieved with nasal lavage with the advantage of being less invasive. The use of multiple samples also appears to increase the viral detection rate. The combination of nose and throat swabs identified 15% more viruses than nose swabs alone in one recent study. The choice of specimen types to be used in either clinical or research practice requires consideration of the cost, ease of collection, tolerability for the patient as well as the diagnostic performance.

2.3 Epidemiology of Viral Respiratory Infections

Much of our understanding of the aetiology and epidemiology of VRI is drawn from a group of large, longitudinal follow-up studies undertaken between the 1950s and 1980s. From these studies, the incidence of VRI can be placed at between 5-8 episodes/year for young children as compared to 2-4 episodes/year for adults. Women have up to a two-fold higher rate of VRI than men. In most of these studies rhinovirus was the most common cause of VRI. It must be noted that these studies differed greatly in design, VRI definition and virological methodology.
The highest viral detection rate amongst these studies was very low at only 25%. It seems unlikely, however, that such large-scale epidemiological studies will be repeated in the era of PCR technology given the immense costs involved.

Respiratory viruses display clear seasonal patterns in the general population with, for example, a peak in rhinovirus infection seen each autumn and other viruses predominating during winter. The seasonality of each virus is discussed in more detail below. Several studies have also shown links between ambient weather conditions and the incidence of viral infections. Laboratory studies have demonstrated that influenza transmission and infectivity are greatest in cold, dry conditions which may explain the typical seasonal pattern of this pathogen. The degree to which these observations apply to the respiratory health of people with CF has not been investigated previously. Chapter Eight of this thesis explores the impact of the seasons and climate on adults with CF in more detail.

2.4 Specific Viral Respiratory Infections

2.4.1 Rhinovirus

Rhinovirus is the most common cause of VRI in most studies. Rhinoviruses belong to the Picornaviridae group and are sub-divided into three species (rhinovirus A, B and C) and then into more than one hundred serotypes. This diversity of rhinoviruses explains why humans are unable to develop immunity to the common cold. Experimental rhinovirus infection has an incubation period of 10-24 hours and colds caused by rhinovirus have a median duration of 9.5-11 days. In elderly patients with lower respiratory tract symptoms and rhinovirus infection, illness may last over two weeks. Rhinovirus is also a major cause of exacerbations of asthma and chronic obstructive pulmonary disease.

Rhinoviruses are isolated throughout the calendar year but in the northern hemisphere a sharp peak in incidence is seen in September. Up to 92% of self-reported colds during the autumn are associated with rhinovirus infection. It is thought that the autumnal outbreak is due to rapid transmission of the virus amongst children returning to school after the summer holidays. Rhinoviruses are spread by direct contact or by droplets.
Most rhinoviruses attach to respiratory epithelial cells through intercellular adhesion molecule 1 (ICAM-1) which facilitates entry of the virus into the cell to allow viral replication. A minority of rhinovirus strains, sometimes termed the “minor group” of rhinoviruses employ the very low density lipoprotein (VLDL) receptor as an alternative means of cell entry. The mechanism of rhinovirus-epithelial cell binding is important as it is a potential target for anti-rhinoviral therapies such as pleconaril. Other laboratory studies into the pathogenesis of the common cold have demonstrated a release of inflammatory cytokines, including IL-6, IL-8 and GM-CSF, in response to rhinovirus infection. It is this process that is believed to drive the symptoms of the common cold.

2.4.2 Respiratory Syncytial Virus (RSV)
Respiratory syncytial virus (RSV) belongs to the Paramyxoviridae family. It is an enveloped, single-stranded RNA virus with two known sub-types, A and B. The sub-types are very similar in virological structure and clinical impact. RSV is most notable for causing approximately 90% of cases of infant bronchiolitis although it is also recognised as a pathogen in the elderly and those with chronic lung disease. RSV infection occurs in a single epidemic each winter lasting for up to 6 months. RSV has an incubation period of 4-5 days and is spread by direct contact or through droplets. Rapid transmission of RSV infection among children attending a nursery school facility has been documented which adds to the evidence that children act as vectors for respiratory viruses.

2.4.3 Parainfluenza Virus
As with RSV, the parainfluenza viruses belong to the Paramyxoviridae and are single-stranded RNA viruses divided into sub-types 1 to 4. The virus is spread by the aerosol route and most often causes disease in infants or the immunocompromised. Parainfluenza virus infection has an incubation period of 2-3 days and may lead to croup, bronchiolitis or pneumonia. Studies have reported varying incidence patterns for parainfluenza virus infection. The Tecumseh study identified annual peaks of parainfluenza virus type 1 and 2 each winter with intermittent occurrences of parainfluenza type 3 throughout the year. More recent data from Rochester, New
York has shown a biennial pattern to parainfluenza type 1 infection with associated outbreaks of croup.  

2.4.4 Influenza

Influenza is an acute respiratory illness caused by a group of RNA viruses belonging to the Orthomyxoviridae family. Influenza viruses are classified as influenza A, B and C with human disease most commonly caused by groups A and B. Influenza viruses display two key surface antigens: haemagglutinin (H) and neuraminidase (N). These antigens are further divided into subtypes H1-15 and N1-9. Each year, circulating influenza viruses are subject to genetic mutations that alter the specific binding sites of the haemagglutinin and neuraminidase antigens. This process is termed “antigenic drift” and produces influenza strains from which the human immune system is not fully protected. Epidemics of these subtly different influenza viruses occur each winter.

“Antigenic shift” occurs when there is a complete change in the type of haemagglutinin or neuraminidase antigens thereby creating a virus which is completely new to the human population. Novel viruses of this kind often originate in pigs or birds. An influenza pandemic results when the new virus develops the ability to be transmitted between humans. Over the last century, pandemics have occurred in 1918-19 (H1N1), 1957 (H2N2), 1968 (H3N2) and 2009 (H1N1). A similar process led to human cases of H5N1 avian influenza in 2005 but the relative inability of this virus to spread effectively between humans prevented a pandemic. More recently, in 2013 an outbreak of a novel influenza A/H7N9 virus was reported in China. The H7N9 influenza virus appears to have arisen from strains circulating in wild birds, ducks and chickens and there has been a suggestion that contact with live poultry markets may be a risk factor for contracting the illness in humans. In common with H5N1 influenza, a high proportion of cases of H7N9 infection have resulted in hospitalisation, acute respiratory distress syndrome (ARDS) and death. The potential for this latest avian influenza virus to cause a pandemic is not yet certain, however, and viral surveillance programs are ongoing.
Influenza viruses are spread through direct contact and by droplets generated through sneezing or coughing. Influenza has an incubation period of 1-2 days. The clinical features of influenza range from asymptomatic cases through to severe pneumonitis complicated by acute respiratory distress syndrome (ARDS) and refractory hypoxia. It is not possible to distinguish influenza from other common viral and bacterial infections on clinical grounds but typical symptoms of influenza include cough, malaise, fever, headache and nasal congestion. Certain presenting features are associated with a varying likelihood of confirmed influenza. Call et al found that the likelihood of influenza was reduced by the absence of fever (likelihood ratio (LR) 0.40, 95% CI 0.25-0.66), cough (LR 0.42, 95% CI 0.31-0.57) or nasal congestion (LR 0.49, 95% CI 0.42-0.59). Amongst elderly patients, sneezing was associated with a lower probability of influenza (LR 0.47, 95% CI 0.24-0.92).

Influenza is a major cause of mortality in the general population, especially during epidemic seasons. Influenza is thought to have caused a mean of over 21 000 excess deaths each year in the USA between 1976 and 1999. Simonsen et al compared death rates during influenza pandemics with typical epidemic seasons in the USA. The 1918-19 influenza H1N1 pandemic was associated with an excess mortality rate of 529 deaths per 100 000 population as compared to 0.7–93 deaths per 100 000 in non-pandemic seasons. Each of the influenza pandemics over the last century demonstrated disproportionately high mortality rates in patients under the age of 65 years. This contrasts sharply with non-pandemic seasons when the highest risk of mortality is amongst elderly patients. It is thought that the high mortality in young people during pandemics is due to the naivety of the immune system to the pandemic virus whereas elderly people are likely to have encountered similar influenza viruses earlier in life. In addition to causing excess mortality, influenza is also associated with considerable morbidity. Thompson et al reported that influenza was associated with an average of 133 900 hospitalisations each year between 1979-2001 in the USA.

### 2.4.5 Coronavirus
Coronaviruses are single-stranded RNA viruses and are responsible for up to 15% of common colds. Coronavirus infection can also be very serious, however, as with
the viruses causing Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). These novel coronaviruses are discussed in detail in Section 2.5.6. The seasonality of coronaviruses varies depending on sub-type: coronavirus NL63, for instance, occurs in autumn and winter whilst sub-types OC43 and 229E are found in the spring months. Human coronaviruses have an incubation period of 3-4 days.

2.4.6 Adenovirus

*Adenoviridae* are a group of double-stranded DNA viruses with over 50 known serotypes. The median incubation period is 5.6 days. Most commonly, adenoviruses cause a mild URTI, gastroenteritis or conjunctivitis but they also cause life-threatening infections in immunocompromised patients. Data on the seasonality of adenovirus has shown contrasting patterns: some investigators have shown a winter predominance whilst others report low level isolation throughout the year. This probably reflects differences in diagnostic techniques.

2.4.7 Metapneumovirus

Human metapneumovirus was first identified in 2001 and belongs to the *Paramyxoviridae* family. It is similar in structure to RSV and predominantly affects young children. Metapneumovirus has been identified in approximately 6% of children presenting to an emergency department with pneumonia or a more broadly defined acute lower respiratory tract infection. Metapneumovirus has also been associated with exacerbations of COPD and outbreaks of respiratory illness in elderly people. In the northern hemisphere, metapneumovirus infection occurs most commonly during the winter and spring months and is thought to be spread by respiratory droplets.

2.4.8 Bocavirus

Bocavirus is a member of the *Parvoviridae* family and was discovered in 2005. The full clinical implications of bocavirus infection are not yet clear but recent studies have shown an association between bocavirus and acute respiratory tract infections in children. In one study using serology, Don et al found evidence of bocavirus infection in 12% of children hospitalised with community acquired pneumonia.
2.4.9 Herpesviruses

The *Herpesviridae* are a family of double-stranded DNA viruses of which there are eight species known to infect humans. Among this group, Epstein-Barr virus is the principle cause of infectious mononucleosis, the triad of fever, pharyngitis and lymphadenopathy.\(^{248}\) Acute infection with cytomegalovirus (CMV) is frequently asymptomatic but may also cause a similar illness to Epstein-Barr virus. Among immunocompromised individuals, herpesviruses such as CMV, herpes simplex and varicella-zoster virus may lead to severe pneumonia and disseminated infection.\(^{249,250}\)

2.5 Clinical Syndromes Caused by Respiratory Viruses

2.5.1 The Common Cold

The common cold refers to an acute contagious illness with prominent upper respiratory tract symptoms. Colds are heterogeneous in terms of presentation, duration and severity. The most frequent features of the common cold are: sore throat, nasal discharge or stuffiness and cough.\(^{198,251-253}\) Other symptoms include: malaise, fatigue, sinus pain, headache and a hoarse voice. Fever and myalgia may occur with colds but are generally more suggestive of influenza.\(^{196,227,254}\) The incubation period of naturally-occurring common colds has not been clearly defined but experimental rhinovirus infection has an incubation period of 10-24 hours.\(^{196,197}\)

2.5.2 Influenza

Influenza is discussed in detail in section 2.4.4.

2.5.3 Croup

Croup refers to a syndrome of acute inflammation of the larynx and trachea as a result of a viral infection.\(^{255}\) Cardinal features of croup are a “barking” cough and stridor, indicating inspiratory airflow limitation,\(^{255,256}\) and it is typically seen in children under the age of 6 years. Most cases of croup are caused by parainfluenza 1 although other parainfluenza viruses, influenza, adenovirus and rhinovirus may be responsible.\(^{209}\) Croup occurs in autumnal epidemics every two years, matching the pattern of parainfluenza 1 incidence.\(^{209,256}\)
2.5.4 Bronchiolitis

Bronchiolitis is the most common acute respiratory illness seen in infants with approximately 2% of babies requiring hospitalisation in the first year of life for this condition.\textsuperscript{257} The clinical syndrome is characterised by respiratory distress, hypoxia, hyperinflation, cough and wheeze due to small airways inflammation and obstruction.\textsuperscript{258,259} Mortality is typically less than 1% in previously healthy patients.\textsuperscript{257} Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and is implicated in up to 90% of cases.\textsuperscript{209} Bronchiolitis may also be caused by influenza, parainfluenza, metapneumovirus and adenovirus.\textsuperscript{241,257} Peaks in rates of hospitalisation for bronchiolitis coincide with winter peaks in RSV incidence\textsuperscript{209} as described in Section 2.4.2.

2.5.5 Community Acquired Pneumonia (CAP)

Community acquired pneumonia (CAP) refers to an acute infective process affecting the lower respiratory tract in an individual not exposed to a healthcare environment. Typical features of CAP include fever, breathlessness and a productive cough although most definitions also include the presence of new abnormalities on chest examination or radiographs.\textsuperscript{260} The significance of viral CAP is still subject to some debate.\textsuperscript{261}

Studies using PCR techniques, with or without additional conventional methods, have detected respiratory viruses in 13.3\% - 73.5\% of cases of CAP.\textsuperscript{246,262-270} Studies of viral CAP do not agree on the most common viral pathogens: influenza,\textsuperscript{270} coronavirus,\textsuperscript{266} rhinovirus,\textsuperscript{271} and RSV\textsuperscript{269} have all been found to be the most common virus in observational studies of CAP. The majority of cases in these studies were found to have a respiratory virus as the sole pathogen but between 4\% and 35\% had co-infection with another virus or bacterium.\textsuperscript{262,264} An interesting finding reported by both Jennings \textit{et al} and Templeton \textit{et al} was that mixed infection, specifically involving rhinovirus in the first study and any co-infection in the second, was a significant predictor of severe CAP.\textsuperscript{262,265} Choi \textit{et al} have reported subsequently important data on the role of viruses in severe pneumonia requiring intensive care therapy.\textsuperscript{272} In this cohort, VRI was detected in 36.4\% of cases with rhinovirus the
most prevalent. Viral pneumonia had a similar mortality rate to bacterial pneumonia in this study. The British Thoracic Society recommends consideration of testing for respiratory viruses in patients with severe CAP and in non-severe CAP at times of outbreaks such as an influenza pandemic.260

2.5.6 Novel Coronavirus-Associated Acute Respiratory Syndromes

Severe Acute Respiratory Syndrome (SARS) refers to a contagious, life-threatening febrile respiratory illness. A global outbreak of SARS began in China in November 2002.273-276 Within six months of the first cases, a novel coronavirus associated with the syndrome, SARS-CoV, had been identified.234,277 The incubation period of SARS-CoV is approximately 4 days.213,273 Typical features of SARS include fever, dyspnoea and dry cough. Up to a quarter of cases developed acute respiratory distress syndrome (ARDS).278,279 Treatment of SARS centres on supportive care and no evidence of benefit from antivirals such as ribavirin or interferon was found.280 Nosocomial transmission of SARS was well-documented but infection control measures such as the use of facemasks reduced the spread of the disease and the outbreak eventually subsided.281

In 2012, a number of patients in Saudi Arabia were diagnosed with a rapidly progressive, contagious pneumonitis that was highly similar to SARS.235 A distinct, novel coronavirus has been identified as the cause of this illness which has been termed Middle East Respiratory Syndrome (MERS). MERS has a mortality rate of over 50% and, in common with SARS, is frequently associated with the development of acute respiratory distress syndrome.282 Person-to-person transmission of the MERS coronavirus (MERS-CoV) has been reported in both the healthcare and domestic settings. Breban et al have suggested, however, that MERS-CoV in its present form is unlikely to be sufficiently transmissible to cause a pandemic but future mutations in the viral genome may increase the likelihood of such a development.284 The epidemiology and virological characteristics of MERS-CoV remain under intense scrutiny.
2.6 The Effect of Respiratory Viruses in Chronic Lung Disease

Respiratory viruses have been strongly associated with exacerbations of asthma although causation has not been proven.\textsuperscript{202} Evidence of viral infection using PCR techniques has been demonstrated in up to 85\% of acute asthma exacerbations.\textsuperscript{285-289} Corne \textit{et al} found that VRI was more severe and of greater duration in asthma patients.\textsuperscript{290} Rhinovirus has consistently been shown to be the most frequent virus associated with asthma exacerbations.\textsuperscript{285-287} Recent data suggests that the rhinovirus C sub-group may cause over 50\% of virus-positive exacerbations.\textsuperscript{291}

The precise pathophysiology of virus-induced asthma exacerbations has not been discovered but there is evidence of enhanced cytokine release, particularly IL-6 and IL-8, with viral infection\textsuperscript{292} and asthmatics may have reduced anti-viral immune responses compared with healthy individuals.\textsuperscript{293,294} There is also evidence to suggest that viruses interact synergistically with inhaled allergens to increase the risk of acute asthma exacerbations.\textsuperscript{295,296} Currently, there is no proven role for anti-viral therapy in the management of virus-induced asthma exacerbations but this remains an area of intense research interest.\textsuperscript{297,298}

As with asthma, there is an association between exacerbations of chronic obstructive pulmonary disease (COPD) and respiratory viruses. Viruses are detected in approximately one third of acute exacerbations of COPD\textsuperscript{299-301} and are significantly more common at exacerbation than when clinically stable.\textsuperscript{300} Seemungal \textit{et al} found that virus-associated COPD exacerbations were more severe than virus-negative events.\textsuperscript{299} Rhinovirus is the most common virus found in exacerbations of COPD.\textsuperscript{202,301}

2.7 Prevention of Viral Respiratory Infection

A key component of strategies to prevent cases of VRI lies in the widespread use of hand-washing and good respiratory hygiene. Barrier nursing measures and the use of respirators by healthcare staff have also been advocated during pandemic episodes. Seasonal influenza vaccination is recommended in the UK for patients aged over 65 years or those aged over six months who are deemed at high-risk for complications of influenza, such as those with chronic respiratory disease.\textsuperscript{302} Influenza vaccination has
not been tested in robust clinical trials in most of these “high-risk” populations, however. Healthcare workers in the UK NHS are also offered the seasonal influenza vaccination each year in an attempt to prevent nosocomial spread of the disease. Rapid development of vaccines for novel influenza strains is a key part of the global response to the onset of a pandemic, as was seen with the 2009 influenza A/H1N1 outbreak.

Prophylactic strategies for other respiratory viruses are lacking, however. Palivizumab is a monoclonal antibody that has been developed for the prevention of RSV infection and is given in repeated doses during the RSV season. In the UK palivizumab is recommended for infants born prematurely who have chronic lung disease or acyanotic congenital heart disease but evidence is lacking for its use beyond these indications. The development of a vaccination against rhinovirus has been investigated but there are considerable technical difficulties in producing a vaccine with sufficient coverage across the breadth of rhinovirus serotypes. It appears clear that there is a need for more research and investment into preventative therapies for use against respiratory viruses.

2.8 Treatment of Viral Respiratory Infections

Despite decades of research looking for effective treatments for the common cold, there remains no proven therapy to prevent it or reduce its duration. Numerous over-the-counter treatments are available including simple analgesics, decongestants, antihistamines and antitussives. These have been shown to have a modest impact on nasal symptoms such as rhinorrhoea. Dietary and herbal supplements, such as vitamin C, Echinacea and garlic have also been used but there is no robust evidence to date that such remedies have a significant clinical effect. A recent Cochrane review did suggest that zinc supplements have modest efficacy in reducing the duration of the common cold and may help prevent respiratory virus infection in healthy children. Oral antibiotics are very commonly prescribed for URTIs but there is no evidence that they reduce the duration or severity of symptoms. Indeed, a recent Cochrane review found that the use of antibiotics for the common cold was harmful as a result of their side-effect profile (hazard ratio 1.8; 95%CI 1.01-
Similarly, nasal corticosteroids are ineffective in relieving the symptoms of the common cold. Antiviral drugs have been used experimentally for the common cold but none have achieved mainstream clinical use as yet. Intranasal interferon alone is ineffective but has some impact on symptoms when used in combination with an antihistamine and non-steroidal anti-inflammatory drug. Pleconaril, which works by binding to the viral capsid, can reduce the duration of colds by up to 1.5 days but resistance develops in a significant proportion of treated cases. Intranasal synthetic ICAM-1 therapy reduces the severity of rhinovirus-related colds in experimental conditions. Given the mild nature of colds in healthy people, however, safety concerns with antivirals usually outweigh the rather modest benefits achieved to date.

Influenza is the only common VRI for which there are licensed antiviral therapies available for routine clinical practice. Amantadine has some efficacy in preventing influenza A through the inhibition of viral entry into epithelial cells. The use of amantadine is severely limited, however, by a poor side-effect profile and development of viral resistance. Neuraminidase inhibitors, of which oseltamivir and zanamivir are commercially available, have been shown to be effective in reducing the duration of symptomatic influenza infection and preventing the transmission of infection during outbreaks. Observational data suggests that neuraminidase inhibitors are at their most effective when given early in the course of influenza, ideally within 48 hours, but some benefit may still be seen with delayed administration.

A systematic review and meta-analysis by Cooper et al, however, showed that neuraminidase inhibitors appear less effective in high-risk populations such as those with lung disease. In this study, oseltamivir reduced the time to alleviation of influenza symptoms by only 0.4 days in high risk patients as compared to 0.9 days in healthy adults. Treatment with zanamivir, licensed in an inhaled form only, is further restricted in chronic lung disease by its associated risk of bronchospasm. It must also be recognised that neuraminidase inhibitors have been the subject of considerable controversy recently as a proportion of the clinical trial data held by the pharmaceutical industry allegedly has not been published.
Chapter Three

Respiratory Viruses in Cystic Fibrosis –
A Literature Review
3.0 Respiratory Viruses in Cystic Fibrosis – A Literature Review

3.1 Introduction
There has been a longstanding emphasis on bacterial infection in CF research to date. This is understandable given the substantial negative impact bacteria such as *P. aeruginosa* have on mortality and morbidity in CF. Since the 1970s, however, there has been a steady interest in the role viruses might play in pulmonary exacerbations, susceptibility to new bacterial infection and disease progression.

The vast majority of studies looking at viruses in CF have been conducted in paediatric populations and to date there has been no large-scale, prospective study in this field amongst adults with CF.

3.2 Diagnosis of Viral Respiratory Infections in CF
As with virology studies in the general population, the reported incidence of VRI in CF has varied greatly depending on the laboratory techniques used to identify viruses. Prior to 1995, researchers relied on a combination of conventional techniques such as serology, cell culture and immunofluorescence. Wang *et al*, for instance, were unable to culture any viruses from 49 children with CF, despite identifying 105 incidences of viral seroconversion. It is likely that clinical studies employing only conventional methods underestimate the true incidence of viral infection. Since Smyth *et al* in 1995, studies have used varying combinations of conventional and PCR-based techniques when investigating viruses in CF.

Researchers have also used a variety of different respiratory specimens when investigating VRI in CF with options including bronchoalveolar lavage, sputum, nasal washes, nose- and throat-swabs. There is no agreed “gold standard” specimen type for diagnosis of VRI but previous data from the Manchester Adult CF Centre has shown that sputum is an appropriate medium for the detection of respiratory viruses using PCR.

3.3 Incidence of Viral Respiratory Infections in CF
The results of observational studies investigating the incidence of symptomatic respiratory illnesses and confirmed VRI in patients with CF are summarised in Tables 3.1 and 3.2. Of the 25 studies identified, 18 were conducted in paediatric populations. Among adults with CF, there has been only one previous prospective study examining
VRI during both clinical stability and acute illness and this study involved just 17 adult participants. The remaining studies in adult cohorts were cross-sectional in design.

Children with CF experience a mean of between 1.45 and 5.3 acute respiratory illnesses per year. When rates of confirmed viral infection are examined, the results depend on the virological techniques used. Conventional methods have identified viruses in 5.1–43% of samples taken at pulmonary exacerbation as compared to 5.6–12.4% of cases when stable. In studies using PCR-based technology the figures were 23–64.1% at exacerbation and 13.2–53% when stable. Overall, the incidence of confirmed VRI in these studies can be placed at between 0.15 – 5.1 episodes per patient per year. The relative frequency of individual viruses varies enormously in these studies as they each included a different panel of viruses and utilised different methodologies. Since the advent of PCR technology, most studies have found that rhinovirus is the most common virus identified during a pulmonary exacerbation of CF. The relative distribution of other viruses varies considerably between studies.

Several groups have compared the incidence of acute upper respiratory tract illnesses in paediatric CF patients with a control group of healthy individuals. Hiatt et al reported that children with CF had similar rates of URTI to controls (mean of 5 URTIs per year in each group). However, CF patients were much more likely to develop lower respiratory tract symptoms with an URTI (OR 4.6, 95% CI 1.3 – 16.5). Similarly, van Ewijk et al reported that CF patients and controls had similar rates of symptomatic URTI (3.8 vs 4.2 episodes/year) but CF patients suffered longer periods with lower respiratory symptoms. Ramsey et al did not separate URTI from LRTI in their study population and found, unsurprisingly, that CF patients had significantly more days per year affected by respiratory illness (22.5 vs 7.8 days/year; p=0.008). Wang et al also reported that CF patients had significantly more respiratory illnesses than sibling controls (3.7 vs 1.7 episodes per year; p<0.001). Hiatt et al found that there was a higher rate of positive viral isolation in the control group than the CF group (28% vs 17%; p<0.05). In each of the other studies with control arms, however, there was no significant difference in the rate of confirmed viral infections between CF patients and healthy controls.
From the above data, it can be concluded that children with CF have more frequent respiratory illnesses than healthy children overall but they probably have similar numbers of viral URTIs per year. While detection of respiratory viruses is common among CF adults presenting with a pulmonary exacerbation, the true incidence of VRI in this population is not known.

3.4 Clinical Features of Viral Respiratory Infections in CF

As with the general population, VRI in children with CF demonstrate a wide spectrum of disease severity. Respiratory viruses have been shown to cause asymptomatic infections through to severe pulmonary exacerbations requiring hospitalisation.\(^{346,348,351,352}\) Upper respiratory tract symptoms are very common in laboratory-confirmed cases of VRI, with between 76-100% of cases displaying at least one typical URTI symptom in prospective studies of children with CF.\(^{342,346}\) Rhinorrhea has consistently been found to be the most common symptom of VRI in CF\(^{342,346,348}\) with a frequency of 76-100%. Other common symptoms of VRI include sore throat (35-69%) and sneezing (40-60%). Fever was recorded in 71% of URTIs in the study by Hordvik \textit{et al}\(^{342}\) but unfortunately there is limited data on pyrexia from other studies. Upper respiratory tract symptoms are not a reliable way of discriminating laboratory-proven viral infection, however. Olesen \textit{et al} reported that URTI symptoms had a positive predictive value of only 0.39 for positive virology tests.\(^{347}\)

In addition to causing URTIs, respiratory viruses may also lead to lower respiratory tract infection in CF. In prospective studies, the association between VRI and pulmonary exacerbations (PEx) is most clearly seen in paediatric CF populations\(^{330,332,346,348}\) The literature regarding adults with CF is largely limited to cross-sectional studies which show that a respiratory virus can be detected in 8-47% of PEx.\(^{333,334,353-355}\) The most common lower respiratory symptoms associated with VRI are increased cough (58%) and breathlessness (43%).\(^{342,346}\) Van Ewijk \textit{et al} found that paediatric CF patients had a significantly longer duration of virus-associated LRTI as compared with a control group (22.4 vs 12.8 days, \(p=0.002\)).\(^{349}\) CF patients and controls with confirmed VRI in this study had similar URTI symptom
scores.\textsuperscript{349} In a retrospective study employing only throat swabs as a means of detecting VRI, Etherington \textit{et al} found that virus-positive PEx were associated with a more substantial fall in FEV\textsubscript{1} than in virus-negative PEx.\textsuperscript{355}

The clinical severity of influenza in CF is variable. For instance, influenza A was associated with marked clinical deterioration in a small case series of UK adults of with CF.\textsuperscript{352} In contrast, infection with 2009 pandemic influenza A/H1N1 generally produced mild, self-limiting illnesses in CF populations.\textsuperscript{353,356-359} Among children with CF, RSV infection is associated with an increased risk of severe pulmonary exacerbation requiring admission to hospital.\textsuperscript{360}

### 3.5 Viral Respiratory Infections and Progression of CF Lung Disease

Several studies have attempted to elicit the effects of viral infections on progression of CF lung disease, although the longest follow-up period reported to date is only of the order of three years.\textsuperscript{335} All studies reporting long-term outcomes have been performed in children so it is difficult to extrapolate the findings to adults with CF.

Using lung function as a measure of disease progression, Wang, Smyth and Collinson all reported significant falls in FEV\textsubscript{1} in CF patients with high rates of viral infection\textsuperscript{330,332,346} although Olesen \textit{et al} found no such effect.\textsuperscript{347} Ramsey \textit{et al} curiously found that patients with a high frequency of virus-positive exacerbations had a higher end-of-study FVC and FEF25-75\% than those with fewer viruses but they did not report data on FEV\textsubscript{1}.\textsuperscript{343} The Schwachman score has been used as an alternative marker of disease severity in CF.\textsuperscript{361} Wang and Collinson found that VRIs were associated with declines in Shwachman scores\textsuperscript{330,346} but Ramsey and Smyth reported no significant change in this parameter.\textsuperscript{332,343} Abman \textit{et al} did not report Shwachman scores but did find that RSV infection was associated with a significant decline in the Brasfield chest radiograph score.\textsuperscript{351}

From the above studies, it would be reasonable to conclude that viral infections in children with CF are probably associated with progression of CF lung disease but the effects in adults are currently uncertain.

54
3.6 Virus-Bacteria Interaction in CF

For nearly three decades there has been a concern that viral infections may interact with bacterial pathogens and increase the risk of colonisation with *P. aeruginosa*. Petersen *et al* observed 116 patients over eight months and reported an increased rate of pulmonary exacerbation in those patients with known chronic *P. aeruginosa* infection. Over the study period, confirmed RSV infection was found in 20% of those with new *P. aeruginosa* infection as compared to 7% in the group with intermittent or no *P. aeruginosa* isolation (p<0.05). The same research group later reported that 68% of new acquisitions of chronic *P. aeruginosa* infection occurred over the winter months when respiratory viruses are at their most prevalent. This observation provides circumstantial support to the idea of a synergistic effect between viruses and bacterial pathogens.

Collinson *et al* found that there was no difference in the rate of acquisition of new bacterial infection between those with frequent (>2.7 per year) and infrequent URTIs. However, of the six patients who isolated *P. aeruginosa* for the first time during the study, five (83%) acquired it whilst suffering symptoms of an URTI. The study also reported that 83% of all new bacterial infections were associated with an URTI.

The pathophysiological interaction between bacterial pathogens in CF and respiratory viruses has yet to be fully unravelled. Laboratory work suggests that RSV infection facilitates adherence of *P. aeruginosa* to respiratory epithelial cells which may help to establish chronic infection. Similarly, Ramphal *et al* reported that tracheal cells infected with influenza in an animal model were more prone to *P. aeruginosa* adherence than healthy cells. Xu *et al* have also demonstrated that CF respiratory epithelial cells demonstrate a more pronounced inflammatory response to influenza infection than cells from people without CF. This enhanced response may be a factor in the development of chronic *P. aeruginosa* infection in CF. A further hypothesis is that viral infections lead to a degree of immunocompromise thereby allowing opportunistic pathogens such as *P. aeruginosa* to prosper. Impairment of neutrophil function with viral infections has been demonstrated in a number of studies. In addition, Strunk *et al* demonstrated that serum complement levels fall following viral exacerbations of CF. It is likely that the interaction between
viruses and bacteria in CF is multifactorial; this interaction may offer a target for therapy to prevent chronic bacterial infection.

3.7 Prevention of Viral Respiratory Infections in CF

Annual influenza vaccination has been recommended for patients with CF but, as recognised in a recent Cochrane systematic review, there is no evidence from RCTs that this strategy is effective in reducing mortality or morbidity. Despite this, RCTs comparing intranasal with intramuscular vaccines and also differing formulations of intramuscular vaccines have been performed in patients with CF. All forms of vaccination were associated with high levels of minor adverse events but immunogenicity of the vaccines was adequate. There was insufficient power in each of these studies to make firm conclusions on clinical efficacy.

A number of observational studies have also shed light on the role of influenza vaccination in CF. Ong et al demonstrated in a non-randomised, non-blinded study that CF patients mounted a satisfactory immunological response to intramuscular influenza vaccination which was maintained at twelve months. Wat et al looked at the effects of influenza vaccination in a paediatric CF population over a seven month period. Among the vaccinated group, 2.4% of patients contracted influenza as compared to 18.2% in the non-vaccinated group. This equates to an absolute and relative risk reduction of 15.8% and 13.2% respectively.

Palivizumab, a monoclonal antibody against RSV, has been shown to be effective in reducing adverse effects of bronchiolitis in children with bronchopulmonary dysplasia and lung disease of prematurity. A 2010 Cochrane systematic review identified only one placebo-controlled RCT of palivizumab therapy in infants with CF which showed no significant differences in rates of hospitalisation or mortality between the treatment and placebo groups. A retrospective review by Giebels et al similarly showed no evidence of improved outcomes with palivizumab. Finally, the effectiveness of amantadine for the prevention of VRIs among children with CF has been evaluated in a double blind RCT. Amantadine was tolerated reasonably well but was no better than placebo in preventing VRIs.
3.8 Treatment of Viral Respiratory Infections in CF

Although widely used, there is no RCT data to support the use of the neuraminidase inhibitors oseltamivir and zanamivir for treatment of symptomatic influenza in patients with CF. A 2010 Cochrane review recommended the use of these drugs in accordance with individual clinicians’ own experience. Caution is advised with the use of zanamivir in chronic lung disease as it is administered by inhalation and may cause bronchoconstriction. A case report of the successful use of inhaled zanamivir in CF has been published. No other antiviral therapy is available for routine clinical use in CF.
### Table 3.1 Incidence of viral respiratory infections in CF – studies using conventional virological techniques

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Age* (years)</th>
<th>Diagnostic techniques</th>
<th>Incidence of acute respiratory illness (episodes/pt/year)</th>
<th>Samples positive for viruses at PEx (%)</th>
<th>Samples positive for viruses when stable (%)</th>
<th>Overall incidence of confirmed viral infection (episodes/pt/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wright 1976</td>
<td>153</td>
<td>12</td>
<td>Serology Culture</td>
<td>NR</td>
<td>22.9</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Petersen 1981</td>
<td>116</td>
<td>NR</td>
<td>Serology</td>
<td>4.35</td>
<td>20.0</td>
<td>NR</td>
<td>0.58</td>
</tr>
<tr>
<td>Wang 1984</td>
<td>49</td>
<td>13.7</td>
<td>Serology Culture</td>
<td>3.7</td>
<td>NR</td>
<td>12.4</td>
<td>1.67</td>
</tr>
<tr>
<td>Efthimiou 1984</td>
<td>46</td>
<td>22.2</td>
<td>Complement fixation</td>
<td>NR</td>
<td>29.0</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Stroobant 1986</td>
<td>30</td>
<td>NR</td>
<td>Serology Culture</td>
<td>3.27</td>
<td>24.7</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Przyklenk 1988</td>
<td>75</td>
<td>15.6</td>
<td>Serology</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0.15</td>
</tr>
<tr>
<td>Ong 1989</td>
<td>36</td>
<td>23.6</td>
<td>Serology Culture</td>
<td>NR</td>
<td>30.6</td>
<td>NR</td>
<td>0.36</td>
</tr>
<tr>
<td>Hordvik 1989</td>
<td>10</td>
<td>14.1</td>
<td>Serology Culture</td>
<td>1.75</td>
<td>37.1</td>
<td>NR</td>
<td>0.5</td>
</tr>
<tr>
<td>Ramsey 1989</td>
<td>19</td>
<td>13.4</td>
<td>Serology Culture</td>
<td>NR</td>
<td>19.1 (serology) 8.8 (culture)</td>
<td>16.2 (serology) 5.6 (culture)</td>
<td>1.29</td>
</tr>
<tr>
<td>Pribble 1990</td>
<td>54</td>
<td>15.4</td>
<td>Serology Culture</td>
<td>NR</td>
<td>25.0</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Hiatt 1999</td>
<td>22</td>
<td>0.83</td>
<td>Serology Culture</td>
<td>5.3</td>
<td>17</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Clifton 2007</td>
<td>305</td>
<td>NR</td>
<td>Serology</td>
<td>NR</td>
<td>5.1</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Abbreviations: PEx: pulmonary exacerbation; NR: not reported. *Age given as mean or median, whichever quoted in the paper.
### Table 3.2  Incidence of viral respiratory infections in CF – studies using polymerase chain reaction (PCR) techniques

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Age* (years)</th>
<th>Diagnostic techniques</th>
<th>Incidence of acute respiratory illness (episodes/pt/year)</th>
<th>Sample positive for viruses at PEx (%)</th>
<th>Samples positive for viruses when stable (%)</th>
<th>Overall incidence of confirmed VRI (episodes/pt/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smyth 1995</td>
<td>108</td>
<td>7.9</td>
<td>Serology Immunofluorescence PCR (rhinovirus)</td>
<td>1.45</td>
<td>28.0</td>
<td>NR</td>
<td>0.41</td>
</tr>
<tr>
<td>Collinson 1996</td>
<td>48</td>
<td>7.3</td>
<td>Culture PCR</td>
<td>2.72</td>
<td>43.0</td>
<td>NR</td>
<td>1.16</td>
</tr>
<tr>
<td>Punch 2005</td>
<td>38</td>
<td>25.9</td>
<td>PCR</td>
<td>NR</td>
<td>23.0</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Olesen 2006</td>
<td>75</td>
<td>8.0</td>
<td>PCR</td>
<td>NR</td>
<td>24.2</td>
<td>13.2</td>
<td>0.16</td>
</tr>
<tr>
<td>Wat 2008</td>
<td>71</td>
<td>9.0</td>
<td>NASBA</td>
<td>1.64</td>
<td>45.7</td>
<td>16.9</td>
<td>0.81</td>
</tr>
<tr>
<td>van Ewijk 2008</td>
<td>20</td>
<td>3.5</td>
<td>PCR</td>
<td>3.8 #</td>
<td>NR</td>
<td>NR</td>
<td>5.1 #</td>
</tr>
<tr>
<td>de Almeida 2010</td>
<td>103</td>
<td>8.9</td>
<td>PCR</td>
<td>NR</td>
<td>64.1</td>
<td>53.0</td>
<td>1.97</td>
</tr>
<tr>
<td>Jones 2011</td>
<td>53</td>
<td>29.0</td>
<td>PCR</td>
<td>NR</td>
<td>47.2</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Burns 2011</td>
<td>44</td>
<td>12.8</td>
<td>PCR</td>
<td>1.48</td>
<td>26.9</td>
<td>21.6</td>
<td>1.23</td>
</tr>
<tr>
<td>Wark 2012</td>
<td>17 adults 9 paeds</td>
<td>27.5 10.2</td>
<td>PCR</td>
<td>2.15</td>
<td>65.0</td>
<td>NR</td>
<td>1.27</td>
</tr>
<tr>
<td>Stelzer-Braid 2012</td>
<td>37</td>
<td>11.4</td>
<td>PCR</td>
<td>NR</td>
<td>64.3</td>
<td>34.8</td>
<td>NR</td>
</tr>
<tr>
<td>Asner 2012</td>
<td>43</td>
<td>&lt;18</td>
<td>PCR</td>
<td>NR</td>
<td>60.5</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Hoek 2013</td>
<td>24</td>
<td>29.7</td>
<td>PCR</td>
<td>NR</td>
<td>33.3</td>
<td>NR</td>
<td>NR</td>
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<tr>
<td>Etherington 2013</td>
<td>180</td>
<td>26.5</td>
<td>PCR</td>
<td>NR</td>
<td>9.7</td>
<td>NR</td>
<td>NR</td>
</tr>
</tbody>
</table>

Abbreviations: PEx: pulmonary exacerbation; NR: not reported; PCR: polymerase chain reaction; NASBA: nucleic acid sequence based amplification.

* Age is given as mean or median, whichever quoted in the paper.

# Quoted figures reflect a 6-month follow-up period – annual incidence likely to be higher.
Chapter Four

Study Aims and Research Questions
4.0 Study Aims and Research Questions

4.1 Aims
To date there has been no large-scale, prospective observational study of respiratory viruses in adults with CF. The primary aim of this research project was to determine the incidence and clinical impact of viral respiratory infection (VRI) among adults with CF. The following research questions were formulated.

4.2 Primary Research Questions

- What is the incidence of VRI in adults with CF?
- What is the prevalence of VRI in adults with CF during a pulmonary exacerbation and during periods of clinically stability?

4.3 Secondary Research Questions

- What is the effect of VRI on lung function, symptoms and inflammatory markers in adult CF patients?
- Do symptoms of an upper respiratory tract infection (URTI) predict laboratory identification of a respiratory virus?
- Are specific rhinovirus sub-types associated with worse clinical outcomes in adults with CF?
- Do changes in the weather affect the epidemiology of respiratory viruses in CF?
- Does VRI lead to changes in bacteria identified in the sputa of patients with CF and is there an associated change in the respiratory microbiome?
Chapter Five

Methods
5.0 Methods

5.1 Study Overview
One hundred adults with CF were recruited to a prospective, single-centre observational study. Participants were seen routinely every two months for twelve months. Between these scheduled visits, patients were asked to contact the investigators by telephone as soon as possible if they developed symptoms suggestive of a cold or pulmonary exacerbation. At each visit, patients underwent a study visit as described in Section 5.4 below. Additional study visits were conducted within 48 hours of patients contacting the unit to report the onset of acute respiratory symptoms. The study was conducted between 22\textsuperscript{nd} December 2010 and 30\textsuperscript{th} March 2012.

5.2 Participants
Participants in the study were identified from the database of patients with CF attending the Manchester Adult Cystic Fibrosis Centre (MACFC). All eligible patients were sent a patient information sheet and letter of invitation to participate in the study in December 2010. The first 100 patients to respond were recruited. Enrolment took place between 22\textsuperscript{nd} December 2010 and 30\textsuperscript{th} March 2011 and participants were each followed for twelve months from their date of recruitment. As a result the study took place over two consecutive winters. Participants were recruited during a standard clinic appointment or hospital admission at MACFC.

Inclusion criteria:
- Patients with cystic fibrosis attending Manchester Adult CF Centre.
- Age \(\geq\)18 years.
- Capable of providing written informed consent.
- Able and willing to provide the required samples and meet the two-monthly visit schedule.

Exclusion criteria:
- Previous lung transplantation.
5.3 Ethical Considerations
All patients gave written informed consent. Ethical approval was granted by the Greater Manchester West NHS Research Ethics Committee (reference 10/H1014/71). Approval was also granted by the Research and Development Department at the University Hospital of South Manchester NHS Foundation Trust. The study was registered with clinicaltrials.gov (reference NCT01238081).

5.4 Study Visits
A medical history and physical examination was performed at recruitment. The following samples and measurements were undertaken at each visit:

- Height (first visit only)
- Weight
- Spirometry – forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC)
- Upper respiratory tract infection (URTI) symptom score
- Pulmonary exacerbation (PEx) score
- Record of development of any new comorbidities or complications of CF
- Record of number of days in hospital in previous 2 months
- Record of number of days on antibiotic treatment in previous 2 months
- Record of number of days on antiviral treatment in previous 2 months
- Record of influenza vaccination within last 2 months
- Sputum sample conventional bacterial and fungal culture
- Sputum sample for virology and culture-independent bacteriology
- Nose and throat swabs for virology
- Venous blood sample for full blood count (FBC), C-reactive protein (CRP) level and serum storage

5.5 Spirometry
Spirometry was performed using the Vitalograph 2150 volumetric spirometer (Vitalograph Ltd, Buckingham UK) in accordance with the 2005 American Thoracic Society - European Respiratory Society spirometry guidelines. The forced
expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) were measured at each visit.

5.6 Upper Respiratory Tract Infection (URTI) Symptom Score

There is no universally agreed definition of upper respiratory tract infection (URTI) and there is no validated URTI symptom score for use in patients with CF. In this study, URTI was defined using the symptom scoring system described by Johnston et al in an observational study of respiratory viruses in children with asthma. The Johnston score was selected for use in this study as it focuses solely on upper airway symptoms, in contrast to most other scores reported in the literature. A further consideration was the ease of use within a routine CF clinic setting.

Each of the following symptoms was given a score between 0 and 3 by the patient (0 = none, 1 = mild, 2 = moderately severe and 3 = severe):

- Runny nose
- Sneezing
- Blocked or stuffy nose
- Itchy, sore or watery eyes
- Sore throat
- Hoarse voice
- Fever or feeling shivery
- Headache or face aches
- Aches or pains elsewhere

The score has a maximum value of 27 and URTI was defined in the original paper by Johnston et al as a score of ≥4.

5.7 Definition of Pulmonary Exacerbation of CF (PEx)

There is no universally agreed definition of a pulmonary exacerbation of cystic fibrosis (PEx). This study used a minor modification of the definition described by Fuchs et al in 1994. The original Fuchs definition required treatment with parenteral antibiotics but this study also included exacerbations treated with oral
antibiotics to reflect current practice at MACFC more closely. The Fuchs criteria were chosen for inclusion in this study as they have been widely used in previous clinical research studies in CF. The Fuchs criteria also have the advantage of providing a quantitative measure of the symptom burden associated with a clinical event.

In this study, PEx was defined as the decision of a CF physician to commence antibiotic therapy for pulmonary symptoms AND any four of the following features:

- Change in sputum
- New/increased haemoptysis
- Increased cough
- Increased dyspnoea
- Malaise/lethargy/fatigue
- Temperature $>38^\circ$C
- Anorexia
- Sinus pain or tenderness
- Change in sinus discharge
- New physical findings in the chest
- Fall in spirometry by $>10$
- New radiographic findings

5.8  Microbiological Specimen Collection & Transport

Nose and throat swabs were taken in accordance with Health Protection Agency standards. Sterile flocked swabs were used in each case. Nose swabs were passed high into the nares and throat swabs were passed firmly over the tonsils and posterior pharynx before being placed in a vial of viral transport medium (Microtest™ M4® Transport, Remel, USA).

Sputum was expectorated spontaneously by the patient into sterile universal containers. Where possible, the patient was asked to produce two separate samples
to allow for both virological and bacteriological analysis. Where only one specimen could be provided, the specimen was divided equally into two portions by a member of the healthcare team under aseptic conditions.

All microbiology samples were transported at room temperature to the microbiology laboratory at the University Hospital of South Manchester (UHSM). Samples for virological analysis were transported within 24 hours to the virology laboratory at Central Manchester University Hospitals. Samples were stored at 4°C prior to analysis.

5.9 Virology
Identification of respiratory viruses from sputum, nose- and throat-swabs was performed using a series of duplex and triplex real-time polymerase-chain reaction (PCR) assays which are described in detail below.

5.9.1 Nucleic Acid Extraction
Respiratory samples, either sputum or the viral transport medium in the case of swabs, were mixed in a 1:1 ratio with AL lysis buffer (Qiagen, Hilden, Germany) to give a total volume of 600 µl. The samples were then inactivated at 80°C for 20 minutes. For sputum samples, the above process was conducted in a class 1 safety cabinet within a category 3 laboratory.

Extraction of nucleic acids from the samples was performed using the fully automated QIAamp® Virus Biorobot® MDx machine (Qiagen, Hilden, Germany). This process involves exposing each sample to proteases, buffers and high temperatures to ensure lysis of the cells within the specimen and inactivation of any contaminating ribonucleases. The instrument adsorbs nucleic acids within the sample onto a silica membrane which then undergoes a two-step washing procedure to purify the extracted nucleic acids. The purified nucleic acids are eluted onto a plate designed for the subsequent PCR amplification assay.
5.9.2 **Real Time Polymerase Chain Reaction (PCR) Technique**

Following nucleic acid extraction, specimens were prepared for PCR amplification by mixing with appropriate forward and reverse primers, gene probes, buffers and enzymes (including *Thermophilus aquaticus* (Taq) polymerase and reverse transcriptase as appropriate). Details of the primers and probes used are given in Table 5.1. The process of mixing the components was performed using the fully automated Biorobot® Universal System (Qiagen, Hilden, Germany) in line with the manufacturer’s instructions.

PCR was undertaken in a series of duplex/triplex reactions:

- Respiratory syncytial virus & metapneumovirus
- Adenovirus & rhinovirus
- Parainfluenza 1, 2 & 3
- Influenza A & B
- Swine lineage influenza – H1 and N1 differentiation

The PCR amplification process itself was performed using the Taqman® SDS 7500 system (Life Technologies, California, USA). The assay requires a total volume of 30 µl. The PCR reaction involved: 15 minutes at 50°C, 2 minutes at 95°C followed by 45 cycles of 95°C for 15 seconds then 60°C for 1 minute (PCR amplification step). Each PCR run included a positive control specimen to exclude false negatives due to inhibitor compounds within specimens. Control specimens were obtained from the National Institute for Biological Standards & Control (NIBSC, Potters Bar).

5.9.3 **Definition of Viral Respiratory Infection**

Viral respiratory infection was defined as the identification of viral nucleic acid in one or more respiratory sample using the PCR method described above. The number of PCR cycles completed before target DNA can be detected is known as the cycle threshold (CT). Lower CT values reflect a stronger degree of PCR positivity and suggest a greater viral load within the specimen. In this study, CT values of <45 were regarded as positive and consistent with VRI. Positive results were further categorised into strong positive (CT <40) and weak positive (CT 40-45) results.
Table 5.1  Primers and probes for respiratory virus polymerase chain reaction assays

<table>
<thead>
<tr>
<th>Virus</th>
<th>FORWARD</th>
<th>REVERSE</th>
<th>PROBE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza A</td>
<td>GAGTCTTCTAACMGAGGTCGAAACGTA</td>
<td>GGGCAACGGTGAGCGGTRAA</td>
<td>FAM–TCCTGTCACCTCTGAC–MGB NFQ</td>
</tr>
<tr>
<td>Influenza B</td>
<td>AATGTYYCAAATATCAGACAAAAACAAAAA</td>
<td>CTGTGTCCCCTCCCAAGAAGAA</td>
<td>VIC–AATTAAGCAGACCATCCC–MGB</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>(1)GACARGGTGTGAAGAGCC</td>
<td>CAAAGTAGTYGGTCCCCATCC</td>
<td>VIC–TCCTCCGCCCCCTGAATGYYGCTAA–TAMRA</td>
</tr>
<tr>
<td></td>
<td>(2)GACATGGTGTGAAGACYC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus</td>
<td>GACATGACTTTTCGAGGTCGATCCCATGGA</td>
<td>CCGGCTGAGAAGGGTGTCGCAGGTA</td>
<td>FAM–CACC CGGC GTCAT–TAMRA</td>
</tr>
<tr>
<td>RSV-A</td>
<td>GTGCAAGGGCAGTGTGATTTAC</td>
<td>CACCCAAATTTTTGGGCATATTCC</td>
<td>FAM–ACA CTTTGTCCCCATTTCTGC–MGB</td>
</tr>
<tr>
<td>RSV-B</td>
<td>TTAGGCAAGTAAATGCTAAGATG</td>
<td>CCTCCCAACTTCTGTCATACTC</td>
<td>VIC–TGGTCGGAGAAATGGGTCTGAATCCTGG–TAMRA</td>
</tr>
<tr>
<td>MPV ALT</td>
<td>CAACAACATAATGCTAGGACATGTATC</td>
<td>CCGAGAACAACACTAGCAAGTTG</td>
<td>VIC–TGGTCGGAGAAATGGGTCTGAATCCTGG–TAMRA</td>
</tr>
<tr>
<td>MPV N</td>
<td>CATATAAGCATGCTATAATTTAAAGAGTCTC</td>
<td>CCTATTCTGCAGCATATTGTAATCAG</td>
<td>VIC–TG YAATGATGAGGGTGTCACCTGGTTG–TAMRA</td>
</tr>
<tr>
<td>Parainfluenza 1</td>
<td>ACAGATGAAATTTTCAGTGCTACTTTAGT</td>
<td>GCTCTTTTTAATGCCATATTATCATTAGA</td>
<td>NED–ATGGTAATAAATGCAGACTCGT–MGB</td>
</tr>
<tr>
<td>Parainfluenza 2</td>
<td>CTTGAAACCACTTTACTTACAGTGATGGA</td>
<td>CCTCCYGGTATRCAGTGACTGGA</td>
<td>VIC–TCAACTGCAAAGCT–MGB</td>
</tr>
<tr>
<td>Parainfluenza 3</td>
<td>ACAGTGGATCAGATTGGGTCAT</td>
<td>ATGTTTGTGGTGTCATTTCTGCT</td>
<td>VIC–TGGTCCTCAACAGAGCT–MGB</td>
</tr>
</tbody>
</table>

RSV: respiratory syncytial virus; MPV: metapneumovirus
5.10 Rhinovirus Sub-Typing

A sub-set of rhinoviruses identified in the study were subjected to genetic sequencing of the 5’ untranslated region (UTR) with the aim of assigning them to the appropriate rhinovirus species and serotype. Specimens were selected for sequencing according to the following rationale:

1. All sputum samples positive for rhinovirus with a CT value of <40 were selected for inclusion in the first batch of sub-typing;

2. The second batch of samples included sequential rhinoviruses identified in sputum, nose- or throat-swabs from patients with consecutive study visits positive for rhinovirus, regardless of CT value. These samples were selected to differentiate persistent infection with the same rhinovirus species from sequential infection with different species.

The sequencing process followed a modification of the method described previously by Lee et al. and is summarised in Figure 5.1. Primers were sourced from Life Technologies (California, USA) and primer details are given in Table 5.2. RNase inhibitor was sourced from Promega (Wisconsin, USA). Other PCR reagents were sourced from Invitrogen (Life Technologies, California, USA) unless specified as being sourced from Qiagen (Hilden, Germany) in the method below.

Table 5.2 Primers used in rhinovirus sequencing assays

<table>
<thead>
<tr>
<th>Primer</th>
<th>Function</th>
<th>Primer Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Forward primer in all assays</td>
<td>CAAGCACCCTCTGTGTYWCCCC</td>
</tr>
<tr>
<td>P3</td>
<td>Reverse primer in first-round PCR</td>
<td>ACGGACACCCAAAGTAG</td>
</tr>
<tr>
<td>P2-1</td>
<td>Reverse primer in semi-nested PCR and sequencing reactions</td>
<td>TTAGCCACATTCAGGGGC</td>
</tr>
<tr>
<td>P2-2</td>
<td>Reverse primer in semi-nested PCR and sequencing reactions</td>
<td>TTAGCCACATTCAGGGAGCC</td>
</tr>
<tr>
<td>P2-3</td>
<td>Reverse primer in semi-nested PCR and sequencing reactions</td>
<td>TTGCGCCATTCAGGGG</td>
</tr>
</tbody>
</table>
5.10.1 Rhinovirus Sub-Typing – Nucleic Acid Extraction

Total nucleic acids were extracted from respiratory specimens using the QIAamp® Virus Biorobot® MDx machine (Qiagen, Hilden, Germany) as described in Section 5.9.1 above.

5.10.2 Rhinovirus Sub-Typing – Reverse Transcriptase Step

Following nucleic acid extraction, a reverse transcriptase (RT) reaction was performed to convert the viral RNA to cDNA. The following reagents were used in the RT step:

- 10x PCR buffer: 2.0 μl
- MgCl₂ (50mM): 3.0 μl
- dNTPs (10mM): 3.0 μl
- Random Hexamer Mix: 1.0 μl
- RNase inhibitor: 0.2 μl
- Moloney Murine Leukaemia Virus (M-MLV) RTase: 0.5 μl
- Nucleic acids extracted from clinical specimen: 11.1 μl

The RT reaction was cycled through the following program: 22°C for 10 min; 37°C for 45 min followed by 99°C for 5 min.

5.10.3 Rhinovirus Sub-Typing – First Round PCR

Following the reverse transcriptase step, a conventional first round PCR assay was performed to amplify the 5’ UTR using the following ingredients:

- 10x Qiagen buffer: 2.5 μl
- dNTPs (2.5 mM): 2.0 μl
- Primer P1-1 (15 mM): 1.5 μl
- Primer P3-1 (15 mM): 1.5 μl
- Qiagen HotStar Taq Polymerase (5 U/μl): 0.25 μl
- Water: 14.25 μl
- cDNA (i.e. product of RT step): 3.0 μl
The first round PCR assay was run on the following program:

1. 95°C for 15 min;
2. 16 cycles of: 95°C for 20 sec; 68°C to 52°C (touchdown by 1°C per cycle) for 30 sec; 72°C for 40 sec;
3. 24 cycles of: 95°C for 20 sec; 52°C for 30 sec; 72°C for 40 sec.

**5.10.4 Rhinovirus Sub-Typing – Semi-Nested Second Round PCR**

A semi-nested PCR was performed with 1.5 µl of the first round PCR product using one forward primer (P1) and three reverse primers (P2-1, P2-2 and P2-3) to amplify the 5’ UTR further. The semi-nested PCR approach maximises the coverage of the target nucleic acid region by increasing the number of DNA fragments available to be incorporated into the subsequent sequencing reaction. The full semi-nested PCR reagents were:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Qiagen buffer</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>dNTPs (2.5 mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>Primer P1-1 (15 mM)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Primer P2-1 (15 mM)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Primer P2-2 (15 mM)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Primer P2-3 (15 mM)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Qiagen HotStar Taq (5 U/μl)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>Water</td>
<td>12.75 μl</td>
</tr>
<tr>
<td>First round PCR product</td>
<td>1.5 μl</td>
</tr>
</tbody>
</table>

The above PCR assay was run on the following program: 95°C for 15 min followed by 30 cycles of 95°C for 20 sec, 52°C for 30 sec and 72°C for 40 sec.

Electrophoresis using a 1.5% agarose gel was performed to ensure successful cDNA amplification and to guide dilution of the PCR products to achieve optimal sequencing resolution. The second round PCR assay product was approximately 300 base pairs in size.
5.10.5 Rhinovirus Sub-Typing – Sequencing Reaction

The second round PCR product was “cleaned up” by adding 3 μl ExoSAP (exonuclease plus shrimp alkaline phosphatase) to remove excess primers and deoxyribonucleotides which would otherwise interfere with the sequencing reaction. The ExoSAP-treated samples were then incubated at 37°C for 15 min and at 80°C for 15 min.

Following the ExoSAP procedure, the second round PCR products were diluted according to the strength of the relevant electrophoresis band. Most specimens were diluted 1:6 with sterile water; three were diluted 1:10; one was diluted 1:2 and one was diluted 1:15.

Four separate sequencing reactions were performed using the diluted, cleaned-up PCR products. Each sequencing reaction utilised a separate primer (P1, P2-1, P2-2 or P2-3). The sequencing reaction mixes contained the following components:

- Big Dye v1.1 2.0 μl
- 5x Qiagen Buffer 2.0 μl
- Primer 2.0 μl
- Water 12.0 μl
- Diluted second round PCR product 2.0 μl

The sequencing reaction program was run on the following program: 96°C for 1 min followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

5.10.6 Rhinovirus Sub-Typing – Sequencing Product Purification

The sequencing reaction products were purified by adding 50 μl of 100% ethanol and 2 μl of 3M sodium acetate to each specimen before being centrifuged at 2000 g for 20 min. The supernatant was discarded and 150 μl of 70% ethanol was added to each well prior to centrifugation at 2000 g for a further 5 min. This step was repeated before adding 20 μl of Hi Di™ formamide to each specimen to re-suspend the sequencing product.
5.10.7 **Rhinovirus Sub-Typing – Sequencing Procedure**

Genetic sequencing of the rhinovirus 5’ UTR was performed using the capillary-based Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, California, USA). The Genetic Analyzer instrument detects fluorescence from the dideoxyribonucleotides that are labelled as part of the sequencing reaction described above. Different bases produce fluorescence of a different frequency which is detected by a camera within the instrument. The in-built Genetic Analyzer software then converts the fluorescence peaks into raw digital sequencing data.

5.10.8 **Rhinovirus Sub-Typing – Data Analysis**

The raw sequencing data was edited manually using Sequencher v4.7 (Gene Codes Corporation, Michigan, USA) to resolve any mis-called bases. Individual DNA fragments were trimmed to match the length of the reference rhinovirus strains reported by Lee et al.\(^\text{385}\) The resulting sequences were compared with the National Center for Biotechnology Information (NCBI) GenBank database using BLASTn software. A local BLAST database was also created using the sequences reported by Lee et al\(^\text{385}\) to allow a further means of identifying individual rhinovirus strains. A phylogenetic tree was created in ClustalW\(^\text{386}\) using the neighbour-joining method with a bootstrap value of 500 to assign individual sequences to the appropriate rhinovirus major group (i.e. rhinovirus A, B or C). Phylogenetic trees were edited in MEGA v5.1.\(^\text{387}\) Sequences identified as part of this study were deposited with the NCBI GenBank database under accession numbers KF112083-KF112142.
Figure 5.1  Overview of the rhinovirus sequencing method
5.11 Bacteriology

5.11.1 Conventional Sputum Culture
Sputum samples were processed at the UHSM microbiology laboratory in accordance with the UK CF Trust’s laboratory standards. Isolates of *Burkholderia cepacia* complex, colistin-resistant *Pseudomonas aeruginosa* and certain other gram-negative organisms were sent to the UK Health Protection Agency Reference Laboratory at Colindale, London for full identification.

5.11.2 Culture-Independent Sputum Bacteriology
Chapters 10 and 11 detail an investigation into the bacterial diversity of CF sputum samples using two culture-independent, molecular techniques. The first such technique was ribosomal intergenic spacer analysis (RISA) which has previously been little investigated in the context of CF. The second method employed was 16S rRNA gene pyrosequencing which is well established as a technique for the investigation of bacterial diversity in the respiratory tract. Culture-independent bacteriology was performed on an aliquot of the same sputum sample used for virological analysis at any given study visit. For these analyses, total nucleic acids were extracted from sputum using the method described in Section 5.9.1 above. Nucleic acid samples were sent by post (next-day delivery) to Prof Mahenthiralingam’s laboratory at Cardiff University and were stored at -80°C prior to further analysis.

5.11.3 16S Ribosomal RNA Gene Pyrosequencing
16S rRNA pyrosequencing is considered the current “gold-standard” method for determining the bacterial diversity of clinical or environmental specimens. This technique involves the PCR amplification of the fragments of DNA within a sample followed by the sequencing of either a portion or the entirety of the bacterial 16S ribosomal gene. The 16S gene is found in the DNA of all bacteria and has sufficient similarity across species to allow reliable PCR amplification using so-called “universal” primers. There is also a degree of variability in the genetic code of the 16S gene between different bacterial species. This variability allows researchers to match the observed genetic sequences in a sample with details of the 16S gene contained in one of several online metagenomic databases. A profile of all the
bacterial species within a sample can then be inferred from the 16S gene sequences detected. The number of sequences identified is termed the number of “reads.”

16S rRNA gene pyrosequencing was performed on a sub-set of sputum samples provided during the study. The criteria by which it was decided which samples were to undergo sequencing are described in Chapters 10 and 11. The 16S rRNA gene pyrosequencing assays were performed on a commercial basis by Research and Testing Laboratory Inc. (Lubbock, Texas, USA). Samples were sent by courier from Cardiff to Lubbock.

The methods used by Research and Testing Laboratory have been described in the scientific literature and are available on the company’s website (http://researchandtesting.com). The technique has been used in the investigation of diabetic foot ulcers, bovine faecal samples and a number of environmental specimens. More recently, the Research and Testing methodology has been applied successfully to respiratory samples from patients with CF.

Extracted sputum nucleic acids were amplified using the universal 16S primers 530F (5’-GTG CCA GCM GCN GCG G) and 1100R (5’-GGG TTN CGN TCG TTG). The Genome Sequencer FLX+ System was used to sequence the V1 to V3 regions of the bacterial 16S ribosomal gene. Observed sequences were subjected to bioinformatic analysis in line with a pre-specified, in-house algorithm in order to “de-noise” the data. “De-noising” refers to the removal of sequences with low quality signals, those clearly due to artefact and also any chimeric products of the PCR amplification process. All sequences less than 250 base pairs in size were excluded. The sequences remaining after these quality-control steps were then evaluated using BLASTn software and compared with the NCBI GenBank database (http://ncbi.nlm.nih.gov). Research and Testing Laboratory provided both the raw data and the product of their in-house bioinformatic analysis. Figures 5.2 and 5.3 summarise the pyrosequencing methodology and the bioinformatic analysis respectively.

In order to quantify bacterial diversity, the Shannon Index was calculated for each sample based on the relative proportion of bacterial taxa within each specimen. The
Shannon Index reflects both the species richness (i.e. how many different species there are within the sample) and the evenness (i.e. the extent to which samples are dominated by one or more species present). The Shannon Index (H) is calculated according to the formula:

\[ H = -\sum (P_i \times \ln P_i) \]

where \( P_i \) represents the proportion of the total number of sequences observed for each individual species within a given sample.
Figure 5.2 Overview of 16S rRNA gene pyrosequencing technique using the 454 FLX+ Genome Sequencer (Roche, Basel, Switzerland)

PCR: polymerase chain reaction
FLX+ Sequencer produces raw data

Sequencing “noise” removed in 4 stage process:
1. Reads with poor quality signals excluded
2. Sequences clustered at 4% divergence → singleton clusters removed
3. Chimeric sequences detected & removed using UCHIIME software
4. Sequences shorter than 250bp or <50% expected length excluded

Data output in .FASTA format (+ supplementary quality and mapping files)

Sequences clustered at 0% divergence to identify Operational Taxonomic Units (OTUs)

Each cluster compared with NCBI GenBank using BLASTn

Sequences classified according to match with GenBank sequences at appropriate taxonomic level:
- >97% identity → species
- 95 – 97% identity → genus
- 90 – 95% identity → family
- 85 – 90% identity → order
- 80 – 85% identity → class
- 77 – 80% identity → phyla
- <77% identity → discarded

Figure 5.3 Overview of post-16S rRNA gene pyrosequencing bioinformatic analysis

NCBI: National Center for Biotechnology Information
BLASTn: Basic local alignment search tool (nucleotides)
5.11.4 Ribosomal Intergenic Spacer Analysis (RISA)

Whilst 16S rRNA pyrosequencing represents the current gold-standard tool for analysing bacterial diversity, it presents a number of drawbacks. For instance, 16S sequencing is expensive, time-consuming and requires considerable bioinformatic processing. A need has been recognised for a rapid, cheap and widely-available technique for measuring the respiratory microbiome. Chapter Ten describes the use of ribosomal intergenic spacer analysis (RISA) as one such option for profiling the bacterial communities within CF sputum.

RISA was originally developed for the investigation of environmental samples such as soil. Two small studies have been conducted previously in which RISA was applied to CF sputum samples but neither of these studies compared RISA with alternative techniques for the analysis of bacterial diversity. RISA itself is a PCR-based technique in which primers are used to amplify the region of DNA between the bacterial 16S and 23S ribosomal genes which is often termed the intergenic transcribed spacer (ITS) region. Individual bacterial species have an ITS region of variable size which can therefore be separated out using chromatography or similar techniques.

The first step in the RISA method was to perform total nucleic acid extraction as described in Section 5.9.1 above. Extracted nucleic acids were posted to Prof Mahenthiralingam’s laboratory at Cardiff University where the remainder of the analysis was conducted. The PCR reagents were sourced from Qiagen (Hilden, Germany) and the primers were ordered from Eurofins (Luxembourg). The primers used in the RISA PCR assay were 1406F-TGYACACACGCCGCGG and 23SR-GGGTTBCCCCATTCRG.
The RISA PCR constituents were:

- Water 9.3 μl
- Qiagen 10x buffer 2.5 μl
- Qiagen 5x solution 5.0 μl
- MgCl₂ (25 mM) 1.5 μl
- dNTP (10 mM) 0.5 μl
- Taq polymerase 0.2 μl
- Primers 5.0 μl
- Sample nucleic acids 1.0 μl

The PCR assay was conducted using the following program: 95°C for 5 min; 34 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec followed by 72°C for 5 min.

The RISA PCR product was analysed using the microfluidics-based Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA) platform. 2 μl aliquots of each sample were loaded onto a microfluidic chips for analysis on the Bioanalyzer. The microfluidic chips held a total of 12 samples. Each run of 12 samples also included a well with a “ladder” containing multiple DNA fragments of known molecular sizes. The Bioanalyzer instrument produces a digital profile of all DNA fragments identified within the sample ordered by molecular size. Individual bacterial species produce one or more of these bands of a fixed fragment size. The number of individual bands detected by the Bioanalyzer instrument was termed the RISA band count and was used as a potential marker of bacterial diversity within each sample. The digital output of the Bioanalyzer was uploaded into Gel Compar II (Applied Maths Inc, Texas, USA) for processing and analysis. Cluster analysis using Pearson correlation coefficients was performed to allow the construction of dendrograms to determine the degree of similarity between individual RISA profiles.
5.12 Inflammatory Markers & Serum Storage
At each visit, venous blood was taken and sent for measurement of C-reactive protein (CRP) levels and full blood count (FBC). These samples were processed in accordance with routine laboratory procedures at UHSM. A further venous blood sample was collected and centrifuged at 3000 rpm for ten minutes to allow collection of serum for storage at -80°C and possible future analysis.

5.13 Meteorological Data
Meteorological data was supplied by the Met Office, Exeter, UK in order to investigate the effect of the weather on the incidence of VRI and pulmonary exacerbations in the study cohort. Climate data was measured at Hulme Library weather station in Manchester, UK (altitude 33m, latitude 54.47 north and longitude 2.25 west). Temperature and relative humidity recordings were included for the entire duration of the observational study.

Hourly ambient temperature readings were provided allowing the calculation of daily mean, minimum and maximum temperatures in °C. Daily mean relative humidity (RH) was also recorded. Values for the mean temperature and RH over the seven days prior to each study visit were also calculated to investigate whether there was a lag effect of these variables.

All study participants living in the north-west of England were included in analysis of the meteorological data. The distance of patients’ homes from Hulme Library weather station was calculated using postal codes. Statistical analysis of the climate data utilised generalised estimating equations with a logistic regression structure as described below.
5.14 Statistical Analysis

5.14.1 Power Calculation
A power calculation was performed prior to the start of the study using McNemar’s test. It was calculated that 94 patients would be required to give 80% power to detect a 20% difference in the proportion of virus-positive events amongst exacerbation visits compared with stable visits, assuming that at least a third of visits are virus positive. A recruitment target of 100 patients was set to allow for patient drop-out.

5.14.2 General Approach to Statistical Analysis
The longitudinal design of this study required an approach to statistical analysis that took into account multiple data points for each patient. Furthermore, patients in this study had variable numbers of visits which took place at varying intervals given the unpredictable nature of pulmonary exacerbations.

Data analysis was performed using SPSS® version 20.0 (IBM, New York, USA). All analysis used two-sided tests and the conventional 0.05 level of significance was assumed unless otherwise stated. Baseline demographic data are presented as mean (standard deviation) for data with a normal distribution and median (inter-quartile range) for non-normally distributed data. Full details of the statistical analyses used are presented in the context of the results chapters that follow in this thesis but a summary of the methods used is presented below.

1. The incidence of VRI and determination of risk factors for VRI was assessed using Poisson regression models (see Chapter 6). This analysis allows for the presence of repeated observations from the participating individuals and also variable durations of follow-up.

2. The association between VRI and clinical outcomes was determined using generalised estimating equation (GEE) models. GEE also takes account of repeated observations from individuals. The presence of VRI was treated as a binary predictor variable and the models used either a logistic regression structure for categorical dependent variables or a linear
regression structure for continuous variables. Variables of interest were selected in advance of the analysis and were left in the model when reporting the results. GEE models are used in Chapters 6, 7, 8, 9 and 11.

3. The effect of the number of viral infections experienced by individuals on the change in lung function measurements over the course of the study was assessed using a multiple linear regression model. The analysis was adjusted for a number of demographic variables and both univariable and multivariable analyses are presented (see Chapter 7). Multiple linear regression was also used in Chapter 10 to investigate the interaction between measures of sputum bacterial diversity and continuous demographic variables.

4. The diagnostic value of the URTI score in the detection of VRI was assessed using receiver operating characteristic (ROC) curves as described in Chapter 7.

5. One-way analysis of variance (ANOVA) was used to compare groups of study participants identified in the cross-sectional study reported in Chapter 10. The Bonferroni correction was applied to take account of multiple comparisons in this analysis.

6. Missing data was not subject to any form of imputation in the analyses described in this thesis.

7. Data that was not normally distributed was subject to log-transformation prior to statistical analysis where appropriate. This applied primarily to data for C-reactive protein values as reported in Chapters 7 and 9. When data required log-transformation, the geometrical mean is reported for each group.
5.15 Individual Contributions to the Work Leading to this Thesis

The research documented in this thesis was conducted at the Manchester Adult Cystic Fibrosis Centre (MACFC) in collaboration with a number of external institutions and individuals. The author of this thesis (WF) was the principle investigator of the study and was supervised by Dr Andrew Jones and Dr Rowland Bright-Thomas.

Individual contributions from the author, the wider study team and other collaborators are summarised here:

- WF designed the study and wrote the study protocol, consent form, patient information leaflet and case report forms.
- WF completed the Research Ethics Committee and R&D applications and attended the ethics committee meeting.
- WF personally recruited and obtained informed consent from all participants in the study.
- Study visits were completed by WF and other doctors working at the MACFC. WF was responsible for tracking patients’ progress through the study and coordinating completion of study visits.
- WF designed the study database and conducted all data entry.
- Clinical specimens were collected by WF and other members of the MACFC medical, nursing and physiotherapy staff.
- Full blood counts, C-reactive protein levels and sputum bacteriology were conducted by the pathology department at the University Hospital of South Manchester NHS Foundation Trust (UHSM) in line with standard clinical practice.
- Viral PCR assays were conducted at the virology department at Central Manchester University Hospitals (CMUH) in line with routine clinical practice. WF spent two weeks shadowing the virology team to understand and undertake the techniques used including specimen preparation, DNA extraction and polymerase chain reaction assays.
- WF conducted the rhinovirus sequencing laboratory assays under the supervision of Peter Tilston at the virology department, CMUH.
WF conducted the sputum nucleic acid extraction for 178 of the samples included in the bacterial diversity analysis (see Chapters 10 and 11).

WF visited Prof Esh Mahenthiralingam’s laboratory at Cardiff University on five occasions to participate in the laboratory work and bioinformatic analysis reported in Chapters 10 and 11. WF conducted four complete RISA runs (12 samples per run) with the remainder of assays performed by Dr Phil Norville from Prof Mahenthiralingam’s team.

16S rRNA gene pyrosequencing assays were performed commercially by Research & Testing Laboratory Inc, Lubbock, Texas.

Bioinformatic analysis of the RISA and 16S rRNA gene pyrosequencing data was performed by WF in collaboration with Prof Mahenthiralingam, Dr Julian Marchesi and Dr Ann Smith at Cardiff University.

Christophe Sarran, Health Research Scientist at the Met Office, Exeter provided daily meteorological data for the duration of the study period (see Chapter 8).

WF conducted statistical analysis of the clinical, meteorological and microbiological data with advice and support from Dr Julie Morris, Head Medical Statistician at UHSM.
Chapter Six

The Incidence of Viral Respiratory Infections in Adults with Cystic Fibrosis
6.0 The Incidence of Viral Respiratory Infections in Adults with Cystic Fibrosis

6.1 Abstract

6.1.1 Introduction

Viral respiratory infection (VRI) is common in children with cystic fibrosis (CF) and is associated with substantial morbidity. The aim of this study was to determine the incidence of VRI in adults with CF.

6.1.2 Methods

One hundred adults with CF were followed for twelve months. Sputum, nose- and throat-swabs were collected every two months and additionally at onset of a pulmonary exacerbation or upper respiratory tract infection. Polymerase chain reaction assays to identify adenovirus, influenza A&B, human metapneumovirus, parainfluenza 1-3, respiratory syncytial virus and human rhinovirus were performed.

6.1.3 Results

One or more respiratory viruses were detected at 191/626 (30.5%) study visits. Nine episodes were positive for two viruses simultaneously. The overall incidence of identifiable VRI was 1.66 (95% CI 1.39 – 1.92) cases/patient-year. The incidence (95% CI) of individual viruses was: rhinovirus 1.17 (0.95 – 1.39), metapneumovirus 0.28 (0.17 – 0.39), influenza A 0.08 (0.02 – 0.13), adenovirus 0.08 (0.02 – 0.13), parainfluenza 1-3 0.05 (0.01 – 0.1), influenza B 0.05 (0.01 – 0.1) and respiratory syncytial virus 0.04 (0.001 – 0.09) cases/patient-year respectively. Patients experienced a mean (SD) of 2.6 (1.8) pulmonary exacerbations/year. 40% of pulmonary exacerbations were positive for a virus compared with 24% of non-exacerbation visits. Identification of a respiratory virus was associated with an increased risk of pulmonary exacerbation (OR 2.19; 95% CI 1.56 – 3.08; p<0.0001) and need for antibiotic therapy (OR 2.26; 95% CI 1.63 – 3.13; p<0.0001).

6.1.4 Conclusions

VRI is common in adults with CF and is strongly associated with pulmonary exacerbation. Respiratory viruses represent a potential therapeutic target for the prevention of pulmonary exacerbations.
6.2 Introduction
Viral respiratory infections (VRI) such as the common cold are frequently experienced by the general population and are associated with substantial morbidity. Respiratory viruses are of particular significance to people with chronic airways diseases and are implicated in up to 80% of asthma exacerbations.\(^{285}\)

Studies in children with CF suggest an incidence of VRI of up to 5.1 cases per patient-year and link viral infection with increased respiratory symptoms, lung function decline and deteriorating chest radiograph scores.\(^{332,346,348-350}\) As many as 64% of pulmonary exacerbations in paediatric CF populations are associated with VRI.\(^{350}\) Small or retrospective studies in CF adults admitted to hospital with a pulmonary exacerbation have identified a respiratory virus in 8 – 47% of cases.\(^{353,355,397}\) The potential for viruses such as influenza to cause severe respiratory infection in adults with CF has long been recognised.\(^{352}\)

Despite the accepted importance of respiratory viruses in paediatric CF lung disease there has been no large-scale longitudinal study examining the frequency of these pathogens in adults. The prevalence of VRI during clinical stability is unknown among adults with CF. We performed a single-centre, prospective observational study to determine the incidence and clinical impact of VRI in a cohort of adults with CF.

6.3 Methods
6.3.1 Study Overview
Detailed methodology is described in Chapter 5. Briefly, all patients over the age of 18 years with CF attending the Manchester Adult Cystic Fibrosis Centre (MACFC) were invited to participate in the study. The first 100 respondents were recruited. The only exclusion criterion was previous lung transplantation. Recruitment was completed between December 2010 and March 2011. The study was approved by the Greater Manchester West Research Ethics Committee (reference 10/H104/71).

Patients were followed for twelve months or until death or lung transplantation. During follow-up, patients were seen routinely every two months. Between these
scheduled visits, patients were asked to contact the investigators as soon as possible if they developed symptoms of a pulmonary exacerbation (PEx) or upper respiratory tract infection (URTI). An additional study visit was conducted within 48 hours of onset of acute respiratory symptoms where possible. It did not prove possible to make a meaningful comparison between scheduled and “unwell” episodes since patients were frequently unwell when attending for routine visits.

6.3.2 Virological Analysis
At each study visit, sputum, nose- and throat-swabs were collected for virological analysis. Sterile flocked swabs were collected according to established guidelines and transported in viral transport medium at room temperature. In-house polymerase chain reaction (PCR) assays were performed to identify the presence of: adenovirus, influenza A&B, human metapneumovirus, parainfluenza 1-3, respiratory syncytial virus (RSV) and human rhinovirus in each specimen. Cycle threshold (CT) values of <45 were considered positive. Consecutive patients admitted to hospital with a viral exacerbation between October 2011 and March 2012 also provided weekly respiratory samples for repeated virological analysis to determine the duration of PCR positivity.

6.3.3 Clinical Outcomes
Spirometry was performed according to accepted standards using the Vitalograph 2150 volumetric spirometer (Vitalograph Ltd, Buckingham UK). A modified version of the definition described by Fuchs et al was used to determine a pulmonary exacerbation with ≥4 symptoms and a decision by the treating physician to prescribe intravenous or oral antibiotics considered a pulmonary exacerbation.

6.3.4 Statistical Analysis
The sample size was determined using McNemar’s test and assuming significance at the 5% level. A sample size of 94 patients would give 80% power to detect a 20% difference in the proportion of virus-positive events amongst exacerbation visits compared with stable visits, assuming at least a third of visits were positive for a virus. A recruitment target of 100 patients was set to allow for patient drop-out.
Where patients remained positive for the same virus at two or more consecutive visits, it was assumed that this represented persistence of a single illness rather than re-infection. Incidence of viral infection is reported as the average number of virus-positive visits (excluding visits classed as persistent infection) per person-year. The incidence of VRI was calculated using a Poisson regression model offset by the natural log of follow-up duration. *A priori* it was decided to exclude patients with a follow-up duration of <6 months from incidence calculations as it was not felt to be reliable to project annual incidence from such short periods of observation. A multivariable Poisson regression model was used to evaluate demographic variables associated with increased incidence of VRI over the study period.

Assessment of the association between VRI and binary outcomes such as pulmonary exacerbation was made using generalised estimating equations based on a logistic regression model. This analysis takes account of repeated longitudinal data and uses an exchangeable correlation structure allowing for within-subject variability. Data are presented as mean (standard deviation) or median (interquartile range) as appropriate. Statistical analysis was performed using SPSS® version 20.0 (IBM, New York, USA).

### 6.4 Results

#### 6.4.1 Study Participants

One hundred patients were recruited. Baseline demographics of the participants are detailed in Table 6.1 with a comparison to the overall MACFC clinic population and national data from the UK CF Registry Report 2011.91 Study participants were broadly representative of the wider MACFC population with the exception of a relative under-representation of male patients and a lower mean FEV$_1$. The study cohort had a higher rate of *P. aeruginosa* and *B. cepacia* complex infection than the national average in common with the Manchester clinic population at large.

One participant was lost to follow-up and one withdrew from the study after the baseline visit. The remaining 98 patients completed a mean of 6.6 (1.7) study visits with a mean follow-up period of 11.5 (2.0) months. Four of these patients were
followed for <6 months leaving a total of 94 patients included in the VRI incidence calculations. During the study period there were three deaths and one patient underwent bilateral lung transplantation. Uptake of the seasonal influenza vaccination in the autumn prior to the start of the study was 89% amongst the study cohort.

### Table 6.1 Baseline demographics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Virology Study Cohort</th>
<th>Overall MACFC Population</th>
<th>UK CF Registry 2011 (Adults)</th>
<th>p value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>100</td>
<td>363</td>
<td>4993</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 (23-36)</td>
<td>27 (23-35)</td>
<td>-</td>
<td>0.828</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.5 (19.7-23.2)</td>
<td>21.5 (19.7-23.3)</td>
<td>21.9</td>
<td>0.762</td>
</tr>
<tr>
<td>Best FEV₁% in previous year</td>
<td>58.9 (41-77)</td>
<td>65.4 (43-66)</td>
<td>65.9</td>
<td>0.043</td>
</tr>
<tr>
<td>Male (%)</td>
<td>48</td>
<td>60</td>
<td>54.3</td>
<td>-</td>
</tr>
<tr>
<td>Chronic <em>P. aeruginosa</em> infection (%)</td>
<td>73</td>
<td>74</td>
<td>55.8</td>
<td>-</td>
</tr>
<tr>
<td>Chronic <em>B. cepacia</em> complex infection (%)</td>
<td>14</td>
<td>9.9</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>Maintenance azithromycin (%)</td>
<td>95</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Maintenance nebulised antibiotic (%)</td>
<td>85</td>
<td>-</td>
<td>81.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are presented as % or median (interquartile range) as appropriate.
* P values compare the study cohort with the overall MACFC population.
MACFC: Manchester Adult Cystic Fibrosis Centre; BMI: body mass index; FEV₁: forced expiratory volume in 1 second.

### 6.4.2 Identification of Respiratory Viruses

A total of 649 study visits were completed of which 238 (36.7%) met the criteria for PEx. Virology results were available for a total of 626 visits. Of these, 191 (30.5%) were positive for ≥1 respiratory virus. The mean PCR cycle threshold (CT) value for virus-positive specimens was 37.1 (SD 5.8) with lower values indicating greater PCR positivity. The PCR assay was strongly positive (i.e. CT <40 cycles) at 109/191 (57.1%) virus-positive visits. Two viruses were detected at the same visit
on nine occasions giving a total of 200 viruses identified during the study. Rhinovirus accounted for 72.5% of all viruses identified. The breakdown of the remaining viruses detected during the study is shown in Figure 6.1. Details of the dual viral infections are given in Table 6.2.

Figure 6.1  Breakdown of all viruses identified during the study
MPV: Metapneumovirus; RSV: Respiratory Syncytial Virus.

Table 6.2  Combinations of viruses seen in dual viral infection

<table>
<thead>
<tr>
<th>Virus 1</th>
<th>Virus 2</th>
<th>Number of Dual Infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinovirus</td>
<td>Metapneumovirus</td>
<td>4</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Adenovirus</td>
<td>1</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Parainfluenza 3</td>
<td>1</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Influenza A/H1N1</td>
<td>1</td>
</tr>
<tr>
<td>Influenza A/H1N1</td>
<td>Influenza B</td>
<td>1</td>
</tr>
<tr>
<td>Influenza A (unidentified)</td>
<td>Metapneumovirus</td>
<td>1</td>
</tr>
</tbody>
</table>
6.4.3 Incidence of Viral Respiratory Infection

The overall incidence of laboratory-confirmed VRI during the study was 1.66 (95% CI 1.39 – 1.92) cases/patient-year. Rhinovirus had the highest incidence at 1.17 (0.95 – 1.39) cases/patient-year followed by metapneumovirus at 0.28 (0.17 – 0.39). The incidence of the remaining less common viruses is presented in Table 6.3 below.

Of the 94 patients included in the VRI incidence calculations, 80 (85%) experienced at least one episode of VRI over the course of the study. There was no significant effect of any demographic variable on the incidence of VRI as analysed using a Poisson regression model. Data for this analysis is presented in Table 6.4.

### Table 6.3 Incidence of viral respiratory infection in adults with CF

<table>
<thead>
<tr>
<th>Incidence (cases/patient-yr)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any respiratory virus</td>
<td>1.66</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>1.17</td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>0.28</td>
</tr>
<tr>
<td>Influenza A</td>
<td>0.08</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>0.08</td>
</tr>
<tr>
<td>Influenza B</td>
<td>0.05</td>
</tr>
<tr>
<td>Parainfluenza Virus 1–3</td>
<td>0.05</td>
</tr>
<tr>
<td>Respiratory Syncytial Virus</td>
<td>0.04</td>
</tr>
</tbody>
</table>

### Table 6.4 Demographic risk factors for viral respiratory infection

<table>
<thead>
<tr>
<th>Rate Ratio#</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female gender</td>
<td>1.07</td>
<td>0.73 – 1.57</td>
</tr>
<tr>
<td>Age</td>
<td>1.00</td>
<td>0.98 – 1.02</td>
</tr>
<tr>
<td>F508del homozygous</td>
<td>1.29</td>
<td>0.88 – 1.84</td>
</tr>
<tr>
<td>Baseline FEV₁</td>
<td>1.01</td>
<td>0.80 – 1.29</td>
</tr>
<tr>
<td>Chronic P. aeruginosa</td>
<td>0.90</td>
<td>0.61 – 1.32</td>
</tr>
<tr>
<td>Children &lt;18yrs at home</td>
<td>1.14</td>
<td>0.71 – 1.81</td>
</tr>
</tbody>
</table>

# Rate ratio calculated using a Poisson regression model
FEV₁: forced expiratory volume in 1 second; CFRD: CF-related diabetes
6.4.4 Persistence of Viral PCR Positivity

Positivity for the same virus at two consecutive visits was seen at a total of 34 study visits, excluding those episodes where rhinovirus sequencing showed the patient to be infected with different rhinovirus strains (see Chapter 9). Of these 34 apparent cases of persistent infection, one was positive for adenovirus and the remainder were positive for rhinovirus.

Persistence of viral PCR positivity was further assessed in eight hospitalised patients involving six cases of rhinovirus and two of RSV infection. Both cases of RSV became PCR-negative by day seven. One case of rhinovirus infection also became PCR-negative within seven days. The minimum duration of PCR-positivity in the other five cases of rhinovirus infection studied ranged from 14 to 40 days.

6.4.5 Association between VRI and Pulmonary Exacerbation (PEx)

Data was available for both exacerbation and virology status in 609 visits. Of these, 229 (37.6%) episodes met the pre-defined criteria for PEx. Patients experienced a mean (SD) of 2.6 (1.8) exacerbations per patient-year. 40% of PEx were positive for a virus compared with 24% of non-exacerbation visits. Identification of a virus at any one visit was associated with an increased risk of PEx (odds ratio 2.19; 95% CI 1.56 – 3.08; p<0.0001). The proportion of virus-positive visits meeting the criteria for PEx varied according to the virus identified. Visits positive for influenza or parainfluenza were associated with PEx in 80% of cases. The equivalent proportions for the other viruses were: RSV 75%; rhinovirus 50.7%; adenovirus 37.5% and metapneumovirus 37.5%.

Overall, 306 (47%) visits resulted in a prescription for antibiotics of which 51% were for oral and 49% intravenous antibiotics. Virus-positive episodes were associated with a greater likelihood of any antibiotic prescription (odds ratio 2.26; 95% CI 1.63 – 3.13; p<0.0001) compared with virus-negative visits and a trend towards increased need for intravenous antibiotics (odds ratio 1.30; 95% CI 0.94 – 1.81; p=0.1).
PCR CT values at virus-positive visits were a significant predictor for meeting the criteria for PEx and requirement for antibiotic therapy. There was a trend towards association between a lower CT value and a need for intravenous antibiotics. Details of these analyses are given in Table 6.5.

Table 6.5   The effect of PCR cycle threshold on pulmonary exacerbation status & need for antibiotic therapy

<table>
<thead>
<tr>
<th>Outcome Variable</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary exacerbation</td>
<td>0.92</td>
<td>0.88 – 0.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Any antibiotic therapy</td>
<td>0.89</td>
<td>0.83 – 0.95</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IV antibiotic therapy</td>
<td>0.96</td>
<td>0.91 – 1.01</td>
<td>0.085</td>
</tr>
</tbody>
</table>

IV: intravenous

6.4.6   Comparison of Specimen Types for Diagnosis of VRI
Complete sets of virology samples (i.e. sputum, nose- and throat-swabs) were collected at 469 visits. In the remaining visits, one or more samples were not sent, most commonly due to the patient being unable to expectorate sputum. 151/469 (32.2%) were positive for ≥1 virus. Of the virus positive visits, 100 (66%) were positive in only one of the three specimens taken, 30 (19.9%) were positive in two samples and only 21 (13.9%) were positive in all three specimens. Sputum had the highest positivity rate of the three specimen types and identified 58.0% of viruses compared with 46.7% for nose-swabs and 43.3% for throat-swabs. The sensitivity of different combinations of sputum, nose- and throat swabs as compared to a “gold standard” of all three specimens is shown in Figure 6.2. As well as picking up a greater number of cases of VRI, sputum samples also identified a wider diversity of viral pathogens than upper airway swabs as illustrated in Figure 6.3. The proportion of cases of each virus detected by the individual specimen types is shown in Table 6.6.

Analysis of CT value data for the different specimen types revealed some important observations. When positive for a virus, sputum samples had a significantly lower
mean CT value than both nose- and throat-swabs, suggesting stronger PCR positivity. The mean (SD) CT value was 33.7 (5.4) for sputum compared with 39.1 (4.5) and 40.1 (3.6) for nose- and throat-swabs respectively (p<0.001 for both comparisons).

![Bar chart showing sensitivity of different combinations of sputum, nose- & throat-swabs for viral respiratory infection in adults with CF.]

**Figure 6.2**  Sensitivity of different combinations of sputum, nose- & throat-swabs for the diagnosis of viral respiratory infection in adults with CF

![Bar chart showing breakdown of viruses identified by each sample type at study visits where only one specimen was positive.]

**Figure 6.3**  Breakdown of viruses identified by each sample type at study visits where only one specimen was positive

RSV: respiratory syncytial virus
### Table 6.6 Percentage of cases of each virus detected by sputum, nose- and throat swabs

<table>
<thead>
<tr>
<th></th>
<th>No. of Cases</th>
<th>Sputum n (%)</th>
<th>Nose Swab n (%)</th>
<th>Throat Swab n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Viruses</td>
<td>199</td>
<td>105 (55.0)</td>
<td>87 (45.5)</td>
<td>77 (40.3)</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>8</td>
<td>4 (50.0)</td>
<td>2 (25.0)</td>
<td>5 (62.5)</td>
</tr>
<tr>
<td>Influenza</td>
<td>11</td>
<td>9 (81.8)</td>
<td>2 (18.2)</td>
<td>3 (27.3)</td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>26</td>
<td>9 (34.6)</td>
<td>11 (42.3)</td>
<td>7 (26.9)</td>
</tr>
<tr>
<td>Parainfluenza 1-3</td>
<td>5</td>
<td>5 (100)</td>
<td>0 (0)</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>Respiratory Syncytial Virus</td>
<td>4</td>
<td>4 (100)</td>
<td>1 (25.0)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>145</td>
<td>76 (52.4)</td>
<td>71 (49.0)</td>
<td>61 (42.1)</td>
</tr>
</tbody>
</table>

### 6.5 Discussion
This chapter presents the results of the first large-scale, prospective study to examine the incidence of viral respiratory infection in adults with CF. The results reveal that VRI is common in this population and is strongly associated with pulmonary exacerbation. The overall incidence in our study of 1.6 cases of laboratory-confirmed VRI per patient-year is consistent with previous studies of children with CF. It is clear from our data that respiratory viruses play an important role in CF lung disease in adults as well as children.

Our study has identified rhinovirus as overwhelmingly the most common viral pathogen in adults with CF. Rhinovirus belongs to the Picornavidae family of viruses and has long been recognised as the principle cause of the common cold. For patients with airway diseases, however, rhinovirus represents a much more sinister problem. Over half of acute asthma attacks and a third of exacerbations of chronic obstructive pulmonary disease (COPD) are associated with rhinovirus...
infection. The predominance of rhinovirus in our study mirrors the findings of observational data in children with CF and supports the hypothesis that rhinovirus is an important pathogen in CF.

Research in the field of CF lung disease to date has had a strong emphasis on bacterial pathogens. Indeed, interest in the role of viral infections in CF first arose from the observation that acquisition of *Pseudomonas aeruginosa* was often associated with a viral upper respiratory tract infection. The interaction between viruses and bacteria in CF lung disease remains poorly understood but it appears likely that there is a degree of synergism between these microorganisms. Chattoraj *et al* have shown that rhinovirus appears to promote the release of *P. aeruginosa* from biofilms in a CF epithelial cell model which could explain an increase in the severity of rhinovirus infections in CF. Co-infection of CF airway cells with both *P. aeruginosa* and rhinovirus has also been shown to lead to impaired production of antiviral cytokines such as interferon which may be of pathogenic importance. In vitro work by other groups has shown that influenza and RSV infection promote adherence of bacteria to respiratory epithelial cells which again supports a role for virus-bacteria interaction.

Further plausibility to the hypothesis that rhinovirus is implicated in bacterial colonisation of the lower airways is provided by the experiments of Papadopoulos and colleagues who demonstrated over a decade ago that rhinovirus is capable of infecting lower airway epithelial cells as well as the those in the nose and throat. This places rhinovirus directly at the site of bacterial infection in CF. Recent work by Kieninger *et al* suggests that rhinovirus infection alone does not produce an enhanced inflammatory response in epithelial cell models which raises the possibility that VRI leads to a flare of bacteria-driven inflammation in CF. Further work is required to unravel the pathophysiology of viral exacerbations in CF and determine the relative extent to which viruses and bacteria contribute to inflammation and lung damage.

This study presents important data on the optimal means of diagnosing VRI in CF. Previous studies have utilised a wide variety of upper and lower airway specimen types for virological diagnosis. There is no agreed “gold standard” sampling
method. We have previously shown that CF sputum is a suitable means for the diagnosis of VRI using PCR techniques. However, data in children with CF suggests that the sensitivity of sputum is lower than that of upper airway samples which presumably reflects the difficulty many children experience in expectorating sputum. Additionally, most previous CF studies did not include sputum sampling in their virological methods. In this study, sputum samples had a higher viral identification rate than both nose- and throat-swabs. Sputum also detected a greater variety of viruses and if the study had relied on upper airway specimens alone then a considerable number of cases of influenza, parainfluenza and RSV infection would have been missed. The data reported in this chapter suggest that a combination of sputum, nose- and throat-swabs is required to maximise diagnostic sensitivity. The relative utility of nasal or bronchoalveolar lavage for viral diagnostics in the adult CF population remains uncertain.

A number of limitations of this study need to be recognised. For several reasons it is to be expected that the incidence figures we have quoted underestimate the true frequency of VRI in adults with CF. As a result, the visits classified as “non-viral” are likely to include both truly virus-free episodes and those at which a viral infection was not detected. Considering this in more detail, the PCR panel of nine respiratory viruses we employed is not exhaustive and it is likely that there were undetected cases of bocavirus, coronavirus and as-yet-undiscovered viral infections in our cohort. In addition, the study design required patients to contact the CF centre at the onset of new respiratory symptoms to arrange an additional visit. It is possible that some instances of colds or mild exacerbations went unreported by participants. A further gap in the dataset is the lack of a record of the number of exacerbations suffered by each patient in the year prior to the study. This variable may have a role in each individual’s risk of future exacerbations but unfortunately was not recorded in a standard way prior to commencement of the study. Finally, the planned strategy of testing sputum, nose- and throat-swabs at each visit was achieved in only 72% of study episodes. Incomplete collection of sample sets was most commonly due to the patient being unable to expectorate sputum. This reflects “real-life” practice, however, where patients often vary in the volume and ease with which they produce sputum.
Allowing for these limitations, our study has demonstrated an important link between VRI and pulmonary exacerbations (PEx) in adults with CF. PEx are increasingly recognised to have a major impact on health outcomes in CF. Several studies have demonstrated that PEx lead to impaired quality of life, declining lung function and, most recently, increased mortality. A number of established CF therapies have been shown to reduce the frequency of PEx such as nebulised antibiotics and mucolytics. Despite widespread availability of these treatments, PEx remain extremely common and hazardous to patients. The need for additional therapies to prevent such infections is clear. Given the link we have demonstrated between viral infection and PEx, it is evident that respiratory viruses, especially rhinovirus, represent a potential target for the prevention and treatment of such exacerbations in the future.

A number of antiviral therapies might be considered for evaluation in future CF clinical trials. Compounds that bind to the rhinovirus capsid, such as the oral drug pleconaril, may be an option for the treatment of VRI in CF. Pleconaril reduces the duration of colds in healthy individuals but development of this drug was suspended due to concerns over its side-effect profile and development of drug resistance. Zinc supplementation has been shown to have a modest effect in reducing the duration and severity of the common cold and may also have a prophylactic effect. A small pilot study in children with CF has suggested that zinc may reduce the number of lower respiratory tract infections and this strategy deserves closer attention. Work on the development of a rhinovirus vaccine is under way but is beset by problems relating to the number of different rhinovirus serotypes. Effective, safe treatments for rhinovirus remain elusive but if achieved would be likely to be effective in the setting of CF lung disease.

In summary, we have demonstrated that respiratory viruses are common pathogens in adults with CF and that they are strongly linked to pulmonary exacerbations. Further research needs to be directed at understanding the pathophysiology of these infections and the interactions between viruses and bacteria within the CF lung. The ultimate aim of this work must be to stimulate a search for effective therapies to prevent virus-induced exacerbations in patients with CF.
Chapter Seven

The Clinical Impact of Viral Respiratory Infection in Adults with Cystic Fibrosis
7.0 The Clinical Impact of Viral Respiratory Infection in Adults with Cystic Fibrosis

7.1 Abstract

7.1.1 Introduction
Viral respiratory infection (VRI) has been associated with increased respiratory symptoms and disease progression in children with cystic fibrosis (CF). The clinical impact of VRI in adults with CF is poorly understood. Chapter 6 has demonstrated that VRI is common in adults with CF and is associated with pulmonary exacerbation (PEx). We aimed to determine the effects of VRI on lung function, symptoms and inflammatory markers.

7.1.2 Methods
One hundred patients were recruited and followed for 12 months or until death/lung transplantation. Details of the study design are given in Chapter 5. Spirometry, symptom scores and inflammatory markers were measured at each visit. Generalised estimating equations and multiple linear regression were used in data analysis, allowing for multiple observations from individual participants where appropriate.

7.1.3 Results
Overall, there was no significant difference in relative fall from baseline FEV₁ at virus-positive visits compared with virus-negative visits (8.7 vs 9.4%, p=0.4). Virus-positive PEx had a less substantial fall in FEV₁ than virus-negative PEx (12.7 vs 15.6%; p=0.04). Number of PEx, but not number of viral infections, was associated with a statistically significant decline in lung function over twelve months (β coefficient -1.79; 95% CI -3.4 to -0.23; p=0.02). Mean C-reactive protein (CRP) levels were higher at virus-positive visits (25.0 vs 17.3 mg/l; p=0.008). Virus-positive visits were associated with higher upper respiratory tract infection (URTI) and PEx symptom scores than virus-negative visits. URTI symptoms had a low predictive value for the diagnosis of VRI (area under receiving operator characteristic curve 0.68; 95% CI 0.54 to 0.81).
7.1.4 Conclusions

Identifiable VRI is associated with increased respiratory symptoms and elevated CRP levels. Pulmonary exacerbations, but not overall incidence of laboratory-confirmed VRI, are associated with long-term decline in FEV$_1$ in adults with CF.
7.2 Introduction

Cystic fibrosis (CF) is a genetic disorder characterised by chronic endobronchial sepsis coupled with progressive decline in lung function. The natural course of CF is punctuated by acute episodes known as pulmonary exacerbations (PEx) which lead to substantial morbidity and an increased risk of death. A clear link between the incidence of viral respiratory infections (VRI) and risk of PEx was documented in Chapter 6. The focus of this chapter centres on the acute and long-term clinical impact of VRIs and their interaction with exacerbations.

Our current knowledge of the clinical effects of VRI in CF largely comes from studies in paediatric patients. Between 76–100% of children with CF who develop a laboratory-confirmed respiratory virus infection experience typical coryzal symptoms such as rhinorrhea or sore throat. Such patients also experience substantial periods of lower airway symptoms such as cough and breathlessness. van Ewijk et al reported that children with CF suffered a mean of 22 days of lower airway symptoms following VRI compared with 13 days in healthy controls. In addition to causing acute ill health it appears likely that VRI is associated with progression of CF lung disease in paediatric cohorts. Several groups have reported that children with frequent VRI have a significant long-term decline in lung function and chest radiograph scores although this has not been a universal finding.

Despite the results of these paediatric studies, the features of VRI in adults with CF are poorly characterised in the literature. Additionally, there is little previous data to indicate the relative virulence of different respiratory viruses. The aim of this chapter was to determine the acute and long-term effects of VRI on lung function, inflammatory markers and symptomatology in an observational study of adults with CF.

7.3 Methods

7.3.1 Study Overview

A detailed description of the methodology is given in Chapter 5. Briefly, all patients over the age of 18 years with CF attending the Manchester Adult Cystic Fibrosis
Centre (MACFC) were invited to participate in the study. The first 100 respondents were recruited. The only exclusion criterion was previous lung transplantation. Recruitment was completed between December 2010 and March 2011. The study was approved by the Greater Manchester West Research Ethics Committee (reference 10/H104/71).

Patients were followed for twelve months or until death/lung transplantation. During follow-up, patients were seen routinely every two months. Between scheduled visits, patients were asked to contact the investigators as soon as possible if they developed symptoms of a pulmonary exacerbation (PEx) or upper respiratory tract infection (URTI). An additional study visit was conducted within 48 hours of onset of acute respiratory symptoms where possible. Clinicians were blinded to the results of virological tests during the study.

7.3.2 Virological Analysis
At each study visit, sputum, nose- and throat-swabs were collected for virological analysis. Sterile flocked swabs were collected according to established guidelines and transported to the regional virology laboratory in viral transport medium at room temperature. In-house polymerase chain reaction (PCR) assays were performed to identify the presence of adenovirus, influenza A&B, human metapneumovirus, parainfluenza 1-3, respiratory syncytial virus (RSV) and human rhinovirus in each specimen. Cycle threshold (CT) values of <45 were considered positive.

7.3.3 Clinical Outcomes
Spirometry was performed at each visit according to accepted standards using the Vitalograph 2150 volumetric spirometer (Vitalograph Ltd, Buckingham UK). Baseline forced expiratory volume in one second (FEV$_1$) was defined as the best value recorded during regular clinic visits over the three months prior to enrolment in the study. End of study FEV$_1$ was taken as the best value recorded in the three months after completion of the follow-up period (or in the month prior to exit from the study in the case of withdrawal, death or lung transplantation). Spirometry was repeated after completion of intravenous antibiotic therapy but not at the end of oral
antibiotic therapy for logistical reasons. “Treatment failure” was defined as a failure of the FEV$_1$ to return to >90% of baseline after a course of intravenous antibiotics given for an exacerbation.

A 27-point upper respiratory tract infection URTI symptom score as described by Johnston et al was recorded at each visit. In the original description, a score of ≥4 was used to define URTI. A modified version of the definition described by Fuchs et al was used to determine a pulmonary exacerbation with ≥4 symptoms and a decision by the treating physician to prescribe intravenous or oral antibiotics being considered a pulmonary exacerbation.

7.3.4 Statistical Analysis
The sample size was determined in advance as described in Chapter 5. Multiple linear regression modelling was used to investigate the relationship between rates of VRI and long-term changes in lung function. Generalised estimating equation (GEE) models were used to investigate longitudinal data, a method which takes into account repeated observations from individuals in the study. GEE models with a logistic regression structure were used for categorical variables while continuous variables were analysed with linear regression-based GEE structures.

The level of significance was set at 0.01 for analysis of the association between VRI and individual symptoms in order to account for multiple comparisons. For all other analyses, the conventional 0.05 level of significance was assumed. Receiver operating characteristic (ROC) curves were used to investigate the diagnostic utility of the URTI score in identifying VRI. For ROC analysis, data from the baseline visit only was used. Data are presented as mean (standard deviation) unless otherwise stated. Statistical analysis was performed using SPSS® version 20.0 (IBM, New York, USA).

7.4 Results
7.4.1 Overview of Study Findings Reported in Chapter Six
One hundred patients were recruited. Two participants withdrew after the baseline visit, three patients died during follow-up and one underwent lung transplantation.
Baseline demographic data for the study participants are provided in Chapter 6. A total of 649 study visits were completed of which 626 had virology results available. 191/626 (30.5%) visits were positive for ≥1 respiratory virus with rhinovirus accounting for 72.5% of all viruses identified. The pre-defined criteria for pulmonary exacerbation (PEx) were met at 229 (37.7%) study episodes. A high rate of antibiotic use was noted during the study with 306 (47%) of visits resulting in a prescription for antibiotics. Of these, 51% were for oral and 49% for intravenous antibiotics.

7.4.2 Acute Changes in FEV$_1$ in Relation to Virology Result
There was no statistically significant difference in the mean fall in FEV$_1$ relative to study baseline when virus-positive visits were compared with virus-negative visits across the whole study. The mean relative fall in FEV$_1$ was 8.7% (standard error 0.90) for virus-positive visits compared with 9.4% (0.89) for virus-negative visits (p=0.4). When only visits meeting the criteria for exacerbation were considered, virus-positive PEx were associated with a less substantial fall in FEV$_1$ than virus-negative PEx (12.7% (SE 1.3) vs 15.6% (1.4); p=0.04).

7.4.3 VRI and Failure of Intravenous Antibiotic Therapy
During the study, 60 patients received intravenous antibiotics for a total of 122 PEx. Pre- and post-intravenous antibiotic spirometry data were available for 90 of these exacerbations of which 31 were virus positive. In 26/90 (29%) exacerbations the FEV$_1$ failed to return to ≥90% of baseline and these were classified as “treatment failures.” Treatment failure occurred in 33.9% of virus-negative PEx compared with 22.6% of virus-positive PEx (odds ratio 0.55; 95% CI 0.1 to 2.7; p=0.46).

7.4.4 Impact of VRI and PEx on Long-term Decline in FEV$_1$
The FEV$_1$ of patients completing the study fell by a mean of 1.48% (SD 12.4) relative to baseline over the course of follow-up. There was no evidence of a strong association between an increasing number of virus-positive visits per year and decline in FEV$_1$ over the study period (β coefficient -0.88, 95% CI -2.7 to +0.95; p=0.3) as shown in Figure 7.1A. The number of PEx per year did show a significant association with decline in FEV$_1$ (see Figure 7.1B). The link between PEx and FEV$_1$
decline persisted after adjustment for baseline demographics and number of viral infections during the study (see Table 7.1).

![Figure 7.1](image)

**Figure 7.1** Relative change in FEV\(_1\) in relation to A) number of virus-positive visits and B) number of pulmonary exacerbations during study follow-up

FEV\(_1\): forced expiratory volume in 1 second; PEx: pulmonary exacerbation

<table>
<thead>
<tr>
<th></th>
<th>Univariable Analysis</th>
<th>Multivariable Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta) coefficient#</td>
<td>95% CI</td>
</tr>
<tr>
<td>PEx per year</td>
<td>-1.65</td>
<td>-3.1 to -0.23</td>
</tr>
<tr>
<td>Virus positive visits per year</td>
<td>-0.88</td>
<td>-2.7 to +0.95</td>
</tr>
<tr>
<td>Age</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sex#</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\# The \(\beta\) coefficient is the gradient of the regression line in a regression model.
PEx: pulmonary exacerbation; FEV\(_1\): forced expiratory volume in 1 second
7.4.5 Effect of VRI on Inflammatory Markers

Virus-positive visits were associated with a significantly higher C-reactive protein (CRP) level and a lower lymphocyte count, although the absolute difference in mean lymphocyte count between groups was small at 0.11 x 10^9/l. There was no significant difference with regard to neutrophil or total white cell count. Further details of these analyses are given in Table 7.2.

Table 7.2 Inflammatory marker levels according to virology result

<table>
<thead>
<tr>
<th></th>
<th>Virus-Positive Visits Mean (95%CI)</th>
<th>Virus-Negative Visits Mean (95%CI)</th>
<th>Adjusted Mean Difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/l)*</td>
<td>10.3 (8.1 – 13.0)</td>
<td>8.4 (6.8 – 10.3)</td>
<td>0.2</td>
<td>0.004</td>
</tr>
<tr>
<td>WCC (x10^9/l)</td>
<td>10.5 (9.8 – 11.2)</td>
<td>10.4 (9.8 – 11.0)</td>
<td>0.08</td>
<td>0.7</td>
</tr>
<tr>
<td>Neutrophils (x10^9/l)</td>
<td>7.63 (7.0 – 8.3)</td>
<td>7.51 (6.9 – 8.1)</td>
<td>0.13</td>
<td>0.6</td>
</tr>
<tr>
<td>Lymphocytes (x10^9/l)</td>
<td>1.83 (1.71 – 1.96)</td>
<td>1.94 (1.71 – 2.07)</td>
<td>-0.11</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* CRP values were log-transformed to correct for non-normal distribution. Geometric means are quoted for CRP values.

GEE: generalized estimating equations; SE: standard error; CRP: C-reactive protein; WCC: white cell count

7.4.6 Symptoms of VRI

Virus-positive visits were associated with a mean (SD) Johnston URTI score of 7.6 (6.3) out of 27 compared with 5.3 (4.6) for virus-negative visits (p<0.001). Mean (SD) Fuchs PEx scores, from a maximum value of 12, were 3.6 (2.3) and 2.9 (2.4) in the virus-positive and negative groups respectively (p<0.001).

The odds of virus-positive visits being associated with each individual component of the symptom scores are shown in Table 7.3. Change in sputum, increased cough, increased dyspnoea, sinus discharge, sore throat and hoarse voice were all significantly more likely at virus-positive visits compared with virus-negative visits. The frequency with which patients complained of each symptom is shown in Figures 7.2 and 7.3. Of note, 41/193 (22.8%) virus-positive visits with full symptom score
data were minimally symptomatic as defined by a total of <4 in both the URTI and PEx scores.

Table 7.3   Odds of individual symptoms being present at virus-positive compared with virus-negative visits

<table>
<thead>
<tr>
<th>PEx Score</th>
<th>OR</th>
<th>95% CI</th>
<th>p value*</th>
<th>URTI Score</th>
<th>OR</th>
<th>95% CI</th>
<th>p value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in sputum</td>
<td>2.04</td>
<td>1.5 – 2.8</td>
<td>&lt;0.001</td>
<td>Runny nose</td>
<td>1.47</td>
<td>1.1 – 2.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Haemoptysis</td>
<td>1.26</td>
<td>0.8 – 2.0</td>
<td>0.3</td>
<td>Sneezing</td>
<td>1.59</td>
<td>1.1 – 2.3</td>
<td>0.013</td>
</tr>
<tr>
<td>↑ Cough</td>
<td>2.08</td>
<td>1.5 – 2.9</td>
<td>&lt;0.001</td>
<td>Blocked nose</td>
<td>1.39</td>
<td>0.99 – 2.0</td>
<td>0.06</td>
</tr>
<tr>
<td>↑ Dyspnoea</td>
<td>1.48</td>
<td>1.1 – 2.0</td>
<td>0.01</td>
<td>Itchy eyes</td>
<td>1.19</td>
<td>0.8 – 1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Malaise</td>
<td>1.20</td>
<td>0.9 – 1.6</td>
<td>0.2</td>
<td>Sore throat</td>
<td>2.52</td>
<td>1.7 – 3.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pyrexia &gt;38°C</td>
<td>1.73</td>
<td>0.95 – 3.2</td>
<td>0.07</td>
<td>Hoarse voice</td>
<td>2.15</td>
<td>1.6 – 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Anorexia</td>
<td>1.72</td>
<td>1.1 – 2.6</td>
<td>0.013</td>
<td>Fever/shivers</td>
<td>1.46</td>
<td>1.02 – 2.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Sinus pain</td>
<td>1.38</td>
<td>0.98 – 1.9</td>
<td>0.06</td>
<td>Headache</td>
<td>1.17</td>
<td>0.9 – 1.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Sinus discharge</td>
<td>2.05</td>
<td>1.4 – 3.0</td>
<td>&lt;0.001</td>
<td>Myalgia</td>
<td>1.19</td>
<td>0.8 – 1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>New signs</td>
<td>0.59</td>
<td>0.3 – 1.06</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall in FEV₁ &gt;10%</td>
<td>0.91</td>
<td>0.6 – 1.4</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New CXR findings</td>
<td>1.3</td>
<td>0.4 – 4.6</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PEx: pulmonary exacerbation; URTI: upper respiratory tract infection; FEV₁: forced expiratory volume in 1 sec; CXR: chest x-ray; OR: odds ratio; CI: confidence interval

# The level of significance was set at 0.01 a priori to take account of multiple comparisons in this analysis.
**Figure 7.2**  Presence of upper respiratory tract infection symptom by virology result

URTI: upper respiratory tract infection

**Figure 7.3**  Presence of pulmonary exacerbation symptoms by virology result

FEV₁: forced expiratory volume in 1 second; CXR: chest xray
7.4.7 The Clinical Impact of Different Respiratory Viruses

The low incidence of individual viruses, other than rhinovirus, hinders an accurate exploration of their relative virulence. In comparison with rhinovirus, the visits positive for other viruses had a similar rate of pulmonary exacerbation (47.8 vs 48.6% respectively). Non-rhinovirus VRI was associated with an increased rate of prescription of intravenous antibiotics (41.3 vs 21.0%; p=0.01). Table 7.4 summarises the other key clinical parameters studied and shows that rhinovirus-positive visits were comparable in severity to visits positive for the combined group of other viruses. Details of the URTI and PEx score components for each individual virus type are given in Appendix 1.

Table 7.4 Clinical outcomes for rhinovirus-positive visits in comparison with visits positive for other viruses

<table>
<thead>
<tr>
<th>Binary Outcomes</th>
<th>Rhinovirus</th>
<th>Other Viruses Combined</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEx (%)</td>
<td>48.6</td>
<td>47.8</td>
<td>1.10</td>
<td>0.60 – 2.06</td>
<td>0.7</td>
</tr>
<tr>
<td>Prescription of any ABx (%)</td>
<td>59.4</td>
<td>65.2</td>
<td>0.92</td>
<td>0.46 – 1.83</td>
<td>0.8</td>
</tr>
<tr>
<td>Prescription of IV ABx (%)</td>
<td>21.0</td>
<td>41.3</td>
<td>0.38</td>
<td>0.18 – 0.80</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Continuous Outcomes*</th>
<th>Rhinovirus</th>
<th>Other Viruses Combined</th>
<th>Adjusted Mean Difference</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URTI score</td>
<td>7.9 (6.6)</td>
<td>6.9 (5.6)</td>
<td>+0.96</td>
<td>-0.96 to +2.89</td>
<td>0.3</td>
</tr>
<tr>
<td>PEx score</td>
<td>3.6 (2.4)</td>
<td>3.6 (2.4)</td>
<td>+0.06</td>
<td>-0.69 to +0.80</td>
<td>0.9</td>
</tr>
<tr>
<td>Acute fall in FEV₁ %</td>
<td>13.5 (10.7)</td>
<td>16.9 (11.8)</td>
<td>-2.09</td>
<td>-5.5 to +1.4</td>
<td>0.23</td>
</tr>
<tr>
<td>log CRP (mg/l)*</td>
<td>2.20 (1.4)</td>
<td>2.21 (1.3)</td>
<td>+0.03</td>
<td>-0.32 to +0.38</td>
<td>0.9</td>
</tr>
<tr>
<td>White cell count (x10⁶)</td>
<td>10.6 (3.9)</td>
<td>10.3 (3.1)</td>
<td>-0.19</td>
<td>-0.99 to +0.61</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Non-rhinovirus VRI was the comparator variable in each analysis.

# Continuous variables are reported as mean (standard deviation)

* CRP values were log-transformed to correct for non-normal distribution.

URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; FEV₁: forced expiratory volume in 1 second; CRP: C-reactive protein
7.4.8  Effect of PCR Cycle Threshold (CT) on Clinical Parameters

Lower CT values, indicating stronger positivity for the viral PCR assays, were associated with significant increases in the URTI and PEx scores as well as higher CRP levels. A non-significant association was also seen between the CT value and the acute fall in FEV$_1$. Further details are given in Table 7.5 below.

Table 7.5  Effect of polymerase chain reaction cycle threshold (CT) value on clinical outcomes amongst all virus-positive visits

<table>
<thead>
<tr>
<th>Outcome Variable</th>
<th>GEE Coefficient</th>
<th>95% CI</th>
<th>$R^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URTI score</td>
<td>-0.43</td>
<td>-0.58 to -0.28</td>
<td>0.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PEx score</td>
<td>-0.11</td>
<td>-0.15 to -0.06</td>
<td>0.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acute fall in FEV$_1$ (%)</td>
<td>-0.15</td>
<td>-0.37 to +0.08</td>
<td>0.003</td>
<td>0.21</td>
</tr>
<tr>
<td>CRP level (mg/l)*</td>
<td>-0.03</td>
<td>-0.06 to -0.002</td>
<td>0.03</td>
<td>0.037</td>
</tr>
<tr>
<td>White cell count (x10^9)</td>
<td>+0.03</td>
<td>-0.03 to +0.09</td>
<td>&lt;0.001</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* CRP values were log-transformed to correct for non-normal distribution.

GEE: generalised estimating equations; URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; FEV$_1$: forced expiratory volume in 1 second;

7.4.9  Effect of Sputum Positivity for VRI on Clinical Parameters

Detection of a respiratory virus in sputum was associated with more severe clinical outcomes than episodes in which a virus was detected only in upper airway swabs (i.e. nose- and/or throat-swabs). Sputum-positive study visits were more likely to meet criteria for PEx and more likely to result in antibiotic prescription. Sputum-positivity was also associated with higher symptom scores, a greater fall in FEV$_1$ and higher CRP values. Details of these analyses are provided in Table 7.6.
Table 7.6  Clinical outcomes of virus-positive study visits when classified by positivity of sputum samples

<table>
<thead>
<tr>
<th>Binary Outcomes</th>
<th>Sputum Positive</th>
<th>Swab Positive Only</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEx (%)</td>
<td>57.1</td>
<td>34.4</td>
<td>3.32</td>
<td>1.7 – 6.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Prescription of any ABx (%)</td>
<td>74.3</td>
<td>40.6</td>
<td>5.38</td>
<td>2.5 – 11.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Prescription of IV ABx (%)</td>
<td>37.1</td>
<td>9.4</td>
<td>5.79</td>
<td>2.4 – 14.0</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Continuous Outcomes*</th>
<th>Sputum Positive</th>
<th>Swab Positive Only</th>
<th>Adjusted Mean Difference</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>URTI score</td>
<td>9.2 (6.7)</td>
<td>5.8 (5.5)</td>
<td>3.8</td>
<td>2.2 – 5.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PEx score</td>
<td>4.2 (2.2)</td>
<td>2.8 (2.4)</td>
<td>1.6</td>
<td>0.9 – 2.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acute fall in FEV₁ (%)</td>
<td>16.3 (11.5)</td>
<td>12.2 (11.0)</td>
<td>4.1</td>
<td>0.4 – 7.8</td>
<td>0.03</td>
</tr>
<tr>
<td>log CRP (mg/l)*</td>
<td>2.5 (1.3)</td>
<td>2.0 (1.2)</td>
<td>0.4</td>
<td>0.05 – 0.8</td>
<td>0.03</td>
</tr>
<tr>
<td>White cell count (x10⁹)</td>
<td>10.6 (3.4)</td>
<td>10.0 (3.0)</td>
<td>0.3</td>
<td>-0.6 – 1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The upper airway swab group was the comparator variable in each analysis.
# Continuous variables are reported as mean (standard deviation)
* CRP values were log-transformed to correct for non-normal distribution.

URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; FEV₁: forced expiratory volume in 1 second; CRP: C-reactive protein

7.4.10  Diagnostic Value of URTI Symptom Score

Despite higher symptom scores at virus positive visits, receiver operating characteristic (ROC) analysis suggests that the URTI score has limited usefulness for the identification of laboratory-confirmed VRI. The ROC curve is shown in Figure 7.4 and reveals an area under the curve of 0.68 (95% CI 0.54 – 0.81). Optimal performance of the URTI score for the diagnosis of VRI was achieved with a cut-off of ≥11/27. The sensitivity, specificity and predictive values of both this cut-off and the threshold of ≥4, as originally described by Johnston et al, are shown in Table 7.7.
Figure 7.4  Receiver operating characteristic (ROC) curve for diagnosis of viral respiratory infection using the Johnston URTI score

AUC: area under the curve; URTI: upper respiratory tract infection

Table 7.7  Diagnostic value of the Johnston URTI score

<table>
<thead>
<tr>
<th></th>
<th>URTI Score Cut-Off</th>
<th>≥4</th>
<th>≥11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.68 (0.45 – 0.86)</td>
<td>0.46 (0.24 – 0.68)</td>
</tr>
<tr>
<td>95% CI</td>
<td></td>
<td>0.51 (0.40 – 0.63)</td>
<td>0.89 (0.79 – 0.95)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.28 (0.17 – 0.42)</td>
<td>0.53 (0.29 – 0.76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.85 (0.72 – 0.94)</td>
<td>0.85 (0.76 – 0.92)</td>
</tr>
</tbody>
</table>

#  ≥4 described in original paper; ≥11 optimal cut-off in ROC analysis

URTI: upper respiratory tract infection
7.5 Discussion

This is the first study to examine the longitudinal clinical impact of viral respiratory infection in adults with CF. Taken together, the results presented here and in Chapter 6 demonstrate that respiratory viruses in these patients are associated with a substantial symptom burden, raised markers of inflammation and have a clear link with pulmonary exacerbation (PEx).

The relationship between PEx, VRI and decline in lung function merits close consideration. Most studies in paediatric CF populations have observed a greater decline in lung function in patients with a higher frequency of viral infection\textsuperscript{330,332,346} although one group found no such association.\textsuperscript{343} In the present study, identifiable VRI was associated with a two-fold increase in the risk of PEx as reported in Chapter 6. The results presented in this chapter have gone on to demonstrate that the number of PEx over one year of follow-up is linked to an increased rate of decline of FEV\textsubscript{1}. It would be intuitive to assume, therefore, that there is also an association between VRI incidence and lung function decline. The data from this study showed a non-significant trend towards such an association, which became weaker after adjustment for age, gender, baseline lung function and incidence of PEx.

At first glance, one might conclude from the above findings that viral infection is not linked to lung function decline in adults with CF. However, there are a number of considerations to be made first. For instance, the study was not fully powered to detect changes in FEV\textsubscript{1} as the primary endpoint. Given the association between identifiable VRI and PEx, it is distinctly possible that a larger study over a longer follow-up period would have unveiled a significant effect on FEV\textsubscript{1} decline with VRI. An extension of this hypothesis is that exacerbations, of any aetiology, are the primary driver of disease progression and that the presence of a viral trigger for the exacerbation is of secondary importance. In addition, respiratory viruses clearly led to events with a very wide spectrum of clinical severity in adults with CF. The “noise” from the significant minority of asymptomatic VRIs may have masked the impact of more harmful viruses. The underlying pathophysiology of VRI needs further exploration to determine the reasons for this variable severity. Finally, the study period included the winter of 2011-12 which was notable for an unusually low incidence of influenza.\textsuperscript{406} Indeed, only one case of influenza was seen over the
second winter of the study, compared with nine cases the previous winter. In conclusion, I would suggest that the data from this study do not exclude an impact of VRI on lung function decline, mediated through a clear link with exacerbations.

Alongside novel findings relating to the effects of viral infection, this study also presents important data on the impact of PEx themselves. The key role that PEx play in the evolution of CF lung disease is increasingly recognised. Our finding of a relationship between the incidence of PEx and reduction in lung function is important and corroborates data from previous studies. Studies in both children and adults have shown that an increased frequency of PEx is linked to more rapid lung function decline.\textsuperscript{24,58} PEx also have a substantial impact on quality of life for patients with CF\textsuperscript{22} and place a considerable financial burden on CF services.\textsuperscript{407} Most starkly, it is now apparent that frequent PEx lead to decreased survival in adult CF populations.\textsuperscript{24}

PEx are clearly significant events in the course of CF lung disease. However, it does appear that exacerbations are considerably variable in terms of severity and response to therapy. Two recent studies have shown that treatment failure after PEx is common. Sanders \textit{et al} analysed the US CF Foundation registry and found that in a quarter of PEx the FEV\textsubscript{1} failed to return to \( \geq 90\% \) of baseline.\textsuperscript{23} Parkins \textit{et al} reported that treatment failure, as defined by a number of clinical parameters, occurred in 28\% of exacerbations with 15\% failing to recover their FEV\textsubscript{1}.\textsuperscript{408} The PEx treatment failure rate of 29\% reported in this chapter is consistent with these previous studies and confirms this as an important clinical issue. The suggestion from the data in this study that viral exacerbations may have a lower incidence of treatment failure invites further work to examine the potential mechanisms behind this finding. This study also sheds new light on the aetiology of PEx but there remains a need to unravel the detailed pathophysiology of PEx in order to improve future management.

Prior to this study, little was known about the symptom burden associated with VRI in adults with CF. Of the symptoms making up the PEx score, increased cough, change in sputum, dyspnoea and sinus discharge were strongly associated with VRI. Similarly, sore throat and hoarse voice were significantly more common in virus-
positive visits. Pyrexia and new findings on physical examination or radiography were seen infrequently throughout the study. From this data, it can be concluded that VRI is associated with significant morbidity and characteristic symptoms in adult patients with CF.

There are important points to consider with regard to the symptom scores reported in this paper. Firstly, no URTI symptom score has been designed or validated for use in adult CF populations. The Johnston URTI score was originally used in an observational study examining the role of respiratory viruses in children with asthma. This score was chosen for its simplicity, ease of use in a routine clinic setting and for its focus solely on upper respiratory tract symptoms. The high positivity rate of each component of the URTI score at virus-negative visits probably explains the poor positive predictive value overall for the diagnosis of VRI. Although the score did have a high negative predictive value for VRI, our findings suggest that symptoms of an URTI should not be used as a surrogate for laboratory-confirmed viral infection in clinical practice or future research studies. Olesen et al reached similar conclusions in paediatric CF patients.

It must be noted that the PEx score described by Fuchs et al was originally used simply as a means of defining exacerbations and was not intended as a severity score. However, it is intuitive to expect that the number of symptoms present at a particular exacerbation correlates with increased severity. This study, therefore, took the pragmatic approach of using the Fuchs criteria as both a diagnostic tool and a marker of the burden of lower respiratory tract symptoms. It is also important to highlight the fact that we employed a minor modification of the original Fuchs score in that we defined PEx as the presence of ≥4 criteria and a decision to treat with intravenous or oral antibiotics. PEx treated with oral antibiotics were included as it was felt that this would reflect real-life practice at MACFC more clearly. The route of antibiotic administration is often not solely determined by clinical severity but also by patient preference, availability of in-patient beds and numerous psychosocial factors. Unfortunately, the lack of follow-up data on the success of oral antibiotic treatment is a limitation of this study design. The number of PEx per year, as defined by the modified criteria, was still associated with a decline in lung function.
and it appears that these seemingly “minor” exacerbations treated with oral antibiotics are of substantial clinical importance.

The limitations of the overall study design have been considered in detail in Chapter 6 and are equally applicable to the results detailed in this chapter. Allowing for these limitations, this paper presents the first prospective data on the long-term clinical impact of viral respiratory infection in adults with CF. In addition, a wealth of observational data on the effect of PEx in CF lung disease has been described. There is a clear need for further research to examine the intricate pathophysiology of viral infection and exacerbations in order to optimise the care of people with CF.
Chapter Eight

The Effect of Climate and Season on Viral Respiratory Infections and Pulmonary Exacerbations in Adults with Cystic Fibrosis
8.0 The Effect of Climate and Season on Viral Respiratory Infections and Pulmonary Exacerbations in Adults with Cystic Fibrosis

8.1 Abstract
8.1.1 Introduction
Changes in the weather are linked with adverse outcomes in asthma and chronic obstructive pulmonary disease but have been little studied in cystic fibrosis (CF). This chapter describes a prospective observational study to determine the impact of climate and season on the incidence of viral respiratory infection (VRI) and pulmonary exacerbations (PEx) in adults with CF.

8.1.2 Methods
Between December 2010 and April 2012, 98 adults with CF living in the northwest of England participated in an observational study. Each patient was seen every 2 months for one year with additional visits at the onset of PEx. Sputum, nose- and throat-swabs were tested at each visit for a panel of 9 respiratory viruses using polymerase chain reaction assays. Hourly temperature and daily relative humidity measurements were recorded throughout the study. Data was analysed using generalised estimating equation (GEE) models.

8.1.3 Results
29% and 37% of visits met criteria for VRI and PEx respectively. Rhinovirus accounted for 72% of identified viruses. Incidence of rhinovirus was high throughout the calendar year but peaked in autumn. Non-rhinovirus VRI rates peaked in winter. Rhinovirus infection was associated with increased mean temperature (odds ratio 1.08; \( p=0.001 \)) whilst non-rhinovirus VRI was strongly associated with a fall in mean temperature (odds ratio 0.87; \( p<0.0001 \)). There was no statistically significant association between incidence of PEx and climate variables. PEx and antibiotic prescription were common throughout the calendar year.
8.1.4 Conclusions
There is a clear seasonal pattern to laboratory-confirmed VRI in adults with CF.
The incidence of VRI but not PEx is associated with changes in ambient temperature.
8.2 Introduction

The incidence of viral respiratory infections (VRI) in the general population follows a characteristic seasonal pattern and typically peaks over the winter months. The effect of the seasons and climate on the rate of acute respiratory illness in cystic fibrosis (CF) is poorly characterised at present. Detailed knowledge of the seasonal patterns of VRI and pulmonary exacerbation (PEx) would help plan provision of CF care and enhance our current understanding of the pathophysiology of CF lung disease.

Outbreaks of respiratory viruses such as influenza, parainfluenza and respiratory syncytial virus (RSV) occur with relative predictability in the wider population each winter, although this is less apparent in tropical climates. The annual “influenza season” occurs between October and April in temperate regions of the northern hemisphere with low influenza activity levels between this yearly epidemic. Common colds due to human rhinovirus, in contrast, tend to occur throughout the year and peak in incidence each autumn. Alongside the characteristic seasonality of respiratory viruses, it is clear that the incidence and virulence of VRI is affected by the climate. Influenza activity, for instance, is correlated with lower temperatures and laboratory studies reveal that the transmission and infectivity of influenza virus is at its greatest in cold, dry conditions. It appears that both cold weather and influenza contribute to excess mortality each winter. The weather also has a demonstrable impact on the health of patients with underlying respiratory conditions. Observational data in chronic obstructive pulmonary disease (COPD), for example, show that exacerbations are much more common during cold weather and that lower temperatures are associated with longer exacerbation recovery times.

The extent to which seasonal and weather variables affect clinical illness in adults with CF remains poorly described. On a population level, Collaco et al have shown that warmer ambient temperatures are associated with increased rates of Pseudomonas aeruginosa infection and lower levels of lung function.
Data from the US CF Foundation registry has also shown that the incidence of PEx in CF is greater over the winter months. However, this study did not include any microbiological or climactic data. This chapter documents a prospective observational study to test the hypothesis that the incidence of VRI and PEx shows a distinct pattern of seasonality and is associated with levels of ambient temperature and humidity.

8.3 Methods
8.3.1 Study Design
Details of the observational study design and methodology are described in Chapter 5. Briefly, all patients over the age of 18 years with CF attending the Manchester Adult Cystic Fibrosis Centre were invited to participate in the study. The first 100 respondents were recruited of whom 98 lived in the north-west of England. The two patients living in other regions of the country (London and Gloucestershire) were excluded from the analysis in this chapter. The only other exclusion criterion was previous lung transplantation. Recruitment was completed between 22nd December 2010 and 30th March 2011. The study was approved by the Greater Manchester West Research Ethics Committee (reference 10/H104/71).

Patients were followed for twelve months or until death/lung transplantation. During follow-up, patients were seen routinely every two months. Between scheduled visits, patients were asked to contact the investigators if they developed symptoms of an exacerbation or upper respiratory tract infection (URTI). An additional study visit was conducted within 48 hours of onset of new respiratory symptoms where possible.

Pulmonary exacerbation (PEx) was defined according to a modified version of the criteria described by Fuchs et al. A total of ≥4 symptoms and a decision by the treating physician to prescribe intravenous or oral antibiotics were required for the diagnosis of PEx. Treatment failure for PEx was defined as a failure of the FEV₁ to return to >90% of baseline following a course of IV antibiotics.
8.3.2 Virological Analysis
Sputum, nose- and throat-swabs were collected at each study visit for virological analysis. Sterile flocked swabs were collected according to established guidelines and transported in viral transport medium at room temperature.\textsuperscript{217,384} In-house polymerase chain reaction (PCR) assays were performed to identify the presence of adenovirus, influenza A&B, human metapneumovirus, parainfluenza 1-3, respiratory syncytial virus (RSV) and human rhinovirus in each specimen. Cycle threshold (CT) values of $<45$ were considered positive for these assays.

8.3.4 Meteorological Data
Climate data were supplied by the UK Met Office and were recorded at Hulme Library in Manchester. Hulme Library is situated at an altitude of 33m and is located at latitude 54.47 north and longitude 2.25 west. Hourly temperature readings were made to determine the minimum, maximum and mean temperature in $^\circ$C for each 24-hour period of the study. Daily mean relative humidity (RH) was also measured. Mean temperature and RH values for the seven days prior to each study visit were calculated. Seasons were defined as follows: spring: March to May inclusive; summer: June to August; autumn: September to November and winter: December to February.

8.3.5 Statistical Analysis
Data with a normal distribution are reported as mean (standard deviation) and non-normally distributed data are given as median (inter-quartile range) unless stated. Statistical significance was assumed at the conventional level of $p<0.05$. Statistical analysis incorporated methods which allow for repeated observations from the same individual. Generalised estimating equation (GEE) models with a logistic regression structure were used to determine the association between climate variables and binary outcomes such as incidence of PEx and VRI.

The seasonality of VRI and PEx was assessed by calculating the proportion of study visits performed in each calendar month that met the outcome of interest. Comparisons were also made between seasons with values for winter used as the
reference variable. A priori VRI was further subdivided into two groups based on the incidence rates reported in Chapter 6 with visits positive for rhinovirus and those positive for any of the other viruses considered separately. Where a visit was positive for both rhinovirus and a second virus, it was included in the non-rhinovirus VRI group.

8.4 Results

8.4.1 Patient Demographics

The baseline characteristics of the 98 patients included in this analysis are shown in Table 8.1. The median distance from patients’ place of residence to Hulme Library weather station was 13.8 (IQR 8 – 37) kilometres. One patient was lost to follow-up and one withdrew from the study after the baseline visit leaving 96 patients who completed a mean of 6.6 (1.8) study visits over a mean follow-up period of 11.5 (2.0) months. During this period, three patients died and one patient underwent bilateral lung transplantation.

Table 8.1 Baseline demographics of patients in the climate study

<table>
<thead>
<tr>
<th></th>
<th>n = 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)*</td>
<td>28 (23-36)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>47</td>
</tr>
<tr>
<td>Distance between home &amp; Hulme Library (km)*</td>
<td>13.8 (8-37)</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>21.8 (2.9)</td>
</tr>
<tr>
<td>Best FEV₁% in previous 12 Months*</td>
<td>53.1 (21)</td>
</tr>
<tr>
<td>Chronic infection with Pseudomonas aeruginosa (%)</td>
<td>72</td>
</tr>
<tr>
<td>Chronic infection with Burkholderia cepacia complex (%)</td>
<td>14</td>
</tr>
</tbody>
</table>

# Median (interquartile range); * Mean (standard deviation)
BMI: body mass index; FEV₁: forced expiratory volume in 1 second.
8.4.2 Climate Data
Mean daily temperature and relative humidity during the study period are presented in Figure 8.1. Temperature varied from -8.1°C at its lowest point in December 2010 to a high of 28.2°C in September 2011. Relative humidity varied from 43.7 to 98.9% during the study and peaked during the winter. The study period was warmer than average in the north west of England; annual mean temperature in 2011 was 9.7°C as compared to average annual values over the period 2001-2010 of 9.3°C (p=0.038). Annual rainfall in 2011 was similar to the average annual value over the preceding decade (1333.7 vs 1303.1 mm respectively; p=0.56).

8.4.3 Seasonality of Viral Respiratory Infection
Within the population included in this analysis, there were 187 visits positive for ≥1 respiratory virus and 196 viruses identified in total. The relative frequency of the viruses identified is shown in Figure 8.2 with a large majority (71.9%) accounted for by rhinovirus. The monthly incidence of laboratory-confirmed VRI expressed as the percentage of study visits positive for a virus is shown in Figure 8.3A. When all viruses were considered together, the incidence appeared fairly constant throughout the year. When broken down into rhinovirus and non-rhinovirus VRI, however, a clear seasonal pattern emerged as shown in Figure 8.3B. Rhinovirus was common throughout the year but displayed a definite peak in late summer and autumn. In contrast, non-rhinovirus VRI was most common during the winter and spring with very low levels of activity during the summer.
Figure 8.1  A) Mean temperature and B) mean relative humidity during the study

Data have been smoothed by plotting weekly mean temperature and relative humidity values in the graphs above.

Figure 8.2  Breakdown of viruses identified during the study

RSV: respiratory syncytial virus; MPV: metapneumovirus
Figures represent percentage of all viruses identified.
Pulmonary exacerbation was a frequent event throughout the calendar year with a mean of 37% of visits meeting the pre-defined criteria for PEx. As shown in Figure 8.4, the incidence of PEx appeared to mirror the overall incidence of identifiable VRI. High usage of antibiotic therapy was noted throughout the study as illustrated in Figure 8.5. 47% of visits resulted in a prescription for antibiotics of which 49% of these were for intravenous therapy. The rate of antibiotic prescription remained high throughout the calendar year and did not fall appreciably during the summer months. Table 8.2 summarises other measures of exacerbation severity by season including lung function, symptom scores and inflammatory markers. The results of these variables for spring, summer and autumn were compared with those for winter using GEE models. There were no statistically significant differences in any measures of
exacerbation severity between seasons. There was, however, a trend towards a greater need for IV antibiotics in winter compared with autumn (OR 0.54, 95% CI 0.28 – 1.04, p=0.065).

Figure 8.4  Incidence of pulmonary exacerbation (PEx) and viral respiratory infection (VRI) by calendar month

PEx: pulmonary exacerbation; VRI: viral respiratory infection

Figure 8.5  Rate of antibiotic prescription by calendar month

IV: intravenous
Table 8.2  Severity of pulmonary exacerbations by season of onset

<table>
<thead>
<tr>
<th></th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of study visits</td>
<td>174</td>
<td>116</td>
<td>139</td>
<td>206</td>
</tr>
<tr>
<td>No. of PEx n (%)</td>
<td>72 (41.4)</td>
<td>44 (37.9)</td>
<td>53 (38.1)</td>
<td>65 (31.6)</td>
</tr>
<tr>
<td>PEx requiring IV ABx n (%)</td>
<td>33 (45.8)</td>
<td>24 (54.6)</td>
<td>22 (41.5)</td>
<td>38 (58.5)</td>
</tr>
<tr>
<td>Failure of IV ABx n (%)</td>
<td>13 (39.4)</td>
<td>15 (62.5)</td>
<td>10 (45.5)</td>
<td>17 (44.7)</td>
</tr>
<tr>
<td>Fall in FEV1 relative to baseline (%) *</td>
<td>20.8 (13.3)</td>
<td>20.8 (12.2)</td>
<td>20.8 (13.0)</td>
<td>20.5 (13.3)</td>
</tr>
<tr>
<td>URTI score #</td>
<td>8.75 (6.0)</td>
<td>8.57 (5.2)</td>
<td>10.66 (6.0)</td>
<td>9.09 (6.0)</td>
</tr>
<tr>
<td>PEx score #</td>
<td>5.42 (1.6)</td>
<td>5.48 (1.0)</td>
<td>5.83 (1.4)</td>
<td>5.55 (1.4)</td>
</tr>
<tr>
<td>C-reactive protein (mg/l)#</td>
<td>33.1 (44.4)</td>
<td>20.8 (23.9)</td>
<td>37.0 (49.8)</td>
<td>28.8 (34.4)</td>
</tr>
<tr>
<td>White cell count (x10^9) #</td>
<td>11.9 (3.6)</td>
<td>11.1 (3.5)</td>
<td>10.3 (3.1)</td>
<td>11.0 (3.9)</td>
</tr>
</tbody>
</table>

# Continuous variables are reported as mean (standard deviation).
PEx: pulmonary exacerbation; IV: intravenous; ABx: antibiotics; FEV1: forced expiratory volume in 1 second; URTI: upper respiratory tract infection

8.4.5  Climate and Viral Respiratory Infection

The results of GEE models assessing the effect of ambient temperature and relative humidity (RH) on the incidence of identifiable VRI are shown in Table 8.3. When all instances of VRI were analysed as a combined group no significant effect of temperature or RH on the day of the visit was seen. Similarly, there was no association between VRI and the mean values for these variables over the seven days prior to the visit.

When VRI was classified as either rhinovirus or non-rhinovirus VRI, several statistically significant effects were revealed. Firstly, rhinovirus incidence was positively associated with both minimum and mean temperature (i.e. the risk of
rhinovirus increased as the ambient temperature increased). A negative association was seen with regard to rhinovirus and RH on the day of visit suggesting that rhinovirus infection was more likely in dryer conditions. In contrast, temperature and RH appeared to have a directly opposite effect on non-rhinovirus VRI compared with rhinovirus. Non-rhinovirus VRI showed a strong negative association with ambient temperature and a positive association with RH over the previous week as detailed in Table 8.3.

8.4.6 Climate Variables and Incidence of Pulmonary Exacerbation

There was no significant association between pulmonary exacerbation (PEx) and either temperature or relative humidity. The values from the GEE model for this analysis are shown in Table 8.4.
Table 8.3  The effect of ambient temperature and relative humidity on the incidence of viral respiratory infection (VRI) using a generalised estimating equation (GEE) model

<table>
<thead>
<tr>
<th></th>
<th>All VRI</th>
<th>Rhinovirus</th>
<th>Non-Rhinovirus VRI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
<td>p value</td>
</tr>
<tr>
<td>Minimum temperature in °C on day of visit</td>
<td>1.015</td>
<td>0.98 – 1.05</td>
<td>0.4</td>
</tr>
<tr>
<td>Mean temperature in °C on day of visit</td>
<td>1.015</td>
<td>0.98 – 1.05</td>
<td>0.4</td>
</tr>
<tr>
<td>Mean temperature in °C over previous 7 days</td>
<td>1.03</td>
<td>0.99 – 1.07</td>
<td>0.17</td>
</tr>
<tr>
<td>Mean relative humidity on day of visit</td>
<td>0.99</td>
<td>0.97 – 1.00</td>
<td>0.13</td>
</tr>
<tr>
<td>Mean relative humidity over previous 7 days</td>
<td>1.0</td>
<td>0.98 – 1.02</td>
<td>0.8</td>
</tr>
</tbody>
</table>

All VRI = all viruses identified during the study (i.e. rhinovirus + non-rhinovirus VRI).
Non-Rhinovirus VRI = all cases of adenovirus, influenza, metapneumovirus, parainfluenza virus and respiratory syncytial virus.
Table 8.4  The effect of ambient temperature and relative humidity on incidence of pulmonary exacerbation

<table>
<thead>
<tr>
<th></th>
<th>Pulmonary Exacerbation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
</tr>
<tr>
<td>Minimum temperature (°C) on day of visit</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean temperature (°C) on day of visit</td>
<td>1.01</td>
</tr>
<tr>
<td>Mean temperature (°C) over previous 7 days</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean relative humidity on day of visit</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean relative humidity over previous 7 days</td>
<td>0.99</td>
</tr>
</tbody>
</table>

GEE: generalised estimating equations; OR: odds ratio

8.5  Discussion

This is the first study to document the seasonality of respiratory illness in CF in conjunction with detailed virological analysis. The results presented above demonstrate that there is a clear seasonal pattern to the incidence of viral infection in adults with CF and that the incidence of such viruses appears to mirror the rate of pulmonary exacerbations. These observations have important implications for the provision of CF care and for the direction of future research into such exacerbations.

In our study, rhinovirus infection was common throughout the year but peaked in the autumn months whilst the combined group of other respiratory viruses showed a marked winter predominance. The observation of an autumn peak in rhinovirus infection is consistent with numerous studies from the general population\textsuperscript{183,198,203} and there has been speculation that this phenomenon is related to enhanced viral circulation as students return to schools after the summer holiday.\textsuperscript{414} As reported in Chapter 6, rhinovirus is associated with increased risk of PEx in CF and the frequency of this infection throughout the year may explain the lack of an expected peak in PEx over the winter months. Other viruses such as influenza, RSV and parainfluenza are known to have well defined periods of activity over the winter months\textsuperscript{209,216} and this pattern was clearly observed in this study.
Several hypotheses have been proposed for the seasonal nature of influenza epidemics\textsuperscript{415} which might reasonably be extended to other winter-predominant viruses. Relevant factors are likely to include behavioural changes, such as the tendency to increased crowding indoors during cold weather, as well as direct biological effects on viral transmission and virulence in relation to changes in the climate. The impact of heating appliances on changes to relative and absolute indoor humidity levels may also be important. Typically, heating leads to indoor relative humidity (RH) being at its lowest in winter when outdoor RH peaks, which hinders interpretation of the effect of humidity on health outcomes.\textsuperscript{188} It is likely that multiple factors contribute to the seasonal nature of viral respiratory infections.

The data in this chapter show for the first time in a CF population that variations in weather conditions are associated with acute respiratory illness. Both rhinovirus and the combined group of other viral pathogens, which might reasonably be termed “winter viruses,” showed a significant but contrary association with ambient temperature. In this study, rhinovirus incidence was increased by higher temperatures whereas other viruses were associated with lower temperatures. These findings held true whether the temperature on the day of the study visit or over the preceding week was considered. The observations are consistent with previous clinical studies showing an increase in influenza and RSV cases in association with lower temperatures.\textsuperscript{185,186} Laboratory work in animal models has also demonstrated that influenza virus shedding is increased at lower temperatures.\textsuperscript{187}

The effect of temperature on rhinovirus infection and transmission is less clear in the literature, however. Lidwell \textit{et al} found that the incidence of common colds was associated with a fall in temperature two to four days prior to onset of illness but this study did not include virological diagnostics to clarify the proportion of rhinovirus cases.\textsuperscript{416} Further research is required to clarify the effects of ambient temperature on rhinovirus infection in CF and investigate whether there is a link with rates of bacterial infection. Collaco and colleagues found that warmer average temperatures were linked with increased rates of \textit{Pseudomonas aeruginosa} in a large observational study of patients with CF.\textsuperscript{69} There is increasing suspicion that
Rhinovirus displays a degree of synergism with Pseudomonas aeruginosa in CF lung disease and it may be that ambient temperature is a risk factor for both pathogens.

In this study, RH on the day of the visit was found to be negatively correlated with rhinovirus incidence whereas RH over the preceding week was positively correlated with incidence of other viruses. In simpler terms, this suggests that rhinovirus favours lower humidity whilst other viruses favour higher humidity in the context of infection of CF patients. This contrasts with previous work which has demonstrated increased rhinovirus infectivity at high relative humidity. Similarly influenza virus transmission appears to be enhanced at low relative humidity in laboratory studies.

The data presented in this study focus on relative rather than absolute humidity. RH refers to the water content of the air relative to the maximum possible water content at a given temperature whereas absolute humidity is a measure of the actual water content of the air. RH was chosen for investigation as there is a considerably greater volume of literature to show it has an effect on the transmission of viruses. Given the consistently high levels of RH seen in the study, with relatively little seasonal variation, it was felt that further analysis of measures of absolute humidity would be of little additional benefit. It must also be noted that the magnitude of the associations with humidity in this study was very small despite reaching statistical significance. It remains possible, however, that the effect of humidity on viral infection in CF differs from the general population, perhaps through changes to sputum consistency and make-up. The importance of changes in atmospheric humidity to the health of patients with CF requires further investigation.

An important application of the data from this study relates to the utilisation of healthcare resources in CF medicine. It is important to note that the Manchester Adult CF Unit does not have a policy of prescribing regular prophylactic courses of intravenous antibiotics and such therapy is given in response to acute symptoms. Given this, we observed that the incidence of PEx and requirement for antibiotics was high throughout the year. This information is of use to CF healthcare providers.
planning the provision of clinic appointments, intravenous antibiotic therapy and hospitalisation. An awareness of the seasonal patterns of VRI and PEx is also important for the design of future clinical trials aimed at treatment or prevention of exacerbations. Future trials of anti-rhinoviral therapies, for instance, would be best conducted in the autumn months.

This study focused on a population of adult CF patients in the north west of England. The climate in this area is temperate with few extremes of temperature during the study period. A high level of RH was seen throughout the study. Our findings may not be fully applicable to CF populations living in other regions with differing climates. The seasonality and incidence of VRI in tropical climates, for instance, differs greatly from that seen in the United Kingdom. In addition, the majority of the participants in this study live in urban areas and variation in the level of air pollution may also be important in CF lung disease.

The detailed microbiological analysis in this study, close follow-up and the consistent treatment strategies afforded by the single centre population are the key strengths of this study. However, a number of limitations of the study must be considered. Firstly, the patients lived a varying distance from the weather station at which the climate data was recorded. There is likely to have been subtle variation in weather conditions across the geographical study area but with a maximum radius of 64 km in this study, the climate data are felt to be generally applicable across the cohort. Similarly, the Hulme Library weather station is centred within the large urban conurbation of Greater Manchester. The prevailing climate at this location may not be fully representative of that in rural locations where some of the study participants reside.

In addition, the data presented here do not take full account of the variable severity of PEx in CF. It is possible that exacerbations occurring at certain points in the year or at times of severe weather events may be more or less severe than those at other times. The study did not include a validated measure of PEx severity and this issue
requires investigation in future studies. Finally, the general limitations of the study design discussed in Chapter 6 are also applicable to the present analysis.

In conclusion, this study has demonstrated that there is a clear seasonal pattern to the incidence of VRI in adults with CF. A highly significant association between changes in the weather and the frequency of viral infections has been demonstrated, both in relation to temperature and RH. This information is of direct relevance to the care of CF patients, development of CF services and the planning of future research into pulmonary exacerbations of CF lung disease.
Chapter Nine

The Spectrum of Rhinovirus Species Affecting Adults with Cystic Fibrosis
9.0 The Spectrum of Rhinovirus Species Affecting Adults with Cystic Fibrosis

9.1 Abstract
9.1.1 Introduction
Rhinovirus is the most common viral respiratory infection to affect patients with cystic fibrosis (CF) and is associated with pulmonary exacerbations. The relative virulence of different rhinovirus species in CF is not clear.

9.1.2 Methods
Genetic sequencing of a sub-set of rhinoviruses identified as part of the main observational study described in this thesis was performed. The rhinovirus 5’ untranslated region (UTR) was sequenced using the Applied Biosystems 3130xl Genetic Analyzer. Sequences were identified through phylogenetic analysis and comparison with the GenBank database. Clinical outcomes of infection with different rhinovirus species were analysed using generalized estimating equations.

9.1.3 Results
Rhinovirus sequencing was completed in 60 respiratory tract samples representing 42 study visits from 27 patients. A total of 35 different rhinovirus sub-types were identified. Rhinovirus A sub-types were detected in 29/42 (69%) visits compared with 11/42 (26%) and 2/42 (5%) belonging to rhinovirus B and C respectively. Visits positive for rhinovirus A were associated with greater upper respiratory tract infection scores and higher C-reactive protein levels than those positive for rhinovirus B. Of nine patients who were positive for rhinovirus at ≥2 consecutive study visits, one appeared to have chronic infection with the same strain of rhinovirus A. The remaining eight patients were found to harbour different rhinovirus sub-types at subsequent visits.

9.1.4 Conclusions
Rhinovirus A is the predominant species of rhinovirus encountered by adults with CF. The clinical features of rhinovirus A infection appear to be more severe than for rhinovirus B.
9.2 Introduction

Human rhinovirus is the principle cause of the common cold and is probably responsible for more ill health in the human population than any other infectious agent.\(^{161}\) It is clear that rhinoviruses are of greatest significance to people with chronic respiratory disease. The majority of exacerbations of asthma and approximately one third of acute episodes of chronic obstructive pulmonary disease (COPD) are associated with a respiratory virus.\(^{285,299}\) The data presented in Chapter 6 of this thesis confirm that rhinovirus is also the principle viral pathogen in adults with cystic fibrosis (CF) which is consistent with data from paediatric CF populations.\(^{332,346-349}\) Rhinovirus represents an underexploited target for therapy to prevent and treat pulmonary exacerbations (PEx) of CF and there is a clear need for a greater understanding of the role this organism plays in CF lung disease.

Over the last decade there have been considerable strides forward in the understanding of human rhinovirus genetics.\(^{206}\) There are over 150 different rhinovirus subtypes recognised which were traditionally divided into groups A and B based on the receptor with which they bind to the respiratory epithelium.\(^{423}\) In 2007 a third, genetically distinct rhinovirus species was identified and named rhinovirus C.\(^{385}\) There are a considerable number of individual serotypes within each of these groups, the genomes of which have been fully sequenced in most cases.\(^{423}\) Data on the relative virulence of individual rhinovirus strains is steadily emerging and there has been considerable recent interest in the importance of rhinovirus C. Some investigators have found an association between rhinovirus C and more severe outcomes in children with asthma\(^{424}\) and CF.\(^{350}\) It increasingly appears that strains belonging to rhinovirus A and C cause more severe respiratory disease in children than those from group B.\(^{425-427}\) At present there is a distinct lack of clinical data to assess whether similar patterns are seen in adult populations with chronic respiratory disease. A greater understanding of the clinical importance of rhinovirus genetic diversity is essential to guide the development of future rhinovirus therapies and vaccines.

The present study had three aims. Firstly, the diversity of rhinoviruses seen in the main observational study described in Chapter 6 was assessed using genetic sequencing methods. Secondly, the resulting data were compared with key clinical
outcomes including symptom scores, lung function parameters and inflammatory markers to determine the relative virulence of the different rhinovirus groups. The final aim of the study was to clarify whether patients with consecutive rhinovirus-positive visits had chronic infection with the same strain of rhinovirus or sequential infection with different serotypes.

9.3 Methods
9.3.1 Patient Selection
Samples for rhinovirus sequencing were selected from the main observational study described in Chapters 6 and 7 above. To recap, the study recruited 100 adults with CF and followed them for twelve months. Participants provided sputum, nose- and throat-swabs every two months for respiratory virus testing using polymerase chain reaction (PCR) assays. Samples were repeated at onset of acute respiratory illness. All patients gave written informed consent. The study was approved by the Greater Manchester West Research Ethics Committee.

Rhinovirus was identified at 145 out of 625 study visits. Specimens were selected from this dataset according to the following rationale: first, all rhinovirus-positive sputum samples with a cycle threshold value of <40 were selected. Secondly, all sputum or swab samples from patients who had consecutive rhinovirus-positive study visits were chosen to differentiate persistent infection with the same rhinovirus species from sequential infections with different rhinovirus strains.

9.3.2 Rhinovirus Sequencing
Total nucleic acids were extracted from respiratory specimens using the QIAamp® Virus Biorobot® MDx machine (Qiagen, Hilden, Germany) as described in Section 5.9.1. The rhinovirus 5’ untranslated region (UTR) was sequenced using a method adapted from Lee et al.\textsuperscript{385} Firstly, rhinovirus RNA was converted to cDNA and amplified using a two-step PCR assay containing the pan-rhinovirus P1 forward primer (CAAGCACTTCTGTYWCCCC) and the P3 reverse primer (ACGGACACCCAAAGTAG). Primers and PCR reagents were sourced from Life Technologies Ltd (Paisley, UK). The product of the first round PCR assay was
amplified using a semi-nested PCR comprising the P1 forward primer and the following three reverse primers (P2-1: TTAGCCACATTCAGGGGC, P2-2: TTAGCCACATTCAGGAGCC and P2-3: TTAGCCGCATTCAGGGG). Electrophoresis using a 1.5% agarose gel was performed to confirm successful amplification of cDNA. The semi-nested PCR product was treated with exonuclease/shrimp alkaline phosphatase to remove excess primers and deoxyribonucleotide triphosphates.

Genetic sequencing of the 5’UTR region was performed using the Applied Biosystems 3130xl Genetic Analyzer (Life Technologies Ltd, Paisley, UK). The P1, P2-1, P2-2 and P2-3 primers were employed in the sequencing reaction. Raw sequencing data was edited manually using Sequencher v4.7 (Gene Codes Corporation, Michigan, USA) to correct mis-called bases. Individual DNA fragments were trimmed to 270 base pairs to match the length of the reference sequences reported by Lee et al. The resulting sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database using the BLASTn interface. A local BLAST database was also created using the sequences reported by Lee et al to allow a further means of identifying individual rhinovirus strains. Phylogenetic analysis using the neighbour-joining method was performed in ClustalW to assign individual sequences to the appropriate rhinovirus group. Phylogenetic trees were edited in MEGA v5.1. All rhinovirus sequences were uploaded to GenBank under accession numbers KF112083-KF112142.

9.3.3 Correlation of Sequencing Results with Clinical Data

Comparisons were made between rhinovirus groups A, B and C for a number of clinical parameters. Symptoms were measured using the upper respiratory tract infection (URTI) score described by Johnston et al and the criteria for pulmonary exacerbation criteria reported by Fuchs et al. The effect of rhinovirus infection on lung function was quantified as the fall in forced expiratory volume in 1 second (FEV1) relative to the patient’s pre-study baseline FEV1. The white cell count and serum C-reactive protein level were measured at each visit.
9.3.4 Statistical Analysis
Data are presented as mean (standard deviation) or median (range) as appropriate. Generalised estimating equation models were used to compare outcomes between rhinovirus groups, taking into account multiple observations from individual participants. The conventional 0.05 level of significance was assumed. Data analysis was conducted using SPSS® version 20.0 (IBM, New York, USA).

9.4 Results
9.4.1 Patient and Sample Selection
110 rhinovirus-positive respiratory tract samples met the pre-specified criteria for sequencing. 76 of these samples were available in sufficient quantity for analysis. The sequencing process was completed successfully in 60 of these specimens representing a total of 42 study visits provided by 27 patients. Sequenced samples included 29 sputum specimens, 20 nose-swabs and 11 throat-swabs. Baseline demographics of the patients providing samples for rhinovirus sequencing are shown in table 9.1.

<table>
<thead>
<tr>
<th>Table 9.1 Baseline demographics of patients providing rhinovirus samples for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
</tr>
<tr>
<td>Median age (yrs)#</td>
</tr>
<tr>
<td>Male : Female</td>
</tr>
<tr>
<td>Body mass index (kg/m$^2$)*</td>
</tr>
<tr>
<td>Baseline FEV$_1$ %-predicted (%)*</td>
</tr>
<tr>
<td>Chronic P. aeruginosa infection (%)</td>
</tr>
<tr>
<td>Chronic B. cepacia complex infection (%)</td>
</tr>
</tbody>
</table>

FEV$_1$: forced expiratory volume in 1 second
# Median (interquartile range); * Mean (standard deviation)
9.4.2  Rhinovirus Sequencing Results
A total of 35 different rhinovirus subtypes were identified. Figure 9.1 shows a neighbour-joining phylogenetic tree to illustrate the genetic relatedness of all rhinoviruses sequenced as part of this study in comparison with the reference sequences reported by Lee et al.\(^\text{385}\) Rhinovirus A was identified in 29/42 (69.0%) study visits and 11/42 (26.2%) were positive for rhinovirus B. Rhinovirus C was detected in only 2/42 (4.8%) visits including one co-infection with adenovirus. Multiple specimens (i.e. at least two of sputum, nose- and throat-swabs) were analysed from 14 visits. In all 14 cases, identical rhinovirus sub-types were detected in the matched specimens with no instances of simultaneous co-infection with different rhinovirus sub-types.

9.4.3  Evidence of the Potential for Chronic Rhinovirus Carriage in CF
Nine patients provided samples for rhinovirus sequencing from two or more study visits. Samples from a median of 2 (range 2 – 4) visits were investigated in this sub-cohort of patients. In eight of these patients each rhinovirus tested was found to belong to a different sub-type. This suggests that, in most cases, repeated rhinovirus PCR-positivity amongst adults with CF is due to new infection with a different rhinovirus strain. However, one patient was found to harbour the same sub-type of rhinovirus A (HRV 33) on three separate occasions separated by ten months. Over twelve months of follow-up in the study, this patient underwent a total of eight study visits of which seven were PCR-positive for rhinovirus. One of these PCR-positive visits met the criteria for a pulmonary exacerbation and only two visits were associated with high URTI scores. Clinical and virological results from this patient are summarised in Table 9.2. Two years after HRV 33 was first identified in this patient, a sputum sample was again positive for the same strain of rhinovirus. Figure 9.2 shows a phylogenetic tree to illustrate the close clustering of rhinovirus sequences from the patient. These findings strongly suggest that the patient has chronic respiratory infection with rhinovirus A.
Figure 9.1  Phylogenetic tree showing rhinoviruses detected in clinical samples from the Manchester CF Virology Study and reference rhinovirus strains reported in Lee et al.\textsuperscript{385}

The tree was constructed in MEGA 5.1 using the neighbour joining method with bootstrap values of 500. ♦ indicates study samples.

HRV: human rhinovirus
Table 9.2  Serial rhinovirus PCR results and clinical outcomes in a single patient with CF and chronic rhinovirus infection

<table>
<thead>
<tr>
<th>Date</th>
<th>Symptoms of URTI?</th>
<th>PEx?</th>
<th>Rhinovirus PCR Result (CT Value)</th>
<th>Sample Sequenced?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nose Swab</td>
<td>Throat Swab</td>
</tr>
<tr>
<td>21/01/2011</td>
<td>No</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>04/03/2011</td>
<td>Yes</td>
<td>Yes</td>
<td>Pos (41)</td>
<td>Pos (38)</td>
</tr>
<tr>
<td>03/06/2011</td>
<td>No</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>08/07/2011</td>
<td>No</td>
<td>Yes</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>31/08/2011</td>
<td>Yes</td>
<td>No</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>02/11/2011</td>
<td>No</td>
<td>No</td>
<td>Pos (39)</td>
<td>Neg</td>
</tr>
<tr>
<td>30/11/2011</td>
<td>Yes</td>
<td>No</td>
<td>Neg</td>
<td>Pos (34)</td>
</tr>
<tr>
<td>04/01/2012</td>
<td>No</td>
<td>No</td>
<td>Pos (39)</td>
<td>Neg</td>
</tr>
<tr>
<td>21/03/2013</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; CT: cycle threshold
Figure 9.2    Expanded phylogenetic tree to illustrate the persistence of rhinovirus A for over two years in an adult with CF

These trees were constructed as described in the legend of Figure 9.1. Specimens marked with a black diamond were all recovered from the same individual between March 2011 and March 2013.
9.4.4 Clinical Effects of Different Rhinovirus Species

Table 9.3 shows the differences in clinical outcomes between episodes of rhinovirus A and B infection. Rhinovirus A was associated with significantly higher URTI scores and greater CRP levels. There was also a trend towards increased requirement for intravenous antibiotic therapy with rhinovirus A infection (27.6% vs 9.1%; \( p=0.2 \)). As there were only two cases of rhinovirus C identified it is not possible to draw conclusions on the clinical impact of this group. Both cases of rhinovirus C infection met the pre-defined criteria for pulmonary exacerbation and one required intravenous antibiotic therapy.

Table 9.3 Comparison of clinical outcomes between rhinovirus A and B infection

<table>
<thead>
<tr>
<th></th>
<th>Rhinovirus A</th>
<th>Rhinovirus B</th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of visits</td>
<td>29</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pulmonary exacerbation n (%)</td>
<td>18 (62.1)</td>
<td>5 (45.5)</td>
<td>1.82</td>
<td>0.37 to 8.97</td>
<td>0.46</td>
</tr>
<tr>
<td>Any ABx; n (%)</td>
<td>24 (82.8)</td>
<td>6 (54.5)</td>
<td>4.44</td>
<td>0.27 to 73.4</td>
<td>0.30</td>
</tr>
<tr>
<td>IV ABx; n (%)</td>
<td>8 (27.6)</td>
<td>1 (9.1)</td>
<td>3.54</td>
<td>0.51 to 24.5</td>
<td>0.20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Rhinovirus A</th>
<th>Rhinovirus B</th>
<th>Adjusted Mean Difference</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent fall in FEV(_1) relative to baseline</td>
<td>13.8 (12.6)</td>
<td>15.8 (9.9)</td>
<td>-3.17</td>
<td>-9.8 to +3.5</td>
<td>0.35</td>
</tr>
<tr>
<td>URTI score (out of 27)</td>
<td>12.5 (7.1)</td>
<td>6.5 (5.6)</td>
<td>3.82</td>
<td>+0.76 to +6.89</td>
<td>0.01</td>
</tr>
<tr>
<td>PEx score (out of 12)</td>
<td>4.5 (2.3)</td>
<td>3.5 (1.8)</td>
<td>0.88</td>
<td>-0.76 to +2.53</td>
<td>0.29</td>
</tr>
<tr>
<td>log CRP (mg/l)</td>
<td>2.77 (1.27)</td>
<td>1.77 (0.75)</td>
<td>0.98</td>
<td>+0.25 to +1.71</td>
<td>0.008</td>
</tr>
<tr>
<td>White cell count (x10(^9))</td>
<td>10.6 (10.6)</td>
<td>9.8 (2.3)</td>
<td>0.88</td>
<td>-0.31 to +2.07</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Data are presented as mean (standard deviation).

GEE: generalized estimating equation; ABx: antibiotics; IV: intravenous; FEV\(_1\): forced expiratory volume in 1 sec; URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; CRP: C-reactive protein
9.5 Discussion
Rhinovirus is the principle viral pathogen affecting patients with CF. This is the largest study to date of the genetic diversity of rhinoviruses recovered from an adult CF population. The results show that rhinovirus A is the dominant species of rhinovirus seen in adults with CF and reveal important data on the relative virulence of rhinovirus A and B infections in this population.

Rhinoviruses belong to the genus Enterovirus and are split into the three species: rhinovirus A, B and C. A considerable number of different sub-types or strains are found within each of these species. The existence rhinovirus C was first reported in 2007 although retrospective analysis of historical sample collections has shown that it has been in circulation for at least three decades. The identification of rhinovirus C requires molecular genetic sequencing technology as it is naturally resistant to conventional viral culture techniques and this accounts for its relatively recent discovery. Since its identification, there have been a plethora of reports on the epidemiology and clinical impact of rhinovirus C in a variety of patient populations. Overall, it appears that rhinovirus A and C account for the majority of rhinovirus infections. When examining clinical outcomes, Bizzintino et al found that rhinovirus C was associated with increased severity of paediatric asthma exacerbations. Other groups, however, have reported that rhinovirus C had a similar clinical impact to rhinovirus A amongst children with acute respiratory infections.

There have been only two previous studies which differentiated the rhinovirus species affecting patients with CF. de Almeida et al performed rhinovirus sequencing in 93 specimens from 103 children with CF of which 39% were found to be rhinovirus A, 25% rhinovirus B and 30% rhinovirus C. In this study, only rhinovirus C was associated with pulmonary exacerbation. Subsequently, Wark et al found that 16/20 (80%) of rhinoviruses in a small cohort of both adults and children with CF were accounted for by rhinovirus A. The results presented in this chapter are complementary to these findings and suggest that rhinovirus A is most common in CF. The data from this study do not support a major role for rhinovirus C in CF lung disease. It has been demonstrated for the first time, however, that there may be a difference in clinical outcomes between rhinovirus A and B in adults with CF.
Statistically significant increases in URTI symptom score and serum C-reactive protein levels were seen in rhinovirus A-positive episodes alongside trends towards a greater need for antibiotic therapy. Differences in the virulence of different rhinovirus species may be important to help guide the future development of therapies for rhinovirus-related exacerbations of lung disease. It may also be possible to determine whether certain rhinovirus sub-types require different antibiotic strategies to prevent a secondary flare of bacterial inflammation within the CF lung.

A further important finding of this study relates to the nature of recurrent rhinovirus infection in adults with CF. We have shown that in the majority of cases, repeated PCR-positivity for rhinovirus is due to acute infection with a new strain. However, the recognition of the potential for chronic rhinovirus infection amongst patients with CF is of considerable significance. We identified a single case of persistent rhinovirus carriage in this study which has parallels with a previous case of chronic influenza A infection in an adult with CF. The patient with chronic rhinovirus infection in this report was 43 years old with moderate CF lung disease, osteoporosis and CF-related diabetes. The patient was diagnosed with adrenal insufficiency and began hydrocortisone replacement therapy midway through the study period. However, the onset of chronic rhinovirus infection pre-dated the addition of oral corticosteroids. The mechanism by which this patient failed to clear the rhinovirus is unclear and requires further investigation. Prolonged or persistent infection with rhinovirus has previously been reported in patients with immune deficiency. The true prevalence and clinical significance of chronic respiratory virus infection in CF, however, is unclear.

The major limitation of this study is that only a subset of all rhinoviruses identified in the full cohort could be subjected to genetic sequencing. This was due primarily to the combination of finite resources and incomplete availability of sufficient sputum DNA to perform the analysis. Furthermore, in a small number of cases sequencing was attempted but no result was obtained as the initial PCR assays failed to amplify the target DNA for unknown reasons.
Despite these limitations, this study has uncovered several novel findings. Firstly, we have demonstrated the potential for rhinovirus infection to enter a chronic state in CF, a feature that requires further exploration. The results confirm that rhinovirus A is the most common rhinovirus species to affect adults with CF and appears to be associated with more severe clinical features than rhinovirus B. These results can help guide future research into rhinovirus-related exacerbations of CF lung disease with the aim of developing effective treatments.
Chapter Ten

Development of Ribosomal Intergenic Spacer Analysis (RISA) as a Tool to Profile the Bacterial Diversity of Cystic Fibrosis Sputum
10.0 Development of Ribosomal Intergenic Spacer Analysis (RISA) as a Tool to Profile the Bacterial Diversity of Cystic Fibrosis Sputum

10.1 Abstract

10.1.1 Introduction

Chronic respiratory infection in cystic fibrosis (CF) is increasingly recognised as complex and polymicrobial. Traditional culture-based techniques underestimate the bacterial diversity of CF sputum and there is a need for rapid, affordable, culture-independent alternatives. We evaluated ribosomal intergenic spacer analysis (RISA) as a means of profiling the bacterial diversity of CF sputum.

10.1.2 Methods

Conventional culture and RISA were performed on paired sputum samples from 93 adults with CF. 16S rRNA gene pyrosequencing was also performed on a single sputum specimen from a sub-set of 60 of these patients. RISA profiles of pure cultures of reference strains of CF-relevant pathogens were generated. Cluster analysis using Pearson correlation was used to compare RISA profiles. Distinct microbiome types were identified on the basis of RISA, pyrosequencing and culture. Differences between microbiome types were compared using one-way analysis of variance.

10.1.3 Results

RISA profiles were generated from 198 sputum samples provided by 93 patients. RISA separated samples into clusters broadly coherent with the results of standard sputum culture. Three distinct microbiome types were identified: 1) *Pseudomonas* culture positive with low bacterial diversity; 2) *Pseudomonas* culture positive with preserved bacterial diversity and 3) low bacterial diversity in association with *B. cepacia* complex and “other” Gram-negative rods. Among patients who provided ≥4 sequential sputum samples, variability of RISA was noted in 9/15 (60%) cases in relation to antibiotic therapy or onset of a pulmonary exacerbation.
10.1.4 Conclusions
RISA holds potential for development as a simple measure of bacterial diversity in CF sputum for application in future research and clinical management.
10.2 Introduction

Cystic fibrosis (CF) is an inherited condition characterised by chronic endobronchial infection leading eventually to respiratory failure. Traditional culture-based microbiological techniques readily identify “typical” CF pathogens in respiratory samples from these patients with *Pseudomonas aeruginosa* and *Staphylococcus aureus* the most common examples. The treatment of CF has evolved to optimise the prevention and suppression of these infections which are clearly associated with clinical deterioration.

Over the last fifteen years, however, culture-independent techniques have revealed the presence of considerably more diverse bacterial populations within the CF lung than can be appreciated by standard culture alone. Anaerobes, for instance, frequently go undetected unless dedicated anaerobic cultures or molecular analyses are used. In addition, molecular diagnostic technologies typically demonstrate exquisite sensitivity which holds promise for the earlier detection and treatment of pathogens such as *P. aeruginosa*. Beyond the simple identification of individual microbial species, it has also been suggested that different lung microbiome patterns might act as pathogenic entities in their own right. Evidence to support this contention is found in the observation of several authors that bacterial diversity appears to reduce as patients age and their lung function declines.

Unfortunately, current techniques for the assessment of respiratory bacterial diversity are restricted by long turnaround times, limited availability, substantial financial costs and a requirement for considerable bioinformatic expertise. There is a clear need, therefore, for a rapid, accessible and cheap means of profiling the bacterial diversity within the CF lung. Ribosomal intergenic spacer analysis (RISA) employs readily-available polymerase chain reaction (PCR) assays to amplify the intergenic transcribed spacer (ITS) region between the bacterial 16S and 23S ribosomal genes in order to differentiate species. RISA has been applied widely to environmental samples such as soil but has been the subject of only preliminary investigation in the context of CF lung infection. We evaluated the performance of RISA in comparison with conventional culture and 16S rRNA gene pyrosequencing in a large cohort of adult CF patients. The aim of this study was to determine whether RISA is a suitable tool for profiling the respiratory microbiome.
in future clinical practice and research. In the context of this thesis, RISA was used as an additional method to determine whether respiratory viruses affect the CF lung microbiome.

10.3 Methods
10.3.1 Clinical Samples
Sputum samples were collected as part of the observational study investigating respiratory viruses in CF described earlier in this thesis. All sputum samples collected in the first five months of the study were included in this analysis. A further batch of samples was subsequently included to expand the dataset and allow for longitudinal analysis of the microbiome as described in Chapter 11. At each study visit, patients provided paired sputum samples for conventional culture and culture-independent analysis. When both techniques were performed, RISA and 16S rRNA gene pyrosequencing were conducted on separate aliquots of the same sputum DNA specimen. Sputum samples were expectorated spontaneously by the patient into sterile containers. All patients gave informed written consent and were aged eighteen years or older. The study was approved by the Greater Manchester West Research Ethics Committee.

10.3.2 Conventional Sputum Culture
Sputum samples for culture-independent analysis were transported on the same day to the on-site microbiology laboratory and processed according to established guidelines published by the United Kingdom Cystic Fibrosis Trust.\textsuperscript{388}

10.3.3 Nucleic Acid Extraction
Sputum specimens for culture-independent analysis were transported within 24 hours to the regional virology laboratory in Manchester for processing. Sputum was mixed in a 1:1 ratio with AL lysis buffer (Qiagen, Hilden, Germany) to a volume of 600 µl before being inactivated at 80°C for 20 minutes. Samples were stored at -80°C before undergoing DNA extraction. Total nucleic acids were extracted from sputum using the automated QIAamp\textsuperscript{®} Virus Biorobot\textsuperscript{®} MDx instrument (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. Extracted
DNA samples were shipped to Cardiff University at room temperature where they were stored at -80°C until further analysis.

10.3.4 Ribosomal Intergenic Spacer Analysis (RISA)
Full details of the RISA technique are described in Chapter 5. In brief, RISA was performed with conventional polymerase chain reaction (PCR) assays using 1 µl (equivalent to 20–40 ng) of nucleic acids extracted from sputum samples as described above. Other PCR constituents included 0.2 µl Taq polymerase, 2.5 µl buffer, and 5 µl of the relevant primers (1406F: TGYACACACCGCCCGT and 23SR: GGTTBCCCATTCRG). 2 µl of amplified DNA from each sample was analysed using the microfluidic chip-based Agilent 2100 Bioanalyzer platform (Agilent Technologies, California, USA) in order to separate DNA fragments and produce a distinct RISA profile for each specimen.

Electronic data from the Agilent 2100 Bioanalyzer were analyzed using Gelcompar II software (Applied Maths, Texas, USA) with the digital RISA profile imported using a custom script as described in the literature previously. Cluster analysis using Pearson correlation coefficients allowed the construction of dendrograms to determine the degree of similarity between individual RISA profiles. Specimens were then separated into sub-groups based on the major clusters identified with a similarity value of >83% taken as an arbitrary cut-off. Quantification of the bacterial diversity revealed by RISA was achieved by counting the number of discrete bands detected by the Bioanalyzer instrument.

Within-sample reproducibility of the RISA technique was assessed in four specimens by repeating the RISA assay on three separate days. Samples were stored at -80°C between repeat assays.

10.3.5 16S rRNA Gene Pyrosequencing
The first available sputum sample from a sub-set of 60 patients was sent for 16S rRNA gene pyrosequencing analysis. Patients were selected for inclusion in this group so that each major RISA cluster and cultured pathogen was represented. A 15 µl aliquot of the stored sputum DNA previously analysed with RISA was sent for
pyrosequencing. 16S rRNA gene pyrosequencing was performed commercially by Research and Testing Laboratory Inc. (Lubbock, Texas, USA) as previously described by Dowd et al. Briefly, 1 µl of extracted nucleic acids was amplified using the universal 16S primers 530F (5’-GTG CCA GCM GCN GCG G) and 1100R (5’-GGG TTN CGN TCG TTG). The V1-V3 regions of the bacterial 16S rRNA gene were sequenced using the Genome Sequencer FLX System (Roche, Nutley, New Jersey). In-house software developed by Research and Testing Laboratory Inc. was used to process data derived from the sequencing process. Sequence data was denoised and stripped of chimeric sequences. Sequences smaller than 250 bp were excluded. The remaining sequences were identified through comparison with the National Center for Biotechnology Information (NCBI) GenBank database using BLASTn. Sequences were resolved to the genus level based on identity values of >95%. Additional bioinformatic analysis was performed in collaboration with Professor Mahenthiralingam, Dr Julian Marchesi and Dr Ann Smith at Cardiff University. Downstream analysis included calculation of species richness and the Shannon diversity index. Further details of the 16S rRNA gene pyrosequencing methodology and analysis are provided in Chapter 5.

10.3.6 Statistical Analysis
Data are presented as mean (standard deviation) or median (interquartile range) as appropriate. One-way analysis of variance (ANOVA) was used to compare subgroups of patients with distinct RISA profiles. The Bonferroni correction was applied to allow for multiple comparisons. Multiple linear regression was used to investigate the effect of demographic variables on indices of sputum bacterial diversity. The conventional level of 0.05 was used to determine statistical significance.

10.4 Results
10.4.1 Patient Demographics
A total of 200 samples were provided by 93 adult CF patients between December 2010 and November 2011 for inclusion in this analysis. Baseline demographics of the full cohort and the subset of the population who were investigated with 16S
rRNA gene pyrosequencing are shown in Table 10.1. Patients contributed a median of 2 (range 1 – 5) samples to the full dataset. 77/200 (38.5%) of specimens were collected at the time of pulmonary exacerbation.

<table>
<thead>
<tr>
<th></th>
<th>Full RISA Cohort</th>
<th>16S Pyrosequencing Subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>93</td>
<td>60</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28 (23 – 35)</td>
<td>28 (23 – 34)</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>53</td>
<td>52</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>21.9 (2.9)</td>
<td>22.0 (3.1)</td>
</tr>
<tr>
<td>Baseline FEV₁ %-predicted</td>
<td>59.5 (21.7)</td>
<td>58.2 (22.4)</td>
</tr>
<tr>
<td>Chronic P. aeruginosa infection (%)</td>
<td>74.2</td>
<td>70.0</td>
</tr>
<tr>
<td>Chronic methicillin-sensitive S. aureus infection (%)</td>
<td>15.1</td>
<td>11.7</td>
</tr>
<tr>
<td>Chronic methicillin-resistant S. aureus infection (%)</td>
<td>4.3</td>
<td>3.3</td>
</tr>
<tr>
<td>Chronic B. cepacia complex infection (%)</td>
<td>14.0</td>
<td>18.3</td>
</tr>
</tbody>
</table>

RISA: ribosomal intergenic spacer analysis; FEV₁: forced expiratory volume in 1s
Data are presented as mean (SD), median (IQR) or percent as appropriate

10.4.2 RISA Analysis
RISA profiles were generated successfully from a total of 198 sputum samples with a paired standard culture result available for 179 of these. Cluster analysis identified two main groups within the sample set. The first group was termed “cluster P” and included 147 samples, the majority of whom were known to be chronically infected with P. aeruginosa as their main sputum pathogen. The second group of 32 individuals was termed “cluster G” and reflected those patients infected with “other” gram negative rods (GNR), defined as all organisms belonging to the genera Burkholderia, Achromobacter, Ralstonia, Pandorea and Stenotrophomonas. Figure 10.1 illustrates these clusters and for ease of interpretation the phylogenetic tree has been limited to include only the first sputum sample provided by each patient.
Within cluster P, 122/147 (83%) samples were culture positive for *P. aeruginosa* compared with 12/32 (37.5%) in cluster G. On this basis, RISA had a sensitivity of 91.0% and a specificity of 44.4% for the detection of *P. aeruginosa*. Samples from cluster G were positive for a non-*Pseudomonas* GNR in 21/32 (65.6%) cases compared with 17/147 (11.6%) in cluster P giving a sensitivity of 55.2% and specificity of 92.2%.

Figure 10.1  Ribosomal intergenic spacer analysis (RISA) profiles of the initial sputum samples provided by 93 study participants
10.4.3 Application of RISA to Pure Cultures of CF Pathogens

Pure cultures of a number of recognised CF pathogens were also subjected to RISA assays in order to determine whether specific bands correspond to the presence of these particular pathogens. Figure 10.2 shows the RISA profiles produced by pure cultures of three strains of *P. aeruginosa* (C3719, PA01 and LES B58) in relation to all clinical samples with paired sputum cultures positive for *P. aeruginosa*. Pure cultures of *P. aeruginosa* produced a double-banded RISA profile with a dominant 753bp band corresponding to the intergenic transcribed spacer (ITS) region of this organism. The characteristic ITS of *P. aeruginosa* was present in multiple clinical RISA profiles (see Figures 10.1, 10.2 and 10.5) but it was also absent in certain patients known to be culture positive for *P. aeruginosa*.

The ITS bands of other key organisms such as *B. cepacia* complex, *A. xylosoxidans* and *R. mannitololytica* were amplified by RISA on pure cultures of these species as shown in Appendix 2. In 9/93 (10%) cases the RISA profile identified a specific pathogen on the basis of a dominant and distinctive ITS amplicon where this pathogen was not isolated by standard culture. Of these nine cases, four clustered with samples known to be culture positive for *Ralstonia* spp, two with *Burkholderia* spp, two with *P. aeruginosa* and one with *Achromobacter* spp.
Figure 10.2  RISA profiles of all sputum samples culture-positive for *Pseudomonas aeruginosa* in relation to profiles from pure cultures of three *P. aeruginosa* strains

ITS: internal transcribed spacer; bp: base pairs
10.4.4 Reproducibility of RISA
RISA demonstrated good within-specimen reproducibility when repeated on separate days between freeze-thaw cycles. Figure 10.3 shows three repeated RISA profiles from four specimens. Within each sample, band number and position were highly conserved on each repeat.

![Figure 10.3](image)

**Figure 10.3** Within-sample reproducibility of RISA when repeated on three separate occasions

bp: base pairs

10.4.5 Longitudinal Changes in RISA Profiles
Fifteen patients provided ≥4 sputum specimens for RISA analysis which allowed an assessment of longitudinal changes in RISA profiles with time. The degree of stability in RISA profiles was highly variable within this population. In 9/15 (60%) cases a clear change in the RISA profile was noted in association with a viral infection or pulmonary exacerbation. Examples of sequential RISA profiles from three patients are shown in Figure 10.4. Certain individuals, such as patients 01 and 15 in Figure 10.4, demonstrated highly stable RISA profiles despite repeated exacerbations. Conversely, patient 18 is notable for the appearance of a dominant *P. aeruginosa* band at the time of exacerbation followed by a substantial change in the RISA profile following intravenous antibiotics (see Figure 10.4C). This shows that RISA has the potential to detect changes in the respiratory microbiome in response to clinical events such as pulmonary exacerbations or courses of antibiotic therapy.
Sequential RISA profiles from three patients known to be chronically infected with *Pseudomonas aeruginosa*

ITS: internal transcribed spacer region; PEx: pulmonary exacerbation; IV: intravenous antibiotics; OR: oral antibiotics

### 10.4.6 Comparison of RISA with 16S rRNA Gene Pyrosequencing & Standard Culture

Figure 10.5 shows the results of 60 sputum RISA profiles matched to the results of 16S rRNA gene pyrosequencing and conventional culture. The first available sputum sample for each of these patients was included in this analysis. The two major RISA clusters identified in Figure 10.1 were separated into a total of 16 sub-clusters based on an arbitrary similarity value of >83%. The RISA cluster analysis, profile morphology, conventional culture results and 16S rRNA gene
pyrosequencing data were considered in combination to define four groups of patients for further analysis:

- “Pseudomonas-Dominant” – clusters P3 and P7
- “Pseudomonas-Diverse” – clusters P1,2,4,5,6,8 and 9
- “Other Gram-Negative Rods” (GNR) – clusters G1 and G2.
- “Undifferentiated” – clusters G3 – G7

Demographic features of each of these groups are shown in Table 10.2. No significant differences in baseline characteristics were noted between the groups. A comparison of measures of bacterial diversity for the groups is shown in Table 10.3.

The *Pseudomonas*-dominant group was notable for a distinctive RISA profile consistent with the profiles produced by *P. aeruginosa* pure cultures (see Figure 10.2). All thirteen samples in this group were culture positive for *P. aeruginosa* and in each case the 16S pyrosequencing data revealed bacterial communities heavily dominated by *P. aeruginosa* (mean (SD) relative abundance 91.4% (20.9)). In contrast, the *Pseudomonas*-diverse group had notably more variable RISA profiles with greater bacterial diversity on 16S rRNA gene pyrosequencing (mean (SD) Shannon index 1.14 (0.23) vs 0.18 (0.23); p<0.001). The non-*Pseudomonas* GNR group was characterised by low diversity RISA profiles, often dominated by a single band corresponding to pure cultures of *Achromobacter xylosoxidans* and *Ralstonia mannitololytica* (see appendix 2). The non-*Pseudomonas* GNR group also had significantly lower bacterial diversity than the *Pseudomonas*-diverse group (mean (SD) Shannon index 0.65 (0.46) vs 1.14 (0.73); p=0.02).
Table 10.2  Demographic characteristics of patients by RISA sub-group

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas-Dominant</th>
<th>Pseudomonas-Diverse</th>
<th>Other GNR</th>
<th>F Ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>13</td>
<td>23</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29 (8.3)</td>
<td>30 (8.1)</td>
<td>32 (10.6)</td>
<td>0.51</td>
<td>0.6</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>21.9 (4.8)</td>
<td>21.6 (2.4)</td>
<td>22.3 (2.6)</td>
<td>0.25</td>
<td>0.7</td>
</tr>
</tbody>
</table>
| Baseline FEV\(_1\) %-
  predicted            | 55.5 (29.6)          | 64.9 (18.5)         | 56.1 (21.3)| 1.07    | 0.35|
| Gender (% female)    | 46.2                 | 47.8                | 55.6      | -       | 0.8 |
| F508del homozygous (%)| 46.2                 | 56.5                | 61.1      | -       | 0.6 |

Data are presented as mean (SD) or % as appropriate.
Continuous variables were analysed with ANOVA and categorical variables with Pearson’s chi-squared test.

GNR: Gram-negative rod; BMI: body mass index; FEV\(_1\): forced expiratory volume in 1 second
Figure 10.5  Comparison of sputum RISA profiles with conventional culture and 16S rRNA pyrosequencing

A: Achromobacter; B: Burkholderia; D: Pandorea; G: Unidentified Gram negative rod; M: Stenotrophomonas; N: normal flora; P: Pseudomonas; R: Ralstonia; S: Staphylococcus

# The 16S rRNA gene pyrosequencing data quoted are the relative abundances of genera in the sample at a relative abundance of >10%. Clusters were defined on the basis of a level of similarity of ≥83%.
Table 10.3  Comparison of measures of bacterial community diversity and composition by RISA sub-group

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas-Dominant</th>
<th>Pseudomonas-Diverse</th>
<th>Other GNR</th>
<th>F Ratio*</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>13</td>
<td>23</td>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Culture positive for ( P. ) aeruginosa n (%)</td>
<td>13 (100)</td>
<td>19 (82.6)</td>
<td>7 (38.9)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Culture positive for Other GNR n (%)</td>
<td>0 (0)</td>
<td>3 (13)</td>
<td>13 (72.2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Measures of Bacterial Diversity:**

<table>
<thead>
<tr>
<th></th>
<th>RISA band count</th>
<th>Richness</th>
<th>Shannon Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>RISA band count</td>
<td>6.2 (2.1)</td>
<td>8.5 (5.8)</td>
<td>0.18 (0.23)</td>
</tr>
<tr>
<td>Richness</td>
<td>6.41 (2.4)</td>
<td>22.3 (11.1)</td>
<td>1.14 (0.73)</td>
</tr>
<tr>
<td>Shannon Index</td>
<td>3.06 (1.9)</td>
<td>13.1 (9.2)</td>
<td>0.65 (0.46)</td>
</tr>
</tbody>
</table>

**Relative Abundance of Key CF Pathogens:**

<table>
<thead>
<tr>
<th></th>
<th>Pseudomonas relative abundace (%)</th>
<th>Burkholderia relative abundance (%)</th>
<th>Streptococcus relative abundance (%)</th>
<th>Staphylococcus relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>91.4 (20.9)</td>
<td>0.25 (0.37)</td>
<td>0.98 (1.9)</td>
<td>0.01 (0.03)</td>
</tr>
<tr>
<td></td>
<td>63.4 (28.7)</td>
<td>0.98 (3.6)</td>
<td>9.8 (10.8)</td>
<td>1.9 (4.5)</td>
</tr>
<tr>
<td></td>
<td>10.5 (20.5)</td>
<td>28.3 (40.6)</td>
<td>1.4 (2.4)</td>
<td>0.005 (0.01)</td>
</tr>
<tr>
<td></td>
<td>45.2</td>
<td>8.3</td>
<td>9.2</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* F ratio and p values relate to the results of one-way ANOVA. Data are presented as mean (SD) or n (%) as appropriate.

Predictors of Bacterial Sputum Diversity

Figure 10.6 shows the relationships between age, baseline FEV\textsubscript{1} and sputum bacterial diversity as quantified by the Shannon index derived from 16S rRNA gene pyrosequencing data. Multiple linear regression analyses revealed that lower FEV\textsubscript{1} was associated with significantly lower Shannon indices which persisted after adjustment for age, gender, body mass index and the presence of CF-related diabetes mellitus (CFRD). Details of the multiple regression analyses are provided in Table 10.4.

**Figure 10.6** Effect of FEV\textsubscript{1} and age on sputum bacterial diversity

FEV\textsubscript{1}: forced expiratory volume in 1 second

**Table 10.4** Relationship between demographic variables and sputum bacterial diversity assessed using multiple linear regression

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariable Analysis</th>
<th>95% CI</th>
<th>p</th>
<th>Multivariable Analysis</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>-0.02</td>
<td>-0.04 to +0.002</td>
<td>0.07</td>
<td>-0.01</td>
<td>-0.03 to +0.004</td>
<td>0.13</td>
</tr>
<tr>
<td>Gender\textsuperscript{#}</td>
<td>-0.06</td>
<td>-0.4 to +0.28</td>
<td>0.7</td>
<td>-0.09</td>
<td>-0.7 to +1.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Baseline FEV\textsubscript{1} %pred</td>
<td>+0.01</td>
<td>+0.003 to +0.02</td>
<td>0.006</td>
<td>+0.01</td>
<td>+0.003 to +0.019</td>
<td>0.006</td>
</tr>
<tr>
<td>BMI</td>
<td>+0.08</td>
<td>-0.28 to +0.43</td>
<td>0.7</td>
<td>+0.21</td>
<td>-0.013 to +0.55</td>
<td>0.2</td>
</tr>
<tr>
<td>CFRD</td>
<td>+0.006</td>
<td>-0.049 to +0.06</td>
<td>0.8</td>
<td>-0.002</td>
<td>-0.06 to +0.05</td>
<td>0.9</td>
</tr>
</tbody>
</table>

FEV\textsubscript{1}: forced expiratory volume in 1 second; BMI: body mass index; CFRD: cystic fibrosis related diabetes mellitus
\textsuperscript{#} reference category = male
10.5 Discussion

This study has demonstrated the potential for RISA to provide a rapid, reproducible and low-cost means of profiling the bacterial diversity within CF sputum samples. The findings have also shed more light on the complexities of the CF microbiome and, with a population of 93 patients, this represents one of the largest studies of culture-independent bacteriology in CF to date. RISA holds potential as a research tool and ultimately as part of the future clinical assessment of CF lung infection.

It is increasingly recognised that traditional culture-based techniques provide an incomplete picture of the microbial complexity of CF lung disease. This has led to considerable interest in the use of culture-independent methods to investigate respiratory infection in CF and recent investigators have hypothesised that certain microbiome patterns might be associated with more severe outcomes. However, technologies such as 16S rRNA gene pyrosequencing are limited by high costs, slow turn-around times and the need for specialist bioinformatic analysis. As a result, most of the studies using molecular techniques in CF have involved very small numbers of patients. As yet, our increasing knowledge of the CF lung microbiome has not been translated into meaningful changes to clinical practice. The development of new techniques that allow the monitoring and potential therapeutic modification of the CF microbiome in routine CF clinical care are a clear priority.

The combination of RISA with microfluidics technology holds a number of advantages over established methods of evaluating the lung microbiome. Firstly, RISA relies on readily available PCR-based technology with which many diagnostic microbiology laboratories are familiar. The technique is quick to perform, especially when combined with automatic DNA extraction of the kind used in this study. RISA is also relatively cheap; the total cost of consumables for the analysis of 200 samples was approximately £2500 (US$4000). At this cost of approximately £12 per sample, RISA represents a much more affordable alternative to 16S rRNA gene pyrosequencing which at the time of writing costs upwards of £60 per specimen in addition to the requirement for substantial bioinformatic analysis. Our experience suggests that, with further refinement, RISA is a strong candidate for development as a clinical diagnostics tool.
The data reported here demonstrate that RISA is able to identify distinct “microbiome types” within CF sputum samples which are broadly consistent with the 16S rRNA gene pyrosequencing data. There appears to be a division amongst patients with chronic *P. aeruginosa* infection into those whose sputum is overwhelmingly dominated by *P. aeruginosa* and those with preserved bacterial diversity. The *Pseudomonas*-dominant subgroup have a microbiome pattern more closely resembling patients with *B. cepacia* complex and other Gram-negative pathogens than patients among the *Pseudomonas*-diverse group. The reasons behind this difference are unclear and probably reflect a combination of bacterial and host factors. The degree of stability of these microbiome types is currently unknown and needs to be addressed in future longitudinal studies.

A novel finding from the data in this chapter relates to the low bacterial diversity associated with less common CF pathogens *Ralstonia* spp. and *Achromobacter* spp. The CF microbiome literature to date holds relatively little information on these pathogens. Harris *et al* reported a single CF individual whose sputum cultured *Achromobacter xylosoxidans*. On molecular testing with restriction fragment length polymorphism (RFLP), 84% of the bacteria in this patient’s sputum were accounted for by *Achromobacter* with only two other bacterial species identified. In contrast, Rudkjøbing and colleagues described another individual with CF who had sputum dominated by *Achromobacter* but also had a further nine species present. Data from a relatively large study by Cox *et al* suggest that reduced bacterial diversity in CF sputum is associated with increased disease severity. Given this finding, the reduced diversity we have observed with *Ralstonia* and *Achromobacter* species may be of prognostic importance.

In addition to providing a comparison with RISA, the 16S rRNA gene pyrosequencing data reported here have uncovered important associations between clinical parameters and the respiratory microbiome. In keeping with the findings of Cox *et al* and Zhao *et al* our data suggest that lower bacterial diversity is associated with worse lung function, an accepted marker of CF lung disease severity. This observed association between advanced disease and less diverse respiratory microbiota urgently needs to be addressed in a large prospective study. If
confirmed, it is possible to conceive a future therapeutic strategy to increase the bacterial diversity of the CF lung with the aim of improving clinical outcomes.

A number of limitations of this study and the RISA method in general must be acknowledged. Firstly, the data presented here are preliminary and further work is required to ensure that RISA has adequate reproducibility and repeatability. Future experiments to determine the reproducibility of RISA should include the analysis of multiple samples from the same patient both at a single time point and over the course of several days. Such experiments would assess the day-to-day variability of RISA profiles in CF sputum which it is important to know when looking at longitudinal changes in RISA in response to VRI, PEx or antibiotic therapy. On a related theme, the data in this chapter are primarily cross-sectional which limits the strength of conclusions that can be made. In addition, 16S pyrosequencing was not performed on all sputum samples due to resource limitations. Despite this, the study remains one of the largest in the literature to use culture independent bacteriology in CF. RISA itself gives a qualitative profile of the bacterial diversity only, however. Semi-quantification of RISA using band counts appears to lack validity when compared with 16S pyrosequencing data and inspection of the profiles themselves. Further correlation with quantitative culture and molecular diagnostics such as real time-PCR will also be required to validate fully the RISA profiling strategy. Such analyses would take into account bacterial abundance as well as diversity.

Although we have been able to identify certain pathogens such as A. xylosoxidans through cluster analysis of RISA profiles incorporating the profiles of pure cultures, the sensitivity and specificity of the technique for the early diagnosis of P. aeruginosa appears sub-optimal. Use of a greater number of P. aeruginosa reference strains to refine the technique may improve this in the future but it may be that RISA is best used to capture the overall state of the microbiome rather than detect individual pathogens. A role for RISA alongside culture and species-specific PCR assays can be envisaged with changes in the RISA profile prompting further assessment of the patient’s clinical status and respiratory microbiology.

Finally, there are a number of limitations common to all culture-independent bacteriological techniques. Firstly, since RISA and 16S rRNA gene pyrosequencing
identify bacteria on the basis of the presence of DNA in a sample, it is very difficult to distinguish live from dead bacteria. Secondly, respiratory tract specimens by necessity have to pass through the upper airway in the process of being sampled leading to the possibility of contamination of sputum from the oropharyngeal flora. Against this is work by Rogers et al which compared CF sputum with mouthwash samples and suggested that such contamination by oral flora is of little importance.

Allowing for these issues, RISA holds potential for development as a rapid, affordable method of profiling the bacterial diversity of CF sputum. RISA is clearly effective in detecting low-diversity microbiota associated with GNRs that may be missed by routine culture. Many such GNRs are associated with adverse prognoses so techniques which improve the identification and management of these pathogens are a priority for CF research. In the context of this broader research project, RISA also has sufficient reproducibility, variability and correlation with 16S rRNA pyrosequencing to be used as a tool to assess the impact of respiratory viruses on the CF lung microbiome.
Chapter Eleven

The Impact of Viral Respiratory Infections on the Respiratory Microbiome of Adults with Cystic Fibrosis
11.0 The Impact of Viral Respiratory Infection on the Respiratory Microbiome of Adults with Cystic Fibrosis

11.1 Abstract
11.1.1 Introduction
It has been hypothesised that viral respiratory infections (VRI) may be linked with acquisition of new bacterial pathogens among patients with CF. We performed a prospective study to determine whether VRI leads to a change in the respiratory microbiome of adults with CF.

11.1.2 Methods
Eighteen patients provided sequential paired sputum samples over a ten month period. One sputum sample from each visit was processed using conventional culture. The second sample was analysed with 16S rRNA gene pyrosequencing, ribosomal intergenic spacer analysis (RISA) and polymerase chain reaction (PCR) assays for nine respiratory viruses. Study visits were classified as “stable,” “viral” or “non-viral exacerbation.” Generalised estimating equation models were used to examine differences between each group of study visits.

11.1.3 Results
Eighteen patients provided a total of 77 paired sputum samples over a median follow-up period of 290 (range 62 – 359) days. Conventional culture did not identify any new bacterial species during the study period. A mean of 5453 (SD 2847) reads were detected by 16S rRNA pyrosequencing with a mean Shannon Index of 0.59 (SD 0.58) and mean richness of 15.1 (SD 7.9) genera. No consistent change in bacterial diversity indices or relative abundance of individual genera was seen in response to either viral infections or non-viral exacerbations. The majority of patients had highly variable RISA and 16S rRNA gene pyrosequencing profiles while a subset of four patients (22%) were found to have a highly stable, low-diversity lung microbiome.
11.1.4 Conclusions
Longitudinal change of the respiratory microbiome varies considerably among adults with CF. No consistent effect of either VRI or pulmonary exacerbation on the lung microbiome was observed.
11.2 Introduction

The hallmark of cystic fibrosis (CF) lung disease is chronic endobronchial infection, most commonly with gram negative bacteria such as *Pseudomonas aeruginosa*.

“Typical” CF pathogens such as *P. aeruginosa* are readily identified through standard sputum culture but it is increasingly recognised that culture alone underestimates the diversity of microbial communities within the CF lung.

Studies using molecular microbiological techniques suggest that there appears to be a link between reduced respiratory bacterial diversity and increased disease severity in CF. The impact of respiratory viruses such as influenza on the CF lung microbiome has not previously been investigated but such viral infections represent a potential mechanism by which bacterial communities might be disturbed.

The suspicion among the CF community that respiratory viruses might precipitate new infection with bacterial pathogens was first voiced over three decades ago. Petersen *et al* documented an increased rate of recent respiratory syncytial virus (RSV) infection amongst CF patients with new isolation of *P. aeruginosa*. Similarly, Collinson *et al* reported that 5 out of 6 cases of new *P. aeruginosa* infection in a prospective paediatric study coincided with an upper respiratory tract infection (URTI).

A number of groups have subsequently performed *in vitro* work to look at mechanisms of virus-bacteria interaction in CF. Chattoraj *et al* have shown, for instance, that rhinovirus infection leads to release of *P. aeruginosa* from biofilms with an associated immune response. Cell models have also demonstrated that both RSV and influenza promote adherence of *P. aeruginosa* to airway epithelial cells.

The above studies suggest mechanisms by which respiratory viruses might affect bacteria in the CF airways. In order to investigate viral-bacterial dynamics within the CF lung, we analysed sequential sputum samples from a cohort of adults with CF using standard culture, 16S rRNA gene pyrosequencing and ribosomal intergenic spacer analysis (RISA) matched to the results of virological testing. The results comprise the first ever investigation of the impact of respiratory viruses on the CF lung microbiome.
11.3 Methods

11.3.1 Study Overview

Patients were identified from a cohort of 100 adults with CF participating in a prospective observational study to investigate the incidence of respiratory virus infection. Details of the wider study design are described in Chapter 5. In brief, patients were seen every two months over a follow-up period of one year with additional study visits completed at the onset of a pulmonary exacerbation or cold. Paired sputum samples were collected at each visit. Conventional culture was performed on one sputum sample and the second was used for both viral polymerase chain reaction (PCR) assays and molecular bacteriological analysis. Nose- and throat-swabs were also collected at each visit for virological testing. All patients were aged eighteen years or older and gave written informed consent. The study was approved by the Greater Manchester West NHS Research Ethics Committee.

11.3.2 Patient Selection

Selection of patients was conducted in a two-step process. Proof-of-principle was demonstrated in an initial group comprising the first five patients who provided a stable baseline study visit followed by laboratory-confirmed infection with rhinovirus. A further 15 patients were then selected from three pre-specified groups within the main study cohort using a random number generator (www.random.org). Five patients were selected from those who did not experience a pulmonary exacerbation (PEx) during the observational study; five patients were selected from those with ≥2 viral PEx but no non-viral PEx and the remaining five patients were taken from those with ≥2 non-viral PEx but no viral PEx. All sputum samples available for these patients were included in the bacteriological analysis.

11.3.3 Classification of Study Visits

Pulmonary exacerbation (PEx) status was defined according to a modified version of the Fuchs criteria: the presence of ≥4 symptoms and a decision to treat with oral or intravenous antibiotics was considered a PEx. Each study visit was further defined on the basis of both PEx and virology status. Visits were classified as follows:
• Stable (S) Virology negative and criteria for PEx not met
• Viral (V) Respiratory virus detected (irrespective of PEx)
• Non-Viral PEx (E) Criteria for PEx met but virology negative

11.3.4 Virological Analysis
Virological analysis using in-house polymerase chain reaction (PCR) assays was performed on sputum, nose- and throat-swab samples taken at each study visit. Specimens were tested for a panel of nine viruses: adenovirus, influenza A&B, metapneumovirus, parainfluenza virus 1-3, respiratory syncytial virus (RSV) and rhinovirus. Samples were transferred to the regional virology laboratory at room temperature and processed within 24 hours of collection. Total nucleic acids were extracted from each specimen using the fully automated QIAamp® Virus Biorobot® MDx instrument (Qiagen, Hilden, Germany). Respiratory virus infection was defined as a positive polymerase chain reaction (PCR) assay from sputum, nose- or throat-swabs at any given visit. PCR cycle threshold values of <45 cycles was considered positive. The PCR primers are detailed in Chapter 5.

11.3.5 Conventional Sputum Culture
At each visit, sputum was sent for standard culture in accordance with the laboratory standards published by the UK CF Trust. Conventional culture was performed at an on-site laboratory and samples were processed within four hours of collection.

11.3.6 16S rRNA Gene Pyrosequencing
Total nucleic acids were extracted from sputum samples as described in the virology methods above. 16S rRNA gene pyrosequencing was performed commercially by Research and Testing Laboratory (Lubbock, Texas, USA) as previously described by Dowd et al. PCR amplification was performed on extracted nucleic acids using the universal 16S primers 530F (5’-GTG CCA GCM GCN GCG G) and 1100R (5’-GGG TTN CGN TCG TTG). The V1-V3 regions of the bacterial 16S rRNA gene were sequenced using the Genome Sequencer FLX System (Roche, New Jersey, USA). In-house software developed by Research and Testing Laboratory was used to process data derived from the sequencing process as described previously. Sequence data was denoised and stripped of chimeric sequences. Sequences smaller
than 250 bp were excluded. The remaining sequences were evaluated using BLASTn.NET software and compared with the NCBI GenBank database. Identified sequences were resolved to the genus level based on a sequence identity of >95%. The Shannon Index and richness (the total number of different genera identified) were calculated in order to quantify the degree of bacterial diversity within each sample.

11.3.7 Ribosomal Intergenic Spacer Analysis (RISA)
RISA was used as a second method to evaluate bacterial diversity within sputum samples. The bacterial intergenic spacer region (ITS) was amplified using a conventional PCR assay comprising 1 µl extracted DNA, 0.2 µl Taq polymerase, 2.5 µl buffer, and 5 µl of the relevant primers. The RISA primers 1406F TGYACACACCCGCGT and 23SR GGTTBCCCCATTG were used. 2 µl of amplified DNA from each sample was analysed using the microfluidic chip-based Agilent 2100 Bioanalyzer platform (Agilent Technologies, California, USA) in order to separate DNA and produce a distinct RISA profile for each specimen. The total number of RISA bands in each sample was calculated as a semi-quantitative measure of sputum bacterial diversity. Bioanalyzer RISA profiles were analyzed using Gelcompar II (Applied Maths, Texas, USA) as described in Section 5.11.4. Pearson correlation was performed to determine the degree of similarity between RISA profiles.

11.3.8 Statistical Analysis
Data are presented as mean (standard deviation) or median (inter-quartile range) as appropriate. Data produced by RISA and 16S rRNA gene pyrosequencing were analysed using generalised estimating equation (GEE) models in order to take account of repeated observations from individual participants. The visit classification (i.e. stable, viral or non-viral exacerbation) was used as an independent variable in these models. The conventional p<0.05 level of significance was assumed. Statistical analyses were conducted using SPSS® 20.0 (IBM, New York, USA).
11.4 Results

11.4.1 Patient Characteristics

Of the 20 patients selected, two had fewer than three sputum samples available for analysis and were excluded from the study. The baseline characteristics of the remaining 18 patients are presented in Table 11.1.

Table 11.1 Baseline characteristics of study participants

<table>
<thead>
<tr>
<th></th>
<th>Microbiome Analysis Cohort</th>
<th>Full Virology Study Cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>Age (yrs)#</td>
<td>30.5 (19–47)</td>
<td>28 (23–36)</td>
</tr>
<tr>
<td>Female gender (%)</td>
<td>39</td>
<td>52</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>22.1 (2.4)</td>
<td>21.8 (2.9)</td>
</tr>
<tr>
<td>Baseline FEV₁ %-predicted</td>
<td>59.7 (23.3)</td>
<td>59.3 (22.0)</td>
</tr>
<tr>
<td>Chronic P. aeruginosa infection (%)</td>
<td>88.9</td>
<td>73.0</td>
</tr>
<tr>
<td>Chronic B. cepacia complex infection (%)</td>
<td>16.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Maintenance azithromycin (%)</td>
<td>100</td>
<td>95.0</td>
</tr>
<tr>
<td>Maintenance nebulised antibiotics (%)</td>
<td>88.9</td>
<td>85.0</td>
</tr>
</tbody>
</table>

FEV₁: forced expiratory volume in 1 second
#: median (IQR). Other data are presented as mean (SD) or %

11.4.2 Study Visits

Analysis was conducted on sputum samples from a total of 77 study visits. Each patient provided a mean of 4.3 (1.0) samples over a median follow-up period of 290 days (range 62 – 359). 46 visits were classified as stable, 23 as viral and 8 as non-viral PEx. Two of the 23 virus-positive episodes were dual viral infections giving a total of 25 viruses identified. Among the virus-positive visits, 11/23 (47.8%) met the pre-defined criteria for PEx. Table 11.2 provides a breakdown of the different viruses identified. The clinical parameters associated with each visit category are shown in Table 11.3.
Table 11.2  Breakdown of viruses identified during the study

<table>
<thead>
<tr>
<th>Virus</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhinovirus*</td>
<td>14</td>
</tr>
<tr>
<td>Metapneumovirus*</td>
<td>4</td>
</tr>
<tr>
<td>Influenza A*</td>
<td>2</td>
</tr>
<tr>
<td>Influenza B*</td>
<td>2</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>2</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

* Figures include one case of metapneumovirus-rhinovirus co-infection and one case of influenza A&B co-infection

Table 11.3  Clinical characteristics of study visits by virological and exacerbation status

<table>
<thead>
<tr>
<th></th>
<th>Stable Visits</th>
<th>Viral Visits</th>
<th>Non-Viral PEx Visits</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of visits</td>
<td>46</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>Fall from baseline FEV&lt;sub&gt;1&lt;/sub&gt; (%)</td>
<td>9.4 (9.0)</td>
<td>8.3 (10.8)</td>
<td>9.3 (6.2)</td>
</tr>
<tr>
<td>URTI score (max = 27)</td>
<td>3.3 (3.4)</td>
<td>6.9 (5.1)</td>
<td>4.6 (1.7)</td>
</tr>
<tr>
<td>PEx score (max = 12)</td>
<td>1.5 (1.7)</td>
<td>3.7 (2.1)</td>
<td>4.6 (0.9)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>11.3 (14.4)</td>
<td>8.5 (6.3)</td>
<td>36.4 (36.1)</td>
</tr>
<tr>
<td>White cell count (x10&lt;sup&gt;9&lt;/sup&gt;)</td>
<td>10.3 (3.4)</td>
<td>9.6 (3.1)</td>
<td>11.6 (3.3)</td>
</tr>
<tr>
<td>Antibiotics prescribed (%)</td>
<td>6.5</td>
<td>78.3</td>
<td>100</td>
</tr>
</tbody>
</table>

FEV<sub>1</sub>: forced expiratory volume in 1 sec; URTI: upper respiratory tract infection; PEx: pulmonary exacerbation; CRP: C-reactive protein

11.4.3  Conventional Sputum Culture Results
In each of the 18 patients included in this analysis, serial standard sputum culture identified only bacterial pathogens with which patients were known to be chronically infected at entry to the study. Four patients had intermittent growth of a
pathogen during the study (three patients with intermittent \textit{S. aureus} and one with \textit{P. aeruginosa}). In each of these four cases, isolation of the intermittent pathogen was associated with clinical stability and was not related to either VRI or PEx.

### 11.4.4 16S rRNA Gene Pyrosequencing Results

16S rRNA gene pyrosequencing detected a mean of 5453 (2847) sequence reads per sputum sample. The mean Shannon Index value was 0.59 (0.58) with a mean richness of 15.1 (7.9) genera per sample. \textit{Pseudomonas} spp was the dominant organism in 67/77 (87\%) samples (mean relative abundance 80.8\%) with a further 6/77 samples heavily dominated by \textit{Burkholderia} spp (mean relative abundance 85.4\%). Table 11.4 and Figure 11.1 show comparisons of the key measures of bacterial diversity at study visits categorised by virology and exacerbation status. Virus-positive visits were associated with a significantly greater number of RISA bands compared with stable visits (4.91 (2.6) vs 3.83 (2.5) bands respectively; \(p=0.04\)). There were no statistically significant differences in the other diversity measures across the three groups.

Scrutiny of the serial 16S rRNA gene pyrosequencing results reveals that most patients had a high degree of variability in both the overall sputum bacterial diversity and the relative abundance of various different organisms within the microbiome. For a sub-set of four participants (patients 1, 11, 53 and 88), the bacterial diversity remained persistently low throughout the study despite the onset of PEx, VRI and antibiotic therapy. Examples of these differences in longitudinal variability of 16S sequencing results are shown in Figure 11.2. Similar data for all patients reported in this chapter are provided in Appendix 3.

Across the cohort, there was no consistent pattern to changes in the respiratory microbiome in response to either VRI or PEx. The main predictor of the bacterial communities identified within a sample appears to be the identity of the patient providing the sample. This is seen most clearly in Figure 11.3 which shows multi-dimensional scaling plots to demonstrate the relatedness of the microbiota within individual sputum samples. Samples from each individual patient clustered tightly together whereas when samples were organised by virology and exacerbation status,
there was considerable overlap with no clear pattern emerging. No significant
correlation was seen between mean Shannon Index values across the whole study
and baseline age, FEV₁ %-predicted or body mass index.

Table 11.4  Comparison of bacterial diversity indices by exacerbation and
virology visit status

<table>
<thead>
<tr>
<th></th>
<th>Stable Visits</th>
<th>Viral Visits</th>
<th>Non-Viral PEx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>No of samples</td>
<td>46</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>No of 16S reads</td>
<td>5271 (2980)</td>
<td>5524 (2760)</td>
<td>5613 (3126)</td>
</tr>
<tr>
<td>Richness</td>
<td>14.7 (8.0)</td>
<td>15.7 (8.4)</td>
<td>15.0 (5.8)</td>
</tr>
<tr>
<td>Shannon Index</td>
<td>0.60 (0.60)</td>
<td>0.57 (0.53)</td>
<td>0.58 (0.72)</td>
</tr>
<tr>
<td>RISA band count</td>
<td>3.83 (2.53)</td>
<td>4.91 (2.56)</td>
<td>5.13 (2.95)</td>
</tr>
</tbody>
</table>

*p values were derived from generalised estimating equation models with stable
visits used as the reference variable.
PEx: pulmonary exacerbation; RISA: ribosomal intergenic spacer analysis

Figure 11.1  Sputum bacterial diversity by visit status as measured by A)
Shannon Index and B) richness (i.e. total number of genera identified)
PEx: pulmonary exacerbation
Figure 11.2  Longitudinal changes in the sputum 16S rRNA gene pyrosequencing results from four representative patients with CF

The results show a variety of patterns: A) minimal change in diversity despite PEx, VRI and antibiotic therapy; B) highly variable diversity in a patient with multiple viral infections; C) increased bacterial diversity in association with rhinovirus infection and D) increased bacterial diversity in association with non-viral PEx

S: stable visit; E: non-viral exacerbation visit; V: virus-positive visit  ●: oral antibiotics; *: intravenous antibiotics
HRV: human rhinovirus; MPV: metapneumovirus; Flu: influenza
Figure 11.3 Multi-dimensional scaling plots to show the degree of similarity of bacterial populations detected by 16S rRNA gene pyrosequencing for sputum samples clustered by A) individual patients and B) visit status

PEx: pulmonary exacerbation

11.4.5 Ribosomal Intergenic Spacer Analysis (RISA) Results
Serial RISA profiles for each patient are given in full in Appendix 4. Of the 23 virus-positive visits captured in the study, four occurred at the baseline visit so RISA profiles from these visits could not be compared with previous samples. Of the remaining 19 virus-positive episodes, 7 (36.8%) were associated with an increase in the complexity of the RISA profile, 6 (31.6%) showed no change compared with the preceding profile and 6 (31.6%) showed a reduction in the number of visible RISA bands. Figure 11.4 shows representative examples of serial RISA profiles from three patients to illustrate the observed changes following VRI. The lack of a predictable effect of VRI on RISA profiles is consistent with results of 16S rRNA gene pyrosequencing reported above.
Figure 11.4  Bacterial diversity within sequential sputum samples as detected by ribosomal intergenic spacer analysis (RISA)

Examples of sequential RISA profiles to show variable response to viral respiratory infection (VRI): A) increased bacterial diversity at time of VRI; B) reduced bacterial diversity at time of VRI; C) no change in RISA profile at time of VRI

IV: intravenous antibiotics; ORAL: oral antibiotics

11.5  Discussion

This study represents the first ever investigation of the in vivo effect of respiratory viruses on the lung microbiome. The data also illustrate the temporal changes in the bacterial communities within the lungs of adults with CF. Our findings suggest that the nature of an individual’s previous respiratory microbiota is a more important
predictor of subsequent changes in bacterial diversity than the onset of a viral infection or PEx. We have not shown a consistent effect of either VRI or PEx on the bacterial populations in CF sputum.

Despite the lack of a clear overall impact of VRI in this study, a number of individual patients did appear to have a change in their personal respiratory microbiome in association with a respiratory virus. Such changes were seen both with 16S rRNA gene pyrosequencing and RISA. Patients 21, 22, 35 and 52, for instance, all appeared to have a considerable increase in sputum bacterial diversity in association with rhinovirus infection. Three of these patients were noted to have an increase in the relative abundance of *Streptococcus* spp at the time of VRI raising the possibility of an interaction with rhinovirus. It must be acknowledged, however, that other patients in the cohort experienced variability in the proportion of *Streptococci* during periods of stability. Similarly, four individuals experienced either no change or a reduction in bacterial diversity at the time of VRI (see patients 18, 31, 51 and 83 in Appendix 3). Given these observations, the true effect of VRI on the respiratory microbiome remains equivocal and may vary considerably between individual patients.

Beyond the assessment of virus-bacteria interactions in the CF lung, this study has provided important new data on longitudinal changes in the CF respiratory microbiome. The majority of studies in this field have, to date, been cross-sectional in design with relatively little prospective data in existence. Our finding of a division between patients with persistently low levels of bacterial diversity and those with more complex, variable bacterial populations is in keeping with the findings of Zhao *et al.* Zhao and colleagues reported serial bacterial diversity analyses in sputum samples collected over a nine year period from six patients with CF. Three of the patients were noted to have a progressive reduction in bacterial diversity associated with accelerated disease progression while the remaining three patients maintained both clinical stability and preserved respiratory bacterial diversity. It remains open to debate whether reduced bacterial diversity has a causative role in the progression of CF lung disease or acts merely as a marker of advanced disease.
A further novel finding of this study relates to the comparison of two culture-independent bacteriological techniques: 16S rRNA gene pyrosequencing and RISA. Both techniques provide measures of bacterial diversity within the respiratory microbiome. RISA may be considered a profiling method with lower discriminatory power while pyrosequencing is accepted as the gold-standard cultivation-independent technique for the measurement of bacterial diversity. Despite these differences, each was able to separate out patients with persistently low levels of diversity. RISA lacks the specificity of pyrosequencing and similar methods but this is offset by its low cost, quick turnaround time and reliance on widely available PCR-based technology. The RISA results suggest that the technique has potential for use in the longitudinal monitoring of bacterial communities in the setting of clinical infection. Further research and refinement of RISA over longer-term studies will be required to validate this conclusion.

A number of features of the study cohort need to be considered when analysing the data presented above. Firstly, the vast majority of episodes of VRI were due to rhinovirus which limits the conclusions which can be drawn regarding other specific viruses such as influenza. Secondly, all of the patients included in this study were known to be chronically infected with either \( P. \) aeruginosa or the \( B. \) cepacia complex. As was shown in Chapter 10, infection with these organisms in a considerable proportion of cases is associated with low levels of bacterial diversity. As a result, many of the patients investigated in this analysis may have had limited potential for change in their respiratory microbiome. There is a pressing need to study the effects of VRI on bacterial communities in the lungs of patients with milder CF lung disease before they become infected with \( Pseudomonas \). If respiratory viruses were found to interact with bacteria in this context, then this may represent an opportunity to intervene to prevent chronic infection with typical CF pathogens.

The study of naturally-occurring viral infection also presents something of a challenge as the timing of onset of infection is never known with certainty. This hampers the interpretation of subsequent clinical and microbiological outcomes. A potential solution to this problem might lie with the use of an experimental rhinovirus challenge model as has been conducted in both asthma and chronic
obstructive pulmonary disease.\textsuperscript{447-450} Such techniques involve nasal inoculation with a known strain of rhinovirus and would allow regular, sequential sampling of the respiratory tract at prescribed time-points before and after onset of rhinovirus infection. The most commonly used strain in challenge models is rhinovirus 16,\textsuperscript{448-451} a member of the species \textit{Human rhinovirus A}, which would be appropriate for use in CF studies given the predominance of rhinovirus A reported in Chapter 9. Potential ethical issues complicate the extension of experimental rhinovirus infection to patients with CF, however. It might be argued, for instance, that the intentional causation of an exacerbat is contrary to the best interests of study participants given the clear association between PEx and both lung function decline and increased risk of death.\textsuperscript{23,24}

Weaknesses of the present study design must be considered. Firstly, it must be acknowledged that the wider research project was set up to determine the incidence of VRI as the main outcome measure. The timing of respiratory specimen collection during the study was not, therefore, ideal to address the question of whether viruses directly affect the lung microbiome. Future studies in this field would benefit from more frequent and regular clinical sampling following VRI as suggested above with regard to experimental rhinovirus models. The study is further limited by small patient numbers although this is equally applicable to most of the previous longitudinal studies in the CF microbiome literature.\textsuperscript{123,124,452,453} A further issue is that the methods employed in this study do not allow for a quantification of bacterial abundance within samples which may be of clinical relevance. Finally, the generic weaknesses of molecular bacteriological techniques were discussed in Chapter 10 and apply equally to this analysis.

Allowing for these limitations, this study represents the first prospective exploration of virus-bacteria interactions at the level of the microbial community. Despite the lack of a consistent, reproducible impact of VRI on the lung microbiome in CF, the degree of variability of bacterial diversity seen suggests that viruses may indeed lead to changes in the microbiome in certain circumstances. Further studies focused on early CF lung disease are required to determine whether respiratory viruses promote acquisition or establishment of key bacterial pathogens such as \textit{P. aeruginosa}. 

193
Chapter Twelve

Summary and Discussion
Summary and Discussion

The work presented in this thesis represents the first large-scale, prospective study to examine the role of respiratory viruses in adult CF lung disease and has uncovered a number of novel findings. Firstly, this study has shown clearly that respiratory viruses are both common among adults with CF and are associated with significant outcomes such as PEx. The combination of detailed clinical, virological and meteorological data gives a new insight into the epidemiology of acute health events affecting patients with CF. Additionally, the work on bacterial diversity reported here is, at the time of writing, the first attempt to document the effect of viral infections on the respiratory microbiome in any chronic lung disease. This summary discusses the novel findings of each results chapter before considering the overall strengths and limitations of the project at large.

Chapter Six – The Incidence of VRI in Adults with CF

Prior to this project, there had been a considerable number of studies investigating the importance of VRI in children with CF.\textsuperscript{330,332,335,343,346-350} Despite this, there was a considerable gap in the evidence base to inform clinicians as to the significance of VRI among adult patients. The results presented in Chapter 6 address this deficit and mirror the paediatric studies in confirming the importance of viruses in adult CF lung disease. Firstly, this thesis has shown that respiratory viruses affect the vast majority of adults with CF each year and that these infections have a significant association with PEx. In addition, this study has confirmed the overwhelming predominance of rhinovirus over the other viruses investigated. Finally, Chapter 6 has revealed important new data on the optimal means of diagnosing VRI in CF and demonstrates that sputum should be tested for viruses alongside upper airway samples in adult patients. This finding has immediate implications for current clinical practice and must be considered when interpreting the results of studies that relied on upper airway samples alone to detect VRI.\textsuperscript{353}

Chapter Seven – The Clinical Impact of VRI in Adults with CF

Chapter 7 documents the clinical impact of laboratory-confirmed VRI among adults with CF. This is the first study to allow an assessment to be made of the long-term...
impact of VRI on the health of adults with CF. Several paediatric studies have reported a link between viral infection and disease progression in the form of lung function decline\textsuperscript{330,332,346} but this has not been universal.\textsuperscript{343,347} While there was no statistically significant association between VRI and FEV\textsubscript{1} decline in the data given in Chapter 7, it must be noted that the study was not powered to detect changes in this outcome. The largest study to investigate VRI in any CF population included just 153 patients\textsuperscript{336} and it is likely that a much bigger cohort followed over a long period of time would be required to determine conclusively the long-term impact of viral infections on rates of lung function decline. Until such a study is performed, the clear link between VRI and PEx in both adults and children with CF suggests that there should still be a strong suspicion that VRI is of prognostic significance.

12.3 Chapter Eight – The Effect of Climate & Season on VRI & PEx in Adults with CF

The results presented in Chapter 8 show the seasonality of viral infections and PEx amongst the study cohort. Rhinovirus infections were found to be frequent throughout the calendar year while other viruses displayed a definite predominance over winter. Perhaps surprisingly, there did not appear to be a strong seasonal pattern to the incidence of PEx which remained common throughout the year. Clearly, this may not be applicable to all populations and geographical locations but it suggests that CF services need to be able to maintain high demand for clinic consultations, hospital admission and intravenous antibiotic therapy. The data also provide CF patients with evidence that changes in weather conditions can indeed have an impact on their respiratory health.

12.4 Chapter Nine – The Spectrum of Rhinovirus Species Affecting Adults with CF

Only two previous studies have included the results of genetic sequencing to determine which rhinovirus sub-species affect patients with CF.\textsuperscript{334,350} While de Almeida and colleagues found a considerable burden of rhinovirus C disease in Brazilian children with CF,\textsuperscript{350} this was not the case in the present study or that of Wark \textit{et al.}\textsuperscript{334} In the Manchester adult cohort, the diversity of rhinoviruses appeared
to mirror that seen in the general population with rhinovirus A overwhelmingly the most prevalent followed by rhinovirus B. Rhinovirus C accounted for only 5% of cases sequenced. The data uncovered here have also shown for the first time a difference in clinical outcomes between different rhinovirus species with rhinovirus A associated with more severe illness. The pathophysiology of infection with different rhinovirus species is known to vary. The majority of rhinovirus A strains enter human cells through ICAM-1 whilst the rhinovirus B group utilise alternative pathways such as LDL-1. The greater incidence and severity of rhinovirus A suggests that this species should be the focus of future therapies and vaccines to prevent rhinovirus-related exacerbations of CF lung disease.

12.5 Chapter Ten – Development of Ribosomal Intergenic Spacer Analysis (RISA) as a Tool to Profile the Bacterial Diversity of CF Sputum

The development of culture-independent microbiology over the last decade has produced rapidly-evolving insights into the complexity of bacterial populations within the CF lung.\textsuperscript{52,454} It is clear that traditional culture is unable to detect changes in the respiratory microbiome and the new PCR-based methods are increasingly attractive as a way of identifying early infection with harmful pathogens such as \textit{P. aeruginosa}. Others have also hypothesised that particular patterns of bacterial diversity may have prognostic importance.\textsuperscript{441} Ribosomal intergenic spacer analysis (RISA) as described in Chapter 10 provides a rapid, cheap option for the profiling of the CF microbiome. With refinement and further work to confirm the reproducibility of the technique, RISA has the potential for translation into the clinical setting to allow much wider access to culture-independent diagnostics to help inform the management of patients with CF.

12.6 Chapter Eleven – The Impact of VRI on the Respiratory Microbiome of Adults with CF

This study did not find a consistent effect of VRI on measures of bacterial diversity using either RISA or 16S rRNA gene pyrosequencing. The data did, however, give an insight into the within-subject patterns of variability of the microbiome over the
course of the study. The prognostic implications of the degree of microbiome stability require further investigation. Given the relatively small sample size included in this study and the long average interval between viral infection and follow-up samples, further research is also needed to confirm the effect of VRI on bacterial communities in the CF lung.

12.7 Limitations of the Study

Having considered the new knowledge gained from this study, it is important to address again the potential limitations of the project. As discussed in Chapter 6, the respiratory virus PCR panel is not exhaustive and excludes both coronavirus and bocavirus. This omission was pragmatic in nature and was based on the availability of resources. Given the recent global emergence of the novel MERS-coronavirus associated with severe pneumonitis, it might have been preferable in retrospect to have included this group of viruses in the PCR panel. However, the majority of CF studies which included coronavirus assays have found the prevalence to be low at between 0.8 and 9% of all samples collected. Van Ewijk et al did report a much higher incidence of coronavirus infection amongst 20 young children with CF; 90% of patients in that study had an episode of coronavirus over a six month period. The reasons for such a high incidence figure are unexplained but may lie with differences in diagnostic techniques. Only two studies have reported the prevalence of bocavirus in CF patients with the largest detecting this virus in 23 out of 408 samples (5.6%). Taking these results as a whole, it would appear that coronavirus and bocavirus are relatively minor players in CF lung disease. The omission of these viruses from the PCR panel in this project does not, therefore, compromise the central conclusions.

A second weakness of the study relates to the reliance on participants to contact the investigators if and when they experienced new symptoms suggestive of an exacerbation or viral infection. It is possible that a number of such events went unrecorded and as a result the incidence of VRI reported here may be underestimated. However, given that patients were seen by the study team on average every six to eight weeks and generally rely on the CF unit for the majority
of their healthcare needs, it is likely that few clinically significant episodes were not captured by the study. An additional limitation that must be acknowledged is that complete sets of specimens for virological analysis were not collected at every visit. The most common reason for this was an inability of participants to produce a sputum sample at every visit. At approximately 3.5% of study visits, the opportunity to collect virology specimens was missed entirely. This lack of adherence to the study protocol highlights the challenge of conducting research in a busy clinical environment where the immediate health needs of the patient take precedence. However, given the low frequency of such lapses, it does not appear that this issue would have had a significant impact on the overall results of the study.

The final limitation to be addressed involves the investigation of the respiratory microbiome. As previously discussed, the primary aim of the project was to determine the incidence and epidemiology of VRI in adults with CF. The study was not, therefore, designed to answer definitively the question of whether VRI leads to a change in bacterial diversity in the CF lung. The ideal study on this topic would almost certainly include more regular sampling of the respiratory tract in the weeks immediately before and after a laboratory confirmed viral infection. Such a study would by design be rather complex, resource-intensive and intrusive to patients. The lack of a clear conclusion from the present study, however, suggests there is a need for targeted studies to try to answer this important question which has direct clinical implications for the treatment of CF exacerbations.

12.8 Strengths of the Study
Of course, it is appropriate to consider the strengths of this study alongside the weaknesses acknowledged above. The principle strength is the large size of the cohort: this project represents the largest prospective study of viral infections in CF adults and there have been only two larger studies in paediatric populations in the era of molecular virology. Alongside the cohort size, the prospective study design was vital for the interpretation of longitudinal outcomes and allows individual study participants to act as their own controls. A very low level of patient drop-out was achieved with only two patients declining to participate beyond the baseline visit. The study also benefits from a wealth of carefully-documented
clinical data which complement and enhance the microbiological, epidemiological and meteorological results. As a result, the stored serum and respiratory specimens provide a highly valuable resource for future research into the pathophysiology of PEx in CF.

12.9 Areas for Future Research

There remain many issues relating to respiratory viruses and CF which require further research. Firstly, the importance of cross-infection with respiratory viruses on the CF unit, both between patients and through staff members, is an important gap in the current evidence base. There is considerable scope for an intervention study to reduce the incidence of such infections among vulnerable CF patients. Components of such an intervention might include education on respiratory and hand hygiene, increased use of patient isolation and the deployment of masks and respirators by healthcare workers. Coupled to this, there is a need to investigate on a larger scale the typical duration of PCR positivity after acute viral infection in CF and address how long such patients remain contagious.

Further work also needs to be done to characterise in more detail the effect of VRI on bacterial diversity in the CF lung. One option for investigating this might be the use of an experimental rhinovirus challenge model as conducted previously in both asthma and COPD. Considerable ethical issues complicate such a strategy, however, given the clear link between exacerbations of CF and both disease progression and increased mortality. An alternative method might involve the regular sampling of sputum, perhaps daily, following onset of both viral and non-viral exacerbations. The inclusion of quantitative bacteriology to determine the bacterial load as well as diversity would be advantageous in such a study as this aspect has not been addressed in this thesis.

Trials of existing prophylactic therapies to prevent VRI should also be considered in future research. Despite widespread acceptance, there has been no randomised controlled trial of influenza vaccination in patients with CF, for instance. A trial of nasal versus intramuscular influenza vaccination might give insights into the protection afforded by such therapies especially if it were considered that a placebo-
controlled trial was not ethically sound. Alternative preventative treatments for VRI that deserve exploration include regular zinc supplementation.

12.10 Conclusions
The work described in this thesis has demonstrated unequivocally that respiratory viruses are a common and clinically important factor in adult CF lung disease. For the first time, the epidemiology and clinical impact of VRI has been characterised in adults with CF both during pulmonary exacerbations and clinical stability. The challenge for CF researchers is to translate this knowledge into the development of therapies to prevent and treat viral exacerbation of CF.
Appendices
13.0 Appendix 1. Presence of Individual Symptom Score Components by Virus Type

Table 13.1 Mean scores for Upper Respiratory Tract Infection (URTI) Score components by virus type

<table>
<thead>
<tr>
<th></th>
<th>No of Cases</th>
<th>Mean URTI Score</th>
<th>Runny Nose</th>
<th>Sneezing</th>
<th>Blocked Nose</th>
<th>Itchy Eyes</th>
<th>Sore Throat</th>
<th>Hoarse Voice</th>
<th>Fever</th>
<th>Headache</th>
<th>Myalgia</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Viruses</td>
<td>191</td>
<td>7.66</td>
<td>1.00</td>
<td>0.88</td>
<td>1.03</td>
<td>0.47</td>
<td>0.83</td>
<td>0.77</td>
<td>0.66</td>
<td>0.80</td>
<td>0.79</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>7</td>
<td>5.71</td>
<td>0.43</td>
<td>1.00</td>
<td>1.14</td>
<td>0.14</td>
<td>0.29</td>
<td>0.57</td>
<td>0.86</td>
<td>0.43</td>
<td>0.86</td>
</tr>
<tr>
<td>Influenza</td>
<td>9</td>
<td>7.78</td>
<td>0.89</td>
<td>1.00</td>
<td>1.00</td>
<td>0.56</td>
<td>1.11</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.89</td>
</tr>
<tr>
<td>MPV</td>
<td>21</td>
<td>4.68</td>
<td>0.79</td>
<td>0.53</td>
<td>0.42</td>
<td>0.26</td>
<td>0.47</td>
<td>0.42</td>
<td>0.53</td>
<td>0.63</td>
<td>0.63</td>
</tr>
<tr>
<td>Parainfluenza</td>
<td>4</td>
<td>10.75</td>
<td>1.50</td>
<td>1.25</td>
<td>1.00</td>
<td>0.25</td>
<td>2.50</td>
<td>1.25</td>
<td>0.75</td>
<td>1.00</td>
<td>1.25</td>
</tr>
<tr>
<td>RSV</td>
<td>4</td>
<td>13.50</td>
<td>2.00</td>
<td>1.50</td>
<td>2.25</td>
<td>0.75</td>
<td>1.50</td>
<td>1.50</td>
<td>1.25</td>
<td>1.50</td>
<td>1.25</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>138</td>
<td>7.85</td>
<td>1.10</td>
<td>0.95</td>
<td>1.14</td>
<td>0.53</td>
<td>0.88</td>
<td>0.86</td>
<td>0.71</td>
<td>0.88</td>
<td>0.85</td>
</tr>
</tbody>
</table>

The maximum total value of the URTI Score is 27. Each symptom component has a maximum score of 3. Dual viral infections are excluded from the above table.

MPV: metapneumovirus
RSV: respiratory syncytial virus
Table 13.2  Mean Fuchs Pulmonary Exacerbation (PEx) Score by virus type and percentage of visits associated with the presence of each component

<table>
<thead>
<tr>
<th></th>
<th>No of Cases</th>
<th>Mean PEx Score</th>
<th>Sputum Change (%)</th>
<th>Haemoptysis (%)</th>
<th>Cough (%)</th>
<th>SOB (%)</th>
<th>Malaise (%)</th>
<th>Temp &gt;38°C (%)</th>
<th>Anorexia (%)</th>
<th>Sinus Pain (%)</th>
<th>Sinus Discharge (%)</th>
<th>New Chest Signs (%)</th>
<th>FEV₁ ↓ 10% (%)</th>
<th>New CXR change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Viruses</td>
<td>191</td>
<td>3.6</td>
<td>57.1</td>
<td>16.8</td>
<td>57.6</td>
<td>47.6</td>
<td>51.3</td>
<td>6.8</td>
<td>27.7</td>
<td>22.5</td>
<td>26.2</td>
<td>5.8</td>
<td>19.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>7</td>
<td>3.0</td>
<td>57.1</td>
<td>0.0</td>
<td>57.1</td>
<td>28.6</td>
<td>57.1</td>
<td>0.0</td>
<td>42.9</td>
<td>0.0</td>
<td>42.9</td>
<td>0.0</td>
<td>14.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Influenza</td>
<td>9</td>
<td>4.6</td>
<td>77.8</td>
<td>11.1</td>
<td>77.8</td>
<td>77.8</td>
<td>77.8</td>
<td>11.1</td>
<td>22.2</td>
<td>11.1</td>
<td>33.3</td>
<td>11.1</td>
<td>33.3</td>
<td>11.1</td>
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<tr>
<td>MPV</td>
<td>21</td>
<td>2.8</td>
<td>42.9</td>
<td>9.5</td>
<td>42.9</td>
<td>33.3</td>
<td>42.9</td>
<td>0.0</td>
<td>19.0</td>
<td>19.0</td>
<td>14.3</td>
<td>4.8</td>
<td>23.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Parainfluenza</td>
<td>4</td>
<td>4.5</td>
<td>50.0</td>
<td>50.0</td>
<td>50.0</td>
<td>75.0</td>
<td>75.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>25.0</td>
<td>0.0</td>
<td>25.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RSV</td>
<td>4</td>
<td>5.5</td>
<td>100.0</td>
<td>0.0</td>
<td>50.0</td>
<td>50.0</td>
<td>75.0</td>
<td>0.0</td>
<td>75.0</td>
<td>75.0</td>
<td>50.0</td>
<td>0.0</td>
<td>50.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>138</td>
<td>3.6</td>
<td>56.5</td>
<td>18.8</td>
<td>60.9</td>
<td>48.6</td>
<td>49.3</td>
<td>8.0</td>
<td>26.1</td>
<td>23.9</td>
<td>24.6</td>
<td>5.8</td>
<td>18.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The maximum total value of the PEx Score is 12. The percentage of visits with the presence of each component is given in the table. Dual viral infections are excluded from the above table.

MPV: metapneumovirus; RSV: respiratory syncytial virus; SOB: shortness of breath; Temp: temperature; FEV₁: forced expiratory volume in 1 sec; CXR: chest x-ray
Appendix 2. Ribosomal intergenic spacer analysis (RISA) profiles of selected clinical samples in comparison with pure cultures of reference strains

A. Achromobacter xylosidans

B. Ralstonia mannitololytica

C. Stenotrophomonas maltophilia
**D. Burkholderia multivorans**

Figure 14.1 Ribosomal intergenic spacer analysis (RISA) profiles of selected clinical samples in comparison with pure cultures of CF pathogen reference strains

Ax: Achromobacter xylosidans
Bm: Burkholderia multivorans
Pa: Pseudomonas aeruginosa
Sa: Staphylococcus aureus

**E. Burkholderia cenocepa**

Bc: Burkholderia cenocepa
Hi: Haemophilus influenzae
Rm: Ralstonia mannitololytica
Sm: Stenotrophomonas maltophilia
Appendix 3. Sequential 16S rRNA pyrosequencing results for individual study participants

Figure 15.1  Sequential 16S rRNA gene pyrosequencing profiles from patients included in Chapter 11 who did not experience a pulmonary exacerbation during follow-up

S: stable; V: viral infection; E: non-viral pulmonary exacerbation; MPV: metapneumovirus

* intravenous antibiotics; • oral antibiotics
Figure 15.2  Sequential 16S rRNA gene pyrosequencing profiles from patients included in Chapter 11 who experienced ≥2 virus-positive exacerbations and no non-viral exacerbations during follow-up

S: stable;  V: viral infection;  E: non-viral pulmonary exacerbation;  MPV: metapneumovirus;  RSV: respiratory syncytial virus

* intravenous antibiotics;  • oral antibiotics
Figure 15.3  Sequential 16S rRNA gene pyrosequencing profiles from patients included in Chapter 11 who experienced ≥2 non-viral exacerbations and no virus-positive exacerbations during follow-up

S: stable; V: viral infection; E: non-viral pulmonary exacerbation

* intravenous antibiotics; ● oral antibiotics
Figure 15.4  Sequential 16S rRNA gene pyrosequencing profiles from patients included in Chapter 11 who experienced both virus-positive and -negative exacerbations during follow-up

S: stable; V: viral infection; E: non-viral pulmonary exacerbation

* intravenous antibiotics; ● oral antibiotics
16.0 Appendix 4. Sequential ribosomal intergenic spacer analysis (RISA) profiles for individual study participants

<table>
<thead>
<tr>
<th>ID</th>
<th>Visit Status</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt 30</td>
<td>30 A</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>30 B</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>30 C</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>30 D</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>30 E</td>
<td>S</td>
</tr>
<tr>
<td>Pt 31</td>
<td>31 A</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>31 B</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>31 C</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>31 D</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>31 E</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>31 F</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>31 G</td>
<td>S</td>
</tr>
<tr>
<td>Pt 51</td>
<td>51 A</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>51 B</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>51 C</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>51 D</td>
<td>S</td>
</tr>
<tr>
<td>Pt 52</td>
<td>52 A</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>52 B</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>52 C</td>
<td>S</td>
</tr>
<tr>
<td>Pt 53</td>
<td>53 A</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>53 B</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>53 C</td>
<td>S</td>
</tr>
<tr>
<td>Pt 58</td>
<td>58 A</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>58 B</td>
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<td>83 A</td>
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</tr>
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</tr>
<tr>
<td></td>
<td>83 C</td>
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<td></td>
<td>83 D</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>83 E</td>
<td>S</td>
</tr>
</tbody>
</table>

Figure 16.1 Sequential RISA profiles from patients included in Chapter 11 who did not experience a pulmonary exacerbation during follow-up
Figure 16.2  Sequential RISA profiles from patients included in Chapter 11 who experienced ≥2 virus-positive exacerbations and no non-viral exacerbations during follow-up
Figure 16.3 Sequential RISA profiles from patients included in Chapter 11 who experienced ≥2 non-viral exacerbations and no virus-positive exacerbations during follow-up

Figure 16.4 Sequential RISA profiles from patients included in Chapter 11 who experienced both virus-positive and -negative exacerbations during follow-up
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