Characterisation and Genetic Analysis of *Mycoplasma hominis* and *Mycoplasma pneumoniae*

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Summary of Thesis:

Mycoplasmas represent some of the smallest and simplest free-living organisms known. *Mycoplasma hominis* and *Mycoplasma pneumoniae* are two human pathogens that colonise the urogenital and respiratory tracts, respectively, causing a diverse range of disease. Detection of *Mycoplasma hominis* is hampered by the fastidious nature and genetic heterogeneity of this organism. Characterisation of mycoplasmas is becoming more important due to increasing antibiotic resistance, particularly in *M. pneumoniae,* and the need for more discriminatory methods to enhance epidemiology and examine transmission chains.

Firstly, this thesis develops a quantitative, multiplex, real-time PCR assay to simultaneously detect *M. hominis* and *Ureaplasma* species in neonatal clinical specimens, where infection is associated with chronic lung disease, bacteremia and other clinical signs. Results showed that the PCR method was clinically more sensitive than culture and has applications for monitoring bacterial load in clinical specimens and characterising bacterial response to antibiotics.

Secondly, genetic characterisation of *M. hominis* was undertaken by the examination of the variable adherence-associated antigen and the development of sequence based typing. Due to the genetic heterogeneity of *M. hominis,* bioinformatics analysis of genomic sequence was used as a novel method to develop a minimum multi-locus sequence typing (MLST) scheme that accurately represented genomic phylogeny of this species.

Finally, an MLST scheme was developed for *M. pneumoniae,* to aid the analysis of epidemic periods and clusters of infection. A successful scheme was developed based on eight housekeeping genes which had increased discrimination of *M. pneumoniae* compared to established typing methods for this organism. Furthermore, the MLST scheme was found to be representative of genomic sequence-derived phylogeny, with two distinct genetic clades identified. Application of this MLST to UK epidemics revealed that no predominant sequence type was responsible for the epidemic periods studied, indicating a polyclonal population, supporting the hypothesis that epidemics are driven by population immunity.

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Abbreviations

ADP	Adenosine diphosphate
AFLP	Amplified length polymorphism
AMP	Adenosine monophosphate
AT	Allelic type
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
C. pneumoniae	*Chlamydia pneumoniae*
C. trachomatis	*Chlamydia trachomatis*
CAP	Community-acquired
CARDS	Community-acquired respiratory distress syndrome
CC	Clonal complexes
CCU	Colour changing units
CDS	Coding sequence
CFU	Colony forming units
CLD	Chronic lung disease
CLSI	Clinical and Laboratory standards Institute
CNS	Central nervous system
CI	Confidence Interval
CS	Caesarean-section
CSF	Cerebrospinal fluid
Ct	Cycling threshold
CV	Coefficient of variation
DI	Diversity Index
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*

ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
ETS	Endotracheal secretions
GC	Genomic clades
GFP	Green fluorescent protein
GP	General Practitioner
GTR	Generalised time-reversible
HHM	Hidden Markov models
HSP	Heat shock protein
IPC	Internal processing Control
L. pneumophilia	*Legionella pneumophilia*
LOD	Limit of detection
M. agalactiae	*Mycoplasma agalactiae*
M. bovis	*Mycoplasma bovis*
M. gallisepticum	*Mycoplasma gallisepticum*
M. genitalium	*Mycoplasma genitalium*
M. hominis	*Mycoplasma hominis*
M. hyopneumoniae	*Mycoplasma hyopneumoniae*
M. hyorhinis	*Mycoplasma hyorhinis*
M. myocides	*Mycoplasma myocides*
M. pneumoniae	*Mycoplasma pneumoniae*
M. pulmonis	*Mycoplasma pulmonis*
M. tuberculosis	*Mycobacterium tuberculosis*
MIC	Minimum inhibitory concentration

MLSK	Macrolide-lincosamide-streptogramin group and ketolide
MLST	Multi-locus sequence typing
mMLST	Minimal multi-locus sequence typing
MLVA	Multiple-locus variable-number tandem-repeat analysis
MM	Mycoplasma specific medium
$NADH_2$	Hydroxylamine reductase
NEC	Necrotising enterocolitis
NPA	Nasopharyngeal aspirates
NHS	National Health Service
NCBI	National Center for Biotechnology Information
N. gonorrhoeae	*Neisseria gonorrhoeae*
N. meningitidis	*Neisseria meningitidis*
NVD	Normal vaginal delivery
Opp	Oligopeptide permease
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel elecrophoresis
PHE	Public Health England
PHS	Public Health Scotland
QRDR	Quinolone resistance-determining region
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*

S. pyogenes	*Streptococcus pyogenes*
SBT	Sequence based typing
SNP	Single nucleotide polymorphism
ST	Sequence type
STI	Sexually transmitted infection
TE	Tris-EDTA
TK	Thymidine kinase
TR	Tandem repeats
tRNA	Transcriptional ribonucleic acid
T. vaginalis	*Trichomonas vaginalis*
U. parvum	*Ureaplasma parvum*
U. urealyticum	*Ureaplasma urealyticum*
UHW	University Hospital Wales
USM	Ureaplasma specific media
Vaa	Variable adherence-associated antigen
VD	Vaginal delivery
VNTR	Variable-number tandem-repeat

Table of Contents

Chapter 1. Introduction 1

1.1 *Mycoplasma hominis* and *Mycoplasma pneumoniae* 2

1.1.1 Classification of *Mycoplasma hominis* and *Mycoplasma pneumoniae* 2

1.1.2 Characteristics of *Mycoplasma hominis* and *Mycoplasma pneumoniae* 2

1.1.3 Genome and coding capacity 7

1.2 Virulence factors 9

1.2.1 Mycoplasma pneumoniae virulence factors 9

1.2.1.1 Hydrogen peroxide release 9

1.2.1.2 Adhesion complex 11

1.2.1.3 CARDS toxin 12

1.2.1.4 Biofilm formation 13

1.2.1.5 Interaction of surface-displayed enzymes with host factors 14

1.2.2 *Mycoplasma hominis* virulence factors 15

1.2.2.1 Cytoadhesive membrane proteins 15

1.2.2.2 Membrane bound lipoproteins 16

1.2.2.3 Modulation of host immune response by *Mycoplasma hominis* 22

1.3 Detection, quantification and typing of *Mycoplasma hominis* and *Mycoplasma pneumoniae* 23

1.3.1 Culture and quantification 23

1.3.2 Serological detection 24

1.3.3 Molecular detection 26

1.3.4 Molecular typing 28

1.4 *Mycoplasma hominis, Mycoplasma pneumoniae* and disease 29

1.4.1 *Mycoplasma pneumoniae* infections 29

1.4.1.1 Role of *Mycoplasma pneumoniae* in persistent cough in children 30

1.4.1.2 Role of *Mycoplasma pneumoniae* in asthma and chronic lung disease 31

1.4.1.3 Extra-pulmonary manifestations of *Mycoplasma pneumoniae* infection 32

1.4.2 *Mycoplasma hominis* infections 34

1.4.2.1 Contribution of *Mycoplasma hominis* infection to bacterial vaginosis and pelvic inflammatory disease 35

1.4.2.2 *Mycoplasma hominis*, pregnancy and postpartum fever 37

1.4.2.3 Extra-genital *Mycoplasma hominis* infection in adults 37

1.4.2.4 *Mycoplasma hominis* infections in neonates 38

1.5 Epidemiology of *Mycoplasma pneumoniae* infections 39

1.5.1 Epidemiology in the United Kingdom 40

1.5.2 Global epidemiology 46

1.6 Antimicrobial therapy against *Mycoplasma hominis* and *Mycoplasma pneumoniae* 47

1.6.1 Intrinsic antibiotic resistance in *Mycoplasma hominis* and *Mycoplasma pneumoniae* and therapeutic considerations 48

1.6.2 Macrolide-lincosamide-streptogramin group and the ketolides 50

1.6.3 The tetracyclines 54

1.6.4 The fluoroquinolones 54

1.7 Hypothesis 56

1.8 Aims 56

Chapter 2. Materials and Methods 57

2.1 *Mycoplasma pneumoniae* and *Mycoplasma hominis* isolate details 58

2.2 Culturing *Mycoplasma pneumoniae* and *Mycoplasma hominis* 68

2.2.1 Storage and recovery of *Mycoplasma* isolates 68

2.3 Detection of *Mycoplasma hominis* and *Ureaplasma* species in clinical samples 68

2.3.1 DNA Extraction from clinical samples 68

2.3.1.1 QIAsymphony® 69

2.3.1.2 MagNA Pure Compact 69

2.3.2 Detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* by qPCR – MUP PCR 70

2.3.2.1 Clinical sample preparation 70

2.3.2.2 Preparation of PCR Internal Processing Control (IPC) 70

2.3.2.3 Preparation of positive controls 70

2.3.2.4 MUP qPCR 71

2.3.3 Multiplex real-time PCR 73

2.3.3.1 GFP amplification control 73

2.3.3.2 Preparation of standard DNA 73

2.3.3.3 qPCR 73

2.4 Molecular typing methods 75

2.4.1 DNA Extraction from purified isolates 75

2.4.2 PCR 75

2.4.2.1 Agarose gel electrophoresis 75

2.4.2.2 Purification of PCR products 76

2.4.3 Sequencing 76

2.4.4 *Mycoplasma pneumoniae* P1-typing 76

2.4.4.1 P1 sequence analysis 77

2.4.5 Multi-locus Sequence Typing (MLST) 78

2.4.5.1 MLST Sequence analysis 80

2.4.6 *Mycoplasma hominis* variable adhesion-associated (Vaa) protein classification 80

2.4.6.1 *Mycoplasma hominis vaa* sequence analysis 81

2.4.7 *Mycoplasma pneumoniae* multi-locus variable-number tandem-repeat analysis (MLVA) 81

2.4.8 Phylogenetic analysis for Molecular Typing Methods 84

2.4.8.1 Phylogenetic analysis of multi-locus sequence typing data 84

2.4.8.2 Phylogenetic analysis of *vaa* sequences 84

2.4.8.3 Relatedness between sequence types 84

2.5 Antibiotic resistance of *Mycoplasma hominis* 85

2.5.1 Minimum inhibitory concentration calculation by microbroth dilution 85

2.5.2 Minimum inhibitory concentration calculation by agar dilution 85

2.5.3 Monitoring minimum inhibitory concentration with qPCR 86

2.5.4 PCR for the *tetM* transferable element associated with tetracycline resistance 86

2.6 Whole Genome Sequencing 87

2.6.1 Extraction of genomic DNA for whole genome sequencing 87

2.6.1.1 Sample preparation 87

2.6.1.2 GenElute™ Bacterial Genomic DNA Kit 87

2.6.1.3 Ethanol precipitation of genomic DNA 88

2.6.2 Sequencing methodology 89

2.6.3 Read quality assessment 89

2.6.4 *de novo* assembly 90

2.6.5 Mapping of sequence data to a reference strain and annotation 90

2.6.6 *Mycoplasma hominis* genome assembly and pan-genome analysis 90

2.6.7 Basic Local Alignment Tool (blast) 91

2.6.8 Phylogenetic analysis of whole genome sequences 91

2.6.8.1 Single nucleotide polymorphism tree 91

2.6.8.2 Whole genome alignment 94

2.6.9 Minimal MLST gene selection 94

2.6.10 Recombination analysis 94

2.7 Statistical methods 95

Chapter 3. Simultaneous real-time PCR detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* 96
3.1 Introduction 97
3.2 Results 98
3.2.1 Assay development and validation 98
3.2.2 Primer and probe design 99
3.2.3 Rotor-Gene® PCR 99
3.2.4 Reproducibility and LOD 102
3.2.5 Specificity 105
3.2.6 Evaluation with patient samples and comparison to culture and MUP PCR 106
3.2.6.1 Comparison to M-duo culture 107
3.2.6.2 Comparison to MUP PCR 109
3.2.7 Detection of *M. hominis* and *Ureaplasma* species in neonates 112
3.3 Discussion 114
3.3.1 Analysis of the multiplex real-time PCR method used 115
3.3.1.1 Choice of gene target 115
3.3.1.2 Sensitivity and specificity 116
3.3.1.3 Analysis of clinical samples and comparison with culture 116
3.3.1.4 Further application of the multiplex real-time PCR assay 119
3.3.2 Review of previously described PCR detection methods for *Mycoplasma hominis* and *Ureaplasma* species 120
3.3.3 Summary 122

Chapter 4. Application of the multiplex real-time PCR – neonate case study and use to monitor response to antibiotics 123
4.1 Introduction 124
4.2 Results 125
4.2.1 South West intubated neonate case studies 125

4.2.1.1 Detection of *Ureaplasma* infection following clarithromycin treatment 135

4.2.2 Determination of minimum inhibitory concentration for *Mycoplasma hominis* 138

4.2.2.1 Determination of tetracycline resistant *M. hominis* 142

4.3 Discussion 144

4.3.1 Neonatal twin case study 144

4.3.2 Effect of steroid treatment on the detection of *M. hominis* and *Ureaplasma* species infection 146

4.3.3 Monitoring of infection with *Ureaplasma* species following clarithromycin treatment 147

4.3.4 Review of methods used to calculate minimum inhibitory concentration 148

4.3.5 Tetracycline resistance 150

4.3.6 Summary 152

Chapter 5. Molecular typing and characterisation of *Mycoplasma hominis* 153

5.1 Introduction 154

5.2 Results 156

5.2.1 Characterisation of *Mycoplasma hominis* using the variable adherence-associated antigen 156

5.2.1.1 Vaa cassette determination and variability 156

5.2.1.2 Phylogenetic analysis of *vaa* 164

5.2.2 Sequence based typing of *M. hominis* 165

5.2.2.1 Determining sequence based typing loci 165

5.2.2.2 Sequence based typing of *M. hominis* 167

5.2.2.3 Phylogenetic analysis of SBT loci 172

5.2.2.4 Determination of synonymous sequence changes in sequence based typing loci 176

5.2.2.5 Assessment of SBT loci stability 181

5.2.2.6 Genomic sequence analysis 184

5.2.3 Genomic sequence analysis and development of minimum MLST scheme 186

5.2.3.1 Pan-genome 186

5.2.3.2 Core-genome MLST 189

5.2.3.3 Seven-gene MLST (mMLST) 191

5.2.3.4 Diversity of mMLST genes and determination of synonymous sequence changes 193

5.2.3.5 Seven gene mMLST stability 195

5.2.3.6 Recombination analysis 196

5.3 Discussion 198

5.3.1 Analysis of the variable adherence-associated antigen of *Mycoplasma hominis* 198

5.3.2 Analysis of SBT for *M. hominis* 199

5.3.3 Fidelity of SBT loci 200

5.3.4 Application of SBT for *Mycoplasma hominis* infection 202

5.3.5 The use of bioinformatics to develop minimum MLST schemes that represent genomic phylogeny 204

5.3.6 Recombination in *Mycoplasma hominis* 206

5.3.7 Consequences of the genetic variability of *Mycoplasma hominis* 206

5.4 Summary 207

Chapter 6. Development of a multi-locus sequence typing scheme for the molecular typing of *Mycoplasma pneumoniae* 208

6.1 Introduction 209

6.2 Results 210

6.2.1 Determining MLST loci 210

6.2.2 MLST of *M. pneumoniae* 211

6.2.3 Phylogenetic analysis of *M. pneumoniae* sequence types 216

6.2.4 Determination of synonymous sequence changes in MLST loci 228

6.2.5 Application of MLST scheme to published genomic sequences of *M. pneumoniae* 232

6.2.6 Assessment of MLST loci stability 232

6.2.7 Genomic sequence analysis 232

6.2.8 Comparison to MLVA and P1 typing methods 237

6.2.9 Molecular typing and epidemiology in the UK 238

6.2.10 Online database 240

6.3 Discussion 240

6.3.1 Analysis of the MLST loci 241

6.3.2 Improved discrimination between *M. pneumoniae* isolates in comparison to MLVA and P1 typing 241

6.3.3 Analysis of *M. pneumoniae* infection within an individual patient 242

6.3.4 Population modelling using MLST sequence types 242

6.3.5 Genomic sequence comparison 243

6.3.6 Clinical relevance and use in epidemiology 246

6.3.7 Review of current molecular typing methods for *M. pneumoniae* 247

6.4 Summary 249

Chapter 7. General discussion 250

7.1 Diagnosis of *mycoplasma* infections 251

7.1.1 Importance of diagnosis in neonates and pregnancy 253

7.2 Genetic characterisation of *Mycoplasma hominis* and *Mycoplasma pneumoniae* 256

7.2.1 The usefulness of molecular typing for *Mycoplasma* epidemiology 257

7.2.2 Is genomic sequencing the way forward? 259

7.3 Scope for future lines of investigation 260

7.3.1 Multiplex real-time PCR 260

7.3.2 *Mycoplasma hominis* antibiotic susceptibilities 263

7.3.3 mMLST 264

7.3.4 *Mycoplasma pneumoniae* MLST 267

7.4 Concluding statement 268

Appendices 269

References 273

Publication list 306

Conference abstracts 341

Table of Figures

Figure 1.1. Micrographs of *Mycoplasma hominis* and *Mycoplasma pneumoniae*. 7

Figure 1.2. Schematic illustration of the uptake and conversion of carbohydrates leading to the formation of glycerol-3-phosphate and hydrogen peroxide in *M. pneumoniae*. 11

Figure 1.3. Schematic representation of the deduced amino acid sequences of the six *vaa* gene types. 19

Figure 1.4. Model of the predicted protein structure for Vaa category 5. 21

Figure 1.5. Laboratory reports of *M. pneumoniae* infection detection by genomic and serological methods in England and Wales from January 1989 to June 2015. 42

Figure 1.6. Number of laboratory reports per year from January 1995 to June 2015 separated by detection methodology. 43

Figure 1.7. Macrolide classification and example antibiotics. 51

Figure 2.1 Workflow used to generate multiple alignments of SNP variants for the generation of a SNP phylogenetic tree 93

Figure 3.1. Fluorescence and standard curves of serially diluted control DNA. 101

Figure 3.2. Alignment of the amplified region of the *yidC* gene of *Mycoplasma hominis* including primer and probe binding sites. 106

Figure 3.3. Incidence of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in intubated neonates. 114

Figure 4.1. Neonatal characteristics and clinical presentation in association with infection by *Mycoplasma hominis* and *Ureaplasma* species 127

Figure 4.2. Quantification of *Ureaplasma* species by multiplex real-time PCR and culture from twins P130 and P131. 129

Figure 4.3. Quantification of *Ureaplasma urealyticum* by multiplex real-time PCR and culture from P128. 131

Figure 4.4. Quantification of *Mycoplasma hominis* and *Ureaplasma parvum* by multiplex real-time PCR and culture from P162. 133

Figure 4.5. Quantification of *Mycoplasma hominis* and *Ureaplasma parvum* by multiplex real-time PCR and culture from P164. 134

Figure 4.6. Detection of *Ureaplasma* species infection in intubated neonates after treatment with clarithromycin. 137

Figure 4.7. Example graphs to calculate the MIC of *M. hominis* for both gentamicin and tetracycline by real-time qPCR. 140

Figure 4.8. Example graphs to calculate the MIC of *M. hominis* containing *tetM* for both gentamicin and tetracycline by real-time qPCR. 143

Figure 5.1. Amino acid alignment of Vaa category 1 strains. Amino acid sequence alignments for 13 *M. hominis* clinical isolates and two prototype strains, FBG and PG21. 159
Figure 5.2. Amino acid alignment of Vaa category 2 strains. 160
Figure 5.3. Amino acid alignment of Vaa category 3 strains. 161
Figure 5.4. Amino acid alignment of Vaa category 4 strains. 162
Figure 5.5. Phylogenetic trees based on the sequence of the *vaa* gene of *M. hominis*. 165
Figure 5.6. Phylogenetic trees based on concatenated sequences of eight SBT loci for *M. hominis*. 173
Figure 5.7. Phylogenetic trees for each of the eight SBT loci. 176
Figure 5.8. Alignments of amino acid sequences of each allelic type at each of the eight SBT loci. 181
Figure 5.9. Phylogenetic trees based on concatenated sequences of eight SBT loci from the original strains tested and the strains after short-term passage in liquid culture. 183
Figure 5.10. Phylogenetic tree based on the single nucleotide polymorphism profile of 19 *M. hominis* whole genome sequences. 185
Figure 5.11. Frequency of genes in the *Mycoplasma hominis* pan-genome. 187
Figure 5.12. Bimodal distribution of alleles per gene in the *Mycoplasma hominis* pan-genome. 188
Figure 5.13. MLST mosaic of 48 core genes found after leave-one-out analysis. 190
Figure 5.14. MLST mosaic indicating stability of the nine genes used in the three proposed mMLST schemes. 195
Figure 5.15. Prediction of recombination in the *M. hominis* isolates chromosomes. 197
Figure 6.1. Phylogenetic trees based on concatenated sequences of eight MLST loci. 217
Figure 6.2. Analysis of *M. pneumoniae* using eBURST. 218
Figure 6.3. Phylogenetic trees based on the adenylate kinase locus for each individual strain. 219
Figure 6.4. Phylogenetic trees based on the carbamate kinase locus for each individual strain. 220
Figure 6.5. Phylogenetic trees based on the ATP synthase subunit α locus for each individual strain. 221
Figure 6.6. Phylogenetic trees based on the serine hydroxymethlytransferase locus for each individual strain. 222
Figure 6.7. Phylogenetic trees based on the DNA gyrase subunit B locus for each individual strain. 223
Figure 6.8. Phylogenetic trees based on the guanylate kinase locus for each individual strain. 224

Figure 6.9 Phylogenetic trees based on the phosphoglycerate mutase locus for each individual strain. 225
Figure 6.10. Phylogenetic trees based on the inorganic phosphatase locus for each individual strain. 226
Figure 6.11. Phylogenetic trees based on concatenated sequences of eight MLST loci showing individual *M. pneumoniae* strain position. 227
Figure 6.12. Alignments of amino acid sequences of each allelic type at each of the eight MLST loci. 231
Figure 6.13. Phylogenetic tree based on whole genome alignments of 49 *M. pneumoniae* genomic sequences. 234
Figure 6.14. Overall sequence identity of the whole genome sequences of 35 *M. pneumoniae* clinical strains to the reference M129. 235
Figure 6.15. Prediction of recombination in the *M. pneumoniae* isolates chromosomes. 236
Figure 6.16. Distribution of MLVA, P1 type and MLST ST for 57 *M. pneumoniae* isolates. 238
Figure 6.17. Distribution of MLST sequence types for 57 *M. pneumoniae* clinical isolates in the four-yearly epidemic cycles observed in the UK. 239
Figure 6.18. Distribution of MLVA types in positive *M. pneumoniae* clinical isolates collated into the four-yearly epidemic cycles observed in the UK. 239
Figure 6.19. Distribution of P1 type in four-yearly epidemic cycles observed in the UK for positive *M. pneumoniae* clinical isolates. 240
Figure 7.1. Work-flows for future validation of the multiplex real-time PCR assay for the detection of *M. hominis* and *Ureaplasma* species. A) Continued validation of the assay incorporating a spiking assay and further analysis of control plasmid stability. B) Prospective validation study using clinical specimens to compare to conventional culture and singleplex PCR assays. 262
Figure 7.2. Work-flows for the development of mMLST by conventional PCR methods and expansion to additional *M. hominis* strains. 265
Figure A.1. Agarose gel showing the results of a PCR for the presence of *tetM* in *Mycoplasma hominis* strains. 272

Table of Tables

Table 1.1. Human *Mycoplasma* species and their colonisation site. 4

Table 1.2. Minimum inhibitory concentration ranges (µg/mL) by antibiotic class for selected *M. pneumoniae* and *M. hominis* [234-236]. 48

Table 2.1. *Mycoplasma pneumoniae* clinical isolate details. 59

Table 2.2. *Mycoplasma hominis* clinical isolate details. 62

Table 2.3. Primers and probes 71

Table 2.4. Thresholds for analysis of each target. 72

Table 2.5. Positive control Ct value ranges to determine positive clinical results 72

Table 2.6. Details of primers and probes used in the multiplex real-time PCR 74

Table 2.7. Primers used for P1-typing of *Mycoplasma pneumoniae*. 77

Table 2.8. Primer pairs developed for both *M. hominis* and *M. pneumoniae* MLST schemes. 79

Table 2.9. Details of primers used for the amplification of *M. hominis vaa* open-reading frame. 80

Table 2.10. Primer sequences, target sequences, repeat size and fragment size (bp) of *M. pneumoniae* MLVA loci MPN13, MPN14, MPN15 and MPN16 according to number of repeats. 83

Table 2.11. Primers used to detect the presence of the transferable element *tetM* in *Mycoplasma hominis* isolates. 87

Table 3.1. Interpretation of positive controls. 100

Table 3.2. Inter-assay reproducibility of the multiplex *U. urealyticum*, *U. parvum* and *M. hominis* PCR assay. 103

Table 3.3. Percentage of replicate PCR results of the standard dilution series for *Mycoplasma hominis, Ureaplasma parvum* and *Ureaplasma urealyticum*. 104

Table 3.4. Comparison of PCR and M-duo culture results for *M. hominis* from 216 clinical specimens. 108

Table 3.5. Comparison of PCR and M-duo culture results for *Ureaplasma* species from 216 clinical specimens. 109

Table 3.6. Comparison of PCR and Mycoplasma Experience culture results for *M. hominis* and *Ureaplasma* species from 61 clinical specimens. 111

Table 4.1. Gentamicin and Tetracycline MIC calculation for *M. hominis* by broth-dilution and agar methods, and monitoring using qPCR. 141

Table 5.1. Variation of amino acid sequence in *M. hominis* Vaa categories. 163

Table 5.2. Choice of MLST loci based on targets used in already established bacterial MLST schemes. 166

Table 5.3. Description of *M. hominis* strains used in this study, their sequence type (ST) and allelic profile. 168

Table 5.4. Primer pairs developed in this study and variability of the different loci. 171

Table 5.5. SNP differences between the original strain and following 10 passages in MM. 182

Table 5.6. Summary of *Mycoplasma hominis* mMLST schemes. 192

Table 5.7. dN/dS ration and Hunter-Gaston Diversity Indices for the three proposed mMLST schemes 194

Table 6.1. MLST loci used in established bacterial MLST schemes also present in *M. pneumoniae*. 211

Table 6.2. *Mycoplasma pneumoniae* strains, their sequence type (ST) and allelic profile, and their MLVA and P1 types. 213

Table 6.3. Variability of the *M. pnueomoniae* MLST loci 215

Table A.1. Reference strains used for specificity testing of multiplex assay. 269

Chapter 1. Introduction

1.1 *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Mycoplasmas represent some of the smallest self-replicating organisms that are capable of cell-free existence. The first report of a *mycoplasma* to be recovered directly from a human in association with a pathological condition occurred in 1937, when *Mycoplasma hominis* was isolated from a Bartholin's gland abscess [1]. Shortly after this, *Mycoplasma pneumoniae* was isolated in the sputum from a patient with primary atypical pneumonia [2].

1.1.1 Classification of *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Mycoplasma species are taxonomically placed in the phylum *Firmicutes*, class *Mollicutes*, order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Mycoplasma*. The taxonomic description of species within the *Mollicutes* class has been complicated and was first established in the 1960s. This class now contains four orders, five families, eight genera and more than 200 known species that have been detected in humans, vertebrate animals, arthropods, fish/shellfish, and plants [3]. In 1989, phylogenetic analysis based on 16S ribosomal ribonucleic acid (rRNA) sequences was reported for the *Mollicutes* [4] where 47 species were included; revealing five distinct phylogenetic clades of descent namely: anaeroplasmas, asteroleplasmas, hominis, pneumoniae and siproplasmas. Subsequently these groups were further divided into several subgroups or clusters with *M. hominis* positioning in the hominis cluster and *M. pneumoniae* positioning in the pneumoniae cluster [5, 6].

1.1.2 Characteristics of *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Mycoplasmas are smaller than conventional bacteria and lack a cell wall, a characteristic that is used to distinguish these microorganisms from ordinary bacteria. The lack of a cell wall prevents mycoplasmas from staining by Gram stain, conferring pleomorphism of their cells, and makes them very susceptible to osmotic pressure.

They have small genomes and limited biosynthetic capabilities, restricting them to a parasitic existence in association with eukaryotic cells of their host [3, 7]. A majority of mycoplasmas exhibit strict host and tissue specificities, where, once they have entered the host, they can multiply and survive for long periods of time. To facilitate this, mycoplasmas have evolved molecular mechanisms to evade the host immune system and to allow transmission and colonisation of a new host. Mechanisms include: mimicry of host antigens, survival within phagocytic and non-phagocytic cells, and generation of phenotype plasticity [8]. Nineteen *Mycoplasma* species (listed in Table 1.1) have been isolated from humans and at least six of these species are considered to be of pathological significance specifically: *Mycoplasma genitalium*, *M. pneumoniae* and *M. hominis* [7].

Table 1.1. Human *Mycoplasma* species and their colonisation site.

Human species	Colonisation site
Mycoplasma salivarium	Oral cavity
Mycoplasma orale	Oral cavity and oropharynx
Mycoplasma pneumoniae	Respiratory tract
Mycoplasma amphoriforme	Respiratory tract
Mycoplasma fermentans	Urogenital tract
Mycoplasma hominis	Urogenital tract
Mycoplasma penetrans	Urogenital tract
Ureaplasma urealyticum	Urogenital tract
Ureaplasma parvum	Urogenital tract
Mycoplasma genitalium	Urogenital tract
Mycoplasma buccale	Oropharynx
Mycoplasma faucium	Oropharynx
Mycoplasma lipophilum	Oral cavity
Mycoplasma pirum	
Mycoplasma primatum	Oral cavity and urogenital tract
Mycoplasma spermatophilum	Genital tract
Acholeplasma laidlawii	Oropharynx
Hemoplasma	
Candidatus *Mycoplasma girerdii*	Urogenital tract

Individual mycoplasmas and their morphological features cannot be visualised by light microscopy therefore observation under an electron microscope is required. Additionally, mycoplasmas do not produce visible turbidity in liquid growth media and the small cellular mass of these organisms means that they can pass through 0.45µm-pore-size filters that are commonly used to filter-sterilise media [9].

M. hominis forms characteristic "fried egg" colonies when grown on agar which results from the bacteria burrowing into the agar and both bacteria and colonies are pleomorphic in size and shape (Figure 1.1A+B). *M. hominis* cells are filamentous- or rod-shaped and are typically 0.25 µm wide [10]. Individual *M. pneumoniae* cells are spindle-shaped and 1 µm -2 µm in length and 0.1 µm-0.2 µm wide, resulting in a cell volume that is approximately 5% of that of a typical bacillus [9].

Another characteristic of the genus of *Mycoplasma* is the need for sterols in the growth media, usually provided by serum. Sterols are critical components of the mycoplasma cell membrane that provide stability and structural support to the organisms in the absence of a cell wall [9, 11].

The metabolic activities of mycoplasmas appear to be primarily associated with the generation of energy rather than the provision of substrates for synthetic pathways. All *Mollicutes* so far examined have truncated respiratory systems, lacking a complete tricarboxylic acid cycle and have no quinones and cytochromes preventing the generation of adenosine triphosphate (ATP) by oxidative phosphorylation. Therefore, it is assumed that mycoplasmas produce low ATP yields and relatively large quantities of metabolic end products [5, 12, 13].

Mycoplasmas can be divided into fermentative and non-fermentative organisms depending on their ability to metabolise carbohydrates. In addition, some

Mycoplasma species contain genes encoding proteins pertaining to both pathways and therefore potentially have the ability to be both fermentative and non-fermentative. Fermentative Mycoplasmas, such as *M. pneumoniae*, produce acids from carbohydrates. *M. pneumoniae* metabolises glucose to lactic acid and acetic acid by substrate phosphorylation. This process is mediated by phosphoglyceric acid kinase, pyruvate kinase, and acetate kinase activities, and generates ATP. *M. pneumoniae* can also utilise glycerol and other small carbohydrates to generate ATP [5, 9]. In comparison, non-fermentative organisms, such as *M. hominis*, metabolise arginine via the arginine dihydrolase pathway. The hydrolysis of arginine by this pathway results in the production of ornithine, carbon dioxide and ammonia, and generates ATP. This pathway utilises three enzymes: arginine deiminase, ornithine carbamoyl transferase, and carbamate kinase [5, 14]. However, it is interesting to note that annotation of the genomes of both *M. pneumoniae* and *M. hominis* have shown genomic evidence for enzymes required for other metabolic pathways. The utilisation of these enzymes for metabolism has not been directly observed [12, 14].

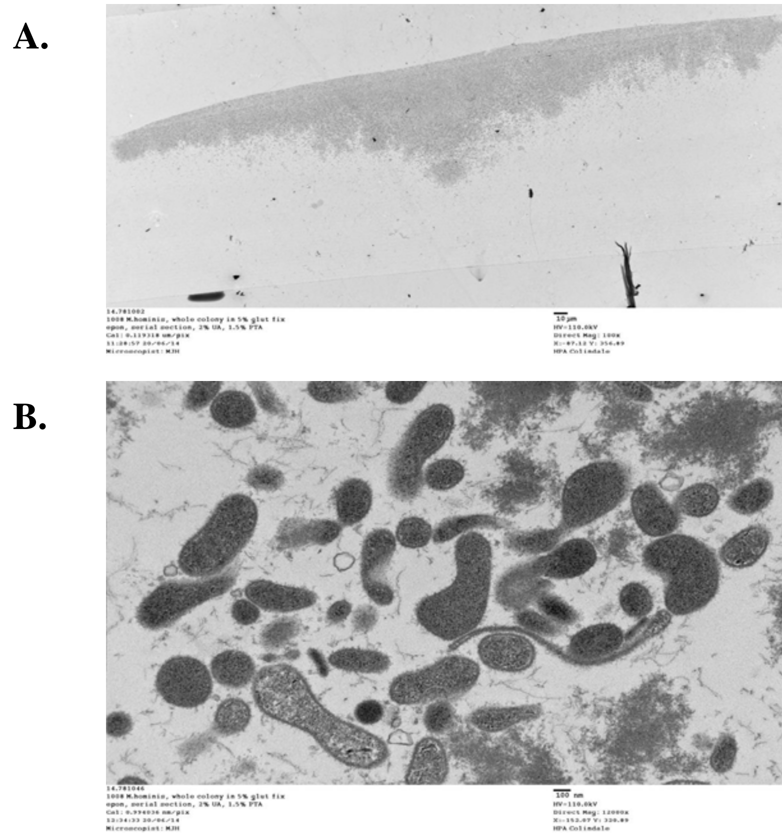

Figure 1.1. Micrographs of *Mycoplasma hominis* and *Mycoplasma pneumoniae*. (A) Electron micrograph of a cross-section of a *M. hominis* colony grown on Mycoplasma agar showing growth into the medium (scale bar = 10 μm). (B) Electron micrograph of *M. hominis* showing small size and pleomorphic cells within a colony grown on Mycoplasma agar (scale bar = 100 nm).

1.1.3 Genome and coding capacity

Mollicutes have small genomes, consisting of a single circular chromosome and low guanine (G) + cytosine (C) content. The small genome is now believed to be the result of a gradual reduction in genome size from a common ancestor in a process known as devolution but the selective pressure for this is unknown. Mycoplasmas are most closely related to the gram-positive bacterial group that includes *Streptococci*, *Bacilli* and *Lactobacilli* [15].

The genome of *M. pneumoniae* was completely sequenced in 1996 and has a size of 816394 base pairs (bp) encoding 687 genes with a G + C content of 40 mol% [16].

In comparison, *M. hominis* has a genome size of 665445 bp with a G+C content of 27.1 mol% [14]. Currently, 19 *M. pneumoniae* and four *M. hominis* complete chromosomes are available on the National Center for Biotechnology Information (NCBI) website. Of the *M. pneumoniae* chromosomes, 14 were sequenced in August 2015 [17] including the re-sequencing of the type strain FH. Additionally, the *M. pneumoniae* M129-B chromosome is a chimera sequence generated by combining the re-sequenced M129 genome (by next generation sequencing) and the first Sanger-based M129 genome. The UGA codon in all other organisms' functions as a terminal stop codon (opal), but in *Mycoplasma* this codon encodes the amino acid tryptophan [18].

The reduced genome size and streamlined metabolism of *M. pneumoniae* provides an ideal model for system-wide studies. The *M. pneumoniae* type strain M129 has been extensively characterised including full analysis of its transcriptome, metabolome, and proteome [19-22]. Since the small genome size results from loss of major anabolic pathways and a reduction in the complexity of gene expression regulation, *mycoplasmas* can still serve as models for other kinds of complex cellular processes such as: genesis of cell polarity, assembly of macromolecular structures, cell division, adherence, and motility [23].

The small genome of mycoplasmas confers their limited biosynthetic capabilities and is responsible for many biological characteristics of the organisms, including the requirement for complex growth medium for cultivation *in vitro* [9]. Mycoplasmas have no ability to synthesise a peptidoglycan cell wall due to many of the genes responsible for this process being absent from the genome. A consequence of such genome reduction is the loss of non-essential genes which can be found in other bacteria, sometimes limiting the organism to an obligate dependence upon a host. One

illustration of the limitations of gene loss is the complete reliance of mycoplasmas, as well as other *Mollicutes*, on the thymidine kinase (TK) enzyme salvage pathway for *de novo* synthesis of deoxyribonucleic acid (DNA) precursors [24-26].

1.2 Virulence factors

Virulence factors are molecules produced by pathogens, such as mycoplasmas, that contribute to the pathogenicity of the organism and enable them to colonise a niche in the host, evade and/or suppress the host's immune response, allow entry into and exit out of cells, and obtain nutrition from the host.

1.2.1 Mycoplasma pneumoniae virulence factors

Pathogenic effects of *M. pneumoniae* infections are assumed to be caused directly (by active interaction with the host), indirectly (by infection-induced immune mechanisms), or by both mechanisms. *M. pneumoniae* is known to cause direct injury to the host through the generation of activated oxygen and the production of a pertussis toxin-like protein, the community-acquired respiratory distress syndrome (CARDS) toxin. In addition to direct damage, *M. pneumoniae* can modulate the immune response of the host, generating inflammatory reactions causing the pulmonary and extra-pulmonary symptoms associated with infection. *M. pneumoniae* expresses a variety of adhesion proteins and glycolipids that share structural homology to host cells which may induce host immune responses that lead to cross-reactive antibodies and autoimmune damage [27].

1.2.1.1 Hydrogen peroxide release

In *M. pneumoniae,* as well as other *Mollicutes*, pathogenicity is closely linked to carbon metabolism [28]. The utilisation of glycerol and phospholipids plays an important role in the virulence of *Mycoplasma* species because the hydrogen peroxide

generated as a product of glycerol metabolism is the major cytotoxic substance produced by these bacteria. In addition to the induction of autoimmune responses, the formation of hydrogen peroxide is the only established mechanism by which *Mycoplasmas* cause damage to their hosts [9, 29]. The role of glycerol metabolism as a mechanism conferring pathogenicity in *Mycoplasma* was first described for *Mycoplasma mycoides*, [30] where the efficient formation of hydrogen peroxide by *M. mycoides* is the major virulence factor of the highly pathogenic strains of this species. Like *M. mycoides*, *M. pneumoniae* possesses the complete set of genes for glycerol utilisation (Figure 1.2) [31, 32].

In addition to glycerol, the metabolism of phospholipids plays an important role in the generation of hydrogen peroxide by *M. pneumoniae*. Glycerophospholipids are the major building blocks of the cell membrane in bacteria and eukaryotes and are degraded in several steps (Figure 1.2), resulting in the formation of glycerol-3-phosphate and the subsequent production of hydrogen peroxide [29, 33].

The formation of *glpD* and *glpQ* mutant strains of *M. pneumoniae* revealed that the utilisation of glycerol and phospholipids and subsequence hydrogen peroxide production plays a particularly important role in the virulence of *M. pneumoniae* as the mutant strains have severely affected pathogenicity [32, 33].

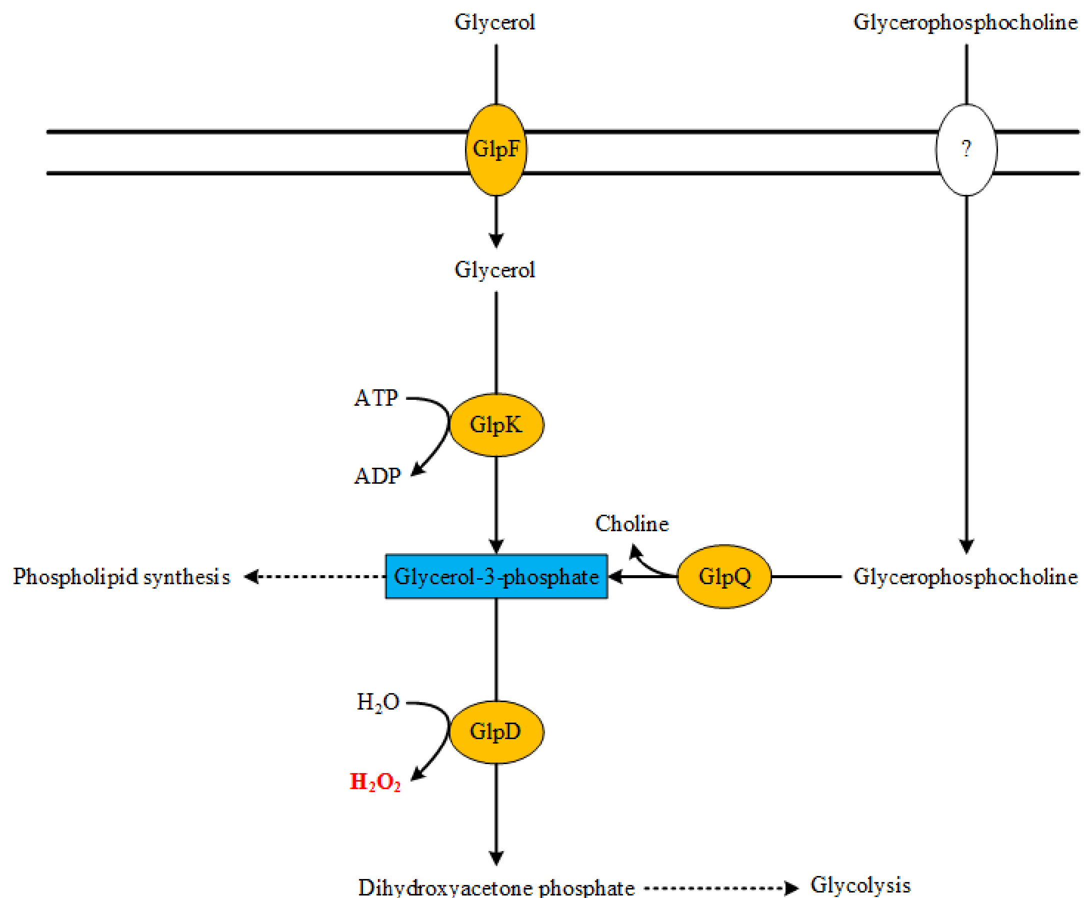

Figure 1.2. Schematic illustration of the uptake and conversion of carbohydrates leading to the formation of glycerol-3-phosphate and hydrogen peroxide in *M. pneumoniae*.

1.2.1.2 Adhesion complex

Following transmission to a new host, *M. pneumoniae* colonises the bronchial passages and is found localised to the base of the cilia where it interacts directly with the host cell surface [5]. *M. pneumoniae* colonisation results from the interaction between adhesin proteins on the mycoplasma surface and sulphated glycolipid or sialoglycoprotein molecules on the host respiratory epithelium [34-37]. Electron micrographs of *M. pneumoniae* colonisation of infected bronchial epithelia revealed a differentiated terminal organelle orientated towards, and directly associated with, the host cell surface, commonly designated the ‘attachment organelle’. This organelle is

a membrane-bound extension of the bacterial cell and is characterised by an electron-dense core (part of the cytoskeleton) orientated lengthwise and enlarging to form a button which interfaces with the inner surface of the *Mycoplasma* membrane at the tip of the cell [38-40]. The attachment organelle is thought to be multi-functional [23] and is involved in gliding motility [16, 41], cell division [42] and adhesion [43].

There are many proteins associated with cytoadherence of *M. pneumoniae*, including: P1, P30, HMW1-5, A, B, and C, of which the membrane protein P1 is thought to have a major receptor-binding role [5, 34, 38]. Antibodies to P1 inhibit *M. pneumoniae* adherence to tracheal epithelium [44]. P1 is primarily localised to the attachment organelle but it is also found distributed over the entire surface of *M. pneumoniae* [45, 46]. Humans mount an aggressive immune response to P1 during infection. Interestingly, that response fails to yield antibodies directed against epitopes responsible for receptor binding and therefore capable of blocking cytoadherence [35]. Biological mimicry and immunological tolerance may account for this, therefore contributing to host specificity.

1.2.1.3 CARDS toxin

The CARDS toxin of *M. pneumoniae* is an adenosine diphosphate (ADP)-ribosylating and vacuolating cytotoxin that provides a mechanism which explains the observed epithelial damage that occurs with infection [47]. This cytotoxin exhibits similarities to pertussis toxin [48]. CARDS toxin binds alveolar surfactant protein A [48, 49] and is likely to contribute to additional colonisation and pathogenic pathways. The CARDS toxin has an enzymatic action on both similar and distinct human cell proteins when compared with the S1 catalytic subunit of pertussis toxin. This leads to a cascade of events such as tissue disorganisation, inflammation and airway dysfunction along with cell vacuolisation [48, 50]. The expression and function of

pathogenic toxins is often mediated by other microbial components and it is postulated that the CARDS toxin relies on other mycoplasma components to fully potentiate its expression, delivery and impact on the host. Other possible microbial components include gene and protein regulatory systems, adherence factors for colonisation and invasion and possible secretion systems for delivery of specific pathogenic products [51, 52].

1.2.1.4 Biofilm formation

Many bacterial species aggregate to form biofilms which are immobile communities of micro-organisms encased within a matrix of polysaccharide, protein and lipid that protects the micro-organisms from harsh interactions with the environment, including the host immune response and antimicrobials [53-55]. It has previously been shown that biofilms are formed by *Mollicutes* [56, 57]. Of the mycoplasmas, biofilms were first described for *Mycoplasma pulmonis,* where the formation of biofilms was shown to protect this organism from the lytic effects of complement and the small antimicrobial peptide gramicidin [57]. Biofilms have been observed for *M. pneumoniae* where, when grown on an abiotic surface in the presence of a serum-containing medium, *M. pneumoniae* adhered to the surface and formed highly differentiated volcano-like biofilm structures [58]. Additionally, the biofilms formed by P1 type 1 and P1 type 2 strains of *M. pneumoniae* differ both qualitatively and quantitatively [59]. The P1 type 1 strain was found to secrete *N*-acetylglucosamine-containing polysaccharide into the culture medium whereas the polysaccharide was found in tight association with the cell surface of P1 type 2 strains [59]. *N*-acetylglucosamine-containing polysaccharides contribute to virulence and to the structure of biofilms produced by a wide range of microbial species [60-63] and

may provide a mechanism behind the difference in biofilms formed by the two major P1 type strains of *M. pneumoniae*.

1.2.1.5 Interaction of surface-displayed enzymes with host factors

The ability of pathogenic micro-organisms to adhere to and colonise host targets involves complex interactions and molecular cross-talk between microbial adhesins and host cell receptors [64, 65]. *M. pneumoniae* utilises glycolysis as the major pathway to produce ATP as it lacks the enzymes for the citric acid cycle. The process takes place in the cytoplasm of the cells; however, glycolytic enzymes can be transported to the surface of the microorganisms by an unknown mechanism [66], where they can interact with host factors [67]. This phenomenon was first reported for *Streptococci* but it appears to be a common feature of micro-organisms as it has been described in phylogenetically different organisms including Gram-negative and Gram-positive bacteria, fungi and parasites [68-70]. An increasing number of microbial glycolytic enzymes have been characterised as involved in interaction with the human extracellular matrix (ECM); glyceraldehyde-3-phsophate and enolase enzymes are typical examples of surface-expressed glycolytic enzymes that interact with proteins of the human ECM [70-72].

Elongation factor Tu, pyruvate dehydrogenase subunit B and glyceraldehyde-3-phosphate dehydrogenase of *M. pneumoniae* have been reported as being surface-localised and function as binding proteins to human fibronectin [73-75]. In addition, pyruvate dehydrogenase subunit B of *M. pneumoniae* has been shown to bind and activate human plasminogen, a key protein of the human fibrinolysis system which interacts with components of ECM after conversion to plasmin [67, 76].

Analysis of the Triton X-100 insoluble proteins of total cells of *M. pneumoniae* indicated the presence of further membrane-associated glycolytic enzymes [77]. It is therefore expected that further interactions of glycolytic enzymes of *M. pneumoniae* with host components can occur.

1.2.2 *Mycoplasma hominis* virulence factors

The mechanisms behind the pathogenic effects of *M. hominis* and the patho-physiological mechanisms that enable commensal *M. hominis* to become pathogenic are mostly unresolved. The ability of mycoplasmas to adhere to host epithelial cells on mucosal surfaces, in the case of *M. hominis* the urogenital tract, is an essential stage to establish successful colonisation and infection. Several mycoplasmas, such as *M. pneumoniae* and *M. genitalium*, have adhesin proteins associated with adhesion concentrated at specific tip structures [43]. In comparison, *M. hominis*, along with other *Mycoplasma* species, lack this attachment organelle. Many surface antigens have been identified in *M. hominis* and some of these play a role in cytoadherence as shown by monoclonal antibody inhibition assays [78, 79]. Adhesion of *M. hominis* is facilitated by cytoadhesive membrane proteins and lipoproteins such as P80 secretin and variable adherence-associated (Vaa) protein, P60 and OppA, respectively [79-81]. Additionally, in bacterial vaginosis, shifts to a higher pH in vaginal flora are often accompanied by higher titres of *M. hominis*. However, whether higher colonisation rates are the consequence or the reason for such changes in the milieu is still unknown.

1.2.2.1 Cytoadhesive membrane proteins

Cytoadhesins of *M. hominis* have been shown to bind to sulphated structures on human cells and ECM molecules [79, 82]. Upregulation of laminin, thrombospondin and collagen as ligands and integrins as receptors for *M. hominis* cytoadherence and internalisation has been shown on the host cell membrane as a

response to infection with *M. hominis* [83]. The method by which *M. hominis* is internalised into host cells is however unknown. Nevertheless, it has been shown by confocal microscopy that *M. hominis* invades HeLa cells and can result in the re-arrangement of the actin cytoskeleton [83].

A surface-expressed, membrane protein of *M. hominis*, P80, was found to function as a cytoadhesin, facilitating attachment of *M. hominis* to host cells in combination with another membrane protein, P60. The masking of P80 or P60 using monoclonal antibodies resulted in a reduction of *M. hominis* adherence to HeLa cells [79]. P80 is also released into the extra-cellular environment [80]. Release of proteins into the surroundings of a *Mycoplasma* cell is thought to lead to an immediate alternation of the cell surface architecture and, as most membrane proteins are targets for the host immune response [79, 84], it may help evade the host's immune system.

The exact role of P80 as a virulence factor is unknown but the release of type II secreted proteins is typically associated with non-invasive organisms that colonise mucosal surfaces, like *M. hominis*, and are considered to be required for the establishment of infection at these sites. A type II protein secretion system has been characterised for *Legionella pneumophila* as a virulence factor linked to intracellular infection [85].

1.2.2.2 Membrane bound lipoproteins

Mycoplasmas are heterogeneous organisms and many display antigenic variation and pronounced variation of surface proteins. This is thought to be an important way of evading the host immune response, particularly the humoral immune response, resulting in the chronic infection characteristic of many *Mycoplasma* infections [5]. Previous studies have indicated that the surface antigenic profiles of

M. hominis strains are highly heterogeneous, expressing both size and phase variants of surface exposed membrane proteins [78, 86-88]. Several *M. hominis* surface proteins have been characterised, including P120, P135 and Vaa; however, the molecular basis of variation in *M. hominis* has only been elucidated in some cases. The mechanisms involved in the diversification of mycoplasma surface proteins are highly complex and include: size variation caused by gain or loss of intragenic repetitive sequences; phase switching by deletion/insertion mutations or DNA inversion affecting promoter activity; and presence of multigene families or multiple copies of partial genes in the mycoplasmal chromosome [89].

***Mycoplasma hominis* Variable adherence-associated antigen (Vaa)**

The presence of a Vaa surface protein of *M. hominis* was initially identified over 20 years ago as a potential adhesin of *M. hominis* by using specific monoclonal antibodies to inhibit mycoplasma adherence to cultured cells [78, 79]. This protein was first identified as; a 49 kDa surface protein in *M. hominis* strain PG21 [90]; an adherence-associated, multiple-banding membrane lipoprotein in strain 1620 [78]; and as a 50 kDa adhesin in strain FBG, known as P50 [79]. While these groups used different nomenclature (Vaa versus P50), the surface lipoprotein in question is the same protein. The original terminology, Vaa antigen will be used, as this lipoprotein often has varying molecular masses with conserved regions and therefore P50 is a less accurate descriptor. Variation in the composition and size of the Vaa proteins results from allelic variant forms of the single copy *vaa* gene in *M. hominis* [91, 92]. The importance of Vaa in the pathogenesis and immune evasion of *M. hominis* infections is still not fully understood. But, Vaa was identified as a potential adhesion of *M. hominis* using monoclonal antibody inhibition, with the masking of Vaa showing prominent difference in the ability of *M. hominis* to adhere to HeLa cells [78, 79].

Differences in the expression of Vaa between strains of *M. hominis* have been observed and Vaa has been shown to undergo high-frequency phase variation resulting in ON/OFF expression [78, 91, 92]. Phase variation of Vaa is controlled at translation, not as a consequence of transcriptional events due to alterations to the promoter sequence seen in other bacteria such as *Neisseria gonorrhoeae* and *Ureaplasma* species [91, 93-95].

Size variation has been observed in Vaa [78], and was initially examined in clonal lineages generated from *M. hominis* strain 1620, wherein three size variants of the Vaa antigen were identified and designated Vaa-2, Vaa-3, and Vaa-4. Sequence analysis showed that this *vaa* gene length variation corresponded to the number of 363 bp intragenic tandem-repeat (TR) elements. The number of repeats was then used in the nomenclature of the different clonal lineages: Vaa-2 (two repeats), Vaa-3 (three repeats), and Vaa-4 (four repeats) [92]. These repeats form the basis of "modules" which provide a platform for further separation of Vaa types into categories (Figure 1.3). This 363 bp repeat became the prototype for what is now referred to as module III.

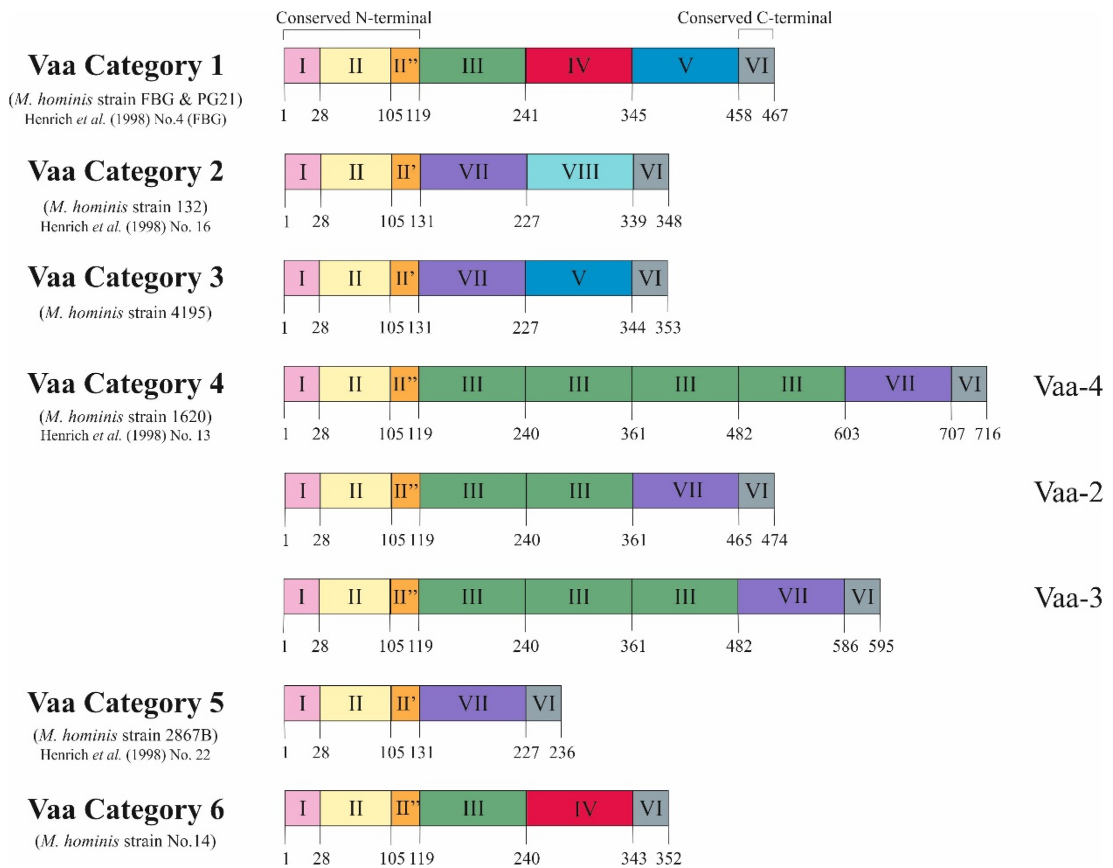

Figure 1.3. Schematic representation of the deduced amino acid sequences of the six *vaa* gene types.
The proteins show a modular composition with homologous modules showing more than 82% amino acid identity. Modules I, II and I'/II'' form the conserved N-terminal of the protein and Module VI represents the 10 amino acids conserved at the C-terminal Modules III, IV, V, VII and VIII form the interchangeable cassettes. Prototype M. hominis strains for each Vaa category are stated along with corresponding strains from Henrich et al. (1998) [96]. Figure modified from Boesen et al. (1998) [97].

All *vaa* genes described thus far all start with highly homologous modules I and II, followed by either module II' or II''. All reported combinations of identified module types analysed in over 100 clinical isolates [96, 97] has resulted in six possible categories (Figure 1.3); however, the number of module III in category 4 have been found to vary between two and four repeats. Sequence homology for each module type was initially restricted to 82% between strains [92, 97].

Modules III, IV, V, VII and VIII form an interchangeable cassette of sequences that provide the size variation observed in Vaa variants. Inter-module homology (38-78%) suggests a common ancestral sequence [97]. A stable, repeated motif of four amino acids (SFKE) was observed in module II, a constant part of the gene. This motif was extended to ELESFKE in almost all of the interchangeable cassettes. Three highly conserved tryptophan residues were also identified in distinct positions in a 16 amino acid region situated in the C-terminal part of the cassette sequence [97].

The presence of these highly homologous interchangeable cassettes in the *vaa* gene suggests a mechanism of variation in which homologous recombination provides insertions or deletions of whole cassettes. Homologous recombination can bring mutations arising in different genomes together and has a strong impact on pathogenic adaptation [98]. The absence of sequences homologous to the *vaa* gene in other members of the *mycoplasma* family, or other bacterial species, suggests that intra-species genetic transfer is responsible for the current array of Vaa categories.

Sequence analysis and modelling of the Vaa protein indicates that Vaa belongs to the group of monomeric microbial surface-exposed coiled-coil proteins similar to Protein A of *Staphylococci* [99-101]. Figure 1.4 shows a hypothetical model of the topology of Vaa. The Figure shows a bacterial membrane lipid anchor that is typical

of prokaryotic lipoproteins attached to the N-terminal cysteine residue of the mature Vaa, with the conserved N-terminal in a triple-helix bundle, extending into an elongated helix. Two β sheets then form a loop region and a C-terminal helix folds back on the elongated helix. This model indicates that Vaa is composed of an N-terminal base domain in close proximity to the membrane and a C-terminal spike cassette domain projecting out from the surface of *M. hominis* [99].

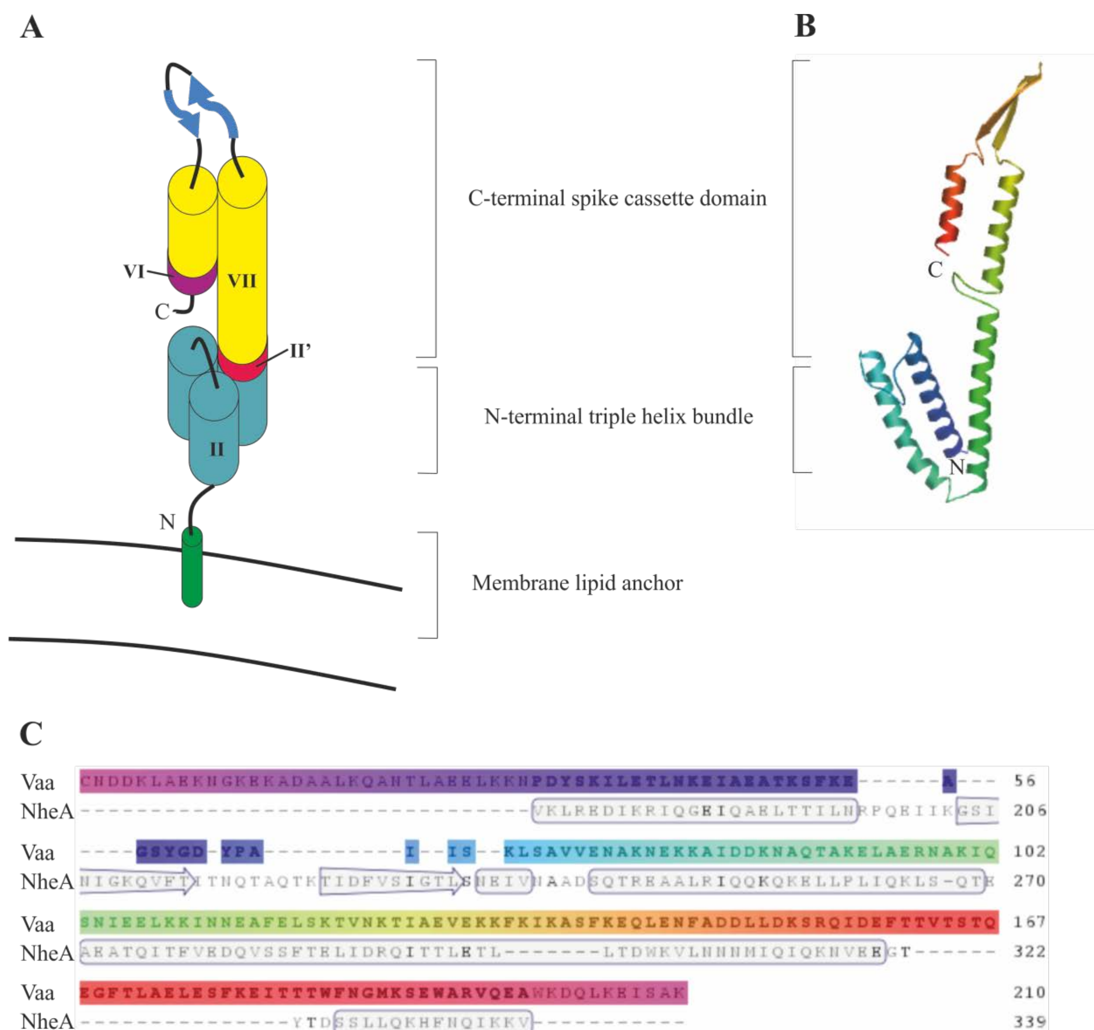

Figure 1.4. Model of the predicted protein structure for Vaa category 5.
(A) Schematic representation of Vaa category 5 modified from Boesen *et al*. [99]. The modules are numbered as outlined in Figure 1.3. (B) Protein homology model of Vaa category 5 strain 2867B. Model was created using www.swissmodel.expasy.org and uses NheA protein as a template. (C) Alignment of Vaa category 5 strain 2867B amino acid sequence with the NheA protein template. Secondary structure is indicated in the template.

OppA

An Opp transport system has also been identified in *M. hominis* [102] which shares little sequence similarity with the respective domains of other species but shares the typical features of these transport systems. It is composed of four domains OppBCDF and the cytoadherence-associated lipoprotein P110 as the substrate binding domain OppA [102]. OppA displays ecto-ATPase activity, which hydrolyses ATP at the external surface of the cell membrane. Extracellular ATP has profound effects on cellular functions and can cause plasma membrane depolarisation and cell death [103, 104]. OppA is proposed to be the main ecto-ATPase of *M. hominis* with 80% of the surface-localised ATPase activity of *M. hominis* derived from OppA [105].

Evidence indicates that *M. hominis* OppA is involved in processes that induce ATP release from and damage to host cells. OppA induces ATP release from HeLa cells and ATP hydrolysis by OppA results in apoptosis of the host cell which is proposed to guarantee the nutrition uptake and survival of *M. hominis* [106]. Additionally, this supports the hypothesis that extracellular colonising pathogens enhance apoptotic cell death. The enzymatic function of OppA as the main ecto-ATPase of *M. hominis* is essential for OppA-mediated adhesion [107]. It is hypothesised that this unique feature of *M. hominis* has an impact on patho-physiological important processes involved in host-pathogen interactions [107].

1.2.2.3 Modulation of host immune response by *Mycoplasma hominis*

M. hominis infection of HeLa cells is initiated by cytoadherence followed by a prominent induction of pro- and anti-apoptotic cytokines and the repression of heat shock protein (HSP) 70 [83]. Heat shock proteins are expressed by cells in response to stress and act as molecular chaperones. They have been documented to modulate

innate and adaptive immune responses after exposure to microbial pathogens. The down-regulation of HSP70 by micro-organisms has been proposed to be a mechanism of immune evasion and to promote chronic infection [108]. The repression of HSP70 by *M. hominis* is in accordance with this observation, as *M. hominis* infection is rarely acute and the *M. hominis* induced cytokine profile in human dendritic cells corresponds to that of a pathogen associated with chronic infections [109].

1.3 Detection, quantification and typing of *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Laboratory based detection of *M. pneumoniae* and *M. hominis* in clinical specimens is gaining greater attention largely due to the availability of improved methods for detection. Traditionally *M. pneumoniae* infections has been detected by serological methods, detecting specific immunoglobulin (Ig) M, IgG, IgA antibodies and cold agglutinins. For *M. hominis,* culture is the most economical and practical means of detection. However, the use of molecular techniques for detection of both *M. pneumoniae* and *M. hominis* is increasing.

1.3.1 Culture and quantification

Mollicutes require an enriched growth medium supplemented with nucleic acid precursors, fatty acids, and amino acids for growth, due to their limited genome capabilities. Detection of *M. pneumoniae* by culture is rarely undertaken due to the length of time necessary for growth to be observed, ranging from days up to three weeks or more. This limiting factor makes culture diagnosis of *M. pneumoniae* impractical for patient management and is therefore rarely offered as a means to diagnose infection by clinical laboratories [110]. However, *M. pneumoniae* can be grown in specialised media containing glucose and colonies can be observed when

grown on agar. Colonies grown on agar must undergo additional testing, usually polymerase chain reaction (PCR), to confirm their identity because several commensal mycoplasma species often inhabit the human oropharynx and they are indistinguishable when grown on agar.

In comparison to *M. pneumoniae*, *M. hominis* grows well in specialised media and on agar, with colonies usually visible under a stereomicroscope within two to three days. There are no phenotypic tests that can distinguish *M. hominis* from other mycoplasma species growing on the agar therefore additional testing is usually performed to confirm diagnosis, such as PCR. Culture is preferred over PCR in laboratories with low to moderate test volumes as cultures can be set up one at a time. For efficient and cost-effective use of PCR, laboratories need to run the assay in batches of several specimens. Culture also has an advantage over PCR as it provides an isolate on which further testing can be performed, e.g. antimicrobial susceptibility, and culture confirms the presence of live bacteria and therefore active infection.

1.3.2 Serological detection

Serological testing was the first method developed for the detection of infection with *M. pneumoniae*. *M. pneumoniae* has both lipid and protein antigens, which elicit an antibody response that can be detected after about one week of illness, with a peak at 3-6 weeks followed by a gradual decline. Initially IgM antibodies occur 6-10 days after infection; however, only about 80% of patients younger than 20 years develop IgM antibodies and this reduces to about 40% in patients that are older than 20 years. This means that a specific IgM response can be absent, especially in older patients. It has also been shown that IgM antibodies can still be detected at least one year after the beginning of symptoms [111]. Specific IgG antibodies appear 9-14 days after infection and may persist for up to four years. Specific IgA antibodies appear one

week after the start of infection and decrease after about five weeks. Due to this antibody response, acute and convalescent patient sera, taken 2-3 weeks apart, are required for confirmation of infection. This is a significant limitation for prompt point-of-care diagnosis, particularly in adults over 40 years of age who may not mount an IgM response.

Complement fixation was the original serological test used for the detection of *M. pneumoniae* infection by clinical laboratories; however, this has now been replaced by newer methods. Complement fixation measures mainly the early IgM response and does not differentiate between antibody classes, which can be used to determine the stage of the infection. This method also suffers from low sensitivity because the glycolipid antigen mixture used may be found in other microorganisms, as well as human tissues; in particular cross-reactions with *M. genitalium* are well recognised.

Immunofluorescent antibody assays, direct and indirect haemagglutination using IgM capture, and other particle agglutination antibody assays have been developed to detected specific anti-*M. pneumoniae* antibodies. However, enzyme immunoassays (EIAs) have become the most widely used commercial methods for detection of *M. pneumoniae*. Enzyme immunoassays allow the class of the antibody to be measured and the sensitivity can be comparable that of the PCR assay, providing sufficient time has elapsed since infection for antibodies to develop and for the patient to have a functioning immune system. These assays may be qualitative or quantitative and can be performed with very small volumes of patient serum.

Comparative studies between commercially available EIAs show variability in diagnosis results, highlighting the need for standardisation and improved sensitivities and specificities among serological reagents used for detecting acute *M. pneumoniae*

infection [112-114]. Other limitations of serological diagnosis of acute *M. pneumoniae* infection include the possibilities that antibody production can be delayed in some infections, or even absent if the patient has an impaired humoral immune response. False-negative tests can also occur if serum is collected after antibiotics have been administered [110].

Serological test methods for *M. hominis* include micro-immunofluoresence, metabolism inhibition and EIA, but the presence of *M. hominis* in healthy people makes interpretation of antibody titres difficult. Serological tests for *M. hominis* are not easily developed due to the significant heterogeneity and antigenic variation among different isolates. However, the measurement of an IgG antibody response to *M. hominis* infection has been performed by enzyme-linked immunosorbent assay (ELISA) using a mixture of membrane proteins from two different *M. hominis* isolates as a preferred antigen [115]. No serological assays for *M. hominis* have been standardised for diagnostic use.

1.3.3 Molecular detection

PCR has been used increasingly for *M. pneumoniae* detection due to the significant reduction in time to obtain a result compared to culture and the removal of the requirement for acute and convalescent sera for serology testing. It can also confirm *M. hominis* infection. The first reports of the use of PCR for the detection of *M. pneumoniae* in clinical samples dates back to 1989 and since then there have been more than 200 publications describing the technique for this use [116, 117].

The sensitivity of PCR is very high, corresponding to the detection of the presence of a single organism when purified DNA is used. Other advantages of using this technique for diagnosis of infection include; the ability to complete the assay in one

day; the possibility of obtaining a positive result more quickly after the onset of illness than when using serology; the need for one specimen containing organisms that do not have to be viable and the ability to detect nucleic acid in preserved tissue [9]. PCR is also a valuable tool for identifying mycoplasma aetiology in people with a variety of extra-pulmonary syndromes, due to the ability to detect the organism in blood and cerebrospinal fluid which has rarely been successful by culture.

Several *M. pneumoniae* gene targets have been used for PCR assays, including the 16s rRNA gene, the elongation factor *tuf*, the P1 cytadhesin gene and repetitive elements located within the latter [118-122]. Comparison of PCR with culture and/or serology has yielded varied results that are not always in agreement. Positive PCR results in culture-negative persons, without evidence of respiratory disease, suggest inadequate assay specificity, persistence of the organism after infection, or asymptomatic carriage of the organism. Positive PCR results in serology negative persons may be due to an inadequate immune response, early successful antibiotic treatment, or to the collection of specimens before the specific antibody response occurs. Negative PCR results in culture or serology proven infections increase the possibility of inhibitors or other technical problems with the assay and its gene target and emphasises the increased sensitivity of culture methods [11].

Conventional PCR assays for *M. hominis* have mainly used 16s rRNA as a gene target. However, due to minor heterogeneity in this gene, other targets including *gap*, *fstY*, and *yidC* have been developed [123-126]. *M. hominis* is a common commensal in the female cervix and vagina and the male urethra. A positive PCR result on specimens from these sites may therefore not be meaningful in the absence of clinical manifestations known to be associated with this organism unless the *M. hominis* load reaches $\geq 10^4$ colony forming units (CFU)/mL, which is considered a crucial criterion

for urogenital infections in women [127]. In the case of extra-genital specimens in adults or neonates, a positive *M. hominis* PCR assay or culture result should be considered clinically significant.

Real-time PCR assays have significant advantages over traditional PCR, including a more rapid turnaround time, less handling of PCR products by electrophoresis analysis and increased sensitivity [110].

1.3.4 Molecular typing

The typing of clinical isolates allows investigation into the epidemiology of *M. pneumoniae* and *M. hominis* infections and analysis of endemic outbreaks.

Molecular typing of *M. pneumoniae* is hindered by the fact that *M. pneumoniae* is a genetically homogenous species. Previous investigations have shown that clinical isolates differ in the sequence of the gene encoding the P1 cytoadhesin and this has been the most common genotyping method used in the past 30 years. PCR-restriction fragment length polymorphism (RFLP) analysis of the P1 gene enabled the separation of *M. pneumoniae* isolates into two types, types 1 and 2 [128, 129]. However, more recent studies use repetitive elements within the P1 gene for molecular typing which, as well as identifying type 1 and 2, identify variants of these sub-types [130].

In addition to these molecular typing methods that target only one gene, other methods based on the whole genome have been adapted to *M. pneumoniae*, such as pulsed-field gel electrophoresis (PFGE), and multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) [128, 131, 132]. PFGE, like P1 gene analysis, can separate *M. pneumoniae* strains into two groups (groups 1 and 2) and can subdivide group 2 into two subgroups, 2a and 2b [128]. However, due to the homogenous nature of the *M. pneumoniae* genome, very little polymorphism had previously been found

in *M. pneumoniae* housekeeping genes leading previous investigators to conclude that multi-locus sequence typing (MLST) was not useful as a molecular typing method for *M. pneumoniae* [131].

MLVA has successfully been used for typing an increasing number of microbial species, including *M. pneumoniae* and, more recently, *M. hominis* [132, 133]. MLVA is a molecular typing method based on the variation in the copy number of tandemly repeated sequences, called VNTRs, found at different loci on the genome. The variation of the copy number of these repeat units varies between isolates. More than 26 differing types have been described for *M. pneumoniae* [130, 132, 134]. However, in comparison to *M. pneumoniae*, *M. hominis* displays high genetic heterogeneity among isolates and 40 MLVA types have been described for 210 isolates tested [133].

Other molecular typing methods have been developed for *M. hominis,* including methods based on sequence analysis of the *p75*, *p120'* and *vaa* genes [135-137]. Isolate groupings based on the sequence analysis of these genes are not comparable, indicating that the three genes vary by different mechanisms.

1.4 *Mycoplasma hominis, Mycoplasma pneumoniae* and disease

1.4.1 *Mycoplasma pneumoniae* infections

M. pneumoniae infections affect both the upper and lower respiratory tracts and occur both endemically and epidemically worldwide in children and adults. *M. pneumoniae* is a leading cause of community-acquired pneumonia (CAP) and is responsible for 15-20 % of CAP cases in adults and up to 40% of cases in children [112, 138, 139].

M. pneumoniae infections are normally mild and in adults cases may be asymptomatic; however, infections are often more serious in children and immunocompromised individuals. Respiratory infections caused by *M. pneumoniae* are often slow to develop, presenting with pharyngitis, sinus congestion, and eventually prolonged lower respiratory involvement up to and including primary atypical pneumonia with fever and bibasilar pulmonary infiltrates. The clinical symptoms that manifest due to infection can be diverse with the most severe infections resulting in CAP and occasionally abscesses persisting for weeks to months [140]. The most common manifestations include a sore throat, fever, a cough which is initially non-productive, headache, and general malaise [9].

Children with hypogammaglobulinemia are known to be at greater risk for development of respiratory and joint infections due to *M. pneumoniae*, demonstrating the importance of functional humoral immunity in protection against infection due to this organism [9].

1.4.1.1 Role of *Mycoplasma pneumoniae* in persistent cough in children

Most coughs in children are caused by acute respiratory tract infections and usually resolve within two weeks [141]; however, a persistent cough following a respiratory tract infection is common in children. A multi-centre parent questionnaire survey undertaken in Europe reported that 12.7% of children aged 7-11 years had experienced a persistent cough lasting three months or longer during the previous autumn/winter season. Of these, 7.6% had a persistent cough in the absence of a cold and 11.5% had a persistent cough at night [142]. Most acute respiratory tract infections are caused by respiratory viruses [143]. *M. pneumoniae* is one of the most common bacterial causes of respiratory tract infections in children with the highest incidence in children aged five to nine years (4/1000 children per year) [144].

A retrospective study was undertaken in England in 2011 to examine the incidence of *M. pneumoniae* in children with a persistent cough and the duration of cough in children infected with *M. pneumoniae* [143]. The patient cohort examined were children aged 5-16 who presented to their General Practitioner (GP) with a cough lasting 14 days or longer that was unexplained or triggered by an acute respiratory tract infection. Samples were obtained from patients from 18 GP surgeries in Oxfordshire, England. *M. pneumoniae* was detected, based on PCR of nasopharyngeal aspirates, in 7.1% (11/155) of children with a persistent cough which was considerably higher than previous estimates reported in children with acute cough and fever (2.6%) [145] and asymptomatic household contacts of *M. pneumoniae* cases (0.25%) [146]. The duration of a persistent cough in *M. pneumoniae* cases was found to be one-third of that in children infected with pertussis [143]. Incidence of *M. pneumoniae* in school-aged children with a persistent cough was found to be increase during periods of high *M. pneumoniae* activity, i.e. during an epidemic [143].

1.4.1.2 Role of *Mycoplasma pneumoniae* in asthma and chronic lung disease

M. pneumoniae has been implicated in both the pathogenesis and the exacerbation of acute attacks of asthma [147]. Asthma is a chronic inflammatory disease of the airways, characterised by hyper-responsiveness to multiple stimuli, reversible airway limitation and chronic eosinophilic infiltration of the airways. Current literature indicates that *M. pneumoniae* infection can be an important trigger for the acute exacerbation of asthma, accounting for between 3.3% and 50% of cases of exacerbations. It is also thought that *M. pneumoniae* can initiate asthma in previously asymptomatic patients [148].

M. pneumoniae can be recovered from the respiratory tract up to several months after recovery from pneumonia, and pulmonary structural abnormalities,

indicating small airway obstruction, have been observed 1-2 years after disease [148]. *M. pneumoniae* can be isolated in higher prevalence from asthmatics than from healthy individuals and follow-up studies in children have demonstrated prolonged airway dysfunction consistent with a persistent infection.

M. pneumoniae induces a number of inflammatory mediators such as IgE, substance P and neurokinin 1, and interleukin (IL)-5, which have been implicated in the pathogenesis of asthma resulting in exacerbations. Children with wheezing and acute *M. pneumoniae* infection have a specific cytokine profile that is characterised by a marked increase in IL-5 [149]. Patients also have increased airway expression of transcription factor (TNF)-α and IL-4, both pro-inflammatory cytokines. Models of chronic respiratory infection in mice have demonstrated that *M. pneumoniae* can produce pneumonia, and stimulate cytokine production, airway hyper-responsiveness resembling chronic asthma and a Th2-dominant airway inflammatory process that enhances organism survival in the lungs [110, 150].

1.4.1.3 Extra-pulmonary manifestations of *Mycoplasma pneumoniae* infection

It is estimated that 25% of people infected with *M. pneumoniae* may experience extra-pulmonary complications at various times after the onset of or even in the absence of respiratory illness [151]. Complications that can arise involve the skin and the nervous, cardiovascular, renal, gastrointestinal, musculoskeletal, and haematologic systems. It is thought that the host immune response that develops after *M. pneumoniae* infection contributes to these complications as well as contributing to autoimmunity [9]. The presence of *M. pneumoniae* in these extra-pulmonary sites has been confirmed by PCR as well as culture [152-156].

Complications that occur within the central nervous system (CNS) are recognised as the most common extra-pulmonary manifestations of *M. pneumoniae* infections. A recent study of 1,988 children with encephalitis showed *M. pneumoniae* as the most common causative agent [157]. *M. pneumoniae* infection is established in 5% to 10% of paediatric encephalitis patients [158, 159] and up to 60% of them show neurologic sequelae [159, 160]. Clusters of encephalitis are occasionally observed in epidemics of *M. pneumoniae* infections. Other CNS complications include: cerebellar syndrome and polyradiculitis, cranial nerve palsy, aseptic meningitis or meningoencephalitis, and acute disseminated encephalomyelitis among others [9]. The mechanisms that result in these neurological manifestations of *M. pneumoniae* infection are not completely understood. However, immune-mediated mechanisms are suspected due to the development of cross-reactive antibodies to the brain and other neurological structures [110]. Anti-GM 1 and galactocerebroside antibodies have been the main autoantibodies associated with *M. pneumoniae* induced encephalitis, and pro-inflammatory cytokines such as IL-6 and IL-8 can be detected in the cerebrospinal fluid at elevated levels in such patients [158, 161, 162]. These pro-inflammatory cytokines are important mediators of inflammation. *M. pneumoniae* RNA has been detected in brain tissue by nucleic acid hybridisation. The presence of the organisms in the cerebrospinal fluid has been confirmed by PCR and culture techniques, indicating that *M. pneumoniae* may disseminate and directly invade the CNS [163]. Most neurological manifestations of *M. pneumoniae* infections resolve completely; however, some incidences can result in chronic debilitating deficits in motor or mental function [9].

Complications involving the CNS are considered to be the most severe extra-pulmonary manifestations of *M. pneumoniae* infections. However,

dermatological disorders are common, clinically significant complications, occurring in approximately 25% of patients. Disorders that occur include; erythematous maculopapular and vesicular rashes which are usually self-limiting. In contrast to this, severe forms of Stevens-Johnson syndrome, conjunctivitis, ulcerative stomatitis, and bullous exanthems have been reported and directly linked to the presence of *M. pneumoniae* at infection sites [140, 164-167].

M. pneumoniae, like other *Mycoplasmas*, can cause chronic infection of the joints. Acute and septic arthritis has occasionally been identified in patients with detection of *M. pneumoniae* in the synovial fluid. This manifestation occurs most commonly in patients with hypogammaglobulinemia [168] but can also occur in patients with normal immunity [169]. Other instances of *M. pneumoniae* infection-associated arthritis may be due to the presence of autoantibodies, resulting in synovial inflammation rather than direct infection of the synovial fluid by *M. pneumoniae* [170, 171].

Haemolytic anaemia is a rare but serious complication of *M. pneumoniae* infection and is more common in children than in adults [172, 173]. This complication is attributed to cross-reactive cold agglutinins, which are primarily IgM antibodies to the Ii blood group. This antigen is found on surface glycoproteins or glycolipids of erythrocytes and other cells but is not present on the *M. pneumoniae* membrane. Instead it acts as a surface receptor for *M. pneumoniae* and is therefore immunogenic [110].

1.4.2 *Mycoplasma hominis* infections

M. hominis can be found on the mucosal surfaces of the cervix or vagina of 21 to 53% of sexually mature, asymptomatic women; this prevalence is somewhat lower in

the urethra of males. Colonisation of *M. hominis* is linked to younger age, lower socioeconomic status, sexual activity with multiple partners, African-American ethnicity, and oral contraceptive use [3, 174].

1.4.2.1 Contribution of *Mycoplasma hominis* infection to bacterial vaginosis and pelvic inflammatory disease

Bacterial vaginosis is the most common cause of abnormal vaginal discharge in women of child-bearing age. The cause of bacterial vaginosis is unknown, but it is characterised by the depletion of the normal *Lactobacillus* population in the commensal flora of the vagina and an overgrowth of vaginal anaerobes, accompanied by the loss of the usual vaginal acidity. Approximately 50% of bacterial vaginosis cases appear to be asymptomatic or women experience only mild symptoms. Women with bacterial vaginosis consistently have up to one thousand fold increase in the concentration of several organisms, namely *Gardnerella vaginalis, Bacteroides (Prevotella)* species, *Mobiluncus* species and *M. hominis* [3, 175].

Not one of these organisms is the sole cause of bacterial vaginosis in women and *M. hominis* is found in the vagina of approximately 60% of women with the condition. Additionally, higher titres of serum IgG antibody to *M. hominis* are found in women with bacterial vaginosis than in healthy women, indicating that *M. hominis* is strongly associated with the condition. However, the mechanism of how *M. hominis* contributes to the pathology of bacterial vaginosis is unknown, if indeed it does at all [175].

A symbiotic relationship between *M. hominis* and *Trichomonas vaginalis*, another micro-organism associated with bacterial vaginosis, has been proposed. It has been demonstrated that *T. vaginalis* is able to pass *M. hominis* infection to

mycoplasma-free trichomonad isolates and human cervical cells *in vitro* [176]. Additionally, *M. hominins* is able to reside and replicate within trichomonad cells [177]. Both *M. hominis* and *T. vaginalis* metabolise arginine, *M. hominis* as its main source of energy and *T. vaginalis* in anaerobic conditions [178]. Co-cultures of *M. hominis* and *T. vaginalis* show increased arginine metabolism by *T. vaginalis* [179], suggesting a possible mechanism that might be important for the establishment of the association between the two micro-organisms. This metabolic interaction might be important in the evasion from the host innate defences. It has been documented that the presence of *M. hominis* in a *T. vaginalis in vitro* infection model upregulated the pro-inflammatory response of macrophages to *T. vaginalis* and that *M. hominis* may be protected from the environmental conditions of the vagina and from antibiotic treatments by *T. vaginalis* [180, 181].

Bacterial vaginosis can be associated with miscarriage in the second-trimester and preterm birth, potentially via increasing the risk of chorioamnionitis which can stimulate preterm birth through the release of pro-inflammatory cytokines. Bacterial vaginosis has also been associated with vaginal cuff cellulitis, wound infection and abscess formation after hysterectomy.

M. hominis has been isolated form the endometrium and fallopian tubes of approximately 10% of women with pelvic inflammatory disease (diagnosed by laparoscopy), and its role in this disorder has been supported by the occurrence of specific antibody responses; up to one quarter of women with pelvic inflammatory disease develop a significant antibody response to *M. hominis* [174].

In men, *M. hominis* is recovered from the lower genital tract, more often in uncircumcised than circumcised men. Bacterial vaginosis has been proposed as one

cause of non-gonococcal urethritis in men, and *M. hominis*, as an important component of the bacterial vaginosis flora, could have a role in non-gonococcal urethritis. However, infection with *M. hominis* alone does not result in disease and appears to behave as a commensal in the majority of patients [174, 182].

1.4.2.2 *Mycoplasma hominis*, pregnancy and postpartum fever

In contrast to *M. pneumoniae*, *M. hominis* may invade the blood stream. *M. hominis* has been isolated from the blood from women diagnosed with febrile abortion and postpartum fevers, indicating that *M. hominis* may be responsible for some of these fevers. These fevers are often transient and self-limiting, but in some cases in which *M. hominis* is involved, trauma or infection in compromised hosts, may result in dissemination of *M. hominis* [3, 174, 183].

M. hominis has been isolated from placentae following pre-term birth, although less frequently than *Ureaplasma* species. A casual relation between different species of genital mycoplasmas and preterm labour has been difficult to prove. However, *M. hominis* has been proven to be the sole pathogen responsible for the induction of preterm birth in pregnant macaque monkeys [184]. Following intra-amniotic injection, there was a short delay in recoverable infectious *M. hominis*, but levels of *M. hominis* then continued to climb without peaking until intra-uterine contractions were stimulated.

1.4.2.3 Extra-genital *Mycoplasma hominis* infection in adults

Mycoplasma hominis is usually associated with infections localised to the urogenital tract. However; it may be pathogenic in non-genitourinary infections such as septicaemia, wound infections, central nervous system infections, joint infections and lower respiratory tract infections particularly in immunocompromised patients

[185, 186]. It is also a rare cause of septic arthritis with predisposing risk factors including underlying joint abnormality and immunocompromised states [187].

Most cases of *M. hominis* septic arthritis have been documented to occur in patients who were post-partum, had recently undergone urogenital surgery or manipulation, or were immunocompromised due to an underlying condition such as hypogammaglobulinemia, solid organ transplant, collagen vascular disease or haematologic malignancy [188]. However, cases of *M. hominis* bone infections, including septic arthritis, have been reported in immune-competent hosts following closed trauma [189]. The knee and hip were found to be the most common joints affected but incidences of *M. hominis* septic arthritis in the shoulder, wrist, ankle and spine have also been reported [190].

M. hominis infections have been documented in organ transplant recipients. *M. hominis* infections following renal transplant have been associated with peritonitis, wound infections, urinary tract infections, septic arthritis and organ rejection [191-194]. Additionally *M. hominis* infections have been reported in recipients of lung [191, 195], heart and lung [196, 197] and liver [198, 199] transplants and after vascular allografts [200]. Post-operative *M. hominis* infections, in addition to transplants, have also been documented [201-203].

1.4.2.4 *Mycoplasma hominis* infections in neonates

M. hominis from the maternal urogenital tract can colonise neonates and has been implicated in cases of meningitis [204, 205], ventriculitis [3], brain abscesses [206], pneumonia, pericarditis, scalp-wound infection complicating foetal monitoring [3, 185], eye infection [207, 208], and supramandibular adenitis in neonates [209].

M. hominis can be transmitted from an infected female to the foetus or neonate by several mechanisms. Firstly, there can be an ascending intrauterine infection in which organisms gain access to the amniotic sac, where they multiply and are then passed into the foetal lung. There have also been numerous reports of *M. hominis* being isolated from cord blood and of *M. hominis* isolation from the bloodstream of neonates and young infants. Secondly and more commonly, acquisition of *M. hominis* by the neonate can occur through passage of an infected maternal birth canal with resultant colonisation of the skin, mucosal membranes and respiratory tract [3, 210].

Isolation of *M. hominis* in the bloodstream of neonates and young infants is sometimes associated with pneumonia and/or meningitis in the infant. Reports of meningitis caused by *M. hominis* have been published since the 1950s with incidences reported in both preterm and full-term newborns with or without neurological defects (e.g. myelomeningocele) [3, 205, 211, 212].

1.5 Epidemiology of *Mycoplasma pneumoniae* infections

As previously described in section 1.4.1, *M. pneumoniae* is a major cause of CAP, with infections occurring globally. *M. pneumoniae* infection is endemic with epidemic peaks occurring every 4 to 7 years [134, 213, 214].

Typical outbreaks of *M. pneumoniae* infection occur in areas of close personal contact for example, schools and military barracks. Individuals with an active *M. pneumoniae* infection carry the organism in their nose, throat, trachea and sputum, and it is transmitted from person to person via aerosols. The spread of *M. pneumoniae* is greatly facilitated by the ubiquitous cough that is associated with the infection [9, 215]. Current opinion suggests that asymptomatic carriage of *M. pneumoniae* occurs, facilitating the spread of infection [27]. Epidemics of *M. pneumoniae* infections can

be prolonged; this could be due to the long incubation period of 1 to 3 weeks of *M. pneumoniae* as well as the relatively low transmission rate of infection. Long-term morbidity due to *M. pneumoniae* infection is uncommon however, the acute illness is often disruptive and can consume significant resources [9].

1.5.1 Epidemiology in the United Kingdom

Epidemics of *M. pneumoniae* infections historically occur every four years in the United Kingdom (UK), with epidemic periods lasting on average 18 months (Figure 1.5) [216, 217]. Sporadic infection occurs at low levels with seasonal peaks from December to February [146]. The most recent epidemic in the UK started in 2014 and continued into 2015, with an increased number of confirmed infections over the winter. Previously, the last documented epidemic was seen in the UK in 2010 and 2011 [134, 218].

In the UK, *M. pneumoniae* is not a notifiable infection and so analysis of infection numbers and epidemics relies on cooperation between the National Health Service (NHS) and the national public health body (Public Health England (PHE); Health Protection Scotland (HPS)). In England and Wales, data is submitted voluntarily from regional laboratories and hospitals and collated by PHE, according to age and region to give an indication of the number of patients testing positive for *M. pneumoniae* by serological, molecular or culture tests each week. Additionally, since 2005, a community surveillance scheme for *M. pneumoniae* using quantitative PCR (qPCR) analysis has been used to monitor infection. Until 2010, this scheme was used for monitoring patients of all ages and from 2010, for children aged under 16 years [134]. In Scotland, reports from NHS laboratories are collated by HPS via a non-mandatory reporting system. Some diagnostic laboratories in Scotland carry out PCR testing for *M. pneumoniae* as part of screening for respiratory viruses [219]. Therefore, young

children presenting with presumed respiratory viral infection to hospitals served by these laboratories also receive concomitant testing for *M. pneumoniae.* Due to the voluntary reporting scheme for information on cases *M. pneumoniae* infection, the number of positive cases is likely to be higher than those documented.

From January 1989 to June 2015, seven epidemics of *M. pneumoniae* were noted in England and Wales of declining amplitude with a recent peak in 2015 (Figure 1.5) [220]. For some epidemic periods, clear annual fluctuations can also be seen, apparent as a double peak over two winter seasons. The clarity of epidemic periods have, in recent years, declined with fewer reported cases overall. From 1975 to 2009, incidence was found to be similar by gender, both during epidemic and inter-epidemic periods. The annual notification rate in 2010-2014 was consistently highest in those aged 15-44 years. the use of culture has declined in recent years and despite serology being the most commonly used methodology, the implementation and increased use of molecular methods has resulted in a proportional increase in reports based on molecular tests from 0.32% in 2010 to 28.5% in the first 6 months of 2015 (Figure 1.6) [220].

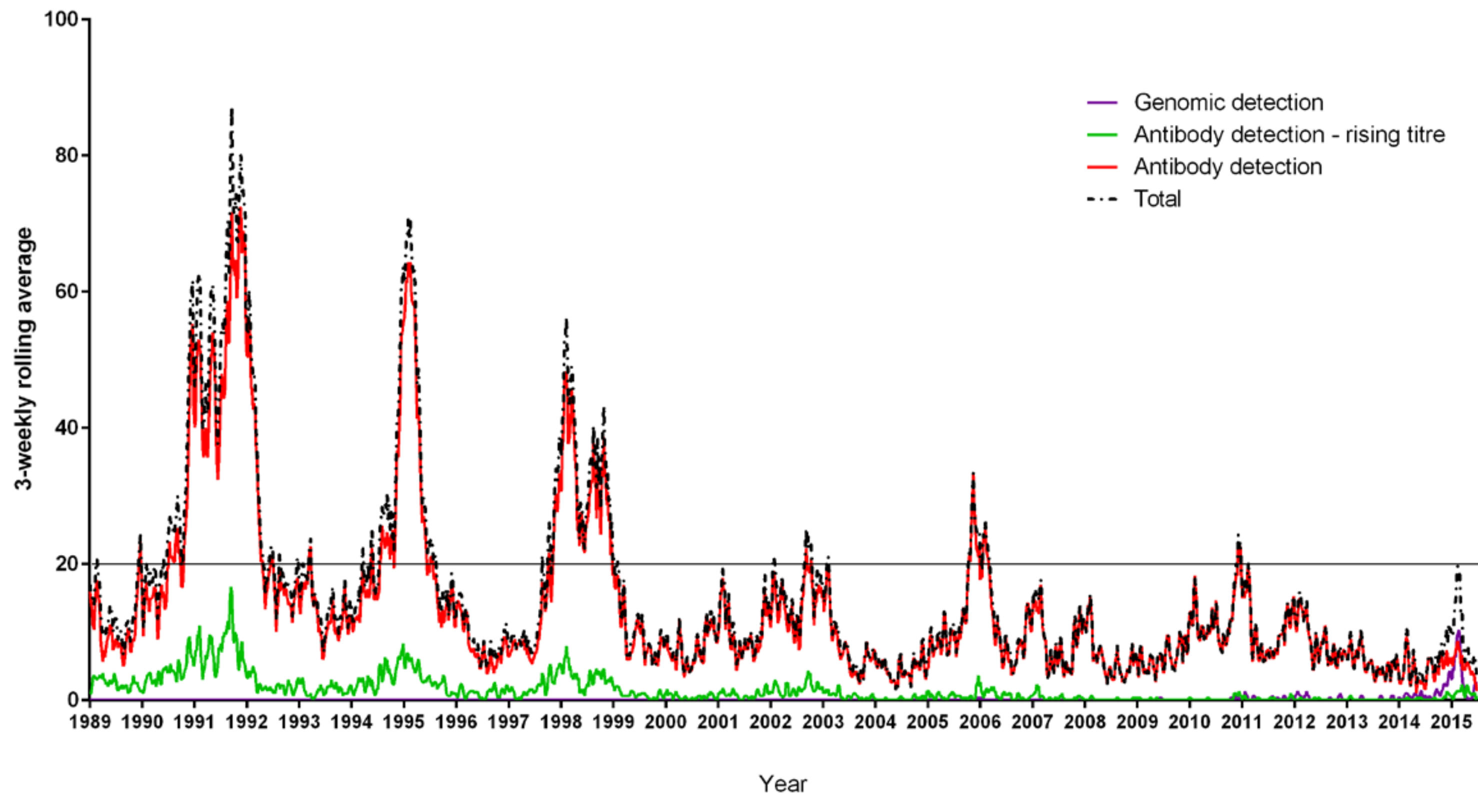

Figure 1.5. Laboratory reports of *M. pneumoniae* infection detection by genomic and serological methods in England and Wales from January 1989 to June 2015.

The line at 20 cases per 3 weekly average rolling period defines seven epidemic periods of declining magnitude and clarity, lasting up to two years (1991-1992, 1994-1995, 1998-1999, 2001-2003, 2005-2006, 2011,2015). National reporting categories include antibody detection and antibody detection – rising titre. A rising titre is defined as a four-fold increase in detectable anti-*M. pneumoniae* antibody level

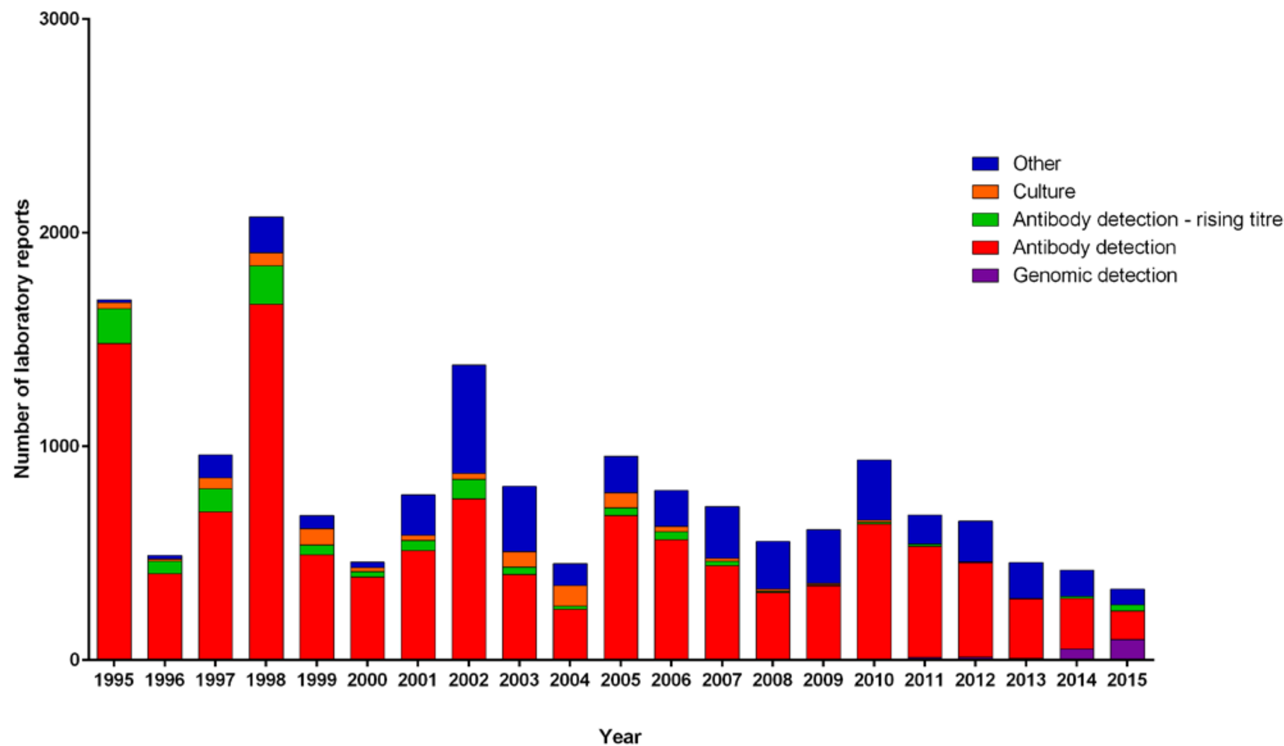

Figure 1.6. Number of laboratory reports per year from January 1995 to June 2015 separated by detection methodology. National reporting categories included are: antibody detection and antibody detection-rising titre. A rising titre is defined as a four-fold increase in detectable anti-*M. penumoniae* antibody level. Other indicates specimens for which *M. pneumoniae* infection was determined using antigen detection, microscopy and unknown categories. Culture indicates cases from which specimens yielded isolates of *M. pneumoniae* and genomic detection those for which DNA of *M. pneumoniae* was detected by PCR.

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Analysis of the epidemic that occurred between 2010 and 2011 in England and Wales showed that *M. pneumoniae* infection was found in all age groups; however, no significant difference between age group affected by *M. pneumoniae* infection was found over this time period. The mean age of positive cases was identified as 8.7 years (standard deviation; SD ± 2.6) with the majority of patients being over five years old [134, 214]. In comparison, in the epidemic that occurred between 2005 and 2006, *M. pneumoniae* infections were mainly reported in children aged 5-14 years in England and Wales. However, in Scotland, *M. pneumoniae* infection had the highest incidence in the age group 0-4 years old in the 2010-2011 epidemics [218].

Molecular typing is used to characterise epidemic outbreaks of *M. pneumoniae*, typically using P1 typing and MLVA. During the outbreak in 2010/2011, a total of 11 distinct MLVA types were identified, with MLVA-M being the most prevalent [134]. However, when new international guidelines for MLVA typing [221] are applied to this study, the number of distinct MLVA types detected reduced to 5 types. At the time of the study, strain type MLVA-M had been found previously in France, Germany and Japan [132]. A study of MLVA types found in children in England and Wales during this epidemic period identified that clonal strains were not detected in the cohort of patients studied [214]. Currently it is unknown whether increases in incidence of *M. pneumoniae* infections are due to an increased incidence of an individual strain or a concurrent increased incidence of several strains. Speculation that a shift in P1 adhesin type may be the cause of epidemics has been disputed [132, 222]. Evidence from studies performed in the UK does not support this hypothesis that a single strain type of *M. pneumoniae* was responsible for the observed increase of infection in England and Wales, specifically in the 2010/2011 epidemic. Instead, it was hypothesised that the outbreak was caused by a decline in immunity or an increase

of the immunologically naïve population that may have triggered the four-year cycle of epidemic periods [134].

From January 2005 to June 2015 eleven cases were referred to the Bacterial Reference Department, PHE that were identified as positive for *M. pneumoniae* that were of particular note [220]. Two cases of Stevens-Johnson syndrome were noted in 2009 and 2010 in male children aged 8 and 6. Two cases were noted in respiratory specimens in immunocompromised patients following extra-pulmonary organ transplantation (2013 and 2015). Infection in donor transplant patient respiratory secretions was also noted in 2015. *M. pneumoniae* was detected by qPCR in the nasopharyngeal aspirate but not the cerebrospinal fluid (CSF) of a child with pneumonia and reactive transverse myelitis in 2005, and in the brochoalveolar lavage of a child with encephalitis and seizures in 2011. In 2011, a young adult patient presented with post respiratory tract infection with encephalitis and transverse myelitis that progressed to tetraplegia with ventilator dependency. In this case, *M. pneumoniae* was confirmed by qPCR on throat swab specimens taken 19 and 21 days post onset but was not detected in concurrent CSF specimens [223]. Detection of *M. pneumoniae* in CSF is unusual and it is postulated that neurological manifestation of *M. pneumoniae* infection is antibody mediated rather than by direct presence of the bacteria itself [9]. Of 68 CSF specimens referred, only one positive case was detected in 2010, in a child with a ventriculoperitoneal shunt, in which contamination of the CSF during sampling could not be excluded. In 2012, *M. penumoniae* was detected by qPCR in the lung of two co-habiting adults that both suffered sudden fatal collapse. This was presumed to be a secondary infection, as one of the two patients also have confirmed *Staphylococcus aureus* infection [220].

1.5.2 Global epidemiology

The global epidemiology and incidence of *M. pneumoniae* infection as a causative agent of CAP has been extensively studied. *M. pneumoniae* was believed to be responsible for 15-20% of all cases of CAP between 1962 and 1975 in Seattle, Washington [138]. A Finnish study [139] reported *M. pneumoniae* detection in 30% of paediatric CAP, and detection in over 50% among children aged five years or older, making it the single most common pathogen detected. A study performed in the USA during the 1990s detected *M. pneumoniae* in 23% of CAP in children 3-4 years of age [224]. In France a study [225] documented occurrence in children under four years of age as having similar infection rates to other children or adults. It was considered that these results may reflect the fact that young children who attend nurseries or child care on a regular basis easily share respiratory secretions with older household members or contacts. Marston *et al.* [226] reported that *M. pneumoniae* was definitely responsible for 5.4% and possibly responsible for 32.5% of 2776 cases of CAP in hospitalised adults in Ohio. An additional finding was their observation that the incidence of mycoplasmal pneumonias in hospitalised adults increased with age and it was second only to *S. pneumoniae* in elderly persons.

Globally, epidemics of *M. pneumoniae* are considered to occur every 3-7 years; however, recent epidemiological studies have documented varying trends in epidemic patterns. Serological studies performed in Denmark showed a pattern of *M. pneumoniae* infections over a 50 year period from 1946 through 1995 with endemic disease transmission punctuated with cyclic epidemics every 3-5 years [227]. As described in section 1.5.1, epidemics occur at approximately four-yearly intervals in the UK. In comparison, recent studies from China have indicated a peak constant level of *M. pneumoniae* infections following the last epidemic in 2012 [228]. In Jerusalem,

historically, epidemics were observed every 3-5 years with seasonal peaks in October and early spring; however, since autumn 2014 a constant rate of infection has been observed, diverging from the historical pattern [229].

1.6 Antimicrobial therapy against *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Antimicrobial therapy is used to treat infections with either *M. pneumoniae* or *M. hominis*. However, because of intrinsic resistance within these species, therapy is restricted to a small number of agents including the tetracyclines, macrolides and fluoroquinolones [27]. Further complications for therapy arise in neonates and children who are colonised with *Mycoplasma*. Tetracycline use in neonates and children younger than 8 years of age is restricted due to effects on bone toxicity and deposition within calcifying tissue, but its use has been reported in a limited number of CSF infections [206, 230]. The usage of quinolones is also restricted in neonates due to effects on cartilage development and should not be administered before adolescence [231]. The susceptibilities of *M. pneumoniae* and *M. hominis* to a selection of antibiotics are listed in Table 1.2. All three classes of antibiotics have the advantage of being active against additional bacteria that may be associated with mycoplasmas in respiratory and genital tract infections.

The first-line antibiotics for treatment of *M. pneumoniae* infections in children are protein synthesis inhibitors of the macrolide class [231]. The 2011 British Thoracic Society guidelines for the management of CAP in children suggest empiric macrolide treatment at any age if there is no response to first-line β-lactam antibiotics, or in the case of very severe disease [232]. In adults, the first-line antibiotics for treatment of hospitalised cases of CAP, recommended by the British Thoracic Society, is empirical

treatment with penicillin/β-lactam antibiotics; however, depending on severity of disease, a macrolide may be added to treatment. If there is no response to first-line penicillin/β-lactam treatment then it is recommended that a macrolide is added or substituted to the treatment. Additionally, in cases of more severe disease that are already being treated with a macrolide, doxycycline or a fluoroquinolone can be added to the treatment when initial empirical treatment has failed [233].

Table 1.2. Minimum inhibitory concentration ranges (μg/mL) by antibiotic class for selected *M. pneumoniae* and *M. hominis* [234-236].

Antibiotic	***M. pneumoniae***	***M. hominis***
Tetracyclines		
Doxycycline	0.03-0.5	0.1-2
Minocycline	0.06-0.25	0.03-1
MLS group		
Erythromycin	≤0.004-0.06	32->1000
Roxithromycin	≤0.01-0.03	>16
Clarithromycin	≤0.004-0.125	16->256
Azithromycin	≤0.004-0.01	4->64
Josamycin	≤0.01-0.03	0.05-2
Clindamycin	≤0.008-2	≤0.008-2
Pristinamycin	0.02-0.5	0.1-0.5
Quinupristin/Dalfopristin	0.008-0.25	0.03-2
Ketolides		
Telithromycin	≤0.001-0.06	2-32
Solithromycin	≤0.000000063-0.000125	0.002-0.008
Fluoroquinolones		
Ciprofloxacin	0.5-2	0.1-4
Ofloxacin	0.05-2	0.1-4
Levofloxacin	0.5-1	0.1-2
Moxifloxacin	0.06-0.3	0.06-0.125
Other agents		
Chloramphenicol	2-10	4225
Gentamicin	4	2-16

1.6.1 Intrinsic antibiotic resistance in *Mycoplasma hominis* and *Mycoplasma pneumoniae* and therapeutic considerations

Two types of intrinsic resistance to antibiotics are found in mycoplasmas; the first is common to all species in the class *Mollicutes* and the other is specific to certain

species. These properties have been used to isolate mycoplasmas from specimens contaminated by other bacteria, and to differentiate *Mycoplasma* species within a specimen [236, 237]. The class *Mollicutes* lack a cell wall and therefore all organisms within the class are resistant to cell wall synthesis inhibitors, such as β-lactams, glycopeptides and fosfomycin. Additionally they are also resistant to polymyxins, sulphonamides, trimethoprim, nalidixic acid and rifampin [238, 239]. *M. pneumoniae* and *M. hominis*, like other human mycoplasmas, are resistant to linezolid. The second type of intrinsic resistance is species-specific and concerns mainly the MLSK antibiotics [238, 239]. Among human pathogenic *Mycoplasmas*, *M. pneumoniae* and *M. genitalium* are sensitive to all MLSK antibiotics with the exception of lincomycin which shows modest activity against these two species. The same is true for *Ureaplasma* species. However, *M. hominis* is resistant to 14- and 15-membered ring macrolides and to telithromycin, but it is susceptible to the 16-membered ring macrolides, josamycin, and to lincosamides [240-243] (Table 1.2). The genetic basis underlying the intrinsic resistance of *M. hominis* to erythromycin has been studied by comparing the genome of *M. hominis* with that of *M. pneumoniae*, resulting in the identification of a single nucleotide difference in the peptidyltransferase loop of the domain V region of 23S rRNA, G (*M. pneumoniae*) to A (*M. hominis*) transition at position 2057 (*E. coli* numbering) [242]. This mutation is associated with macrolide resistance in other bacteria.

Various mechanisms of acquired antibiotic resistance have been elucidated, however, the only methods described *in vivo* for mycoplasmas are antimicrobial target modification or protection [236]. An active efflux mechanism has also been demonstrated *in vitro* [244]. Resistance in *M. pneumoniae* and *M. hominis* is mediated either by chromosomal mutations or acquisition of a transposon and no extra-

chromosomal element has been described in either species. In *M. pneumoniae*, only target alterations by acquired mutations have been associated with antibiotic resistance [245].

Mycoplasmas are characterised by high mutation rates and sequencing studies have revealed only a small amount of genetic information is dedicated to DNA repair [246]. It is hypothesised that there is an association between high mutation rates and antibiotic resistance in mycoplasmas, as has been found for *Pseudomonas aeruginosa* [247]. Resistance through mutation concerns all classes of antibiotics used to treat *M. pneumoniae* and *M. hominis* infections [238].

1.6.2 Macrolide-lincosamide-streptogramin group and the ketolides

The MLSK group of antibiotics are of great clinical significance in many patient groups but their use is becoming compromised due to increasing numbers of antibiotic resistant organisms [248].

Macrolides, lincosamides and streptogramins are classified in the same group of antibiotics although they are chemically distinct (Figure 1.7). Antibiotics constituting the MLSK group inhibit protein synthesis at the ribosomal level, by binding to the 50S ribosomal subunit and blocking peptide bind formation and/or translation. They bind to specific nucleotides in domains II and V or 23S rRNA [236, 249-252]. All MLSK antibiotics are bacteriostatic except the streptogramin combinations and ketolides, which are bactericidal.

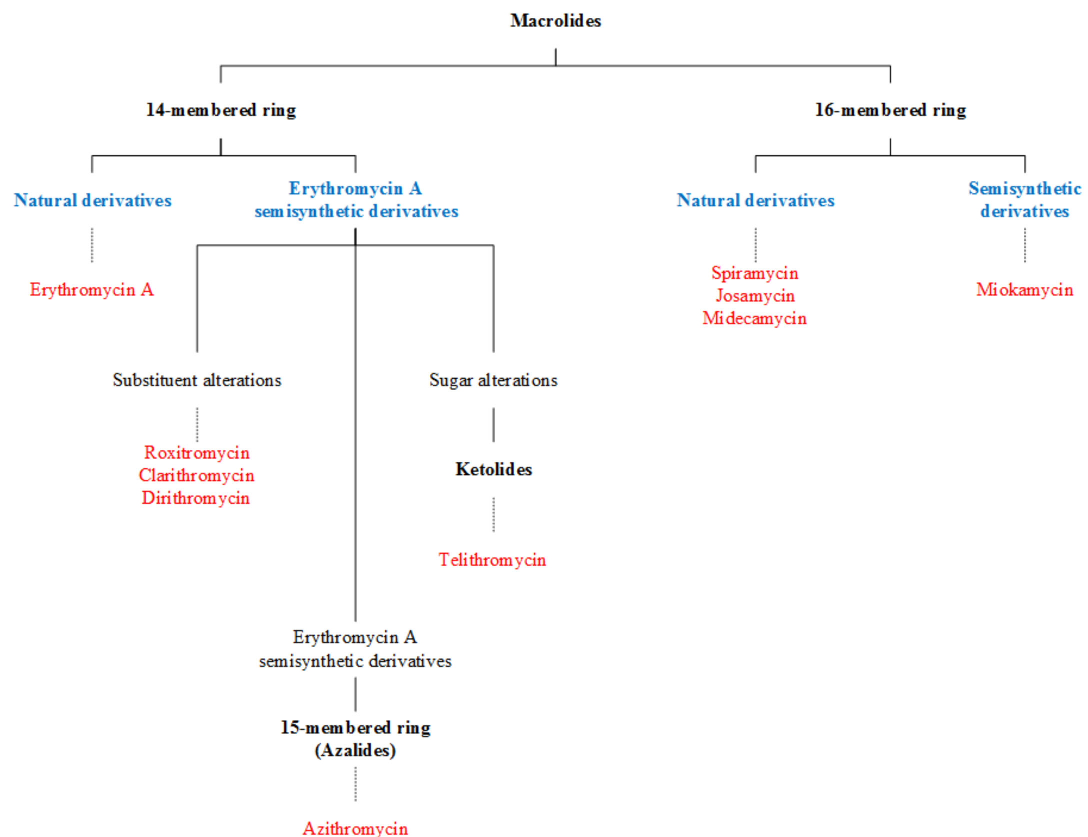

Figure 1.7. Macrolide classification and example antibiotics.

Macrolide resistance in mycoplasmas, which have a small number of ribosomal operons, is conferred by mutations in the ribosomal target (23S rRNA and ribosomal proteins L4 and L22) [253]. Resistance has been described mainly in *M. pneumoniae* [236] but resistant strains of *M. hominis*, *Ureaplasma* species [238, 239] and, more recently, *M. genitalium* [254, 255], have been reported.

Most resistant strains of *M. pneumoniae* that have been reported harbour an A to G mutation at position 2058 in the peptidyltransferase loop of 23S rRNA (*E. coli* numbering) [245] although, mutations have also been observed in clinical isolates at positions 2059 and 2611 [256, 257]. These mutation positions have also been identified as macrolide resistant hot spots in other bacteria. To date, no mutations have been described for *M. pneumoniae* in domain II or in the ribosomal proteins L4

and L22 in clinical isolates. Macrolide-resistant strains of *M. pneumoniae* do not show cross-resistance to other classes of antibiotics [236].

Resistant mutant strains of *M. pneumoniae* have been obtained *in vitro* against different macrolides, streptogramins and a ketolide. Resistance has been shown to be due to mutations at positions 2058 and 2059 of the 23S rRNA, as seen *in vivo* [257, 258]; positions 2611 and 2062 of the 23S rRNA; and in the genes encoding ribosomal proteins L4 and L22 [259].

M. hominis is intrinsically resistant to 14- and 15-membered ring macrolides through inherent sequence determination within its 23S rRNA as described in Section 1.6.1 [242].

Prior to the year 2000, very few macrolide-resistant clinical isolates of *M. pneumoniae* were identified [239, 260-263]. By contrast, since 2000 a significant increase in macrolide-resistant strains has been reported. The global increase in macrolide resistance observed in cases of *M. pneumoniae* infection is of increasing concern and importance to the international community [264]. In China resistance has been documented in over 90% of clinical isolates of *M. pneumoniae* studied [228]; however, resistance is lower in European counties including France, Germany, Switzerland and Sweden [265-268] and North America [269]. This increase in resistance has paralleled a similar rise in macrolide resistance in other respiratory pathogens as a result of antibiotic selective pressure in children during a period of extensive macrolide use in many parts of the world, especially in Asia [270-272].

Prior to 2014, seven cases of macrolide-resistant *M. pneumoniae* strains were reported in the UK, one case in England and Wales and six cases in Scotland [273, 274]. Between 1st September 2014 and 1st September 2015, *M. pneumoniae* was

detected by PCR in 61 clinical specimens submitted to PHE. These were screened for point mutations that are known to confer macrolide resistance in domain V of the 23S rRNA by a modified version of the method described by Li *et al.*, 2009 [275, 276]. Of the 61 specimens, 18 (29.5%, 95% CI: 19.5-42.0) contained insufficient DNA to determine macrolide resistance-conferring mutations. Of the remaining 43 specimens, mutations in the 23S rRNA known to confer macrolide-resistance were found in 9.3% (95% CI: 3.1-22.2; 4/43) [276].

Macrolide-resistance in *M. pneumoniae* has been reported in Scotland at 19% (6/32) [274], higher than the 9.3% documented in the 2014-2015 England and Wales study [276] but this is not statistically significant. This may reflect sampling differences and it is important to note that the specimen cohort examined for macrolide resistance in Scotland were from patients in whom macrolide resistance was considered most likely based on their clinical presentation.

It is important to note that in the 2014-2015 England and Wales study [276] *M. pneumoniae* was not isolated from those specimens where *M. pneumoniae* was detected by PCR, and therefore it was not possible to confirm macrolide resistance *in vitro*. However, point mutations within the 23SrRNA gene in clinical specimens and isolates from specimens have previously been shown to confer resistance [245]. Acquisition of resistance has been documented in patients receiving macrolides and resistance may develop as a consequence of antibiotic selective pressure [277]. This is supported by the highest macrolide-resistance rates being reported in countries with extensive macrolide usage [236].

1.6.3 The tetracyclines

Tetracyclines have a broad spectrum of activity against most gram-positive and gram-negative bacteria. Tetracyclines and the related glycylcylines inhibit bacterial protein synthesis. They act as bacteriostatic antibiotics and exert their activity by binding to the ribosomal 20S subunit which is composed of 16S rRNA and several ribosomal proteins. The major tetracycline binding site is located in a pocket formed by some residues of helix 34 and 31 in 16S rRNA [278]. Binding of tetracyclines to this site prevents the attachment of charged transcription RNA (tRNA) to the A site, therefore preventing protein synthesis.

Resistance to tetracyclines has been well documented for both *M. hominis* and *Ureaplasma* species but not for *M. pneumoniae*. One mechanism by which bacteria mediate tetracycline resistance is due to the acquisition of a tetracycline resistance gene such as *tetM* or *tetO*, which have been found in both gram-positive and gram-negative bacteria, or *tetA-tetF* which are confined to gram-negative bacteria [279]. High-level resistance to tetracyclines in *M. hominis* and *Ureaplasma* species has been associated with the presence of *tetM* [280, 281], the sole tetracycline resistance mechanism so far documented in clinical isolates of human mycoplasmas [239].

1.6.4 The fluoroquinolones

The quinolones are a group of synthetic, broad-spectrum antibiotics used to treat both gram-positive and gram-negative bacterial infections. Fluoroquinolones play an important role in the treatment of serious bacterial infections, especially hospital-acquired infections and infections in which resistance to other classes of antibiotics is suspected. Minimal usage of fluoroquinolones is recommended for the treatment of CAP to discourage the spread and development of multi-drug resistant bacterial strains due to the broad-spectrum activity of fluoroquinolones [232, 282-284]. Newer

fluoroquinolones show enhanced activity against all human mycoplasmas studied, including *M. pneumoniae* and *M. hominis*, compared with the older ones [236].

Fluoroquinolones are bactericidal antibiotics which disrupt DNA replication by binding to enzyme-DNA complexes during replication leading to the death of the cell. The intracellular targets of fluoroquinolones in bacteria are considered to be the type II topoisomerases, DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*), which are essential for bacterial DNA replication [285]. Although the two enzymes are structurally homologous they have differences in activity and preferential sites of action; DNA gyrase proteins in gram-negative bacteria and topoisomerase IV in gram-positive bacteria [286].

Resistance mechanisms to fluoroquinolones have been described and fall into three categories: mutations in the four target genes, *gyrA*, *gyrB*, *parC* and *parE*; reduction in the level of quinolone accumulation inside the cells either by efflux of lack of penetration; and target protection [287]. However, mutations in the target genes are the main mechanism conferring fluoroquinolone resistance in mycoplasmas. Mutations in the gene targets for each gene cluster within a conserved region referred to as the quinolone resistance-determining region (QRDR). QRDRs were first described for GyrA and GyrB and homologous regions were later identified in ParC and ParE [287, 288]. Resistance *in vivo* has only been described in genital mycoplasmas including *M. hominis*, *M. genitalium* and *Ureaplasma* species [238, 239], but mutant strains of *M. pneumoniae* have been selected *in vitro* [289].

1.7 Hypothesis

Detection of *M. hominis* and *Ureaplasma* species in clinical samples is hampered by the fastidious nature of these organisms. The development of a sensitive and specific PCR assay will increase detection of these organisms in clinical samples and can be utilised for further examination of clinical data sets. Molecular typing schemes can be used to differentiate between strains of a bacterial species, allowing the characterisation of isolates into distinct groups and can be representative of the entire genome.

1.8 Aims

- To develop a quantitative, real-time PCR assay to simultaneously detect *M. hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* for clinical diagnosis of infection and determination of MIC
- To characterise *M. hominis* isolates based on their major surface antigen, Vaa, develop a molecular typing scheme to differentiate between *M. hominis* strains, and examine the genetic diversity of *M. hominis* isolates using whole genome sequence analysis
- To develop a molecular typing scheme to differentiate between *M. pneumoniae* strains, and compare with whole genome sequence analysis and other established molecular typing methods for *M. pneumoniae*

Chapter 2. Materials and Methods

2.1 *Mycoplasma pneumoniae* and *Mycoplasma hominis* isolate details

Clinical samples were submitted to PHE for diagnosis of *M. pneumoniae* or *M. hominis* infection. Clinical samples originate from a range of anatomical sites, vary in the type of sample and differ in clinical background. *M. pneumoniae* and *M. hominis* were isolated from positive clinical samples and stored at -80°C. Clinical samples from Wales were also submitted to the University Hospital Wales (UHW) for diagnosis of *Ureaplasma* species infection. Endo-tracheal secretions were submitted to UHW as part of the UREAtrack study from a South West England hospital, for *Ureaplasma* species diagnosis. Mixed infections with *M. hominis* were identified and *M. hominis* cultures were stored at -80°C. All *Mycoplasma* isolates were isolated between 1967 and 2014. Full details of *M. pneumoniae* and *M. hominis* isolates can be found in Table 2.1 and Table 2.2, respectively.

Table 2.1. ***Mycoplasma pneumoniae*** **clinical isolate details.**
All *M. pneumoniae* isolates were submitted to PHE. Isolates from the same patient are indicated by coloured shading.

Isolate	Year of Isolation	Isolation site	Further clinical information
M129 (ATCC 29342)	1969		
FH (ATCC 15531)	1944		
MPN003	1983	Sputum	Chest infection
MPN004	1981	Sputum	Bronchopneumonia
MPN005	1983	Sputum	Pneumonia
MPN006	1982	Sputum	Persistent productive cough
MPN007	1978	Throat swab	Pneumonia
MPN008	1981	Sputum	Pneumonia
MPN010	1983	Sputum	Abnormal chest X-rays; night sweats; lung function loss
MPN011	1983	Sputum	Two month cough
MPN012	1981	Brain cystic cavity	Probable congenital disease; meningitis
MPN013	2009	Nose and throat swabs	Stevens-Johnson syndrome; cough
MPN014	2009	Nose and throat swabs	Stevens-Johnson syndrome; cough
MPN015	2009	Nose and throat swabs	Stevens-Johnson syndrome; cough
MPN016	2009	Nose and throat swabs	Stevens-Johnson syndrome; cough
MPN017	2009	Nose and throat swabs	Stevens-Johnson syndrome; cough
MPN018	1981	Sputum	Pneumonia
MPN019	1983	Sputum	Pneumonia
MPN020	1982	Sputum	Bronchopneumonia

Isolate	Year of Isolation	Isolation site	Further clinical information
MPN021	1983	Unknown	Unknown
MPN022	2010	Sputum	Atypical pneumonia; serology positive
MPN023	1983	Sputum	Pneumonia
MPN101	1978	Throat swab	Lobar pneumonia
MPN102	1981	Brain frontal lobe	Unknown
MPN103	1982	Sputum	Persistent, productive cough
MPN104	1981	Sputum	Bronchopneumonia (2nd sample, 4 days later)
MPN105	1983	Sputum	Unknown
MPN106	1981	Sputum	Bronchopneumonia (1st sample)
MPN107	1983	Sputum	Symptoms look like tuberculosis; night sweats
MPN108	1983	Sputum	Unknown
MPN109	1982	Sputum	Bronchopneumonia
MPN110	1981	Sputum	Recurring chest infection
MPN111	1968	Unknown	Unknown
MPN112	1983	Sputum	Cough
MPN113	1967	Unknown	Pneumonia
MPN114	1983	Sputum	Unknown
MPN116	1982	Sputum	Pneumonia
MPN117	1982	Sputum	Unknown
MPN118	1996	Sputum	Unknown
MPN119	1982	Sputum	Pneumonia
MPN120	1982	Sputum	Cough
MPN121	1983	Sputum	Unknown

Isolate	Year of Isolation	Isolation site	Further clinical information
MPN122	1982	Sputum	Unknown
MPN123	1983	Sputum	Unknown
MPN124	1981	Sputum	Bronchopneumonia
MPN125	1983	Sputum	Chest infection
MPN126	1979	Unknown	Chest infections
MPN127	1982	Sputum	Persistent wet chest
MPN128	1976	Unknown	Unknown
MPN129	1983	Sputum	Productive cough; flu for 5 days
MPN130	1983	Sputum	Unknown
MPN131	1981	Sputum	Un-resolving pneumonia
MPN132	1982	Sputum	Chest infection
MPN133	1982	Sputum	Pneumonia
MPN134	1982	Sputum	Pneumonia
MPN135	1986	Unknown	*M. pneumoniae* strain M1 from Dr McKoy.
MPN136	1982	Sputum	Unknown

Table 2.2. *Mycoplasma hominis* clinical isolate details.
All *M. hominis* isolates were submitted to PHE unless otherwise stated. Isolates from the same patient are indicated by coloured shading and linked patients are indicated by grey shading.

Isolate	Year of Isolation	Isolation site	Further clinical information
PG21 (NCTCC 10111; ATCC 23114)			
MH002	1989	Cerebral spinal fluid	Neonate; haemorrhagic
MH008	2012	Genital isolate	Isolate from outside C-section wound
MH009	2012	Genital isolate	Isolate from outside C-section wound
MH010	2012	Genital isolate	Isolate from outside C-section wound
MH011	2012	Peritoneal fluid	Renal transplant
MH012	2012	Peritoneal fluid	Renal transplant
MH013	2012	Peritoneal fluid	Renal transplant
MH015	2004	Blood culture	Post-termination operation
MH016	2004	Neck swab	Unknown
MH017	2004	Unknown	Unknown

Isolate	Year of Isolation	Isolation site	Further clinical information
MH018	1993	Genital	Hepatitis C Virus infection
MH020	1990	Cervical swab	Unknown
MH021	1986	Knee aspirate	Unknown
MH022	1990	Breast abscess	Unknown
MH023	2005	Endo-tracheal secretions	Neonate; 25 weeks gestation
MH025	2006	Ear swab	Neonate; respiratory distress syndrome; twin
MH026	2008	Cerebral abscess	Unknown
MH027	2008	Abdominal pus	Renal transplant
MH028	2004	Pelvic aspirate	Pelvic haematoma after hysterectomy
MH029	1989	Pleural fluid	Cardiac failure
MH041	2012	Endo-tracheal secretions	Neonate
MH043	2013	Spinal tissue	Post-operative; deep tissue infection & superficial irritation; spinal abscess; spinal curdle C5; scoliosis instrumentation
MH044	2013	Spinal tissue	Post-operative; deep tissue infection & superficial irritation; spinal abscess; spinal curdle C5; scoliosis instrumentation

Isolate	Year of Isolation	Isolation site	Further clinical information
MH045	2013	Spinal tissue	Post-operative; deep tissue infection & superficial irritation; spinal abscess; spinal curdle C5; scoliosis instrumentation
MH101	2013	Genital isolate	Post-natal pyrexia
MH102	2013	Pelvic collection fluid	Post lower segment C-section; laparoscopy and drainage
MH103	2013	Endo-tracheal secretions	Neonate
MH104	2013	Cerebral spinal fluid	Neonate; query meningitis
MH105	2013	Renal isolate	End-stage renal failure; haemodialysis; anti-phospholipid syndrome; query sepsis
MH106	2013	Sputum	Neonate
MH107	2013	Vaginal pus	Gynaecology emergency
MH108	2013	Uterine tissue	Eight days post-natal bleeding
MH109	2013	Pleural fluid	Abdominal/chest stab wound
MH110	2013	Endo-tracheal secretions	Neonate
MH111	2013	Tracheoscopy swab	Sepsis
MH112	2013	Spinal isolate	Operation three weeks prior; posterior spine instrumentation; deep infection & superficial irritation; spinal abscess; superficial curdle; scoliosis

Isolate	Year of Isolation	Isolation site	Further clinical information
MH113	2013	Spinal isolate[a]	Operation three weeks prior; posterior spine instrumentation; deep infection & superficial irritation; spinal abscess; superficial curdle; scoliosis
MH114	2013	Respiratory isolate	Neonate; severe lung disease
MH115	2013	Spinal isolate[a]	Post extensive spinal surgery; spinal abscess
MH116	2013	Endo-tracheal secretions	Neonate; severe lung disease
MH117	2013	Pelvic collection fluid	Puerperal sepsis; query endometriosis
MH118	2012	Knee fluid	Painful knee
MH119	2012	Peritoneal fluid	Unknown
MH120	2012	Pelvic pus	13 days post emergency C-section
MH121	2013	Mid-line drain fluid	Unknown
MH122	2014	Higher vaginal swab	Unknown
MH123	2014	Pelvic fluid	Unknown
MH124	2014	Abdominal fluid	Unknown
MH125	2014	Pelvic abscess fluid	Acute myeloid leukaemia; diagnosed post-partum

Isolate	Year of Isolation	Isolation site	Further clinical information
MH126	2014	Isolate	Sample triggered bactalert; no staining in gram stain; negative for EBV/HSV/Candida/ASP; sever acute respiratory distress syndrome; post influenza A (H3 not H1V1); patient had Tamiflu; multiple haemorrhagic skin lesions.
MH127	1998	Pleural fluid	Unknown
MH128	2004	Uterine swab	Unknown
MH129	2004	Placental isolate	Miscarriage
MH130	2003	Wound swab	Post-laparotomy
MH131	1992	Unknown	*U. urealyticum* FD culture interim batches prior to accession
MH132	1993	High vaginal swab	Unknown
MH133	1986	Blood culture	Renal transplant; pyrexia; inflamed wound
MH134	1990	Isolate	*Mycoplasma* isolate for identification
MH135	2004	High vaginal swab	Post-natal; post evacuation of retained products of conception; late miscarriage; increased temperature; patient on β-lactam
MH136	2003	Endo-tracheal tube isolate	Neonate
MH137	1990	Unknown	Unknown
MH138	1993	Genital isolate	Spontaneous miscarriage at 21 weeks; high temperature

Isolate	Year of Isolation	Isolation site	Further clinical information
MH139	1989	Cerebral spinal fluid	Premature neonate; spontaneous haemorrhagic hydrocephalus
MH140	1990	Genital isolate	Post-abortive sepsis
MH141	2013	Endo-tracheal secretions	P162E; neonate

2.2 Culturing *Mycoplasma pneumoniae* and *Mycoplasma hominis*

Mycoplasmas were cultured in Mycoplasma-specific Medium (MM; Mycoplasma Experience Ltd; Blenchingly, UK) or on Mycoplasma Agar (Mycoplasma Experience Ltd; Blenchingly, UK). *Mycoplasma* cultures were incubated aerobically at 37°C. All *Mycoplasma* strains analysed in this thesis that were isolated from clinical specimens were triple cloned on Mycoplasma Agar to ensure a pure culture.

2.2.1 Storage and recovery of *Mycoplasma* isolates

Mycoplasmas were stored at -80°C in MM. Usually the lowest dilution giving colour change or a triple cloned pure strain was stored by placing in a sealed tube. Aliquots of 20 µL were stored to prevent repeated freeze-thawing along with a larger volume stock. Mycoplasmas grown on agar were stored at -80°C by placing blocks of agar containing colonies in MM.

Isolates frozen in MM were recovered by thawing followed by inoculation and microbroth dilution in fresh MM. Alternatively, the isolate was inoculated directly onto Mycoplasma agar. Isolates frozen on agar blocks were inoculated on Mycoplasma agar using the push block technique and a small volume of the MM was also inoculated onto the plate. Cultures were incubated at 37°C until either colour change in MM or colonies were observed.

2.3 Detection of *Mycoplasma hominis* and *Ureaplasma* species in clinical samples

2.3.1 DNA Extraction from clinical samples

Prior to DNA extraction, all samples were heat-inactivated by incubating at 95°C for 30 minutes.

2.3.1.1 QIAsymphony®

The QIAsymphony® (Qiagen; Manchester, UK) was used to extract DNA from a large number of samples at one time (up 96 samples per extraction run). The QIAsymphony® was set up according to manufacturer's instructions. Briefly, 240 µL of sample was added per well of a 96 well S-Block (Qiagen; Manchester, UK) and the S-Block was loaded onto the QIAsymphony®. DNA was extracted by a four-step process: lysis, binding to magnetic particles, washing and elution, using the automated bacteria DNA protocol. DNA was extracted in a final volume of 100 µL in buffer ATE (Qiagen; Manchester, UK; low-ethylene diaminetetraacetic acid (EDTA) elution buffer containing sodium azide).

2.3.1.2 MagNA Pure Compact

The MagNa Pure Compact System (Roche; Welwyn Garden City, UK) was used to extract DNA from up to eight clinical samples at one time. The MagNa Pure Compact was set up according to manufacturer's instructions. Briefly, 180 µL of MagNa Pure Bacterial Lysis Buffer (Roche; Welwyn Garden City, UK) and 20 µL of Proteinase K recombinant PCR grade (Roche; Welwyn Garden City, UK) were added to 200 µL of clinical sample and thoroughly mixed. The sample was incubated at 65°C for 10 minutes to lyse. DNA was extracted using the MagNa Pure Compact Nucleic acid Isolation Kit I (Roche; Welwyn Garden City, UK) with automated protocol DNA_Bacteria in a four-step process: sample lysis, immobilisation of nucleic acids on magnetic glass particles, removal by washing of unbound substances, and elution of purified nucleic acids from the magnetic particles. DNA was extracted in a final volume of 100 µL in MagNa Pure Compact Elution Buffer (Roche; Welwyn Garden City, UK).

2.3.2 Detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* by qPCR – MUP PCR

This assay simultaneously detected and identified *Ureaplasma* species and *M. hominis* in DNA extracts. It was based on Yi *et al.,* 2005 [290] and targets the *urease* gene of *Ureaplasma* species and on Baczynska *et al.*, 2004 [124] with a modified in-house probe design to the *gap* gene of *M. hominis*.

2.3.2.1 Clinical sample preparation

Clinical DNA extracts were diluted 1:10 in nuclease free water. In addition, unusual and highly cellular samples such as lung tissue were diluted 1:10 and 1:100.

2.3.2.2 Preparation of PCR Internal Processing Control (IPC)

An IPC was previously constructed in the plasmid TOPO TA by amplification of a selected sequence (13825-13844, NC_001416) from bacteriophage lambda DNA (Genbank J02459) with extension at their 5' ends consisting of primer sequences to be used to amplify the *Ureaplasma* template. The plasmid pU-IPC-TOPO (clone 1, 2 and 3) were stored on *E. coli* at -80°C. The plasmid was diluted in TE to 0.01 ng/µL, for use in this assay.

2.3.2.3 Preparation of positive controls

Positive control DNA for *M. hominis* and *U. urealyticum* was obtained from Minerva Biolabs (Berlin, Germany) at a concentration of 10 ng/µL. Positive control DNA for *U. parvum* was obtained by inoculating a 5 mL liquid broth culture and incubating at 37°C until a colour change was observed in the media. The broth culture was then centrifuged at 10,000 x g for 15 minutes and the pellet was re-suspended in 200 µL of nuclease free water. DNA was extracted using the MagNa Pure compact as described in section 2.3.1 and then diluted to 10 pg/µL.

A standard curve of the positive control DNA for each *M. hominis*, *U. parvum* and *U. urealyticum* was used in each qPCR run: 1 pg/µL, 0.1 pg/µL and 0.01 pg/µL.

2.3.2.4 MUP qPCR

Amplification was performed in a Rotor-Gene® Q series thermocycler (Qiagen; Manchester, UK) in 25 µL reactions containing: 1 x Rotor-Gene® multiplex Mastermix (Qiagen; Manchester, UK), 0.5 pmol/µL of each primer, 0.2 pmol/µL of each probe, 0.01 ng/mL IPC DNA and 5 µL of sample DNA. Primers and probes are detailed in Table 2.3. A water blank was included in every PCR run.

Table 2.3. Primers and probes

Name	**Sequence (5'-3')**
Primer UUPF	AAGGTCAAGGTATGGAAGATCCAA
Primer UUPR	TTCCTGTTGCCCCTCAGTCT
Probe UP	FAM-TCCACAAGCTCCAGCAGCAATTTG-BHQ1
Probe UU	ROX-ACCACAAGCACCTGCTACGATTTGTTC-BHQ2
Primer MHF	GGAAGA-TATGTAACAAAAGAAGGTGCTG
Primer MHR	TTTATCTTCTGGCGTAATGATATCTTCG
Probe MH	Cy5-CTGCTCCAGCTAAAAGCGAAGGTGTT-BHQ3
Probe UIPC	Yakima Yellow-AGCCGCTGGCGCATTGAGCA-BHQ1

The Rotor-Gene® was set up to measure in the channels: FAM, Yakima Yellow, ROX and CY5. Cycling conditions were as follows: initial Taq activation step of 5 minutes at 95°C, followed by quantification of 45 cycles of 15 seconds at 95°C and 15 seconds at 60°C.

Thresholds were set to 0.15 (Table 2.4) and the resulting standard curve was used to interpret the results. If positive and negative controls were satisfactory, the Ct values of the clinical samples were examined and were determined to be positive if they fell in the ranges indicated in Table 2.5.

Table 2.4. Thresholds for analysis of each target.

Channel and dye	Target	Threshold for analysis
1 – Green – FAM	*U. parvum*	0.15
2 – Yellow – Yakima Yellow	UIPC	0.15
3 – Orange – ROX	*U. urealyticum*	0.15
4 – Red – Cy5	*M. hominis*	0.15

Table 2.5. Positive control Ct value ranges to determine positive clinical results

	***U. parvum* Ct**		***U. urealyticum* Ct**		***M. hominis* Ct**		**UIPC Ct**	
pg/µL	**min**	**max**	**min**	**max**	**min**	**max**	**min**	**max**
1000	19.8	21.2	18.0	26.9			33.5	41.6
100	23.4	24.7	24.6	25.7	24.8	26.7		
10	26.7	28.5	27.2	30.7	28.2	30.2		
1	30.4	34.1	30.5	34.6	29.5	35.9		
0.1	33.0	37.4	31.9	34.8	31.4	40.7		
0.01	34.5	40.0	32.3	44.1	31.8	39.4		
0.001	35.5	39.6	35.3	38.6				

2.3.3 Multiplex real-time PCR

This assay was developed to simultaneously detect and identify *Ureaplasma* species and *M. hominis* in clinical DNA extracts. It was based on Yi *et al.*, 2005 [290] which targets the *urease* gene of *Ureaplasma* and on Ferandon *et al.*, 2010 [125] which targets the *yidC* gene of *M. hominis*. This multiplex assay included an internal processing control modified from Murphy *et al.*, 2007 [291] and utilised a plasmid containing a single copy of each gene target as a positive control.

2.3.3.1 GFP amplification control

An IPC was constructed by inserting the *gfp* gene into the chromosome of a non-pathogenic strain of *E. coli* as described by Murphy *et al.*, (2007) [291] (Section 2.3.2.2). The control primers outlined by Murphy *et al.*, did not work with the *Ureaplasma* species primers used in this assay, therefore new primers were designed (Table 2.6).

2.3.3.2 Preparation of standard DNA

A plasmid (vector pUC57; cloning sites BamHI-HindIII; RSBS_control_plasmid_01; Genscript, Piscataway, USA) containing one copy of the gene targets for the three species was used as a positive control. The plasmid was supplied lyophilized therefore it required reconstitution by the addition of 20 µL of nuclease free water resulting in a plasmid concentration of 200 ng/µL ($4x10^{10}$ copies/µL). The plasmid was serially diluted to $1x10^4$, $1x10^3$, $1x10^2$, and 10 copies/µL respectively using nuclease free water.

2.3.3.3 qPCR

Amplification was performed in a Rotor-Gene® Q series thermocycler (Qiagen; Manchester, UK) in 25 µL reactions containing: 1 x Rotor-Gene multiplex

Mastermix (Qiagen; Manchester, UK), 0.25 pmol/µL of *Ureaplasma* and *M. hominis* primers, 0.05 pmol/µL of *gfp* primers, 0.1 pmol/µL of each probe, 0.1 pg/µL *gfp* DNA and 5 µL of sample DNA. Primers and probes are detailed in Table 2.6. A water blank was included in every PCR run.

Table 2.6. Details of primers and probes used in the multiplex real-time PCR

Name	Sequence (5'-3')	Length (bp)	G+C (%)[a]	Tm (°C)[a]	Amplicon size (bp)
UUP_FP	AAGGTCAAGGTATGGAAGATCCAA	24	41.7	59.3	90
UUP_RP	TTCCTGTTGCCCCTCAGTCT	20	55.0	59.4	
UP_HP	FAM-TCCACAAGCTCCAGCAGCAATTTG-BHQ1	24	50.0	62.7	
UU_HP	HEX-ACCACAAGCACCTGCTACGATTTGTTC-BHQ1	27	48.1	65	90
gfp_FP	CCTGTCCTTTTACCAGACAACCA	23	47.8	60.6	125
gfp_RP2	ATGCCATGTGTAATCCCAGCAG	22	50.0	60.3	
gfp_HP	Cy5-TACCTGTCCACACAATCTGCCCTTTCG-BHQ3	27	51.9	66.5	
MH_FP	TCACTAAACCGGGTATTTTCTAACAA	26	34.6	58.5	94
MH_RP	TTGGCATATATTGCGATAGTGCTT	24	37.5	57.6	
MH_HP	ATTO680-CTACCAATAATTTTAATATCTGTCGGTATG-BHQ3	30	30.0	59.9	

Rotor-Gene® cycling conditions were as follows: initial activation step of five minutes at 95°C, followed by quantification of 45 cycles of 15 seconds at 95°C and 15 seconds at 60°C with acquisition in channels Green, Yellow, red and Crimson.

Data was analysed using Rotor-Gene® Q series Software version 2.3.1 (Qiagen; Manchester, UK).

2.4 Molecular typing methods

2.4.1 DNA Extraction from purified isolates

DNA was extracted from approximately 500 µL of growing *Mycoplasma* culture. Briefly, the *Mycoplasma* culture was centrifuged at 17,000 x g for 10 minutes, supernatant discarded and the pellet was re-suspended in 50 µL of molecular grade water (Sigma, Dorset, UK). DNA was released by boiling lysis by incubating at 95 °C for 10 minutes. The sample was then vortexed and stored at -20°C until required.

2.4.2 PCR

PCR amplifications were performed in a DNA Thermal Cycler (Techne Prime; Stone, UK) in 50 µL reactions containing: 1 x GoTaq Flexi Buffer (Promega; Southampton, UK), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 pmol/µL each primer, 1.56 units GoTaq DNA Polymerase (Promega; Southampton, UK), and 2.5 µL template DNA. PCR reactions (unless otherwise stated) consisted of an initial denaturation step of 3 minutes at 94°C, followed by 35 cycles of 60 seconds at 94°C, 60 seconds at the corresponding T_m and 60 seconds 72°C. A final extension step was maintained for 10 minutes at 72°C.

2.4.2.1 Agarose gel electrophoresis

PCR products were analysed on 1.5% agarose gels with ethidium bromide visualisation.

2.4.2.2 Purification of PCR products

PCR products were purified using the QIAprep® Spin Miniprep kit (Qiagen; Manchester, UK). The PCR sample was diluted by adding 5 volumes of Buffer PB to 1 volume of the PCR sample and mixed. The QIAquick spin columns were placed in the provided 2 mL collection tubes. The DNA was bound to the column by adding the diluted PCR sample to the column and was centrifuged at 17,000 x g for 60 seconds. The flow-through was discarded and the column placed back in the same collection tube. The column was then washed by adding 0.75 mL of Buffer PE to the column. The column was then centrifuged at 17,000 x g for 60 seconds. The flow-through was discarded and the column centrifuged again at 17,000 x g for 60 seconds to remove any residual ethanol from Buffer PE. The column was then placed in a clean 1.5 mL microcentrifuge tube. The DNA was eluted from the column by adding 40 µL of PCR dH_2O to the centre of the column, ensuring the water was dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The column was centrifuged at 17,000 x g for 60 seconds.

2.4.3 Sequencing

PCR products were sequenced by Eurofins MWG Operon (Ebersberg, Germany) using forward and reverse primers as for PCR reactions unless otherwise stated. Open-reading frame amino acid sequences were identified using Expasy translation tool (*Mycoplasma* setting; web.expasy.org/translate/).

2.4.4 *Mycoplasma pneumoniae* P1-typing

Mycoplasma pneumoniae P1 typing was performed by PCR followed by sequencing as detailed in sections 2.4.2 and 2.4.3. P1-typing included a nested step, where the PCR product from the first amplification round was used as the template DNA in the second round. Primers used are detailed in Table 2.7.

Table 2.7. Primers used for P1-typing of *Mycoplasma pneumoniae*.

P1-subtype	Purpose	Name	Sequence (5’-3’)	Tm (°C)	Product size (bp)
1, 2, V1, V2a, V2b [292]	First amplification	Mp5f	TTGACAAGACCGTCCAATCC	58	2465
		Mp16r	TTGGTTGGGTATCTTGATCAGG		
	Nested PCR	Mp11f	CCTCGTTGTCAGTGGCACC	61	724
		Mp19r	GTTTCCGTCACTCGTGCTTG		
3 [262]	Nested PCR[a]	Mp16f	GGGAATGGGTACAGGTATGG	60	743
		Mp20r	CCAAAATAGGTTTCCACCAAC		
V2c [293]	First amplification	Mp111f	AAGCAAGCGCGACATAATTC	60	1739
		Mp17r	ACCCGTCAGTTTGTTTGTTG		
	Nested PCR	MpV2cf	CTGTACGATGCGCCTTATGC	61	453
		MpV2cr	CACCCTAAAGACACCTATACTCAAAC		

[a] First amplification primers are Mp5f and Mp16r

2.4.4.1 P1 sequence analysis

The sequences obtained from each corresponding forward and reverse primer were assembled and trimmed for double-stranded, high quality sequence. Sequence was compared to published P1 sequences and variation regions to determine P1-type. For determination of types 1, 2, V1, V2a and V2b, sequences were converted to amino acid sequences. Open-reading frame amino acid sequences were identified using

Expasy translation tool (*Mycoplasma* setting; web.expasy.org/translate/) and deduced amino acid sequences were compared to the published sequences [292].

2.4.5 Multi-locus Sequence Typing (MLST)

Ten genes were included for *M. hominis*: adenylate kinase (*adk*), carbamate kinase (*arcC*), ATP synthase subunit α (*atpA*), cell division protein FtsZ (*ftsZ*), serine hydroxymethyltransferase (*glyA*), lysyl-tRNA synthetase (*lysS*), methionine-tRNA ligase (*metG*), inorganic pyrophosphatase (*ppa*), nitrogen fixation protein (*nifS*) and recA protein (*recA*).

Eight genes were included for *M. pneumoniae*: guanylate kinase (*gmk*), serine hydroxymethytransferase (*glyA*), phosphoglyceromutase (*pgm*), adenylate kinase (*adk*), DNA gyrase subunit B (*gyrB*), ATP synthase subunit α (*atpA*), inorganic pyrophosphatase (*ppa*) and carbamate kinase (*arcC*).

Both *M. hominis* and *M. pneumoniae* MLST was performed by PCR and sequencing as detailed in sections 2.4.2 and 2.4.3. PCR reactions were performed in duplicate and then pooled prior to gel electrophoresis and purification. The primers used are detailed in Table 2.8.

Table 2.8. Primer pairs developed for both *M. hominis* and *M. pneumoniae* MLST schemes.

	Name		Primer sequence (5'-3')	Amplicon (bp)	Location of MLST locus in CDS	Tm (°C)
Mycoplasma hominis	*adk*	F	GAAGTGTACAACGAACACGC	676	234-639	56
		R	ATGACCATTTTAAAGCCTCTTCT			
	arcC	F	AGTTATTGTCGGACACGGAA	735	384-766	57
		R	CCGCCTTCTTCAACGAATTT			
	atpA	F	ATTGCCGAAGAATGAATGGC	712	978-1304	57
		R	CATTTGCTTCAGAAACGGCT			
	glyA	F	CATGGCAATGGTTGATCCTG	785	450-860	57
		R	TTGCCGCAGATCCTATTCTT			
	recA	F	ACGCTATTGCCGAAATACAA	648	457-765	56
		R	TGAAACTATATCACGAGCCCT			
	ftsZ	F	GCAAAACTGCTGCTGAATCT	714	492-705	57
		R	TGGCGATTACCGAGACAAAT			
	lys*S*	F	TTCGTGGCCCATTTATTGTT	656	307-649	56
		R	ATGTTTGAAAACGCCTTCAGT			
	metG	F	CCAAGTGGTAATCTTCACATAGG	646	304-649	56
		R	TGTGTTGCTTTTCAGCATCTT			
Mycoplasma pneumoniae	*ppa*	F	CGCTGACCAAGCCTTTCTAC	249	192-440	60
		R	CACTCCAAACTTTGCACTCCC			
	pgm	F	AGCACCTTGCACGATGAAGA	1197	456-1652	60
		R	CCTGCGCCTTCGTTAATTGG			
	gyrB	F	TTGTCCCGGACTTTACCGTG	429	524-952	60
		R	TGTTTTCGACAGCAAAGCGG			
	gmk	F	GAGCGGTGTTGGCAAAAGTA	394	189-582	60
		R	TGCATCCTCGTCATTACGCTT			
	glyA	F	CAGAGAACTATGTGAGTAGGGACA	676	74-749	60
		R	TGACAACCCGGAAAGACACC			
	atpA	F	GTCGCTGATGGCATTGCTAAG	796	100-895	60
		R	CCAGTAAACGCGAGTGCAAG			
	arcC	F	CCCCATCAAGCCGTGTACTT	570	304-873	60
		R	TTGGGCAATAATGGCCGTCT			
	adk	F	GTAGCCAACACCACCGGATT	473	70-542	60
		R	ACGGTGTCTTCGTAAAGCGT			

2.4.5.1 MLST Sequence analysis

The sequences obtained from each corresponding forward and reverse primer were assembled using BioNumerics (version 6.1; Applied Maths, Belgium) and trimmed for double stranded high quality sequence. All the sequences obtained for each locus were aligned using ClustalW (Vector NTI; Paisley, U.K.) and the sequences were trimmed to the same size to allow direct comparison (Table 6.3). Different allelic types (AT; sequences with at least a one-nucleotide difference) were assigned arbitrary numbers. The combination of the eight alleles determined a strain's allelic profile, and each unique allelic profile was designated a unique sequence type (ST). Open-reading frame amino acid sequences were identified using Expasy translation tool (*Mycoplasma* setting; web.expasy.org/translate/) for each AT. Deduced amino acid sequences were aligned using ClustalW (Vector NTI; Paisley, U.K.) for each locus and synonymous changes were identified.

2.4.6 *Mycoplasma hominis* variable adhesion-associated (Vaa) protein classification

Mycoplasma hominis vaa gene was amplified by PCR followed by sequencing (sections 2.4.2 and 2.4.3) to determine module composition and therefore Vaa category. Primers used for amplification and sequencing are detailed in Table 2.9.

Table 2.9. Details of primers used for the amplification of *M. hominis vaa* open-reading frame.

Primer name	Sequence	Tm (°C)	Target	Reference
VaaF1	5'-CCCCGGAGATTATTAAGTCT-3'	38	Flank the entire open-reading frame encoded by the *vaa* gene	Zhang and Wise [92]
VaaR1	5'-GTGCCCATTAGTAGCACTAT-3'			

2.4.6.1 *Mycoplasma hominis vaa* sequence analysis

Open-reading frame amino acid sequence was identified using Expasy translation tool (mycoplasma setting; web.expasy.org/translate/). Sequences were aligned with CLUSTAL omega (www.ebi.ac.uk/tools/msa/clustalo/) and deduced amino acid sequences were compared to published Vaa amino acid sequences. Module composition was based on published sequence data by Boesen *et al*. [97].

2.4.7 *Mycoplasma pneumoniae* multi-locus variable-number tandem-repeat analysis (MLVA)

Multi-locus variable-number tandem-repeat analysis was performed for all *M. pneumoniae* isolates using the previously described method by Dégrange *et al.*, [132] with interpretation and nomenclature clarification from Chalker *et al.*, [221]. Briefly, the four loci selected for MLVA were MPN14, MPN16, MPN13 and MPN15. Each amplification reaction was performed in a final volume of 20 µL containing 1 x HotStarTaq® Master Mix (Qiagen), 0.5 pmol/µL of each primer, 2 mM $MgCl_2$ and 2 µL template DNA. For loci MPN13 and MPN15, 3mM $MgCl_2$ was included in each amplification reaction. Primer sequences, repeat sequence and repeat size are detailed in Table 2.10. Amplifications were performed in a PCR thermocycler under the following cycling conditions: initial denaturation step of 15 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C, and a final elongation step of 10 minutes at 75°C. PCR products were analysed to examine expected size by loading 5 µL onto a 2% pre-cast agarose E-gel (Oxoid; Basingstoke, UK). The remaining PCR product was submitted for in house (PHE) fragment analysis. The four MLVA loci were multiplexed in two solutions, M1 and M2, for fragment analysis consisting of MPN14 and MPN16, and MPN13 and MPN15, respectively. A final volume of 15 µL was submitted for analysis containing 5 µL of

each PCR product and 5 µL of nuclease-free water. Fragment size was calculated using a Peak Scanner and repeat number was determined based on fragment size as shown in Table 2.10. Alternatively, tandem repeat finder software (http://tandem.bu.edu/trf/trf.htmL) was used to determine repeat numbers using the settings: match, mismatch, indel (2,3,5). The naming system, MLVA 1,2,3,4, where each digit corresponds to repeat numbers at each locus MPN13, MPN14, MPN15 and MPN16, respectively, was used.

Table 2.10. Primer sequences, target sequences, repeat size and fragment size (bp) of *M. pneumoniae* MLVA loci MPN13, MPN14, MPN15 and MPN16 according to number of repeats.

		MPN13	MPN14	MPN15	MPN16
Forward primer sequence (5'-3')		GACCAGCATTAGATTGCTATG	CTCAGGGCGAAACCTTAAAG	HEX-CAACAGCACCACATCTTTAG	NED-GACGCGTTCGCTAAAAGAG
Reverse primer sequence (5'-3')		NED-AACAAATTAAGCAGCTCACG	6-FAM-GCAATGGCTTTCAGCACAAC	GCTAATCTTGCAAACGCTGC	CAGGCTCAACCAAATAATGG
Repeat sequence based on M129 TRF (2,3,5)[a]		TATTAATAACTATTCT	TGGACAAAATGGAAGT AAAAA	TTGTCCATTTTTTCTT CCATC	ATTTTTTAAAAGTTTT TATTTATCCGTTTTGA CAACTGCTTTTTGTT
Repeat size (bp)		16	21	21	47
Repeat number	0	364	294	108	259
	1	380	315	129	306
	2	396	336	150	353
	3	412	357	171	400
	4	428	378	192	447
	5	444	399	213	494
	6	460	420	234	541
	7	476	441	255	588
	8	492	462	276	635
	9	508	483	297	682
M129 fragment size		415	399	241	353
M129 repeat number		4	5	7	2

[a] Tandem repeat finder software. The settings used were 2,3,5 (match, mismatch, indel).

2.4.8 Phylogenetic analysis for Molecular Typing Methods

2.4.8.1 Phylogenetic analysis of multi-locus sequence typing data

The locus sequences corresponding to each strain were concatenated head-to-tail for diversity analysis and loci were assessed for stability after 10 passages. Sequence analyses and tree construction were performed using MEGA 6.0. Neighbour-joining trees were constructed for each individual locus and concatenated sequences using Kimura's two-parameter model [294, 295]. Maximum-likelihood trees were constructed for each individual locus using the Jukes-Cantor model of sequence evolution [296]. Maximum-likelihood trees were constructed from concatenated sequences of the eight MLST loci using the generalised time-reversible (GTR) model of sequence evolution with uniform rates of variation [297]. Bootstrap analyses with 1000 replicates were performed for all phylogenetic analyses. [298]

2.4.8.2 Phylogenetic analysis of *vaa* sequences

Phylogenetic trees were constructed based on the nucleotide sequence of the *vaa* gene using maximum-likelihood, Jukes-Cantor model of sequence evolution, and neighbour-joining, Kimura's two-parameter model, methods. Bootstrap analyses with 1000 replicates were performed.

2.4.8.3 Relatedness between sequence types

Relatedness between MLST STs and MLVA types were analysed based on allelic profiles/MLVA profiles for *M. pneumoniae* using eBURST version 3.

2.5 Antibiotic resistance of *Mycoplasma hominis*

2.5.1 Minimum inhibitory concentration calculation by microbroth dilution

Minimum inhibitory concentration was determined based on Beeton *et al.,* [299], where determination of bacterial load in a sample can be performed simultaneously with the determination of antibiotic resistance without prior knowledge of bacterial load. Briefly, in a 96-well plate, wells A1-H1 received 360 µL of 128 µg/mL gentamicin or tetracycline in MM, and 180 µL sterile MM was added to the remaining wells (A2-H12) of the plate. Using a multichannel pipette, rows of doubling dilutions were made by transferring 180 µL from row A1-H11 (an excess of 180 µL was discarded from row A11 to H11). Rows A12-H12 remained free of antibiotic for unrestricted growth comparison. Thus, an antibiotic gradient was created from 128 µg/mL to 0.125 µg/mL. Twenty microliters of *Mycoplasma* from a 48 hour culture (*M. hominis*) of unknown CCU was added to each well in the columns A1-A12 (1:10 dilution). A 10-fold dilution curve of bacteria was then titrated at 90 degrees across the antibiotic gradient. Plates were sealed and incubated at 37°C for 72 hours (*M. hominis*, at which time colour change within the growth control had ceased. The MIC was defined as the lowest concentration of antibiotic that prevented colour change when read at 10^4 CCU (relative to growth in the antibiotic-free medium). MM (with and without antibiotics) was also incubated in the absence of added *Mycoplasma* isolates to serve as a negative colour-changing control.

2.5.2 Minimum inhibitory concentration calculation by agar dilution

Minimum inhibitory concentration was determined as described by Waites *et al.,* [235]. Doubling-dilutions of tetracycline or gentamicin were incorporated into molten mycoplasma agar plates (Mycoplasma Experience; Blenchingly, UK), with each plate containing a different concentration. Concentrations of antibiotics used

were the same as described for the microbroth dilution technique (Section 2.5.1). After the agar plates had solidified, they were inoculated with 10 µL of a *Mycoplasma.* Plates were then incubated at 37°C under anaerobic conditions for 48 hours. Growth of *M. hominis* was assessed by counting colonies and converting to CFU/mL. MIC was defined as the lowest concentration that prevented growth of bacteria.

2.5.3 Monitoring minimum inhibitory concentration with qPCR

Serial dilutions of antibiotic (gentamicin/tetracycline) in MM were made in a total volume of 900 µL. Concentrations included for gentamicin were: 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL, 16 µg/mL and 32 µg/mL; and concentrations for tetracycline were: 0.125 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL. Antibiotic-containing MM was inoculated with 100 µL of growing *Mycoplasma*. An unrestricted growth control was included by inoculating 900 µL of sterile MM with 100 µL of the same *mycoplasma* culture. Cultures were incubated in sealed tubes at 37°C for 72 hours or until colour change was observed in the unrestricted growth culture.

Cultures were then heat-inactivated by incubating for 30 minutes at 95°C; and DNA extracted from 240 µL using the Qiasymphony (Qiagen; section 2.3.1). *Mycoplasma* was then detected and quantified in the sample using the multiplex real-time PCR assay described in section 2.3.3.

2.5.4 PCR for the *tetM* transferable element associated with tetracycline resistance

The presence of the *tetM* transferable element in *M. hominis* isolates was determined by PCR using the primers described by Blanchard *et al.*, [279] (Table 2.11), which amplified the 397 bp product. PCR was performed as described in Section 2.4.2.

Table 2.11. Primers used to detect the presence of the transferable element *tetM* in *Mycoplasma hominis* isolates.

Primer Name	Sequence	Tm (°C)	Size (bp)	Reference
TetMF	5'-TTATCAACGGTTTATCAGG-3'	48	397	Blanchard *et al.*, [279]
TetMR	5'-CGTATATATGCAAGACG-3'			

2.6 Whole Genome Sequencing

Whole Genome Sequencing was performed by PHE (London, UK). Sequencing data analysis was performed using Galaxy (PHE) and Geneious® version 8.

2.6.1 Extraction of genomic DNA for whole genome sequencing

Genomic DNA was extracted from *Mycoplasma* using the GenElute™ Bacterial Genomic DNA Kit (Sigma; Dorset, UK).

2.6.1.1 Sample preparation

In order to obtain sufficient DNA for whole genome sequencing, 100 mL of MM was inoculated with a specific *Mycoplasma* isolate. Cultures were incubated at 37°C until colour change was observed in the media. Cultures were then centrifuged at 17,000 x g for 45 minutes, supernatant removed and pellets stored at -20°C until required.

2.6.1.2 GenElute™ Bacterial Genomic DNA Kit

Genomic DNA extraction was performed as described by the manufacturer. Briefly, the *Mycoplasma* pellet was re-suspended thoroughly in 180 µL of Lysis Solution T. RNA-free genomic DNA was required, therefore 20 µL of RNase A Solution was added to the sample, mixed thoroughly and incubated at room temperature for two minutes. The sample was then incubated with 20 µL of Proteinase

K Solution for 30 minutes at 55°C. The sample was then incubated for 10 minutes at 55°C with 200 µL of Lysis Solution C, following thorough vortexing as a homogenous mixture was essential for efficient cell lysis.

GenElute Miniprep Binding Columns were then prepared by adding 500 µL of Column Preparation Solution followed by centrifugation at 12,000 x g for one minute. The eluate was discarded.

To the lysate, 200 µL of 100% ethanol was then added to the lysate and mixed thoroughly by vortexing. This was then added to the binding column using a wide bore pipette tip to reduce shearing of the DNA, followed by centrifugation at 6,500 x g for one minute. The eluate was discarded. The column was then washed by first adding 500 µL of Wash Solution 1 to the column, centrifugation for one minute at 6,500 x g and second by adding 500 µL of Wash Solution to the column followed by centrifugation for three minutes at maximum speed (16,000 x g) to dry the column. To remove all ethanol from the column, the column was centrifuged for an additional one minute at 16,000 x g. The collection tube was discarded and the column placed in a new 2 mL collection tube.

Genomic DNA was eluted from the column by adding 200 µL of the Elution Solution directly onto the centre of the column. This was then incubated for five minutes at room temperature to increase the elution efficiency. The column was then centrifuged for one minute at 6,500 x g to elute the DNA. The elution step was then repeated twice more to increase the yield of DNA.

2.6.1.3 Ethanol precipitation of genomic DNA

To increase the concentration of genomic DNA, ethanol precipitation was performed. The sample was diluted in sodium acetate (pH 5.2; final concentration of

0.3 M) and mixed well, followed by the addition of 2.5 volumes of cold 100% ethanol. The sample was then incubated on ice or at -20°C for > 20 minutes and then centrifuged at maximum speed (16,000 x g) for 15 minutes. The supernatant was carefully decanted and the genomic DNA pellet was then washed by adding 1 mL of 70% ethanol. It was then centrifuged for 5 minutes at maximum speed and the supernatant carefully decanted. The pellet was then air-dried. The resulting pellet was then re-suspended in the appropriate volume of Tris-EDTA (TE) buffer, typically 70 μL, and stored at -20°C until required.

DNA was quantified prior to sequencing using the GloMax fluorometer (Promega; Southampton, UK).

2.6.2 Sequencing methodology

Genomic sequence was obtained using the Illumina Nextera XT sample prep kit (Illumina, Cambridge, UK) and sequenced on an Illumina HiSeq 2500 platform with TruSeq rapid SBS kits (200 cycles; Illumina) and cBOT for cluster generation (Illumina). Fastq reads were trimmed using Trimmomatic 0.32, with the following parameters: leading, 30; trailing, 30; slidingwindow, 10:30; and minlen, 50 [300].

2.6.3 Read quality assessment

Read quality was assessed using FASTQC, an internal tool on the PHE Galaxy platform and reads of poor quality were discarded. It was important to note that most reads failed G+C content assessment due to low levels; this was to be expected due to the known low G+C content of *mycoplasma* genomes. Sequence was confirmed as *M. hominis* or *M. pneumoniae* using KmerID (internal tool on PHE Galaxy), by computing the similarity between reads in Next Generation Sequencing (NGS) sample and a predefined set of reference genomes from NCBI.

2.6.4 *de novo* assembly

de novo assembly was performed using SPAdes version 2.5.0, which was specifically designed for small genomes [301, 302]. This software was available as a pipeline provided by PHE Galaxy. The settings used were:

Careful correction	No
Use rectangle correction for repeat resolution	No
Number of threads to use	16
Number of iterations for read error correction	1
K-mers to use, separated by commas	21,33,55
Style type of reads	Paired-end, separate inputs

Two sequence files (.fasta) were produced by spades; a contig file and a scaffold file of assembled contigs. This last file was used for mapping to reference genomes.

2.6.5 Mapping of sequence data to a reference strain and annotation

Scaffolds produced by *de novo* assembly were mapped to a reference genome; *M. pneumoniae* M129 (NC_000912) or *M. hominis* ATCC 23114 (NCTC 10111/PG21; NC_013511), using Geneious® (version 8.0.4).

2.6.6 *Mycoplasma hominis* genome assembly and pan-genome analysis

Whole genome assembly of *M. hominis* was performed using SPAdes version 3.6.1, without error correction, and kmers: 21, 33, 45, 53, 65, 77, 83, 93 [301, 302]. For pan-genome analysis, four reference sequences (NZ_CP009652.1, NC_013511.1,

NZ_CP011538.1 and NZ_CP009677.1) were first clustered together using ggPRO (PHE internal tool). Briefly, coding gene sequences were extracted from the annotated GenBank files for the strains listed above and each gene was checked against the database of hidden Markov models (HMMs) using HMMER version 3.2 [303]. A hit was considered significant if the ratio of score over HMM length was greater or equal to 0.85. Alleles were clustered into the same gene family; new alleles were added to a gene family if they introduced a gap of less than 10% into the gene family alignment. The HMM database is only updated when a new gene or allele is identified. After clustering the four reference sequences, the pan-genome contained 777 genes occurring in at least one of the reference genomes. Each clinical *M. hominis* strain was individually scanned against the pan-genome HMM database using HMMER version 3.2 and significant hits (score/HMM length ration >0.85) were recorded in the database.

2.6.7 Basic Local Alignment Tool (blast)

Genes of interest were identified in scaffolds using NCBI Blast (http://blast.ncbi.nlm.nih.gov/) with the setting: align two sequences. The gene sequence from the reference genome was used to identify the same/similar sequence in the scaffolds.

2.6.8 Phylogenetic analysis of whole genome sequences

2.6.8.1 Single nucleotide polymorphism tree

Phylogenetic trees based on single nucleotide polymorphism (SNP) variants in whole genome sequences were constructed using a workflow provided by PHE Galaxy (Figure 2.1). Briefly, after mapping, variants were parsed to retain high quality SNPs based on the following conditions: positions having a depth of greater than 5 reads, a

consensus base call confirmed by at least 90% of the reads, an overall mapping quality score of greater than 40 and a distance to nearest SNP of greater than 5. Software used was: Unified Genotyper from GATK2 (version 0.0.7) [304], Annotate VCF (version 1.0), Combine VCF (version 1.0) VCF to VCF compendium file (version 1.0) VCF to FASTA (version 1.0). A phylogenetic tree was produced from the SNP alignment using RaxML version 8.1.17 with the GTPGAMMA model, seed 12345 and –f d option.

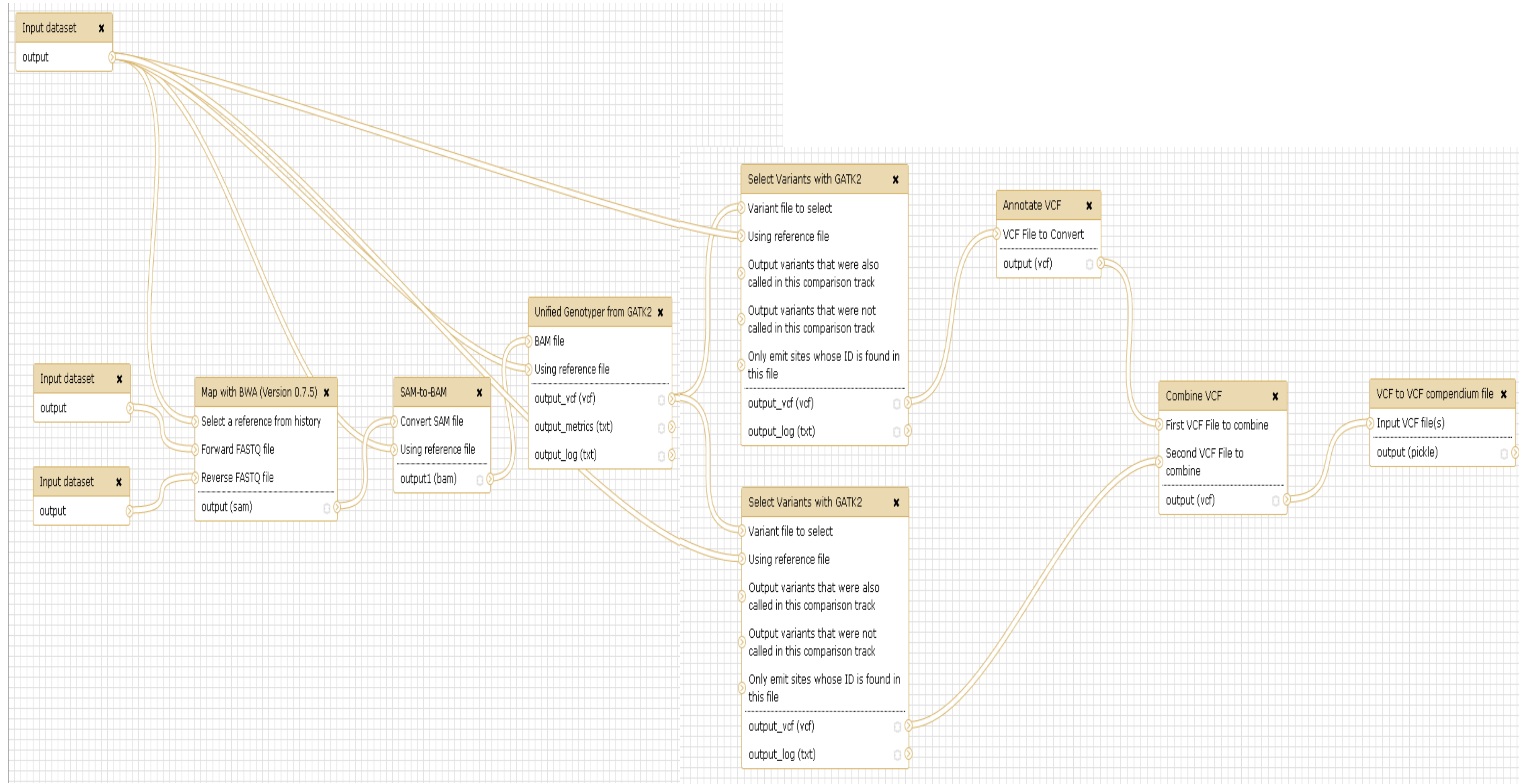

Figure 2.1 Workflow used to generate multiple alignments of SNP variants for the generation of a SNP phylogenetic tree

2.6.8.2 Whole genome alignment

Neighbour-joining trees were constructed from whole genome sequence alignments using MEGA (version 6).

2.6.9 Minimal MLST gene selection

Mycoplasma hominis genomic sequences were used to develop a minimal MLST scheme. Following pan-genome assembly, alleles of 427 genes were extracted into individual multi-Fasta files and aligned using Muscle software [305, 306]. Leave-one-out analysis was performed to identify genes required to maintain whole genome sequence SNP phylogeny, whereby one gene was removed at a time and the remaining genes were concatenated into a single multi-fasta file, with one entry per sample. The topology of the leave-one-out tree was compared to the whole genome tree using the following formula:

$$D = {}^{S_t}\!/_{S_{ref}}$$

Where D is the similarity score between the reference and target trees; S_{ref} and S_t are the number of sets of leafs under each internal node in reference and target trees, respectively. Distance, D, has a strict range (0-1) and represents overall similarity of a target tree to the reference tree. This value can be calculated for each internal node for more refined similarity measure. The results were analysed and plotted using custom scripts written for R statistical package.

2.6.10 Recombination analysis

Illumina reads for all isolates were mapped against the reference chromosome M129 or ATCC 27545 (NZ_CP009652) for *M. pneumoniae* and *M. hominis*, respectively, using SMALT (http://www.sanger.ac.uk/resources/software/smalt/) to identify SNPs, as previously described [307]. Regions of recombination in the whole

chromosomes of the isolates were analysed using Genealogies Unbiased by recomBinations In Nucleotide Sequences (GUBBINS) [308].

2.7 Statistical methods

The limit of detection (LOD) for quantitative real-timer PCR assays were determined using either probit or logistic regression models with 95% confidence intervals. Confidence intervals were calculated based on either robust or conventional standard errors.

Comparison between culture and real-time PCR results was performed using Fisher's exact test. Confidence intervals (95%) were calculated for data presented as a proportion using the modified Wald method [309] (GraphPad Quick Clacs; http://graphpad.com/quickcalcs/ConfInterval1.cfm). Diagnostic tests were evaluated be examining specificity, sensitivity and positive and negative predictive values calculated using MedCalc (https://www.medcalc.org/calc/diagnostic_test.php).

Diversity of MLST STs, loci, P1 types and MLVA profiles was assessed by using Hunter-Gaston Diversity Index. This was performed using VNTR Diversity and Confidence Extractor (V-DICE; http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). A diversity index (DI) of zero indicated no diversity compared to a DI of one indicating complete diversity. The Hunter-Gaston estimate of diversity incorporated a finite sample adjustment. Results included 95% Confidence Intervals (CI) for each locus, giving precision to the DI by providing the upper and lower boundaries

Chapter 3. Simultaneous real-time PCR detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum*

3.1 Introduction

Mycoplasma hominis, *U. parvum* and *U. urealyticum* are considered urogenital commensals or opportunistic pathogens in adults and can be isolated from the urogenital tract of 21% to 53% and up to 80% of asymptomatic women for *M. hominis* and *Ureaplasma* species, respectively [3, 310]. Additionally, in adults, *M. hominis* has been associated with clinically diverse diseases including; urogenital diseases [311, 312], postpartum fever [313], pneumonia [191], meningitis [186, 205] and septic arthritis [187, 189].

Culture methods to establish infection/colonisation with *M. hominis* and *Ureaplasma* species require specialised media due to the fastidious nature of *Mollicutes*, which are notoriously difficult to isolate. This capability is not available in most clinical laboratories, although definitive detection and identification of active infection with either of these bacteria requires culture. Conventional PCR assays for *M. hominis* and *Ureaplasma* species have mainly used 16S rRNA as a gene target [314]; however, other targets including *gap*, *fstY*, and *yidC* have been also developed [123-126]. Variation in the *gap* gene of *M. hominis* has been described and the extent of variation was found to be larger than that observed in the 16S rRNA gene [124, 315]. Other gene targets in use for the detection of *Ureaplasma* species include the multiple-banded antigen (MBA) and the *urease* gene [290, 316].

The objective of the work described in this chapter was to establish a reliable, specific, sensitive and quantitative real-time PCR to simultaneously detect *M. hominis*, *U. urealyticum* and *U. parvum* in clinical specimens, specifically neonate specimens.

3.2 Results

3.2.1 Assay development and validation

The aim of the work was to improve the current diagnostic assay used by the PHE National Reference Laboratory through the development of a multiplex assay to detect *M. hominis* and *Ureaplasma* species in clinical specimens. PHE receives respiratory and CSF samples from neonates and occasionally samples from respiratory, invasive and systemic infections in adults for testing for the presence of *M. hominis*, *Ureaplasma* species, or both. At the time of this study specimens were routinely tested using an in-house multiplex real-time PCR (MUP PCR) targeting the *gap* gene of *M. hominis* and the *urease* gene of *Ureaplasma* species (described in section 2.3.2), in addition to simple culture using the commercial kit, M-duo, for screening of all clinical specimens. Following a positive MUP PCR result, culture using Mycoplasma-selective Medium (MM; Mycoplasma Experience Ltd.; Bletchingly, UK) was performed on the clinical specimen to enable isolation of either *M hominis* or *Ureaplasma* species or both (described in section 2.2). A publication detailing failings in the use of the *gap* gene for the amplification of all *M. hominis* strains [125] and PHE data indicating that culture positive, *gap* PCR negative specimens were occurring (data not shown) resulted in an urgent need to redesign and validate a multiplex neonate qPCR test for use at the National Reference Laboratory (PHE) that could simultaneously detect *M. hominis* and *Ureaplasma* species. Furthermore, reference laboratory tests are required to be different and bespoke to those available commercially to enable independent confirmation of infection. This chapter presents an improved multiplex qPCR assay developed for use on neonate specimens to simultaneously detect *M. hominis*, *U. parvum* and *U. urealyticum.* The assay by Yi *et al.*, [290] has been supplemented by the addition of an *M. hominis*

specific target identified by Ferandon *et al.*, [125] (*yidC*), and a GFP amplification control to identify PCR inhibitors [291].

3.2.2 Primer and probe design

Each oligonucleotide in the qPCR was assessed using blastn (NCBI; section 2.6.7) to examine specificity. Analysis of the *yidC* target oligonucleotides and probe gave maximum similarity (100%) over the entire length against the available, complete putative inner membrane protein translocase component *yidC* gene sequence of *M. hominis* (ATCC 23114; PG21). To check for intra-species heterogeneity at the nucleotide level, sequence analysis of the entire *yidC* gene from 19 *M. hominis* clinical isolates and the reference strain PG21 was undertaken. Only 70 of 1,995 nucleotides varied among the 20 strains studied, and were found to be outside the sequences of primers and probe used for amplification in this assay. This is in concordance with results for strains from other countries as published by Ferandon *et al.,* [125]. The *urease* target oligonucleotides gave maximum similarity (100%) over the entire length against available complete urease complex component gene sequences of *U. parvum* (serovar 1, serovar 3 [ATCC 700970, ATCC 27815, SV3F4], serovar 6, serovar 14) and *U. urealyticum* (serovar 2 serovar 4, serovar 10). The probes gave maximum similarity (100%) to *U. parum* and *U. urealyticum*, respectively.

3.2.3 Rotor-Gene® PCR

The multiplex real-time PCR was performed as described in section 2.3.3. A standard dilution series of the plasmid control was amplified in each Rotor-Gene® PCR run for validation and DNA quantification. For detection in clinical samples, standards of 10^4, 10^3, 10^2 and 10^1 copies/µL were included, and a run was deemed successful if the positive controls fell within the thresholds outline in Table 3.1. Fluorescence curves down to 1 copy/µL could be detected. The slope of the standard

curve for each target was -3.375, -3.018 and -3.195 for *M. hominis*, *U. parvum* and *U. urealyticum*, respectively, with calculated R^2 values of 0.975, 0.957, and 0.983 (Figure 3.1). The concentration of *M. hominis*, *U. urealyticum* and *U. parvum* in patient samples was calculated from the standard curves.

Table 3.1. Interpretation of positive controls.
Ct value ranges (average Ct ± standard deviation) for positive control plasmid for each *U. parvum*, *U. urealyticum*, *M. hominis* and IPC.

	Ct values							
	U. parvum		*U. urealyticum*		*M. hominis*		IPC	
Copies/µl	min	Max	Min	max	min	max	min	max
10000	22.0	26.0	23.3	26.7	23.1	29.0	29.3	32.7
1000	26.0	28.0	27.2	30.8	27.2	30.8		
100	28.0	34.0	29.6	35.4	29.5	35.7		
10	29.2	40.2	30.5	42.5	30.3	42.3		

[a]UIPC Ct average and standard deviation calculated from 502 samples

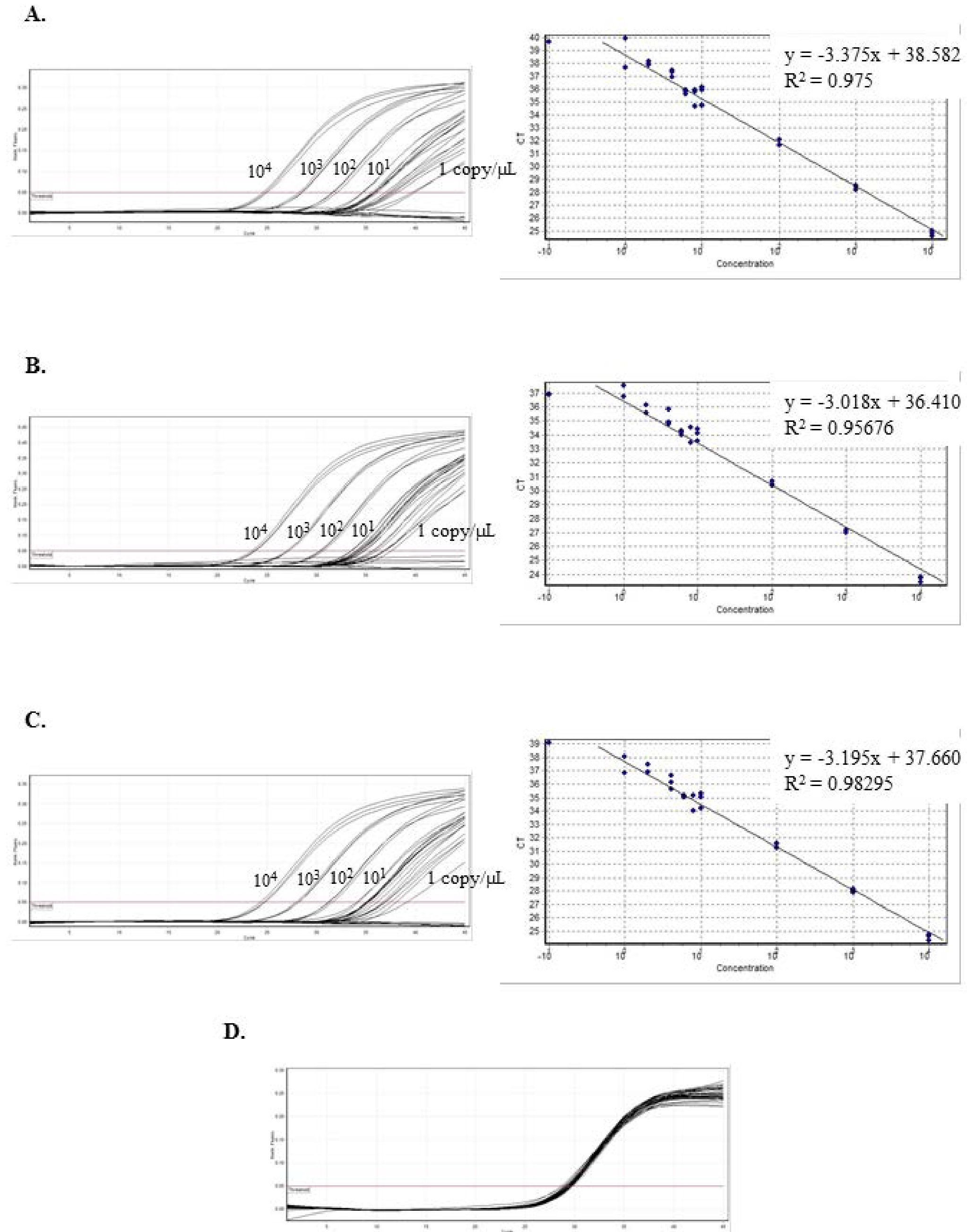

Figure 3.1. Fluorescence and standard curves of serially diluted control DNA. Fluorescence and standard curves of serially diluted standard DNA for *M. hominis* (A), *U. parvum* (B), *U. urealyticum* (C), and fluorescence curve for the GFP amplification control (D). The equation of the standard curve and calculated R^2 values are shown for the multiplex real-time PCR.

3.2.4 Reproducibility and LOD

To determine the reproducibility of the multiplex real-time PCR, the intra- and inter-assay variations were measured with respect to cycling threshold and concentration. To determine the intra-assay variation, triplicate standard dilution series of 10^4, 10^3, 10^2 and 10^1 copies/µL were tested in one run. The coefficients of variation (CV) of the cycling threshold (Ct) were in the range of 0.6% to 2.1% for *M. hominis*, 0.4% to 1.3% for *U. parvum* and 0.5% to 1.6% for *U. urealyticum*. The CVs of the concentrations were in the range of 12.5% to 54.9% for *M. hominis*, 8.9% to 34.0% for *U. parvum*, and 10.6% to 42.1% for *U. urealyticum*, with the highest values in the lower concentrations.

Twenty-two standard curves were evaluated for the determination of the inter-assay variation. The standard curves were chosen from runs performed on different days on four different Rotor-Gene® Q thermocyclers. The CV of the Ct varied from 3.0% to 7.0% for *M. hominis*, 4.0% to 7.0% for *U. parvum* and 3.2% to 8.3% for *U. urealyticum*, and the CV of concentration varied from 24.5% to 106.0% for *M. hominis*, 24.3% to 148.6% for *U. parvum* and 23.8% to 92.0% for *U. urealyticum*, with the highest values for the lowest concentration in the standard curve (10^1 copies/µl; Table 3.2). Concentrations of 10^4 and 10^3 copies/µL were detected in all of the 22 runs of the standard dilution series; however, 21 out of 22 runs (95.5%) detected 10^2 copies/µL of *M. hominis* and this concentration was detected in 22 out of 22 runs for both *U. parvum* and *U. urealyticum*. Additionally, 10^1 copies/µL was detected in 19 out of 22 runs (86.4%) for *M. hominis* and 20 out of 22 runs (90.9%) for both *U. parvum* and *U. urealyticum* (Table 3.2).

Table 3.2. Inter-assay reproducibility of the multiplex *U. urealyticum*, *U. parvum* and *M. hominis* PCR assay.

	Ureaplasma urealyticum					***Ureaplasma parvum***					***Mycoplasma hominis***				
Genome copy no./µl	**Observed no.**	**Mean Ct[a]**	**CV[b] Ct (%)**	**Mean concn.**	**CV concn. (%)**	**Observed no.**	**Mean Ct[a]**	**CV[b] Ct (%)**	**Mean concn.**	**CV concn. (%)**	**Observed no.**	**Mean Ct[a]**	**CV[b] Ct (%)**	**Mean concn.**	**CV concn. (%)**
10^4	22/22	25.0	3.4	1.01×10^4	25.3	22/22	25.0	4.0	1.04×10^4	26.6	22/22	26.0	5.0	1.07×10^4	24.5
10^3	22/22	29.0	3.2	1.04×10^3	23.8	22/22	28.0	4.0	1.02×10^3	24.3	22/22	30.0	3.0	1.00×10^3	28.2
10^2	22/22	32.5	4.5	1.19×10^2	55.9	22/22	31.4	5.0	1.15×10^2	55.2	21/22	33.1	5.0	1.08×10^2	48.1
10^1	20/22	36.5	8.3	1.40×10^1	92.0	20/22	34.9	7.0	1.03×10^1	148.6	19/22	36.5	7.0	1.52×10^1	106.0

[a] Ct, cycling threshold

[b] CV, coefficient of variation

The LOD was approached by including additional concentrations of 8, 6, 4, 2 and 1 copies/µL into the standard dilution series. LOD was calculated both statistically and empirically as recommended by Burd [317] and Saunders *et al*. [318] (described in section 2.7) using six runs, with triplicate reactions in each run, resulting in a total of 18 replicates. Details of positive replicate results for *M. hominis, U. parvum* and *U. urealyticum* can be found in Table 3.3 Empirical LOD determination resulted in 40 copies/reaction, 20 copies/reaction and 30 copies/reaction for *M. hominis*, *U. parvum* and *U. urealyticum*, respectively, where all replicates above at or above these values were detected excluding a single outlier. Statistically, the LOD 95, the number of copies of each target that the assay has a 95% chance of detecting, was estimated as 45 copies/reaction (95% CI: 25%-63%) for *M. hominis*, 22 copies/reaction (95% CI: 18%-36%) for *U. parvum*, and 25 copies/reaction (95% CI: 14%-32%) for *U. urealyticum.*

Table 3.3. Percentage of replicate PCR results of the standard dilution series for *Mycoplasma hominis, Ureaplasma parvum* and *Ureaplasma urealyticum*.

	Percentage of replicates detected (%)		
Concentration of control plasmid (copies/µL)	***M. hominis***	***U. parvum***	***U. urealyticum***
10^4	100	100	100
10^3	100	100	100
10^2	100	100	100
10^1	95	100	100
8	100	100	100
6	83	94	95
4	83	94	89
2	55	67	67
1	28	22	28

3.2.5 Specificity

The sequence of the *yidC* gene of *M. hominis* was examined in 20 *M. hominis* strains to examine the variability in the amplicon region. All the sequences were identical except for one strain which has a single nucleotide polymorphism within the amplicon; however, this was not within the primer and probe binding sites (Figure 3.2). All 20 *M. hominis* strains were also tested in the assay. In agreement with the results from sequencing, all the 20 *M. hominis* strains were detected, and the fluorescence curves were comparable to the 10^4 copies/µL standard. Additionally, 20 *Ureaplasma* species (ten *U. parvum* and ten *U. urealyticum*) isolates were tested using the multiplex real-time PCR assay. All isolates were detected and the fluorescence curves were comparable to the 10^4 and 10^3 copies/µL standards.

The assay proved to be specific for *M. hominis, U. parvum* and *U. urealyticum,* as no cross-reactions of the assay were detected against DNA from other human mycoplasmas and 58 other respiratory bacteria and viruses (listed in Appendices Table A.1) that are commonly found in respiratory specimens.

```
                          Forward primer
MH2     TCAATTCAATTATCAGCAAATTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH21    TCAATTCAATTATCAGCAAATTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH23    TCAATTCAATTATCAGCAAATTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH11    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH12    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH15    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH17    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH29    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH28    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH20    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH18    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAGGAATTCATATAT     1680
MH41    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH43    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH44    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH27    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH26    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH25    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
PG21    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH9     TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
MH10    TCAATTCAATTATCAGCAAACTCACTAAACCGGGTATTTTCTAACAAAGAATTCATATAT     1680
        ******************** *************************** ************

            Probe - ATTO680/BHQ3             Reverse primer
MH2     CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH21    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH23    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH11    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH12    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH15    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH17    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH29    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH28    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH20    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH18    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH41    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH43    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH44    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH27    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH26    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH25    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
PG21    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH9     CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
MH10    CTACCAATAATTTTAATATCTGTCGGTATGCAAGCACTATCGCAATATATGCCAAAGATT     1740
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Figure 3.2. Alignment of the amplified region of the *yidC* gene of *Mycoplasma hominis* including primer and probe binding sites.
The *yidC* sequence of *M. hominis* PG21 was compared to the *yidC* sequence of 19 additional *M. hominis* strains and aligned using clustalW. * (asterix) indicate completely conserved residues. Single nucleotide polymorphisms within the PCR amplicon are indicated by grey shading. The selected primers and probe in the *yidC* sequence are underlined.

3.2.6 Evaluation with patient samples and comparison to culture and MUP PCR

The multiplex real-time PCR assay was evaluated with retrospective testing of 216 clinical specimens submitted to PHE in 2014 for *Ureaplasma* species and *M. hominis* testing and compared to M-duo culture and MUP PCR results. The M-duo culture test cannot distinguish *Ureaplasma* species therefore *U. parvum* and

U. urealyticum detection totals were combined for specificity and sensitivity analysis. Additionally, MUP PCR positive specimens were subsequently cultured using MM and *ureaplasma*-specific (USM) media.

This multiplex real-time PCR assay was not validated against conventional culture using *Mycoplasma/Ureaplasma*-specific media due to the retrospective nature of this study. Conventional culture was not routinely performed on all clinical specimens sent to PHE for *Mycoplasma/Ureaplasma* testing and only DNA extracts were stored for further analysis. M-duo culture however, was used to screen all specimens, therefore this data was used for evaluation.

3.2.6.1 Comparison to M-duo culture

The multiplex real-time PCR assay detected a higher number of positive samples than the M-duo screening. The PCR assay detected 22/216 (10.2%; 95% CI: 0.07-0.15) positive *M. hominis* and 63/216 (29.2%; 95% CI: 0.24-0.36) positive *Ureaplasma* species (40/216 [18.5%; 95% CI: 0.14-0.24] positive *U. parvum* and 23/216 [10.7%; 95% CI: 0.07-0.16] positive *U. urealyticum*) clinical samples. M-duo screening detected 5/216 (2.3%; 95% CI: 0.01-0.05) positive *M. hominis*, 29/216 (13.4%; 95% CI: 0.10-0.19) positive *Ureaplasma* species clinical samples. The results for the multiplex real-time PCR assay and M-duo screening are shown in Table 3.4 and Table 3.5, respectively. It is important to note that three clinical samples were positive for *Ureaplasma* species and two samples were positive for *M. hominis* by M-duo screening but were negative in the multiplex real-time PCR assay, three of which could not be confirmed by subsequent culture. The two samples that were culture positive but negative in this PCR were originally found to be positive using the MUP PCR assay at the time of DNA extraction but on re-testing with the MUP PCR assay

negative results were obtained. The discrepancy is attributed to degradation, as has been documented for detection of other *Mollicutes* [319].

The multiplex real-time PCR had a statistically higher clinical sensitivity than the culture technique for all targets (*M. hominis*, *p*=0.0081; *Ureaplasma* species, *p*=0.0001). When M-duo culture was considered as the reference method, the multiplex real-time PCR has sensitivities of 60.0% (95% CI: 14.7-94.7) and 89.3% (95% CI: 71.8-97.7) for *M. hominis* and *Ureaplasma* species, respectively, and specificities of 91.0% (95% CI: 86.3-94.5) and 80.3% (95% CI: 73.9-85.8). Mixed infections were detected in patient clinical specimens with the multiplex real-time PCR assay, however this was uncommon with 4/216 (1.9%; 95% CI: 0.006-0.05) *M. hominis* and *U. urealyticum* infections, 5/216 (2.3%; 95% CI: 0.008-0.06) *M. hominis* and *U. parvum* infections, and 1/216 (0.46%; 95% CI: 0.0001-0.03) *U. parvum* and *U. urealyticum* infection.

Table 3.4. Comparison of PCR and M-duo culture results for *M. hominis* from 216 clinical specimens.

		M-duo culture		MUP PCR	
		Positive	Negative	Positive	Negative
Multiplex real-time PCR	Positive	3	19	10	12
	Negative	2	192	1	193
MUP PCR	Positive	2	9		
	Negative	3	202		

Table 3.5. Comparison of PCR and M-duo culture results for *Ureaplasma* species from 216 clinical specimens.

		M-duo culture		MUP PCR	
		Positive	Negative	Positive	Negative
Multiplex real-time PCR	Positive	26	37	49	14
	Negative	2	151	3	150
MUP PCR	Positive	28	24		
	Negative	0	164		

3.2.6.2 Comparison to MUP PCR

The multiplex real-time PCR assay detected a higher number of positive samples than the MUP PCR (Table 3.4-Table 3.5; method described in section 2.3.2). Using the MUP PCR, 11/216 (5.1%; 95% CI: 2.8-9.0) specimens were positive for *M. hominis* and 52/216 (24.1%; 95% CI: 18.8-30.2) specimens were positive for *Ureaplasma* species (34/216 [15.7%; 95% CI: 11.5-21.2] specimens were positive for *U. parvum* and 18/216 [8.3%; 95% CI: 5.3-12.9] specimens were positive for *U. urealyticum*). It is important to note that 26/216 (12.0%; 95% CI: 8.3-17.1) clinical specimens were found to be positive for either *M. hominis* or *Ureaplasma* species by the multiplex real-time PCR but were reported as negative using the MUP PCR. Of these, the 11/216 (5.1%; 95% CI: 2.8-9.0) *U. parvum* and 5/216 (2.3%; 95% CI: 0.8-5.5) *U. urealyticum* positive samples had very low copy numbers (6.5 to 25.6 copies/µl) but the 12/216 (5.6%; 95% CI: 3.1-9.6) *M. hominis* positive samples had copy numbers of 9.0 to 7.6 x 10^3 copies/µl. The multiplex real-time PCR was repeated with the positive samples and all samples remained positive with consistent

copy numbers. Additionally, three samples that were positive for *M. hominis* with the real-time multiplex PCR were confirmed as positive by 16S rRNA sequencing.

The multiplex real-time PCR had a statistically higher clinical specificity than the MUP PCR for all targets (*M. hominis*, p=0.0001; *U. parvum*, p=0.0001; *U. urealyticum*, p=0.0001). When MUP PCR was considered as the reference method, the multiplex real-time PCR had sensitivities of 90.9% (95% CI: 58.7-99.8), 72.5% (95% CI: 56.1-85.4), and 78.3% (95% CI: 56.3-92.5) for *M. hominis*, *U. parvum* and *U. urealyticum*, respectively and specificities of 94.2% (95% CI: 90.0-96.9), 98.3% (95% CI: 95.1-99.7), and 100% (95% CI: 98.1-100).

Sixty-one clinical specimens were subjected to culture using MM or USM media, following a positive detection result from the MUP PCR, as per standard diagnostic procedure performed by PHE. A total of 63 positive results were recorded due to the detection of both *M. hominis* and *Ureaplasma* species two clinical specimens. Of the 63 MUP PCR positive results, 26/63 (41.3%; 95% CI: 30.0-53.6) were confirmed by MM/USM culture. Of these, 10/63 (15.9%; 95% CI: 8.7-27.0) were positive for *M. hominis* and 16/63 (25.4%; 95% CI: 16.2-37.4) were positive for *Ureaplasma* species (Table 3.6). These 61 clinical specimens were also subjected to the multiplex real-time assay and comparison was made between results from the assay and MM/USM culture. Of the 63 MUP PCR positive results, 23/63 (36.5%; 95% CI: 25.7-48.9) were positive by both the multiplex real-time PCR and MM/USM culture; 8/63 (12.7%; 95% CI: 6.3-23.4) were found to be positive for *M. hominis* and 15/63 (23.8%; 95% CI: 14.9-35.7) were positive for *Ureaplasma* species (Table 3.6). Three clinical specimens were found to be positive for *M. hominis* (1/63; 1.6%; 95% CI: 0.01-9.3) and *Ureaplasma* species (2/63; 3.2%; 95% CI: 0.23-11.5) by MUP PCR but this was not confirmed by MM culture or the multiplex real-time PCR (Table 3.6).

The two clinical specimens identified as having mixed infections by: MUP PCR, multiplex real-time PCR and M-duo screening were not confirmed by MM culture. MM culture returned one positive *M. hominis* result in a single clinical specimen.

The GFP control included in the assay allowed assessment of inhibition within the clinical specimens. No inhibitory specimens were identified out of the 216 clinical specimens tested.

Table 3.6. Comparison of PCR and Mycoplasma Experience culture results for *M. hominis* and *Ureaplasma* species from 61 clinical specimens. Mycoplasma experience culture was performed on samples that were found positive for *Ureaplasma* species (52 clinical specimens) or *M. hominis* (11 clinical specimens) by the MUP PCR. Note that two clinical specimens were found positive for both *Ureaplasma* species and *M. hominis*.

			MM/USM Culture			
			M. hominis		*Ureaplasma* species	
			Positive	Negative	Positive	Negative
Multiplex real-time PCR	*M. hominis*	Positive	8	2		
		Negative	0	1		
	Ureaplasma species	Positive			15	34
		Negative			1	2
MUP PCR	*M. hominis*	Positive	10	1		
	Ureaplasma species	Positive			16	36

3.2.7 Detection of *M. hominis* and *Ureaplasma* species in neonates

Endo-tracheal secretions from 45 intubated neonates (anonymised; South West) were tested for the presence of *M. hominis* and *Ureaplasma* species. A total of 194 clinical specimens were tested. An additional 34 clinical specimens, collected in a hospital in Wales, from eight neonates were tested. These eight neonates were treated with clarithromycin after collection of the first sample. Multiplex real-time PCR results were compared to culture results for *Ureaplasma* species performed using specialised Ureaplasma-specific medium (USM) followed by differentiation into *U. parvum* or *U. urealyticum* using primers that generate different sizes of amplicons by standard PCR [320]. Culture for *M. hominis* was not performed.

The multiplex real-time PCR detected a higher number of positive samples than culture; detecting 5/228 (2.2%; 95% CI: 0.78-5.2) positive *M. hominis*, 46/228 (20.2%; 95% CI: 15.5-25.9) positive *U. parvum* and 46/228 (11.8%; 95% CI: 8.2-16.7) positive *U. urealyticum* clinical samples compared to 39/228 (17.1%; 95% CI: 12.7-22.6) positive *U. parvum* and 24/228 (10.5%; 95% CI: 7.1-15.2) positive *U. urealyticum* clinical samples detected by culture (Figure 3.3). Mixed infections of *M. hominis* and *U. parvum* were detected in 3/228 (1.3%; 95% CI: 0.3-4.0) clinical specimens from two neonates (P162 and P164) using the multiplex real-time PCR. The multiplex real-time PCR had a statistically higher clinical sensitivity ($p = 0.0001$) than culture using USM for the detection of *Ureaplasma* species in the clinical specimens.

Interestingly, when clinical samples from the South West and Wales were examined separately, a higher proportion of the samples were positive for *Ureaplasma* species in the Wales samples than the South West samples. The multiplex real-time

PCR detected 13/34 (38.2%; 95% CI: 23.9-55.0) positive for *U. parvum* and 5/34 (14.7%; 95% CI: 6.0-30.6) positive for *U. urealyticum* in the Wales clinical samples compared to 33/194 (17.0%; 95% CI: 12.3-23.0) positive for *U. parvum* and 22/194 (11.3%; 95% CI: 7.6-16.6) positive for *U. urealyticum* in the South West clinical samples. Additionally, *M. hominis* was only detected in the South West clinical samples (5/194; 2.6%; 95% CI: 0.9-6.1). The higher proportion of positive results observed in the Wales clinical samples can be attributed to the sampling. In Wales, clinical samples were only collected following prolonged ventilation requirement of the neonate and when sepsis/respiratory infection caused by other bacteria was ruled out by standard microbial culture. However in the South West hospital, all ventilated neonates born at less than 28 weeks gestation that consented to study, were examined.

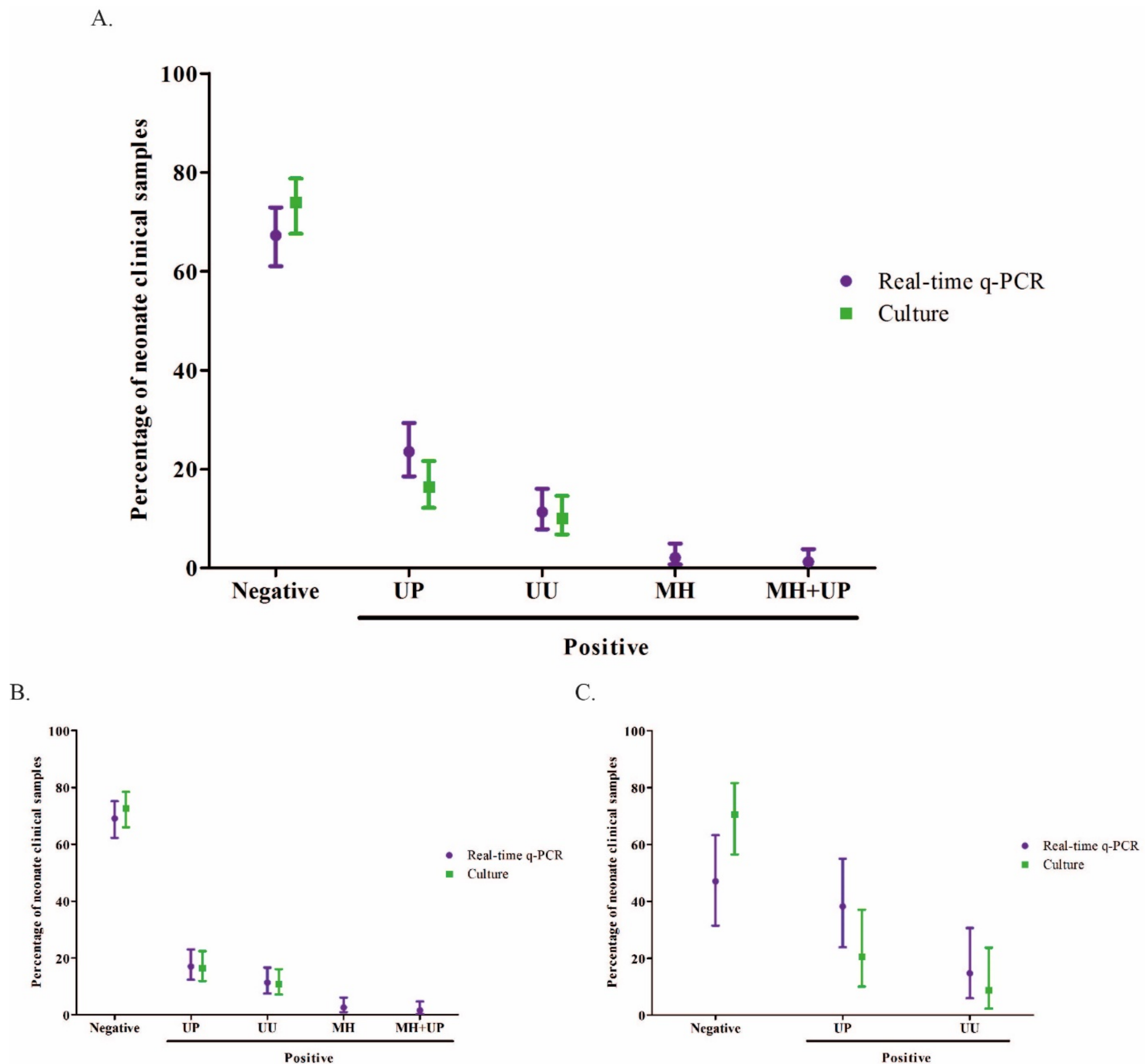


Figure 3.3. Incidence of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in intubated neonates.
M. hominis, U. parvum and *U. urealyticum* were detected in neonatal clinical specimens (endotracheal secretions) by multiplex real-time PCR (purple) and by culture (green). Data is presented as a percentage of neonatal clinical samples with 95% CI. A) Neonatal samples from both the South West and Wales; B) Neonatal samples from the South West only; C) Neonatal samples from Wales only.

3.3 Discussion

Direct detection techniques are the only methods adequate for genital mycoplasmas. Although culture is considered to be the reference technique for the

detection of *M. hominis*, it requires specialised media and expertise and does not yield results before 2-5 days [321].

3.3.1 Analysis of the multiplex real-time PCR method used

This chapter has described the successful combination of two previously published single-plex PCRs for the detection of *M. hominis* and *Ureaplasma* species. The combined assay is a rapid, sensitive, and specific multiplex real-time PCR for the simultaneous detection of *M. hominis*, *U. urealyticum* and *U. parvum*. An increased number of positive clinical samples were detected for all three targets in comparison to M-duo culture and the MUP PCR and the assay also detected mixed infections within a clinical sample. This is an improvement that was essential to the National Reference Laboratory for detection of *M. hominis*, *U. parvum* and *U. urealyticum* by qPCR in neonates. Furthermore, this assay contains an internal, GFP control that can be used to monitor the efficiency of each PCR reaction and assess PCR inhibition that could adversely affect the result of the PCR.

3.3.1.1 Choice of gene target

Most PCR techniques detecting *M. hominis* target the 16S rRNA gene [322-327]; however, minor sequence variations were observed in the 16S rRNA gene sequences [123] and may lead to a lower clinical sensitivity of these techniques. Additionally, variation has been described in the *gap* gene and the extent of variation was found to be larger than in the 16S rRNA gene [124, 315]. Sequence analysis of the *gap* gene from *M. hominis* isolated from MUP PCR negative clinical specimens revealed nucleotide mutations within the primer and probe binding sites, indicating a reason for amplification failure. In comparison, a Taqman assay has been developed for the detection of *M. hominis* targeting the *yidC* gene [125], in which the *yidC* gene has been shown to have relatively low intra-species heterogeneity at the nucleotide

level, with no sequence polymorphisms identified within the primer and probe binding sites. Therefore, the primer and probe sequences from this assay were used to develop the multiplex assay described in this chapter. These primers and probes have been validated for use on urogenital clinical specimens [125] and this chapter has validated their use for detection of *M. hominis* in respiratory specimens.

3.3.1.2 Sensitivity and specificity

The LOD of the multiplex real-time PCR for *M. hominis* was 9 copies/µL in comparison to 7 copies/µL in the *yidC* single-plex assay (performed in another lab under different PCR conditions [125]). However, both of these LOD were consistent with the sensitivities previously reported for real-time PCR techniques detecting *M. hominis* [124, 126]. However, as previously shown for *M. pneumoniae* [328], the analytical sensitivity may not predict the ability to detect the bacterial target in clinical specimens. In highly variable species such as *M. hominis* [329], the PCR target conservation at the nucleotide level is essential for providing a high clinical sensitivity. Thus, it could be speculated that using the *yidC* gene as a target may result in a PCR assay with a higher clinical sensitivity than the previously reported PCRs.

Additional validation could be performed to assess the robustness of the multiplex real-time PCR in the form of a spiking assay. Analysis of the ability of the assay to detect low levels of *M. hominis* in the presence of high concentrations of *Ureaplasma* species (and vice-versa) will determine if there is any interference between detection of *M. hominis* and *Ureaplasma* species in clinical specimens.

3.3.1.3 Analysis of clinical samples and comparison with culture

The M-duo culture method has previously been evaluated for use in clinical diagnosis and is considered to have sensitivity comparable to conventional PCR and a

higher detection rate than culture using conventional A7 differential agar [330, 331]. Higher rates of detection using PCR-based methodologies compared to culture have previously been reported for the detection of human *Mycoplasma* species and *Ureaplasma* species [125, 290, 332-335]; however, these have not always been statistically significant. Nevertheless, this multiplex real-time PCR assay resulted in a significant increase in detection of all three targets in clinical samples in comparison to M-duo. When the M-duo culture method was considered as a reference method, the new PCR assay showed 60% and 89.29% sensitivity, and 91% and 80.32% specificity for *M. hominis* and *Ureaplasma* species, respectively. Conversely, when the MUP PCR assay is used as the reference method, the M-duo culture showed 13.64% and 40.32% sensitivity, and 98.97% and 98.05% specificity for *M. hominis* and *Ureaplasma* species, respectively.

When neonate clinical samples were tested for *Ureaplasma* species, the multiplex real-time PCR had a statistically higher clinical sensitivity ($p = 0.0001$) than culture on USM. However, published data on the *ureaseB* primers for the detection and speciation of *Ureaplasma* (single-plex assay), found that a higher number of positive clinical specimens were detected using the assay than culture but that the difference in clinical sensitivity was not significant [290]. Furthermore, the sensitivity documented for the single-plex assay when culture was considered the reference method was 44.4% [290] compared to 83.3% when the multiplex real-time PCR was compared to USM culture. However, different samples and PCR conditions could be responsible, in part, for the differences observed.

Discrepancies between culture and PCR results have been documented for *M. hominis* [125]. When the Taqman assay was developed using the *yidC* gene as a target, 10 clinical specimens were found to be positive for *M. hominis* using the assay

but were negative using the culture method, resulting in a statistically higher clinical sensitivity, as observed in this chapter. This is in contrast to results reported for assays utilising the *gap* gene as a target for *M. hominis* detection where 100% agreement between culture and PCR results has been documented [124]. However, an increase in detection using real-time PCR is consistent with results reported for conventional PCR techniques [336-338] and higher clinical sensitivity of PCR compared to culture has been widely reported [339-341].

One advantage of this multiplex real-time PCR over culture methods is the ability to determine the species of *Ureaplasma* present in the clinical sample, either *U. parvum* or *U. urealyticum* or mixed infections, without requiring any further testing. This could be important as it has been proposed that the two different species may be responsible for differences in pathogenicity, ability to culture, or response to treatment and several studies [342-346] propose that certain species are more commonly associated with invasive disease and adverse pregnancy outcomes. Furthermore, mixed infections may have differing antibiotic resistance profiles depending on the species present. However, in other studies [347-351], no correlation has been found between *Ureaplasma* species and pathogenicity. It has also been widely reported that *U. parvum* is the more common of the two species to be isolated from clinical specimens [352], but both species may occur simultaneously in some people [3]. Previous studies used various methods such as liquid DNA hybridisation [326], PCR of 16S rRNA gene [343], or PCR of the MBA gene [348] to differentiate between *U. parvum* and *U. urealyticum*. The results of these studies have indicated that *U. parvum* is present in 76-82% of *Ureaplasma* species positive clinical specimens whereas *U. urealyticum* is only present in 13.5-30%. Results from the single-plex real-time assay distinguishing between *U. parvum* and *U. urealyticum* by

targeting the *urease* gene detected *U. parvum* and *U. urealyticum* in the same proportions as previously documented, 64.7% and 35.3%, respectively [290]. Results regarding numbers of *U. parvum* and *U. urealyticum* positive clinical specimens detected in this chapter are consistent with these observations.

3.3.1.4 Further application of the multiplex real-time PCR assay

This multiplex real-time PCR assay has been validated for respiratory, neonatal clinical samples; nevertheless, it has a wider application and could be used for urogenital and sexually transmitted infections as well as other invasive infections. This would, however require further validation. *Ureaplasma* species and *M. hominis* have been included as targets in other multiplex assays and genital screening assays [314, 353, 354]; however, this is the first assay to simultaneously detect *M, hominis* and *Ureaplasma* species, and differentiate between *U. urealyticum* and *U. parvum*. The assay would be ideal for multiplexing with additional targets to be used as a wider screen for common pathogens in neonates and sepsis/meningitis patients, such as *Streptococcus agalactiae*, *E. coli*, coagulase-negative Staphylococci, *S. pneumoniae* and *Listeria* [355].

The assay developed in this study could be used to monitor the quantity of DNA of each target and may therefore have utility to monitor bacterial load in sequential patient specimens and growth in culture media. The role of *Ureaplasma* species as a sexually transmitted pathogen has not yet been defined but both *M. hominis* and *Ureaplasma* species are found in a high percentage of female cervix and vaginal specimens and are present in the male urethra. A positive PCR result on specimens from these sites may not be meaningful in the absence of clinical manifestations associated with these organisms, unless the bacterial load is significantly high [127]. However, an increased importance of infection based on

detected load has not been definitively associated with increased severity of clinical signs. Therefore, it may be important to quantify bacterial load in relation to other bacterial flora and pathogens allowing for informed diagnosis of importance of infection with these species. In the case of extra-genital specimens in adults or neonates positive PCR or culture result for *M. hominis* or *Ureaplasma* species should be considered clinically significant, especially in immunocompromised hosts. This multiplex real-time PCR assay that can simultaneously detect and quantify *M. hominis*, *U. parvum* and *U. urealyticum* in neonate clinical specimens has further importance for detection of mixed infections that may inform relevant and appropriate therapeutic intervention.

3.3.2 Review of previously described PCR detection methods for *Mycoplasma hominis* and *Ureaplasma* species

Many PCR assays have been developed to detect *M. hominis* and *Ureaplasma* species in clinical specimens. These are most commonly associated with other urogenital pathogens such as *Chlamydia trachomatis*, *N. gonorrhoeae* and *M. genitalium* in sexually transmitted infections (STI) and urethritis screening tests [314, 326, 356, 357]. In many cases, only *U. urealyticum* is included as a target, excluding *U. parvum* which is more commonly isolated from clinical specimens. However, it may be more common that the term *U. urealyticum* is misused as both species were defined as biovars of *U. urealyticum* prior to 2001 and therefore both species are identified by these PCRs but not speciated. The most common gene target utilised for the detection of *M. hominis* and *Ureaplasma* species is the 16S rRNA [314, 326, 358]; however, there is increasing use of the *urease* gene as a target for *Ureaplasma* species [338, 341, 358, 359]. In 2005, J. Yi, et al. (290) utilised the *urease* gene for the discrimination between *U. urealyticum* and *U. parvum* through the

use of a species-specific probe. Since then discrimination using this gene target has been included in other multiplex assays with modifications to primers and probes [352, 360, 361].

As previously mentioned, sequence variation in the 16S rRNA gene of *M. hominis* has been documented, along with variation in another gene target, the *gap* gene. This instability has been shown to reduce the clinical sensitivity of PCR assays utilising these gene targets, therefore use of alternative targets, such as *yidC*, for *M. hominis* detection should be encouraged. A real-time PCR assay has been developed targeting the *ftsY* gene of *M. hominis* [126] and although intra-species heterogeneity was not examined when the assay was developed, previous examination of recombination within *M. hominis* revealed low levels in *ftsY* in comparison to other genes such as *gap* [362].

There are now several commercial assays that detect *M. hominis* and *Ureaplasma* species (Seegene, Korea; Fast-Track Diagnostics, Malta; SpeeDx, Australia; Sarace, Italy). These are often combined with other pathogens involved in STI and urethritis. The gene targets utilised by these assays are unknown (not present in manufacturer documentation) therefore direct comparison with the multiplex real-time assay developed in this chapter cannot be performed. The Fast-Track assay is a multiplex assay targeting common causes of urethritis, including *M. hominis*, *U. parvum* and *U. urealyticum* and reports sensitivities of 100% for all targets and specificities of 100%, 96% and 96%, respectively [363]. Additionally, Sacace have developed a multiplex assay targeting only *M. hominis*, *U. parvum* and *U. urealyticum* with reported sensitivities and specificities of 100% for all targets. However, this diagnostic assay has been validated for genital rather than respiratory clinical

specimens with cross-reactivity only assessed against other common genital pathogens.

Due to the availability of multiple diagnostic assays for *M. hominis* and *Ureaplasma* species, there is need for an independent reference test that can be used as the 'gold standard' for comparison of other diagnostic PCR assays. This chapter has described the development of such an assay for the National Reference Laboratory that can be used an independent test to confirm diagnostic results from externally tested clinical specimens.

3.3.3 Summary

In conclusion, the multiplex real-time PCR developed in this chapter provides an improved nucleotide-based method, including an amplification control, for the detection of *M. hominis* and *Ureaplasma* species with a significantly higher clinical sensitivity than culture.

Chapter 4. Application of the multiplex real-time PCR – neonate case study and use to monitor response to antibiotics

4.1 Introduction

Definitive association of *Ureaplasma* species with disease in humans remains inconclusive, however evidence is increasingly being reported on an association with non-gonococcal urethritis [364], and with preterm birth and vertical transmission occurs in new-borns [365]. A study of *U. parvum* and *M. hominis* infection in pregnant macaque monkeys demonstrated that these bacteria induced preterm birth within six days of infection and caused bronchopulmonary dysplasia in the neonates, as sole pathogens [184]. Furthermore, two studies in 2016 continue to substantiate the association of *Ureaplasma* species with disease in humans [358, 366]. In neonates, these bacteria are associated with serious infections [3] including: pneumonia [367], bacteremia [368], meningitis [369], abscesses [206], intraventricular haemorrhage [370], necrotising enterocolitis (NEC) [371] and chronic lung disease (CLD) [372].

Antibiotic resistance among human pathogens continues to have a major impact on patient morbidity, mortality and economic cost to the health service [373]. Due to the limited number of antibiotics that are affective against *M. hominis* as well as the further treatment limitations in neonates, the emergence of resistant strains further complicates treatment. Due to the technical difficulties in cultivation of *M. hominis*, antimicrobial susceptibility testing is predominantly carried out by the micro-broth dilution technique using a standardised 10^4-10^5 CCU [374]. *M. hominis* is resistant to 14- and 15-membered ring macrolides and therefore treatment options are restricted to 16-membered ring macrolides, lincosamides, tetracyclines and fluorquinolones. Due to differences in intrinsic antibiotic resistance of both *M. hominis* and *Ureaplasma* species, targeted treatment and detection of mixed infections is important. Clindamycin (a lincosamide) is used to treat *M. hominis* infections however, this antibiotic has no effect on *Ureaplasma* species which are resistant to it. Conversely

erythromycin (a 14-membered ring macrolide) is commonly used to treat *Ureaplasma* species infection but has no effect on *M. hominis* [321]. Acquired antibiotic resistance has also been documented for *M. hominis*; in clinical *M. hominis* isolates, tetracycline resistance is conferred by the expression of the horizontally acquired *tetM* gene.

The objective of the work described in this chapter was to apply the multiplex real-time PCR developed in Chapter 3 to a cohort of neonatal specimens, quantifying bacterial load, with specific focus on several interesting cases. Furthermore, this technique was applied to monitor the response of the bacteria to differing antibiotic classes. Tetracycline resistance of *M. hominis* was also examined.

4.2 Results

4.2.1 South West intubated neonate case studies

The association between a positive PCR result for *M. hominis*, *U. parvum* and *U. urealyticum* and sex; gestation; method of delivery; clinical presentation; treatment with macrolides or steroids; being a twin; and survival is shown in Figure 4.1. The highest number of neonates with detected *M. hominis* or *Ureaplasma* species infection were born between 23 and 25 weeks gestation (16/20; 80.0%; 95% CI: 57.8-92.5), with lower numbers detected at 26-28 weeks gestation (3/20; 15.0%; 95% CI: 4.4-36.9) and one positive case at 31 weeks gestation (1/20; 5.0%; 95% CI: 0.01-25.4). Neonates were significantly more likely to be positive for *Ureaplasma* species and *M. hominis* if they were born earlier than 26 weeks gestation ($p = 0.0141$). There was no association between sex and prevalence of *M. hominis*, *U. parvum* or *U. urealyticum* in the 20 neonates with positive PCR results. Additionally, the highest number of positive neonates (for either *M. hominis*, *U parvum* or *U. urealyticum*) were detected in neonates delivered by vaginal delivery (VD; 6/20; 80.0%; 95% CI: 57.8-

92.5), either normal vaginal delivery (NVD; 13/20; 65.0%; 95% CI: 43.2-82.0) or breech vaginal delivery (3/20; 15.0%; 95% CI: 4.4-36.9). A significant increase (p = 0.0273) in detection of *Ureaplasma* species and *M. hominis* was observed in neonates delivered by vaginal delivery (including: NVD, breech VD and, forceps VD) in comparison to neonates delivered by Caesarean-section (CS). *U. parvum* was detected in four neonates that were delivered via CS (20.0%; 95% CI: 7.5-42.2). A majority of the neonates where *M. hominis* or *Ureaplasma* species were detected survived (15/20; 75.0%; 95% CI: 52.8-89.2). Thirty-five of the neonates had chronic lung disease (CLD) at 36 weeks corrected gestational age (considered to reflect severe CLD), of these 16/35 (45.7%; 95% CI: 30.5-61.8) returned positive results using multiplex real-time PCR: *M. hominis* and *U. parvum* (3/35; 8.6%; 95% CI: 2.2-23.1), *U. parvum* (9/35; 25.7%; 95% CI: 14.0-42.3), *U. urealyticum* (4/35; 11.4%; 95% CI: 3.9-26.6).

A more detailed analysis of five intubated neonates from the South West sample set was undertaken due to either interesting clinical presentation or the presence of a mixed infection.

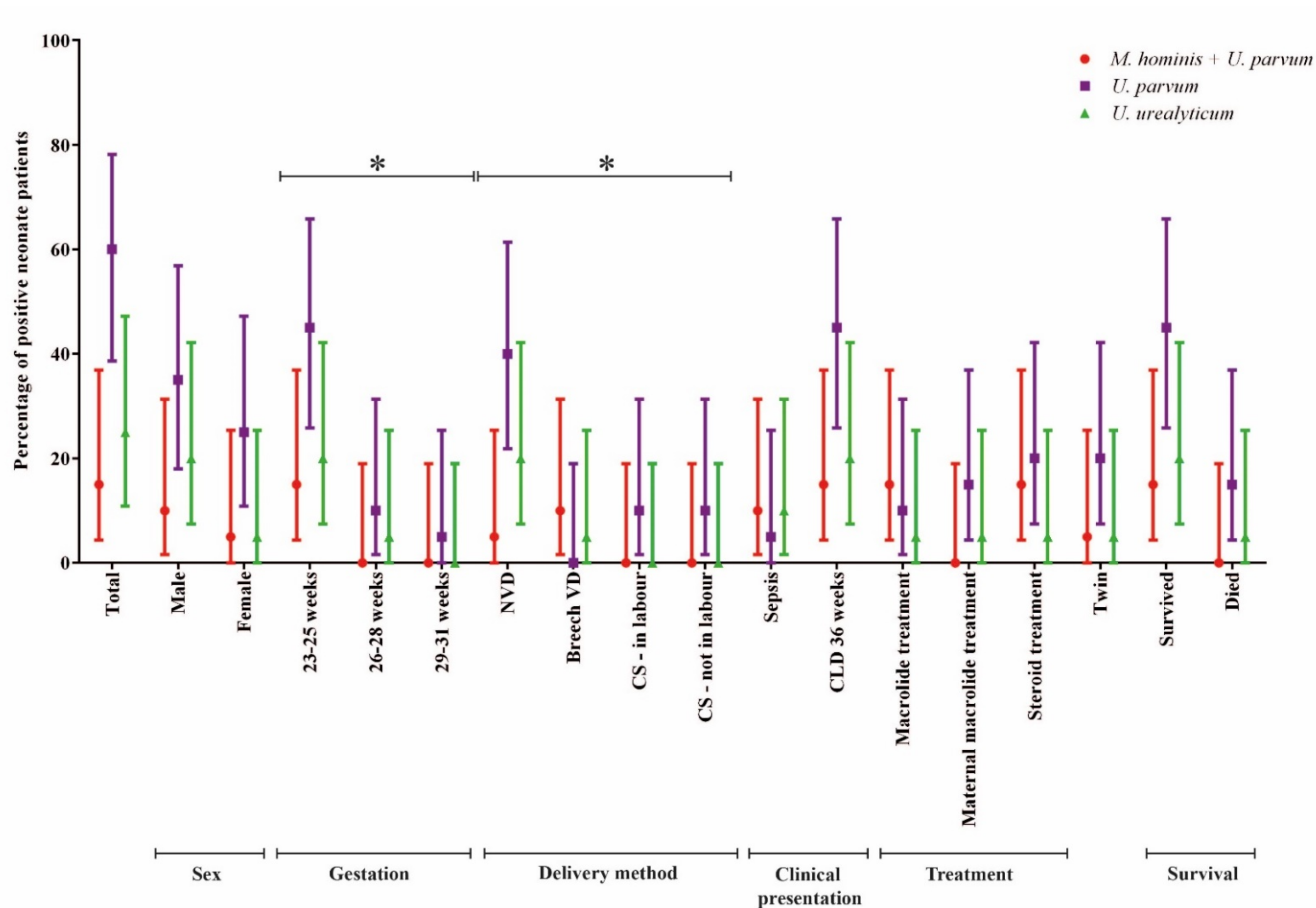


Figure 4.1. Neonatal characteristics and clinical presentation in association with infection by *Mycoplasma hominis* and *Ureaplasma* species

Proportion of positive neonate cases for each *M. hominis* (red), *U. parvum* (purple) and *U. urealyticum* (green) in relation to: sex, gestation, method of delivery, clinical concerns and presentation, treatment, twin, and survival. NVD = normal vaginal delivery; VD = vaginal delivery; CS = Caesarean-section; CLD = chronic lung disease. Data is presented as percentage of positive neonates with 95% CI.* (asterix) indicates significant difference ($p < 0.01$) within the indicated group.

Twin patients

Two neonates from the South West were a set of twins and presented a unique case study where the presenting twin (P130) was positive for *U. parvum* in endotracheal secretions in comparison to the second non-presenting twin (P131) that was positive for *U. urealyticum*.

Twin P130 was transferred from birth hospital to a South West hospital on day 1 post-birth. She was extubated on day 20 to continuous positive airway pressure after commencing diuretics, but as a result of increasing apnoeas, bradycardias and desaturations, mandated re-intubation was commenced on day 23. Re-intubation was accompanied by administration of antibiotics (teicoplanin and gentamicin). Twin P130 was extubated on day 31. In comparison, due to high ventilation requirements and recurrent pulmonary haemorrhages twin P131 was too unstable to be transferred on day 1 post-birth and arrived at a South West hospital on day 5 post-birth. The twin remained on ventilation until death on day 32. Primary cause of death was determined as necrotising enterocolitis with secondary causes listed as prematurity, chronic lung disease of prematurity and intracranial haemorrhage.

Ureaplasma species were detected in endotracheal secretions by both culture and multiplex real-time PCR described in this chapter (Figure 4.2). Samples from P130 were weakly positive by culture for *Ureaplasma* on day 2 post-birth (1 CCU) but progressively increased in titre with a maximum load of 10^4 CCU on day 6 post-birth. *Ureaplasma* titres then decreased and a negative result was obtained by day 24 post-birth. *U. parvum* was detected at day 6 post-birth by multiplex real-time PCR (139.26 copies/µl) with the maximum bacterial load observed at this time point. The bacterial load was 121.02 copies/µl by day 10 post-birth and a negative result was

obtained by day 15 post-birth. However, in contrast to culture, a low positive result (13.18 copies/µl) for *U. parvum* was detected by multiplex real-time PCR on day 24 post-birth, the last sample tested. Samples from P131 were positive for *Ureaplasma* species by culture on day 18 post-birth (10^2 CCU) and increased to high titres (10^7 CCU) by day 24 post-birth which were maintained until the neonate died on day 32 post-birth. High levels *U. urealyticum* were detected at day 24 post-birth (2.84×10^5 copies/µl) by multiplex real-time PCR and the bacterial then decreased to 7.68×10^4 copies/µl by day 31 post-birth.

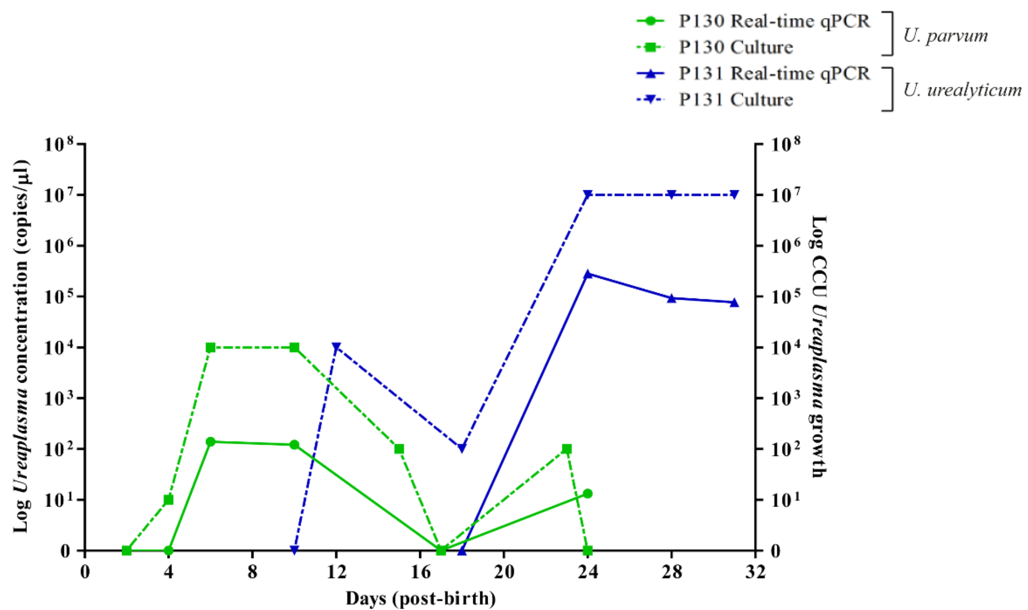


Figure 4.2. Quantification of *Ureaplasma* species by multiplex real-time PCR and culture from twins P130 and P131.
Multiplex real-time PCR (circle + solid line) and culture (square + dashed line) results from endotracheal secretions during the course of infection for a set of neonatal twins. *U. parvum* was detected and isolated from P130 (green) and U. urealyticum was detected and isolated from P131 (blue). CCU = colour changing units.

Effect of steroid administration

Ureaplasma urealyticum was detected in and isolated from a male neonate (P128), delivered via NVD at 25.1 weeks gestation. Neonate P128 had CLD at 36 weeks post-birth and was still supported by a ventilator at this time.

Ureaplasma urealyticum was first detected in ETS at day four post-birth by both culture (50 CCU) and multiplex real-time PCR (2109.51 copies/µL; Figure 4.3). The maximum bacterial load was detected at day 21 post-birth; 1×10^6 CCU and 9.73×10^5 copies/µL for culture and multiplex real-time PCR, respectively. Levels of *U. urealyticum* then decreased and samples at day 30 and day 34 post-birth were found to be negative for *U. urealyticum* by both culture and multiplex real-time PCR. However, at day 35 post-birth, *U. urealyticum* was detected in ETS by both culture (1×10^2 CCU) and multiplex real-time PCR (47.46 copies/µL). By day 41 post-birth culture results indicated the clearance of infection but *U. urealyticum* was still detected by multiplex real-time PCR (1.88×10^3 copies/µL).

Clearance of *U. urealyticum* infection by day 30 post-birth coincides with the administration of azithromycin at day 26 post-birth however the increase in bacterial load detected at day 35 post-birth occurred whilst azithromycin was still being administered (Figure 4.3). Additionally, neonate P128 was also treated with two courses of steroids. The increase of *U. urealyticum* at day 35 post-birth coincided with the re-administration of steroids (Figure 4.3).

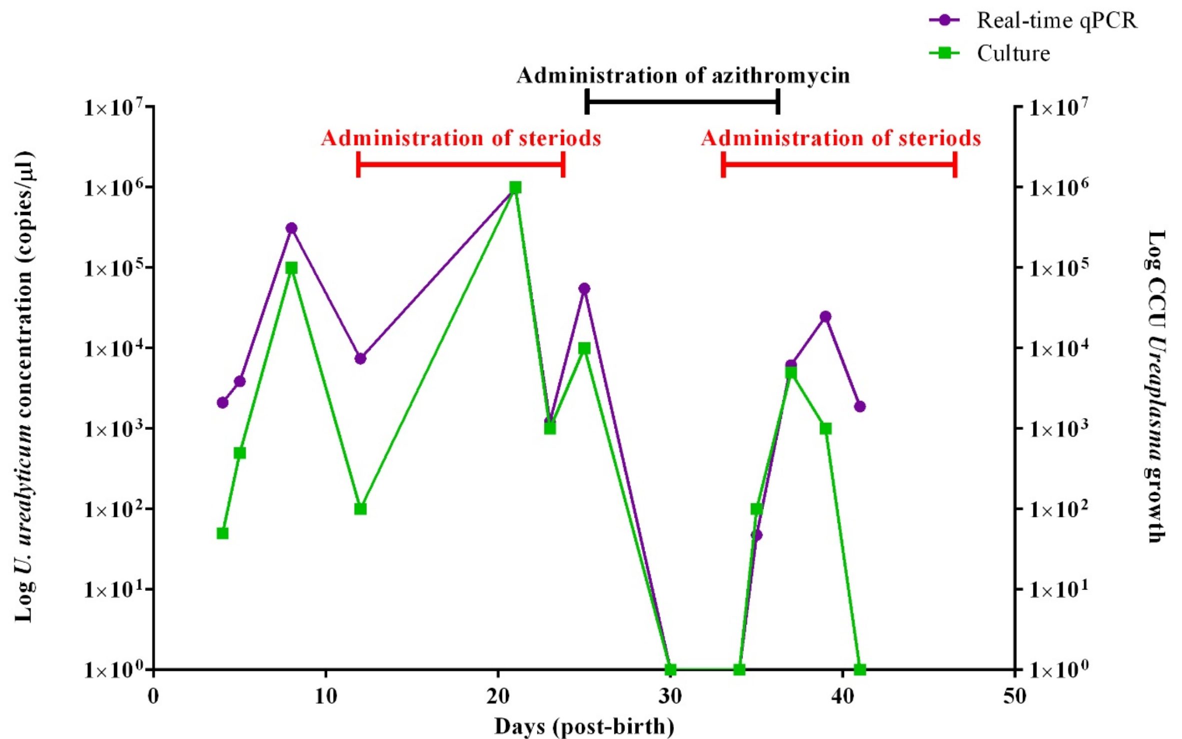


Figure 4.3. Quantification of *Ureaplasma urealyticum* by multiplex real-time PCR and culture from P128.
Multiplex real-time PCR (purple) and culture (green) results from endotracheal secretions during the course of infection for neonate P128. *U. urealyticum* was detected and isolated from P128. CCU = colour changing units. Steroid (red) and macrolide (black) treatment are indicated by square brackets.

Mixed infections

Ureaplasma parvum and *M. hominis* were isolated from two male neonates (P162 and P164), both delivered via breech VD at 24.6 and 23.1 weeks gestation, respectively. Both neonates had CLD at 36 weeks post-birth and both were supported by a ventilator at this time. Both neonates survived.

Neonate P162 was born eight days after rupture of membranes. The first clinical specimen in which either *Ureaplasma* species or *M. hominis* was detected was at day seven post-birth, where *U. parvum* was detected by culture (1 x 10^2 CCU; Figure 4.4). It is important to note that this specimen was not tested using multiplex real-time

PCR. Titres of *U. parvum* increased until day nine post-birth, where *U. parvum* was detected by both culture (1 x 10^4 CCU) and multiplex real-time PCR (3.82 x 10^4 copies/µL). Additionally, at day 12 post-birth, *M. hominis* was detected by real-time qPCR (2.78 x 10^2 copies/µL). Titres of both *M. hominis* and *U. parvum* then declined with negative results at day 14 post-birth for *M. hominis* and at days 12 and 21 post-birth for *U. parvum* detected using multiplex real-time PCR and culture, respectively. This decline in titre coincides with administration of a ten-day course of erythromycin (Figure 4.4) but this is would not have an effect on *M. hominis* titre due to inherent resistance of this bacteria to erythromycin. Upon the end of the course of erythromycin treatment, titres of both *M. hominis* and *U. parvum* then spiked at day 38 post-birth at 3.63 x 10^5 copies/µL and 4.22 x 10^4 copies/µL (1 x 10^3 CCU), respectively. This could be due to treatment failure or, less likely, re-infection. A second course of antibiotics (azithromycin) was administered at day 42 post-birth, coinciding with negative multiplex real-time PCR and culture results for both *M. hominis* and *U. parvum* at day 44 post-birth (Figure 4.4).

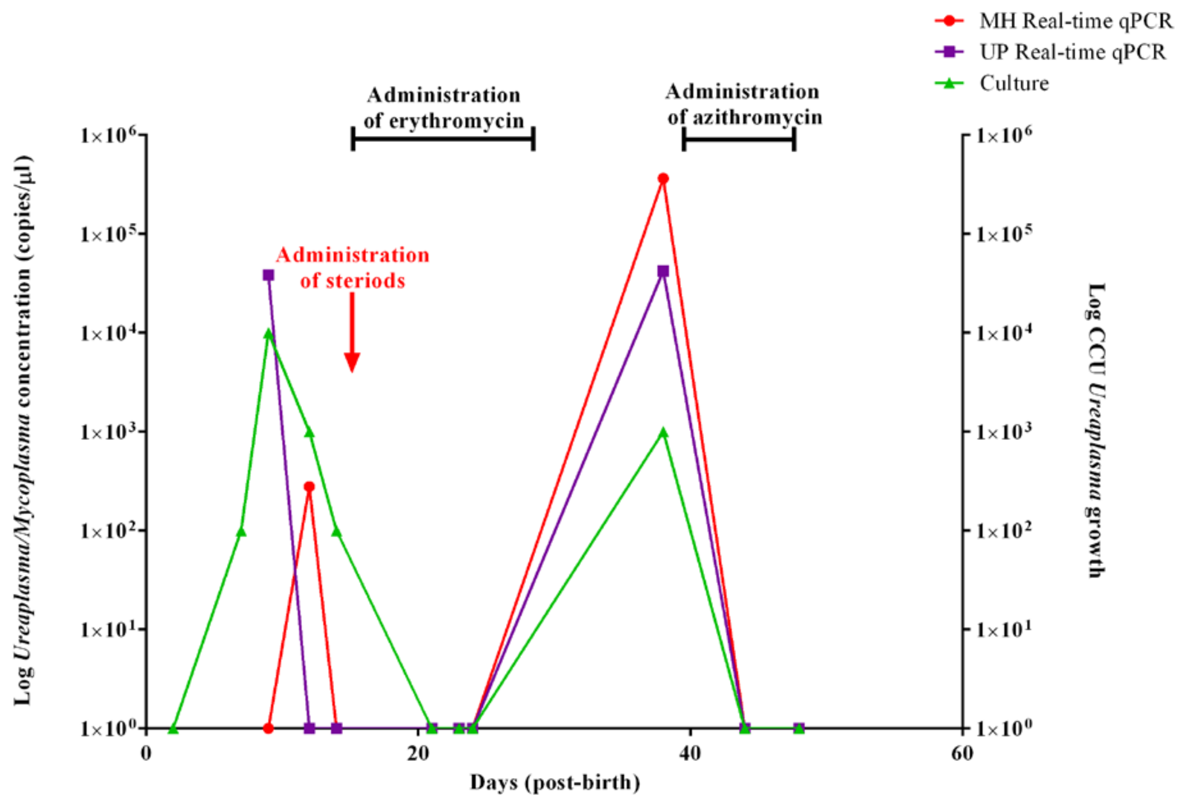


Figure 4.4. Quantification of *Mycoplasma hominis* and *Ureaplasma parvum* by multiplex real-time PCR and culture from P162.
Multiplex real-time PCR *M. hominis* (red), *U. parvum* (purple) and culture (green) results from endotracheal secretions during the course of infection for neonate P162. *U. urealyticum* and *M. hominis* was detected and isolated from P162. CCU = colour changing units. Steroid (red) and macrolide (black) treatment are indicated by square brackets.

Ureaplasma parvum was first detected in ETS from neonate P164 at day four post-birth by culture (1 x 10^1 CCU; Figure 4.5). Titres of *U. parvum* then rose to a peak at day 11 (plus 18 hours) post-birth (1 x 10^2 CCU; 2.76 x 10^3 copies/µL) and then rapidly dropped resulting in negative results by both multiplex real-time PCR and culture at day 12 post-birth. Following this initial peak, *U. parvum* was again detected in ETS at day 17 post-birth by multiplex real-time PCR (4.38 x 10^4 copies/µL) and by day 19 post-birth using culture (1 x 10^2 CCU). Additionally, *M. hominis* was detected in the ETS at day 17 post-birth by multiplex real-time PCR (1.39 x 10^2 copies/µL;

Figure 4.5). Both titres of *U. parvum* and *M. hominis* peaked at day 19 post-birth, 7.45 x 10^5 copies/µL and 1.13 x 10^5 copies/µL, respectively. By day 22 post-birth multiplex real-time PCR and culture results were negative for *U. parvum* and *M. hominis*. However, *U. parvum* was detected by multiplex real-time PCR at day 45 post-birth (4.19 x 10^3 copies/µL) but this re-infection was not consistent with culture results (Figure 4.5).

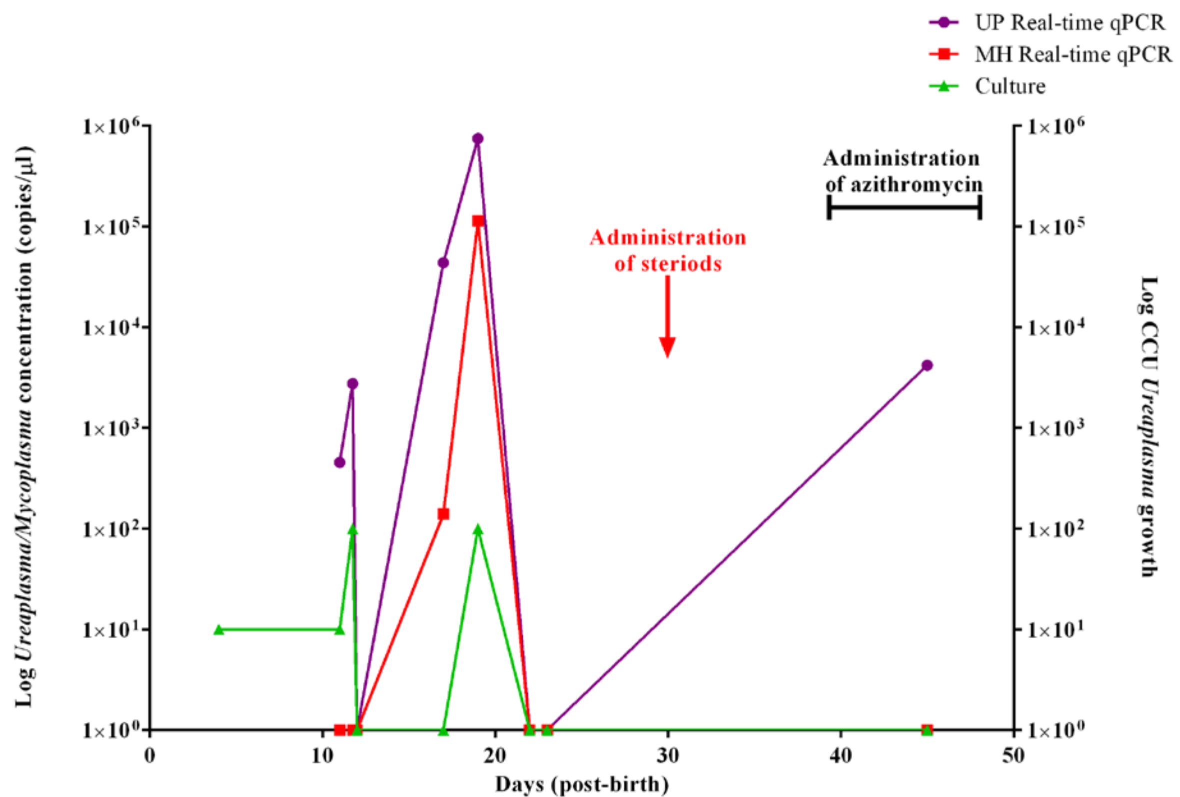


Figure 4.5. Quantification of *Mycoplasma hominis* and *Ureaplasma parvum* by multiplex real-time PCR and culture from P164.
Multiplex real-time PCR *M. hominis* (red), *U. parvum* (purple) and culture (green) results from endotracheal secretions during the course of infection for neonate P164. *U. urealyticum* and *M. hominis* was detected and isolated from P164. CCU = colour changing units. Steroid (red) and macrolide (black) treatment are indicated by square brackets.

4.2.1.1 Detection of *Ureaplasma* infection following clarithromycin treatment

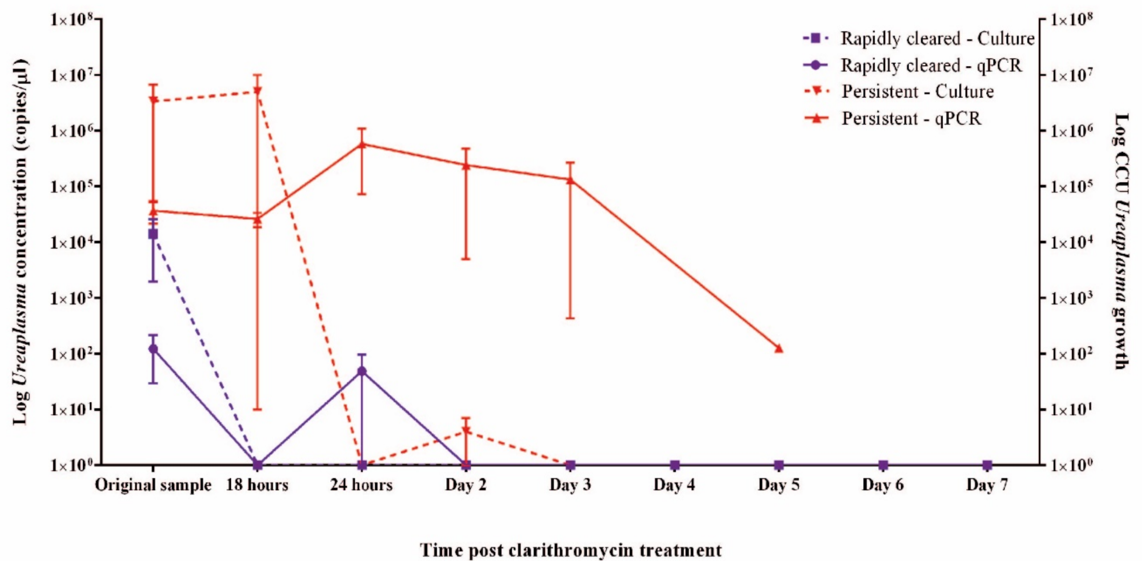
Thirty-four clinical specimens, collected in Wales, from eight intubated neonates were investigated to examine clearance of infection following treatment with clarithromycin. Clinical specimens (ETS) were taken before (time-point 0) and at multiple time points following clarithromycin treatment.

Ureaplasma species were detected in seven of the eight neonates, of which six were positive for *U. parvum* and only one positive for *U. urealyticum*. *M. hominis* was not detected in any of the clinical specimens. The positive neonates could be grouped into two groups: those that had no detectable infection by day two post clarithromycin treatment (rapidly cleared *Ureaplasma* DNA) and those for which reduction in detectable infection took longer than two days (persistent *Ureaplasma* DNA). The rapidly cleared group contained four neonates, in which *U. parvum* was detected in ETS specimens by both multiplex real-time PCR and culture. Of these four neonates, all but one had no detectable *U. parvum* DNA in the ETS by 24 hours post clarithromycin treatment (Figure 4.6). Indeed, one neonate had non-detectable *U. parvum* infection by 18 hours post clarithromycin treatment, with negative results at this time point by both multiplex real-time PCR and culture.

The persistent group contained three neonates, in which *U. parvum* was detected in two neonates and *U. urealyticum* in one. When examining culture results for these three neonates, infection with *Ureaplasma* species was undetectable by day three post clarithromycin treatment, with no growth observed (Figure 4.6). However, when examining multiplex real-time PCR results, *Ureaplasma* genomic DNA continued to persist with no negative result detected in the specimens collected (Figure 4.6 B) up to day 5 post-treatment. For two of the three neonates the latest time-point

at which a sample was obtained was day three post clarithromycin treatment, at which point titres for *U. parvum* were still high by multiplex real-time PCR (7.77×10^3 copies/µL and 2.66×10^5 copies/µL) in contrast to the negative culture results. The latest time-point collected was for the third neonate in this group, which was positive for *U. urealyticum*, where a specimen was collected at day five post clarithromycin treatment. This specimen was positive for *U. urealyticum* by multiplex real-time PCR (1.27×10^2 copies/µL) however, it was negative by culture.

A.



B.

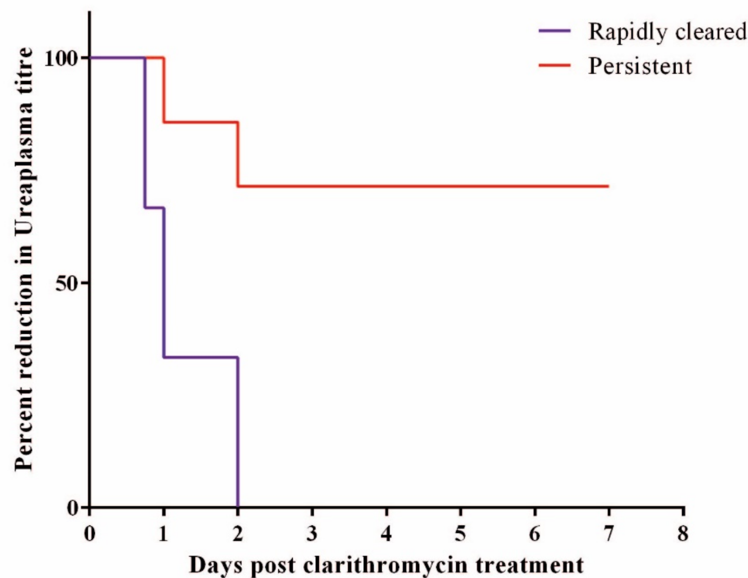


Figure 4.6. Detection of *Ureaplasma* species infection in intubated neonates after treatment with clarithromycin.
Samples from seven neonates from Wales were grouped into "rapidly cleared" (purple) and "persistent" (red) depending on the length of time post clarithromycin treatment for *Ureaplasma* infection to clear. The sensitive group contain samples from four neonates and the resistant group contained samples from three neonates. A. qPCR (solid line) and culture (dashed line) results for sensitive and resistant groups plotted as mean ± SEM. B. Survival curve showing percent reduction in *Ureaplasma* titre determined by qPCR for sensitive and resistant groups. Reduction in titre was defined as a 10-fold reduction in DNA concentration or a negative qPCR result.

4.2.2 Determination of minimum inhibitory concentration for *Mycoplasma hominis*

Broth-dilution and agar methods can be used for susceptibility testing of *M. hominis* to different antimicrobial agents; however; the subtle colour change caused by the growth of *M. hominis* in MM renders MIC determination by broth-dilution challenging. Additionally, the supplementation of Mycoplasma-selective Agar with antimicrobial agents can have varying, batch-dependent results.

The MIC was determined for 16 *M. hominis* strains using the antimicrobial agents: gentamicin and tetracycline, by broth-dilution and agar methods as described in section 2.5. Additionally, MIC was monitored using the multiplex real-time PCR method described in this chapter by assessing *M. hominis* DNA concentration after incubation in MM supplemented with varying concentrations of antimicrobial agent. Using this method, DNA concentration is indicative of *M. hominis* growth and a reduction in DNA concentration to the base-line level (same inoculum) indicates lack of growth at that particular antimicrobial concentration (Figure 4.7).

Minimum inhibitory concentrations of gentamicin and tetracycline for *M. hominis* can be seen in Table 4.1, including the control strain PG21. Results between all three methods were consistent for six (6/16; 35.7%) *M. hominis* strains for gentamicin MIC determination and for seven (7/16; 43.75%) *M. hominis* strains for monitoring the response to tetracycline. Of the ten strains where MIC calculations were inconsistent for gentamicin, eight (8/10; 80%) *M. hominis* strains had consistent results between broth-dilution and qPCR methods and only two (2/10; 20%) *M. hominis* strains had differing results for each method used. The Clinical & Laboratory Standards Institute (CLSI) breakpoints define any isolate that has an MIC by broth or agar ≥ 8 µg/mL as resistant, while an MIC of ≤ 4 µg/mL is defined as

sensitive [374]. All *M. hominis* strains, except one strain, were sensitive to gentamicin with a range of MICs between 1 µg/mL to 4 µg/mL using the broth-dilution method. The exception, MH12 had an MIC of 8 µg/mL by both broth-dilution and qPCR methods, however an MIC of 2 µg/mL was calculated using the agar monitoring method. Of the nine strains where results between MIC determination methods were inconsistent for tetracycline, eight (8/9; 88.89%) *M. hominis* strains had consistent results between broth-dilution MIC determination and qPCR monitoring methods. One (1/9; 11.11%) *M. hominis* strain had differing results using all three methods for tetracycline MIC determination. Fifteen *M. hominis* strains were sensitive to tetracycline with a range of MICs between 0.125 µg/mL to 8 µg/mL however, one strain (MH9) has a high tetracycline MIC determined as 16 µg/mL by broth-dilution and agar-based methods, and a level indicative of >16 µg/mL by qPCR monitoring.

Inconsistency in MIC calculation using the methods described is predominantly due to the agar-based method. In all, bar two inconsistent results, the MIC for either gentamicin or tetracycline was higher when determined using the agar-based method in comparison to the broth-dilution method and qPCR monitoring. This is confirmed by the CLSI guidelines, where MIC ranges calculated from broth-dilution are 2-fold dilution lower than the agar method ranges for susceptibility for moxifloxacin, levofloxacin and clindamycin [374]. However, only both-dilution MIC ranges are available for tetracycline susceptibility and gentamicin was not examined.

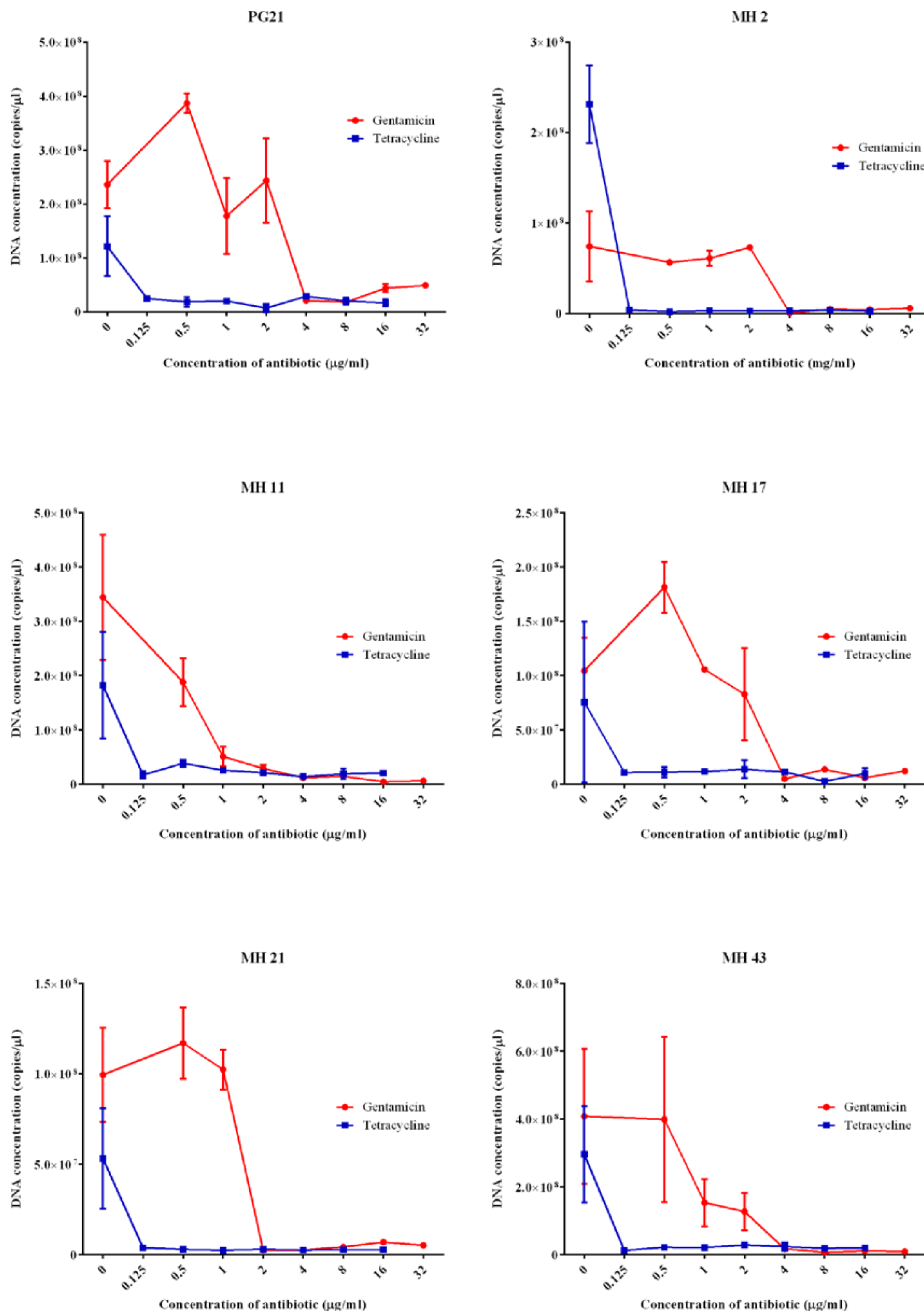


Figure 4.7. Example graphs to calculate the MIC of *M. hominis* for both gentamicin and tetracycline by real-time qPCR.
M. hominis DNA concentration following treatment with gentamicin (red) or tetracycline (blue) is indicated for six representative *M. hominis* strains.

Table 4.1. Gentamicin and Tetracycline MIC calculation for *M. hominis* by broth-dilution and agar methods, and monitoring using qPCR.

Strain	Gentamicin MIC (µg/ml)			Tetracycline MIC (µg/ml)		
	Broth-dilution	Agar	qPCR	Broth-dilution	Agar	qPCR
PG21	4	4	4	0.125	0.125	0.125
MH2	4	4	4	0.125	8	0.125
MH9	4	4	4	16	16	>16
MH10	2	2	2	0.125	4	8
MH11	2	8	2	0.125	0.125	0.125
MH12	8	2	8	0.125	1	0.125
MH17	2	8	4	0.125	0.125	0.125
MH18	2	4	2	0.125	1	0.125
MH20	2	4	2	0.125	0.5	0.125
MH21	2	2	2	0.125	1	0.125
MH23	2	4	2	0.125	1	0.125
MH26	2	8	2	0.125	0.125	0.125
MH27	2	4	2	0.125	0.125	0.125
MH41	2	2	2	0.125	0.125	0.125
MH43	1	16	4	0.125	2	0.125
MH44	4	32	4	0.125	8	0.125

4.2.2.1 Determination of tetracycline resistant *M. hominis*

A total of 54 *M. hominis* isolates were examined for the presence of antibiotic resistance to tetracycline by PCR. The presence of the tetracycline-resistance mediating transposon, *tetM*, was detected by PCR (described in section 2.5.4) in five *M. hominis* strains (MH9, MH104, MH108, MH110 and MH111; in Appendices Figure A.1). Sensitivity to tetracycline was determined for four of these strains using the qPCR method. Additionally, the MIC for tetracycline for MH9 was calculated by broth-dilution and agar-based methods.

The MIC for three of the *M. hominis* strains (MH9, MH108 and MH110) was determined to be >16 µg/mL, higher than the published standardised range (0.12-1 µg/mL) [235] and higher than the CLSI guidelines that determine resistant strains as having an MIC ≥ 8µg/mL [374], indicating that these strains are resistant to tetracycline (3/20; 15% incidence). One strain, MH111, was sensitive to tetracycline (MIC: 1 µg/mL) despite the presence of *tetM* (Figure 4.8).

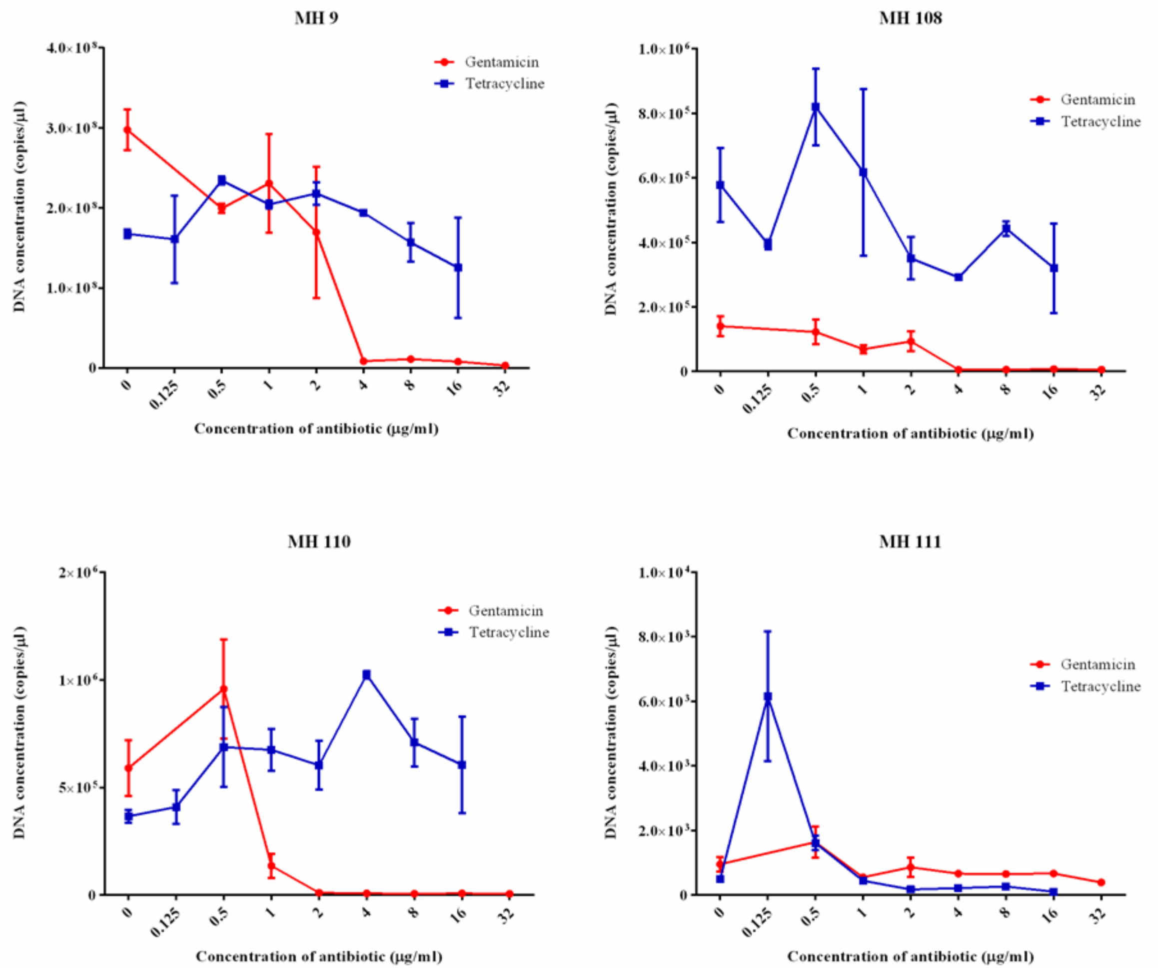


Figure 4.8. Example graphs to calculate the MIC of *M. hominis* containing *tetM* for both gentamicin and tetracycline by real-time qPCR.
M. hominis DNA concentration following treatment with gentamicin (red) or tetracycline (blue) is indicated for four representative *M. hominis* strains containing *tetM*.

4.3 Discussion

Ureaplasma species have been linked to disease such as non-gonococcal urethritis as well as septic arthritis in hypogammaglobulinemic patients, but the patient group which has been the focus of this chapter have been premature neonates. *Ureaplasma* species have been strongly linked as an infectious cause of premature birth where the organism can additionally be transmitted from mother to child either *in utero*, as a result of an ascending infection from the mother's urogenital tract, or acquisition during birth.

4.3.1 Neonatal twin case study

This chapter presents a case study of dizygotic twins both with *Ureaplasma* positive endotracheal aspirates samples, but interestingly harbouring two separate species.

For P130 the titres of *U. parvum* obtained from samples rapidly increased from the first sample, but a sample taken following reintubation on day 23 indicated complete natural clearance of *Ureaplasma*. Although this negative result coincided with the administration of teicoplanin and gentamicin these will not have an impact on *Ureaplasma* due to the pathogens intrinsic resistance to these antibiotics [375]. The MIC of gentamicin for *U. parvum* is given as > 32 µg/mL which is not achievable *in vivo* due to nephrotoxicity [375].

With respect to P131, the first sample was not taken until day 10 post-birth as poor patient clinical status precluded transport to the research hospital. This sample was weakly positive for *U. urealyticum*. From day 24 through to death samples were positive for a high titre of *U. urealyticum*. This high titre coincided with death as a result of NEC. The role of *Ureaplasma* species in the development of NEC has

received greater attention in recent years. *Ureaplasma* are thought to induce NEC by both direct and indirect mechanisms. *Ureaplasma* have been detected directly in gastric aspirate samples which may then result in the generation of a local inflammatory response within the intestinal region. In addition, the ingestion of amniotic fluid containing pro-inflammatory mediators, as a result of *in utero* infection, also have a contributory role [376, 377]. Work by Okogbule-Wonodi *et al.*, have reported that neonates born <33 weeks and colonised with Ureaplasma have a 2-fold greater risk of developing NEC with the risk increasing to 3.3-fold in those born <28 weeks [371].

Previous reports have shown discordance in microbial infection between twins and have examined the role of *in utreo* infection during twin pregnancies. Romero *et al.*, carried out amniocentesis and culture on a cohort of 46 women presenting with twin deliveries [378]. They identified 11% with at least one sac positive for microbial growth to then result in preterm labour and 12% to result in premature birth. Ureaplasma were identified in three out of the five mothers and always in the presenting twin (never from the non-presenting alone). Unfortunately this study predated the differentiation of Ureaplasma species. In a second study examining microbial invasion of preterm twins, only the presenting twin was infected in five out of nine cases, and microbial titre was always higher for the presenting twin in the remaining cases where both twins were infected [379]. *Ureaplasma* species were the most commonly observed pathogen in this study. A retrospective analysis study examining a greater number of twins (1156 twin placentas) further confirmed that the non-presenting twin was significantly less likely to experience chorioamnionitis when compared with the presenting twin [380].

4.3.2 Effect of steroid treatment on the detection of *M. hominis* and *Ureaplasma* species infection

The single case study reported in this chapter regarding neonate P128 provides no clear evidence to the effect of steroid treatment on the clearance of *Ureaplasma* infection. This study showed an increase in *U. urealyticum* titres, following previous clearance of infection, by both q-PCR and culture with re-administration of steroids. This occurred whilst a course of azithromycin was still being administered. However, titres of *U. urealyticum* also declined during steroid treatment.

Antibiotic administration is an effective standard therapy for bacterial lung infections, like pneumonia. However, rapid release of bacterial cell wall components has been implicated in exaggerated host inflammation-induced lung injury [381, 382]. Systemic glucocorticoid steroids have diverse anti-inflammatory and immunomodulatory properties [383, 384], and administering them in addition to antibiotics may constitute a useful method for treating pneumonia. However, adjunctive glucocorticoid steroid therapy for pneumonia has long been a subject of debate. A randomised placebo-controlled clinical trial showed that a significant reduction in hospital stay was associated with adjunctive steroid treatment [385]. In addition, studies have suggested that administration of steroids in patients with CAP might be beneficial, particularly in severe cases [386, 387]. Conversely, other studies have shown no benefit, or indeed harmful outcomes, of steroid treatment for pneumonia [388-390]. Furthermore, glucocorticoid steroids have been shown to impair host bacterial clearance from the pulmonary system, particularly at high doses [391, 392].

Currently, there is no documentation of the effect of steroid treatment on *Ureaplasma* species or *M. hominis* infection. However, the use of steroids as

combination therapy with antibiotics has been examined for *M. pneumoniae* [393]. In an experimental model of *M. pneumoniae* respiratory tract infection, it was found that monotherapy with clarithromycin had the greatest effect on reducing concentrations of *M. pneumoniae* in bronchoalveolar lavage (BAL) but combination therapy had the greatest effect on decreasing levels of cytokines and chemokines as well as pulmonary histologic inflammation [393].

4.3.3 Monitoring of infection with *Ureaplasma* species following clarithromycin treatment

Ureaplasma species infection was not detected in six of the seven neonates studied in this chapter following clarithromycin treatment. Four neonates (rapidly cleared *Ureaplasma* DNA group) did not have detectable infection after two days post-clarithromycin treatment whereas the other three neonates (persistant group) did not have detectable infection by day 3 (culture results). However, PCR results for the persistent group indicated high levels of *Ureaplasma* species DNA at day 3 post-clarithromycin treatment (last sample for two of the neonates). It should be noted that treatment with clarithromycin was a two-week course and therefore the continuing course of antibiotics could have an effect on bacterial load at a later time-point. As this was a retrospective study, further clinical samples at later time-points were not available. However, it would be interesting to assess *Ureaplasma* species detection and reduction in detectable load/colony isolation numbers following clarithromycin treatment with a larger patient cohort and over a time period that would allow reduction of less sensitive strains of *Ureaplasma* to be observed. This would also allow discrepancies between culture and PCR methods to be further addressed.

Macrolide resistance has been described in *Ureaplasma* species, with *in vitro* resistance associated with mutations in the 23S rRNA and L4 and L22 ribosome-

associated proteins [394]. Further characterisation for antibiotic resistance of the resistant group of clinical specimens would provide further evidence for delayed clearance of infection by clarithromycin. Research previously published within the research group has analysed *Ureaplasma* isolated from one of these neonates from the resistant group (UHWO10). This isolate was found to be resistant to erythromycin, azithromycin and clarithromycin with a mutation documented in the L4 protein gene; 6 bp deletion resulting in the deletion of two amino acids [299].

Due to toxic effects of tetracyclines and fluoroquinolones on bone and cartilage development, their use in the treatment of neonates is limited, therefore macrolides are the antibiotic of choice for the treatment of *Ureaplasma* and *M. hominis* infections. Clarithromycin, a 14-membered ring macrolide, is commonly used for the treatment of *Ureaplasma* infection in neonates. Several studies show that macrolides affect many inflammatory processes including the migration of neutrophils, oxidative burst in phagocytes, and production of pro-inflammatory cytokines [395]. Clarithromycin has been reported to inhibit superoxide production by activated neutrophils and also has a membrane-stabilising activity [396]. In addition to this, clarithromycin has also been found to suppress interleukin 1β gene expression in human nasal epithelial cells [397]. These anti-inflammatory effects may contribute to successful treatment of severe pulmonary conditions caused by *Ureaplasma* [398]. Previously, clarithromycin treatment for neonates in which *Ureaplasma* species were detected by PCR, showed an improved clinical outcome and ultimately eradication of *Ureaplasma* species infection [399].

4.3.4 Review of methods used to calculate minimum inhibitory concentration

Methods for *in vitro* antimicrobial susceptibility testing of mycoplasmas were first described in the 1960s [400] however, until 2001 there was no universally

accepted or standardised broth dilution- or agar-based methods. These were further clarified and limited break points for four to five antibiotics for each *M. hominis*, *U. urealyticum* and *M. pneumoniae* were published in 2011 and 2012 [235, 374]. Prior to this the lack of a consensus method for MIC determination, coupled with the complex growth requirements of mycoplasmas, resulted in considerable confusion regarding the antimicrobial activities of various drugs against mycoplasmas.

Standardised antimicrobial susceptibility testing methods and designated quality-control parameters for human *Mycoplasmas* and *Ureaplasmas* were deemed necessary because culture is seldom performed for diagnostic purposes, and *in vitro* testing of individual isolates is even more rarely obtained. Antimicrobial susceptibilities can vary geographically and in response to selective antimicrobial pressure. Moreover, clinically significant acquired drug resistance, potentially affecting multiple antimicrobial classes, can occur in all of the *Mycoplasma* and *Ureaplasma* human pathogens. Most *Mycoplasma* and *Ureaplasma* infections are treated empirically, therefore obtaining accurate and reproducible antimicrobial resistance surveillance data for currently available antibiotics and new therapeutics is important.

The standardised methods described by Waites *et al*., [235] and the CLSI guidelines [374] require a defined inoculum of 10^4 to 10^5 CFU/mL. The broth-dilution technique utilised in this chapter, as described by Beeton *et al*., [299], allows for simultaneous quantification of bacteria in the presence and absence of antibiotic, providing a more rapid approach for sensitivity determination. However, broth-dilution techniques require the accurate reading of colour changes in liquid media, which for *M. hominis* can be difficult due to subtle colour changes. Using quality-controlled media, like Mycoplasma Experience Ltd. Mycoplasma-selective Media

(used throughout this thesis), aids with this challenge but batch variation and inconsistencies in colour change still proved a challenge.

To aid the determination of MIC for *M. hominis*, real-time PCR was used to monitor the quantity of the bacteria in the presence and absence of antibiotic. Consistent results from agar-, broth- and PCR-based methods were obtained for 36% and 44% of *M. hominis* isolates for gentamicin and tetracycline, respectively. Higher consistency was observed between broth- and PCR-based methods; 88% and 94% of *M. hominis* isolates for gentamicin and tetracycline, respectively. The inconsistency observed with the agar-based method may be due to the necessary incubation time required for growth of *M. hominis* and storage of media; it is generally accepted that agar containing antibiotics has a shelf-life of 72 hours before the antibiotics start to denature [235]. Therefore, incubation or storage of the media for longer than 72 hours would alter the concentration of antibiotic in the media, affecting the MIC. It is important to note that the use of PCR to monitor response to antibiotics in this chapter represents a method to putatively detect and monitor phenotypic resistance, as bacteria are grown in medium containing antibiotic and quantified using qPCR.

Quantitative PCR has been used to monitor the MIC for *C. trachomatis* [401] and the chlamydia-related bacterium *Waddlia chondrophila* [402]. Results from these studies showed that the MIC calculated using qPCR were within already published ranges and proved a reliable, less time-consuming and more sensitive method for monitoring the bacterial response to antibiotics.

4.3.5 Tetracycline resistance

Tetracyclines are one of the first-line treatments of *M. hominis* infections [239], with the exception of paediatric treatment. High-level resistance to

tetracyclines in *M. hominis* has been associated with the presence of the *tetM* transposable element [280, 281], which is the only observed mechanism of tetracycline resistance in clinical isolates of *M. hominis* [239]. The presence of *tetM* was identified in five *M. hominis* strains with phenotypic resistance observed in three of the five strains (Section 4.2.2.1). An increase in tetracycline-resistant *M. hominis* has been observed, with 18.75% resistance (1999-2002) compared to 2.8% (1992) in Bordeaux, France [403]. Indeed, increased antimicrobial resistance in genital mycoplasmas has been reported globally [404-406]; for example 40% of *M. hominis* detected in vaginal specimens in Iran were tetracycline-resistant (2015) [407]. Country-dependent antibiotic prescription guidelines and recommendations may account for the varying tetracycline resistance patterns observed globally. Antibiotic resistance is often determined using molecular methods, such as PCR followed by sequencing, to identify mutations associated with resistance, and phenotypic susceptibility testing is not always undertaken.

As shown in this chapter, the presence of the *tetM* gene does not necessarily confer phenotypic resistance to tetracyclines. Degrange *et al* identified two *tetM*-positive *M. hominis* isolates that were tetracycline susceptible [403]. One of these *M. hominis* isolates had a 1,260 bp insertion in the leader peptide sequence (likely preventing successful transcription), while no mutations were found within the *tetM* gene or promoter region of the second isolate; no mutations were observed within the *tetM* sequence itself. This has also been observed in *Ureaplasma* [403]. Therefore interrogation of the sequence of *tetM* did not elucidate the lack of function and subsequent susceptibility to tetracyclines. This confirms the requirement for phenotypically determined susceptibility of an organism to antimicrobial agents, as genotypic resistance does not necessarily confer phenotypic resistance. Conversely,

in one *tetM* positive, tetracycline-sensitive *M. hominis* strain, tetracycline resistance was induced *in vivo* during short-term passage in media containing tetracycline with no changes in sequence observed [403]. This observation led to suggestions that *tetM* regulation might involve transcriptional attenuation, as described for *Enterococcus faecalis* [408], and that tetracycline-susceptible strains that carry *tetM* should be reported as potentially resistant to all tetracyclines [403].

A further explanation for the lack of expression may be due to gene silencing. A study by Enne *et al.*, demonstrated that in a selection or *E. coli* isolates, all of which containing the pVE46 resistance plasmid, a sub-population were phenotypically sensitive [409]. The plasmid DNA sequence of the sensitive isolates were found to be identical to that of the resistant strains, but interestingly when the plasmid from the sensitive strain were introduced to a different *E. coli* strain, the resistant phenotype was restored. This suggested that chromosomal differences were to explain these differences in gene silencing and could potentially be the mechanism for *tetM* silencing in *M. hominis*.

4.3.6 Summary

The multiplex real-time PCR assay developed in Chapter 3 has multiple uses, along with a diagnostic purpose. It can be used to quantify bacterial load within clinical specimens and monitor clearance of infection, and be used as a novel method for monitoring the bacterial response to antibiotics, enhancing information obtained by traditional MIC determination in *M. hominis*. Tetracycline resistance was observed in *M. hominis* and the presence of the transferable genetic element *tetM* was confirmed in resistant isolates. In addition, a *tetM* positive, phenotypically sensitive *M. hominis* isolate was identified.

Chapter 5. Molecular typing and characterisation of *Mycoplasma hominis*

5.1 Introduction

Mycoplasma hominis is an opportunistic human pathogen and resides as a commensal on the mucosal surfaces of the cervix or vagina in 21 to 53% of sexually mature, asymptomatic women; this is somewhat lower in the urethra of males [3]. However, *M. hominis* has also been associated with clinically diverse diseases including; urogenital diseases [311, 312], postpartum fever [313], pneumonia [410], meningitis [186, 205], post-operative wound infection [201], post-organ transplant infection [411] and septic arthritis [187, 189]. Although this organism has only been isolated from humans, the capacity of *M. hominis* to cause disease as a sole pathogen has been proven by induction of preterm labour and development of foetal chronic lung disease following experimental *in utero* administration of *M. hominis* to pregnant macaque monkeys [184].

Current discriminatory methods for typing of *M. hominis* to improve understanding of epidemiology of infection and genetic diversity are not in clinical use. Several molecular typing mechanisms have been developed for *M. hominis* including: pulsed-field, gel electrophoresis (PFGE) [329, 412], restriction fragment length polymorphism (RFLP) analysis [413], amplified length polymorphism (AFLP) [414] and random amplified polymorphic DNA (RAPD) [415]. These techniques have displayed poor reproducibility (RAPD), requirement for specialised equipment and time required (PFGE) and large quantities of biological material. Typing based on sequence analysis of the *p75*, *p120'* and *vaa* genes was not comparable, indicating that the three genes vary by different mechanisms (16-19).

Previous studies have indicated that the surface antigenic profiles of *M. hominis* strains are highly heterogeneous, expressing both size and phase variants of surface exposed membrane proteins [78, 86-88]. Several *M. hominis* surface proteins have

been characterised including P120, P135 and Vaa; however, the molecular basis of variation in *M. hominis* has only been elucidated in some cases. The mechanisms involved in the diversification of mycoplasma surface proteins are highly complex and include: size variation caused by gain or loss of intragenic repetitive sequences; phase switching by deletion/insertion mutations or DNA inversion affecting promoter activity; and presence of multigene families or multiple copies of partial genes in the mycoplasmal chromosome [89].

Variation in the composition and size of the Vaa proteins results from allelic variant forms of the single copy *vaa* gene in *M. hominis* [91, 92]. Size variation has been observed in Vaa using the monoclonal antibody H3 that was used to: initially identify this protein, inhibit the growth of *M. hominis*, and block attachment to host cells [78]. The size of Vaa observed in different isolates ranged from 28 kDa to 72 kDa and resulted from the gain or loss of intragenic repetitive sequences. These repeats form the basis of “modules” which provide a platform for further separation of Vaa types into categories. *M. hominis* can be characterised based on the size variation observed in Vaa variants. This variation derives from interchangeable cassette sequences within Vaa; Modules III, IV, V, VII and VIII.

Multiple locus variable-number tandem-repeat (VNTR) analysis (MLVA) has been used to successfully subtype other *Mycoplasma* species [132, 416-419]. However, when used for *M. hominis,* high genetic heterogeneity was found resulting in a method that was limited for use at individual level studies, it being too discriminatory for large epidemic studies [133].

Multi-locus sequence typing (MLST) is a typing system using housekeeping genes considered to be under less selective pressure than other genes. It has been

successfully employed for many mycoplasma species such as *M. bovis* [420], *Mycoplasma agalactiae* [421], *M. hyorhinis* [422], *M. hyopneumoniae* [423] and *M. pneumoniae* [424]. A previous study examined six housekeeping gene sequences to investigate evidence of genomic recombination in *M. hominis* [362] and revealed a high degree of variability between these genes. However, the authors did not utilise the data to create a genotyping scheme.

The ability to discriminate strains of *M. hominis* can provide information to study transmission chains in nosocomial, maternal and neonate infection, and increase understanding of sexual transmission of the organism in the community.

In this results chapter, *M. hominis* was characterised based on sequences of the *vaa* gene and derived amino acid sequences, resulting in the assignment of a Vaa type. Genetic diversity of *M. hominis* was investigated using: sequence based typing (SBT) of eight housekeeping genes, genomic sequence SNP analysis and, bioinformatics analysis of genomic sequence to further characterise *M. hominis*.

5.2 Results

5.2.1 Characterisation of *Mycoplasma hominis* using the variable adherence-associated antigen

5.2.1.1 Vaa cassette determination and variability

Vaa category was determined as described in Section 2.4.6, where Vaa type is based on module composition shown in Figure 1.3, Vaa category was determined either by PCR of the entire open reading frame or from whole genome sequence and subsequent amino acid composition prediction for 31 *M. hominis* isolates. Thirteen isolates belonged to category 1, 12 to category 2, one to category 3 and five to category

4. Deduced amino acid sequences were aligned against prototype strains for each Vaa category observed (Figure 5.1-Figure 5.4). Strains in Vaa category 1 showed very high homology for modules I, II, and II" with 92.6%, 94.8% and 100% homology, respectively (Table 5.1). Lower levels of homology were observed in modules III and IV; however this was still above the 82% homology threshold suggested by Boesen *et al* [97]. As observed in category 1, high levels of homology were observed for modules I, II and II' for strains belonging to categories 2, 3 and 4 (Table 5.1). In the strains analysed, the modules that contain the highest levels of variation occur at the C-terminal end of the interchangeable cassette region, module V and module VIII in Vaa category 1 and Vaa category 2, respectively. In both cases, these modules fail to maintain the 82% homology between isolates required for inclusion in a particular module, with observed homology of 72.0% and 77.0% for module V of category 1 and module VIII of category 2, respectively. However, the sequences of these strains share higher homology with the module to which they are assigned than to other described modules present in the interchangeable cassette region. In Vaa category 4, varying numbers of Module III have been documented and in the five clinical isolates determined to have Vaa category 4 proteins, only two repeats of the module are present. In comparison, the prototype for Vaa category 4 used as a comparison, *M. hominis* 1620, had three repeats of Module III.

In all strains analysed, the SFKE and ELESFKE motifs were conserved in module II and in the interchangeable cassettes, respectively. The conserved tryptophan residues can also be observed in the interchangeable cassettes (Figure 5.1-Figure 5.4).

```
                Module I                                        Module II                                     Module II''
MH11     MKKSKKIFITLCGIAATAVLPVATISCNDDKLAEKNGKEKADAALKQANALVEELKKNPDYSKILETLNKEIAEATKSFKEAGSYSDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH13     MKKSKKIFITLCGIAATAVLPVATISCNDDKLAEKNGKEKADAALKQANALVEELKKNPDYSKILETLNKEIAEATKSFKEAGSYSDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
PG21     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH28     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH27     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPEIISKLSAAVENAKSEQQKVDQANKKIADEN
MH107    MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH106    MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYSDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH127    MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
FBG      MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
MH120    MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANTLAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
MH131    MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANTLAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
MH16     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKSEQQKVDQANKKIADEN
MH29     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANTLAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
MH20     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN
MH41     MKKSKKIFITLCGIATTAILPVATISCNDDKLAEKNGKEKADAALKQANALAEELKKNPDYSKILETLNKEIAEATKSFKEAGSYGDYPAIISKLSAAVENAKNEQQKVDQANKKIADEN

                                                              Module III
MH11     LKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSAEVKTFATVNTIKKDFVLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKAEDDKKLAE
MH13     LKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSAEVKTFATVNTIKKDFVLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKAEDDKKLAE
PG21     LKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFEIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH28     LKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFEIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH27     QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH107    QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKEQLISTIELLNKKSVEVKTFATVNTIKKDFVLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKAEDDKKLAE
MH106    QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFVLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKAEDDKKLAE
MH127    QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
FBG      QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH120    QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH131    QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSGLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH16     QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNNSWLEKIVSEWEEVKKAWSKELAEIKSDDDKKLAE
MH29     QKIKEGAKELLKLSEKTQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH20     QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE
MH41     QKIKEGAKELLKLSEKIQSFADTIALTITKLEGKKFQIDETFKKQLISTIELLNKKSVEVKTFATVNTIKKDFLLSELESFKEFNTSWLEKIVSEWEEVKKAWSKELAEIKADDDKKLAE

                                                 Module IV
MH11     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKSLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKAEWVKVQEAWKNELAEI----NGVEE
MH13     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKSLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKAEWVKVQEAWKNELAEI----NGVEE
PG21     ENQKIQNGIAELTKLSKENSDLAQTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELTEINSIIKGVEE
MH28     ENQKIQNGIAELTKLSKENSDLAQTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELTEINSIIKGVEE
MH27     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELTEINSIIKGVEE
MH107    ENQKIQNGIAELSKLSKENSDLEKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFISTISKKTEFVLEELESFKELNTLQFNEIKTEWAKVQETWKNELAEI----NGVEE
MH106    ENQKIQNGIAELSKLSKENSDLEKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFISTISKKTEFVLEELESFKELNTLQFNEIKTEWAKVQETWKNELAEI----NGVEE
MH127    ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NDVEE
FBG      ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH120    ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH131    ENQKIQNGIAELSKLSKENSDLAQTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH16     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH29     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH20     ENQKIQNGIAELSKLSKENSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
MH41     ENQKIQNGIAELSKLSKKNSDLAKTIEETIAKLEKKFQIPKDFKEKLTSTIKLLNKKANEINTFVSTVSKKTEFVLEELESFKELNTLQFNEIKTEWAKVQEAWKNELAEI----NGVEE
```

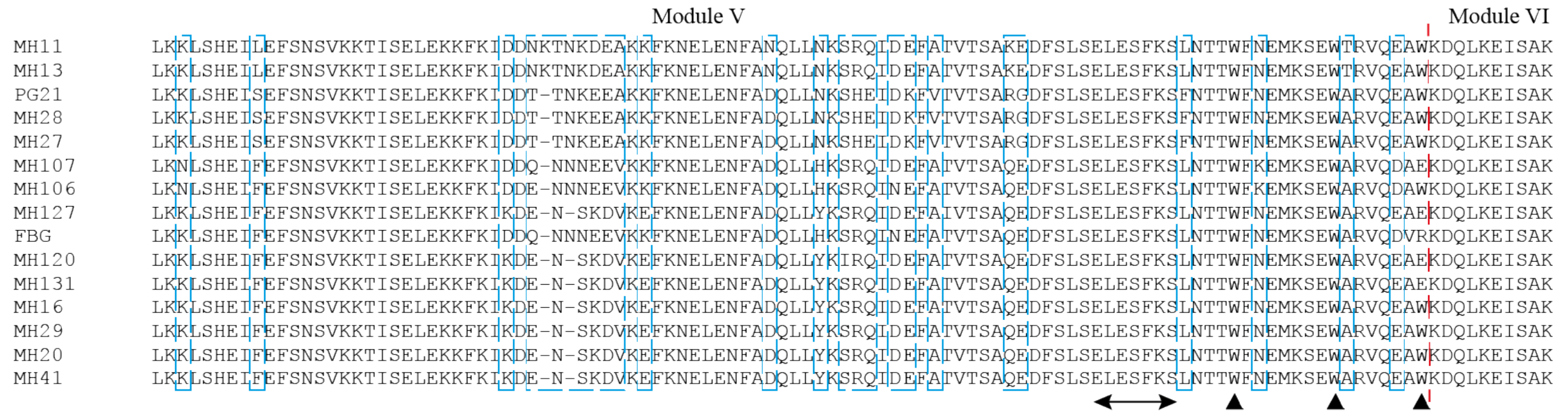


Figure 5.1. Amino acid alignment of Vaa category 1 strains. Amino acid sequence alignments for 13 *M. hominis* clinical isolates and two prototype strains, FBG and PG21.

Modules are separated by red dotted lines; polymorphisms are highlighted by blue boxes; conserved tryptophan residues indicated by triangle; and conserved ELESFKE motif indicated by arrow.

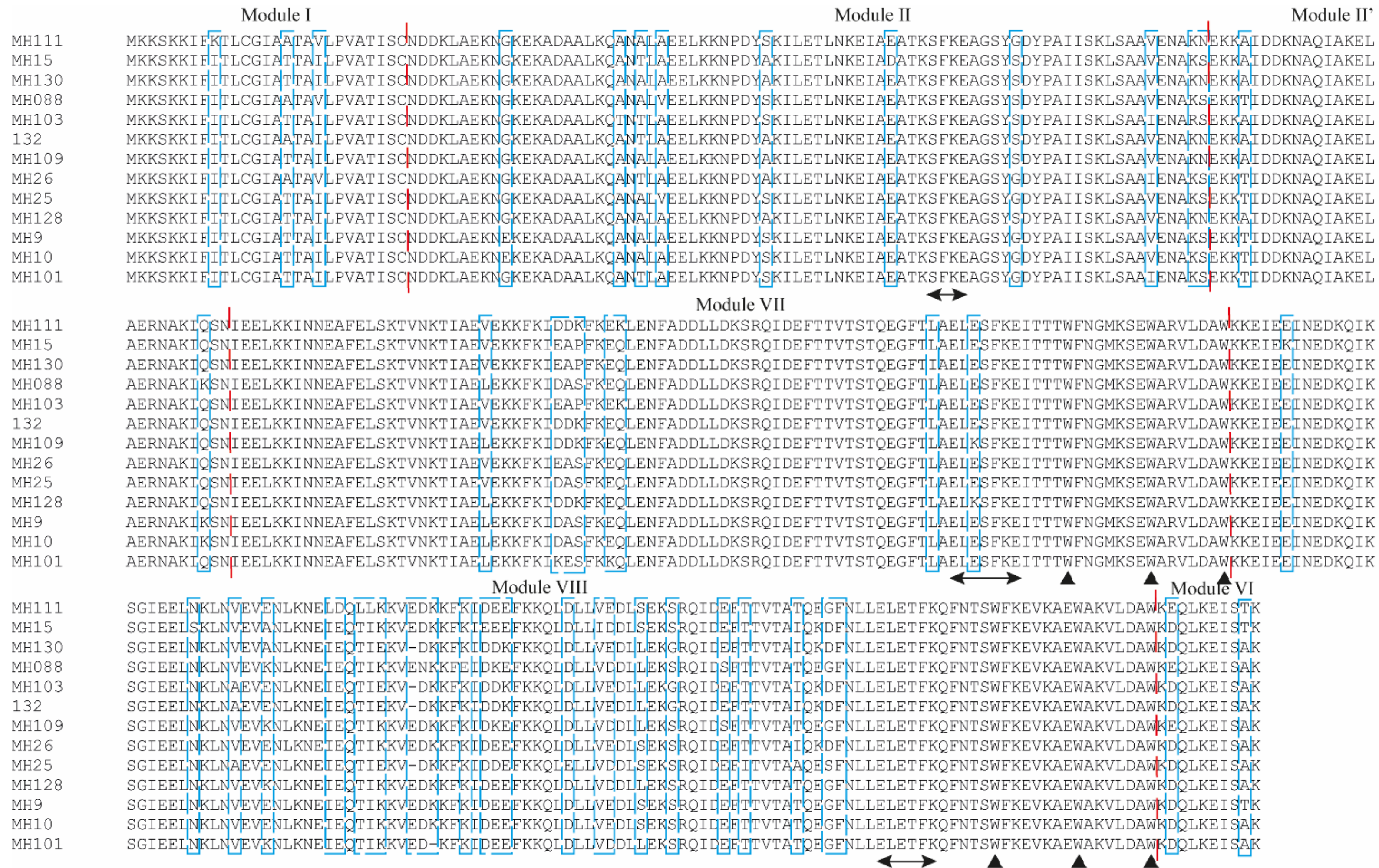


Figure 5.2. Amino acid alignment of Vaa category 2 strains.
Amino acid sequence alignments for 12 *M. hominis* clinical isolates and a prototype strain, 132. Modules are separated by red dotted lines; polymorphisms are highlighted by blue boxes; conserved tryptophan residues indicated by triangle; and conserved ELESFKE motif indicated by arrow.

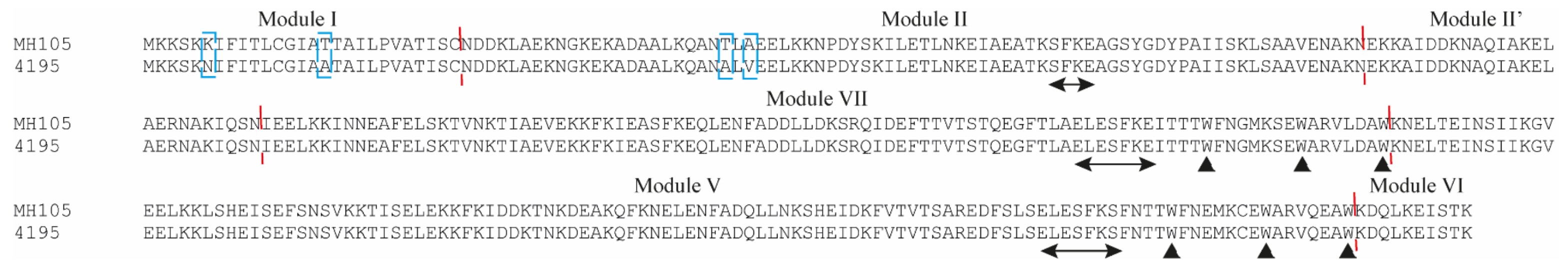


Figure 5.3. Amino acid alignment of Vaa category 3 strains.
Amino acid sequence alignment for a single *M. hominis* clinical isolates and one prototype strain, 4195. Modules are separated by red dotted lines; polymorphisms are highlighted by blue boxes; conserved tryptophan residues indicated by triangle; and conserved ELESFKE motif indicated by arrow.

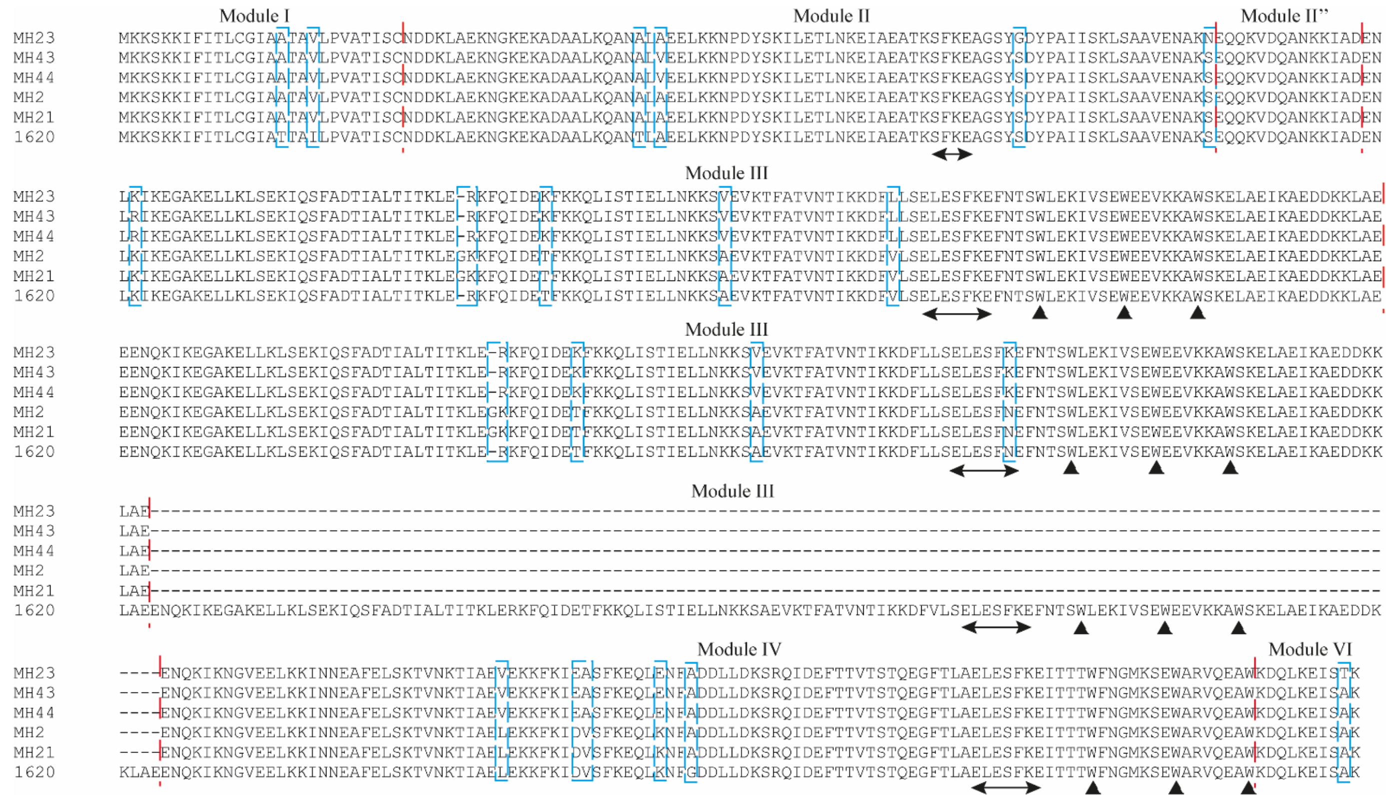


Figure 5.4. Amino acid alignment of Vaa category 4 strains.
Amino acid sequence alignments for 5 *M. hominis* clinical isolates and a prototype strain, 1620. Modules are separated by red dotted lines; polymorphisms are highlighted by blue boxes; conserved tryptophan residues indicated by triangle; and conserved ELESFKE motif indicated by arrow.

Table 5.1. Variation of amino acid sequence in *M. hominis* Vaa categories.
The number of polymorphic sites in the amino acid sequence for each module is shown with the percentage homology shown in brackets, between the Vaa sequences determined in this study and prototype strains. Total number of strains analysed, including prototype strains, are shown in brackets.

		Number of polymorphic sites (% polymorphic sites)									
Vaa Category	**No. of *M. hominis* strains**	**Module I**	**Module II**	**Module II'**	**Module II''**	**Module III**	**Module IV**	**Module V**	**Module VI**	**Module VII**	**Module VIII**
1	13 (15)	2 (92.6%)	4 (94.8%)		0 (100.0%)	10 (91.8%)	10 (90.4%)	33 (72.0%)	0 (100.0%)		
2	12 (13)	3 (89.9%)	10 (87.0%)	2 (92.3%)					2 (80.0%)	8 (91.7%)	26 (77.0%)
3	1 (2)	2 (92.6%)	2 (97.4%)	0 (100%)				0 (100.0%)	0 (100.0%)	0 (100.0%)	
4	5 (6)	2 (92.6%)	4 (94.8%)		0 (100.0%)	6 (95.1%)	5 (95.2%)		1 (90.0%)		

Blank spaces indicate that the module is not present in the Vaa category

5.2.1.2 Phylogenetic analysis of *vaa*

Phylogenetic analysis of the *vaa* gene of 31 *M. hominis* clinical strains and five prototype strains showed four distinct clusters (Figure 5.5; method described in section 2.4.8.2), corresponding to the four Vaa categories observed. Phylogenetic relationships were more apparent using the Neighbour-joining method, where clear separation between Vaa categories could be observed. Vaa categories 1 and 2 and Vaa categories 3 and 4 co-localised to the same major clade, respectively. SNP analysis of the *vaa* gene for the 36 *M. hominis* strains resulted in 26 ATs with a discriminatory ability of 0.72 ATs per strain. Examination of the Hunter-Gaston DI (DI, ranges from 0.0 = no diversity to 1.0 = complete diversity; method described in Section 2.7) confirmed large diversity in this gene (DI: 0.981; 95% CI: 0.967-0.994). However, when Vaa category, based on amino acid sequence, was examined as a discriminatory method it resulted in a discriminatory ability of 0.11 categories per strain and Hunter-Gaston DI showed reduced diversity in the protein (DI: 0.684; 95% CI: 0.612-0.756). The Vaa antigen is a major adhesin of *M. hominis* and displays pronounced mutational variation in size as well as sequence and antigenic variation, therefore the heterogeneity observed in the nucleotide sequence of the 36 *M. hominis* isolates examined is not unexpected.

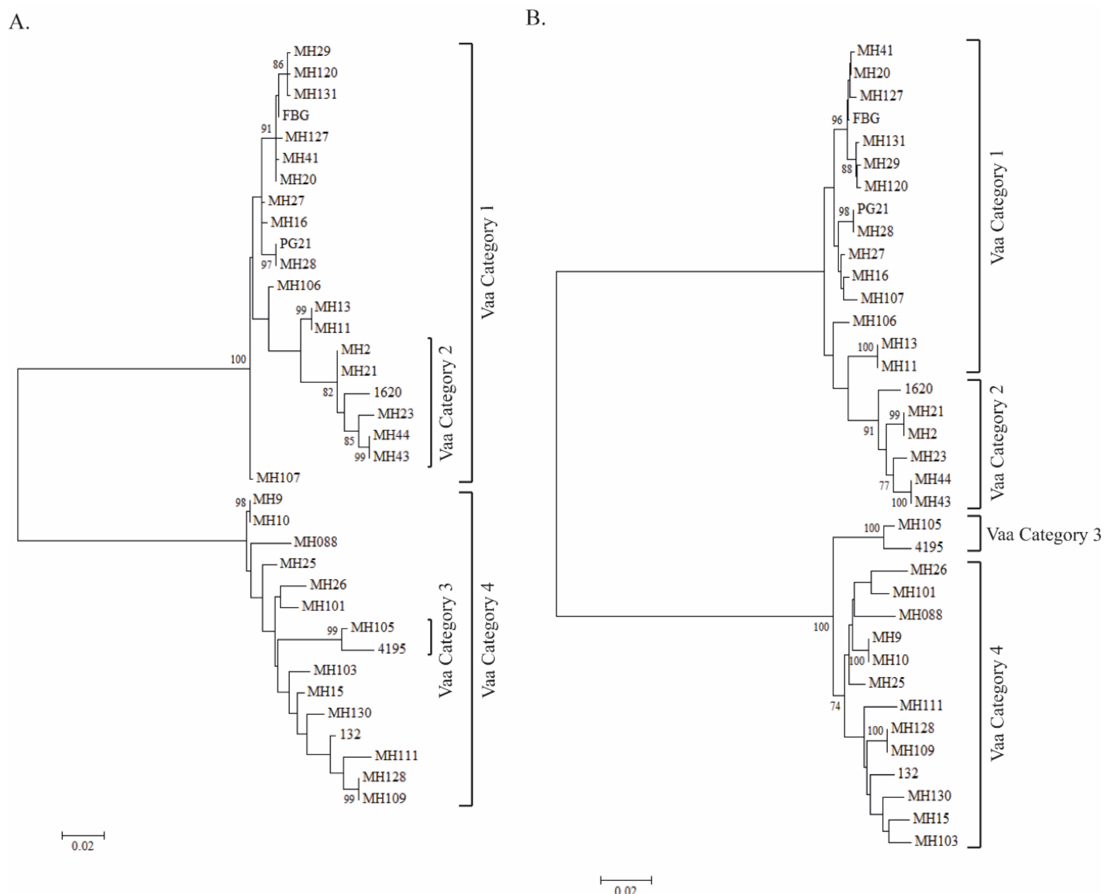


Figure 5.5. Phylogenetic trees based on the sequence of the *vaa* gene of *M. hominis*.
Phylogenetic trees were constructed based on the nucleotide sequence of the *vaa* gene for 31 clinical *M. hominis* strains and five prototype strains using Maximum likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown. Vaa categories are indicated by square brackets.

5.2.2 Sequence based typing of *M. hominis*

5.2.2.1 Determining sequence based typing loci

Housekeeping genes considered conserved in other bacterial species under a low rate of selective pressure were chosen for analysis (Table 5.2). Locus sequences were selected using the available genome sequence of *M. homins* PG21 (NC_013511.1). Ten genes were included for initial analysis: recA protein (*recA*), inorganic phosphatase (*ppa*), cell division protein FtsZ (*ftsZ*), lysyl-tRNA synthetase (*lysS*), methionine-tRNA ligase (*metG*), serine hydroxymethyltransferase (*glyA*),

nitrogen fixation protein NifS (*nifS*), ATP synthase subunit α (*atpA*), carbamate kinase (*arcC*), and adenylate kinase (*adk*). After initial PCR amplification and sequencing of all loci (methods detailed in sections 2.4.2, 2.4.3 and 2.4.5) with the available *M. hominis* strains, *ppa* and *nifS* were excluded from the SBT scheme due to amplification failure in multiple strains.

Table 5.2. Choice of MLST loci based on targets used in already established bacterial MLST schemes.

Bacterial species	MLST loci [a]							
	adk	***arcC***	***atpA***	***glyA***	***recA***	***ftsZ***	***lysS***	***metG***
Escherichia coli	✓				✓			
Bartonella henselae						✓		
Chlamydia trachomatis				✓			✓	
Campylobacter jejuni				✓				
Enterococcus faecium	✓		✓					
Haemophilus influenzae	✓				✓			
Helicobacter pylori			✓					
Moraxella catarrhalis	✓							
Neisseria meningitidis	✓							
Staphylococcus aureus		✓						
Staphylococcus epidermidis		✓						
Streptococcus suis					✓			
Vibrio vulnificus								✓
Yersinia pseudotuberculosis	✓							

[a] MLST loci were chosen based on the frequency of use in other bacterial MLST schemes (http://www.mlst.net/) and the presence of the gene in the published PG21 whole genome.

5.2.2.2 Sequence based typing of *M. hominis*

PCR and sequencing (described in section 2.4.5) of all eight targets for the 21 isolates and type strain resolved a total of 20 STs (Table 5.3). The discriminatory typing ability for *M. hominis* was 0.9 ST per isolate. The number of SNPs observed within each individual locus and the percentage of polymorphic sites are indicated in Table 5.4, with *glyA* having the highest number of SNPs (26 SNPs) and *recA* having the highest percentage of polymorphic sites corrected for sequence length (6.8%). The number of alleles per locus ranged from six (*atpA*) to 13 (*glyA*) (Table 5.4). Examination of the Hunter-Gaston DI indicated large diversity between the STs (DI: 0.987; 95% CI: 0.961-1.000) with the greatest diversity shown in *glyA* (DI: 0.944; 95% CI: 0.903-0.984) and the lowest diversity in *ftsZ* (DI: 0.597; 95% CI 0.369-0.826).

Table 5.3. Description of *M. hominis* strains used in this study, their sequence type (ST) and allelic profile.

Strain	Year of isolation	Isolation site	ST	Allelic profile								MLST Amino Acid ST
				adk	*arcC*	*atpA*	*glyA*	*recA*	*ftsZ*	*lysS*	*metG*	
NCTC 10111	1953	Rectal swab	1	7	3	3	12	4	2	4	2	A
MH2	1989	CSF	2	1	4	2	13	8	5	5	1	B
MH8	2012	Genital	3	8	3	2	6	10	6	7	3	C
MH9	2012	Genital	4	6	5	3	8	5	6	1	3	D
MH10	2012	Genital	5	8	3	2	8	5	6	1	2	E
MH11	2012	Peritoneal fluid	6	5	3	4	6	9	6	8	4	F
MH12	2012	Peritoneal fluid	6	5	3	4	6	9	6	8	4	F
MH13	2012	Peritoneal fluid	6	5	3	4	6	9	6	8	4	F
MH15	2004	Blood culture	7	6	7	5	13	9	6	2	7	C

Strain	Year of isolation	Isolation site	ST	Allelic profile								MLST Amino Acid ST
				adk	***arcC***	***atpA***	***glyA***	***recA***	***ftsZ***	***lysS***	***metG***	
MH16	2004	Neck swab	8	8	4	3	3	2	6	6	5	C
MH17	2004	Unknown	9	8	5	3	2	2	6	6	5	G
MH18	1993	Genital	10	4	8	3	5	1	4	6	6	H
MH20	1990	Genital	11	8	7	6	10	5	6	9	7	C
MH21	1986	Knee aspirate	12	1	4	2	13	8	5	6	1	I
MH22	1990	Breast abscess	13	4	3	2	7	1	4	6	6	H
MH23	2005	Endo-tracheal secretions	14	8	5	1	11	3	3	10	7	G
MH25	2006	Ear swab	15	3	3	2	5	7	1	4	7	J
MH26	2008	Cerebral abscess	16	1	1	2	9	6	6	4	7	K
MH27	2009	Abdominal pus	17	10	2	3	1	9	6	11	7	L
MH28	2004	Pelvic aspirate	18	9	5	1	11	3	7	3	7	M

Strain	Year of isolation	Isolation site	ST	Allelic profile								MLST Amino Acid ST
				adk	***arcC***	***atpA***	***glyA***	***recA***	***ftsZ***	***lysS***	***metG***	
MH29	1989	Pleural fluid	19	6	5	3	9	1	6	10	7	G
MH41	2012	Endo-tracheal secretions	20	2	6	2	4	9	6	4	6	N

Table 5.4. Primer pairs developed in this study and variability of the different loci.

Name		Primer Sequence (5'-3')	T_m (°C)	Amplicon [a] (bp)	Sequence [b] (bp)	Location of MLST locus in CDS	No. of alleles	No. of polymorphic sites	% of polymorphic sites	Average G + C content (%)	Hunter-Gaston Diversity Index [c]	95% confidence interval
adk	F	GAAGTGTACAACGAACACGC	56	676	406	234-639	10	14	3.45	26.85	0.892	0.822-0.962
	R	ATGACCATTTTAAAGCCTCTTCT										
arcC	F	AGTTATTGTCGGACACGGAA	57	735	383	384-766	8	10	2.61	36.03	0.818	0.714-0.922
	R	CCGCCTTCTTCAACGAATTT										
atpA	F	ATTGCCGAAGAATGAATGGC	57	712	327	978-1304	6	6	1.83	33.94	0.771	0.672-0.869
	R	CATTTGCTTCAGAAACGGCT										
glyA	F	CATGGCAATGGTTGATCCTG	57	785	411	450-860	13	26	6.32	34.55	0.944	0.903-0.984
	R	TTGCCGCAGATCCTATTCTT										
recA	F	ACGCTATTGCCGAAATACAA	56	648	309	457-765	10	21	6.80	33.33	0.896	0.826-0.966
	R	TGAAACTATATCACGAGCCCT										
ftsZ	F	GCAAAACTGCTGCTGAATCT	57	714	214	492-705	7	8	3.74	31.78	0.597	0.369-0.826
	R	TGGCGATTACCGAGACAAAT										
lys*S*	F	TTCGTGGCCCATTTATTGTT	56	656	343	307-649	11	19	5.54	30.61	0.909	0.851-0.967
	R	ATGTTTGAAAACGCCTTCAGT										
metG	F	CCAAGTGGTAATCTTCACATAGG	56	646	230	304-649	7	11	4.78	25.65	0.835	0.733-0.938
	R	TGTGTTGCTTTTCAGCATCTT										

[a] Amplicon sequence sizes in base pairs according to PG21 data

[b] Trimmed sequence sizes in base pairs after alignment of the different loci

[c] Hunter-Gaston diversity index (DI, ranges from 0.0 no diversity to 1.0 complete diversity)

5.2.2.3 Phylogenetic analysis of SBT loci

Neighbour-joining and maximum-likelihood trees constructed from concatenated sequences of the eight loci for the 22 *M. hominis* isolates (Figure 5.6; method described in section 2.4.8.1) confirmed a high level of intra-species diversity as indicated by the Hunter-Gaston DI. Three homogenous strains (MH11, M12 and MH13) with identical STs originated from peritoneal fluid of the same patient following a recent renal transplant undergoing organ rejection. Conversely, MH8, MH9 and MH10, originated from a Caesarean-section patient clinical specimen, taken from an area outside, yet proximal to, the surgical wound and had different STs: ST3, ST4 and ST5, respectively. This indicates population variation within a single clinical sample. ST4 and ST5 co-located to the same phylogenetic clade whereas ST3 located to a distal clade suggesting two distinct lineages of *M. hominis* within a single patient specimen.

Neighbour-joining and maximum-likelihood trees constructed for the eight loci individually (Figure 5.7). MH11, MH12 and MH13 consistently co-located to the same clade and were found to be indistinguishable for all loci. Topology of both neighbour-joining and maximum-likelihood trees was consistent for all loci and concatenated sequences.

Unfortunately, other paired specimens including: MH42-MH44, MH13-MH15, and MH123-MH124 were not examined due to poor growth and subsequent DNA yield of the *M. hominis* strains.

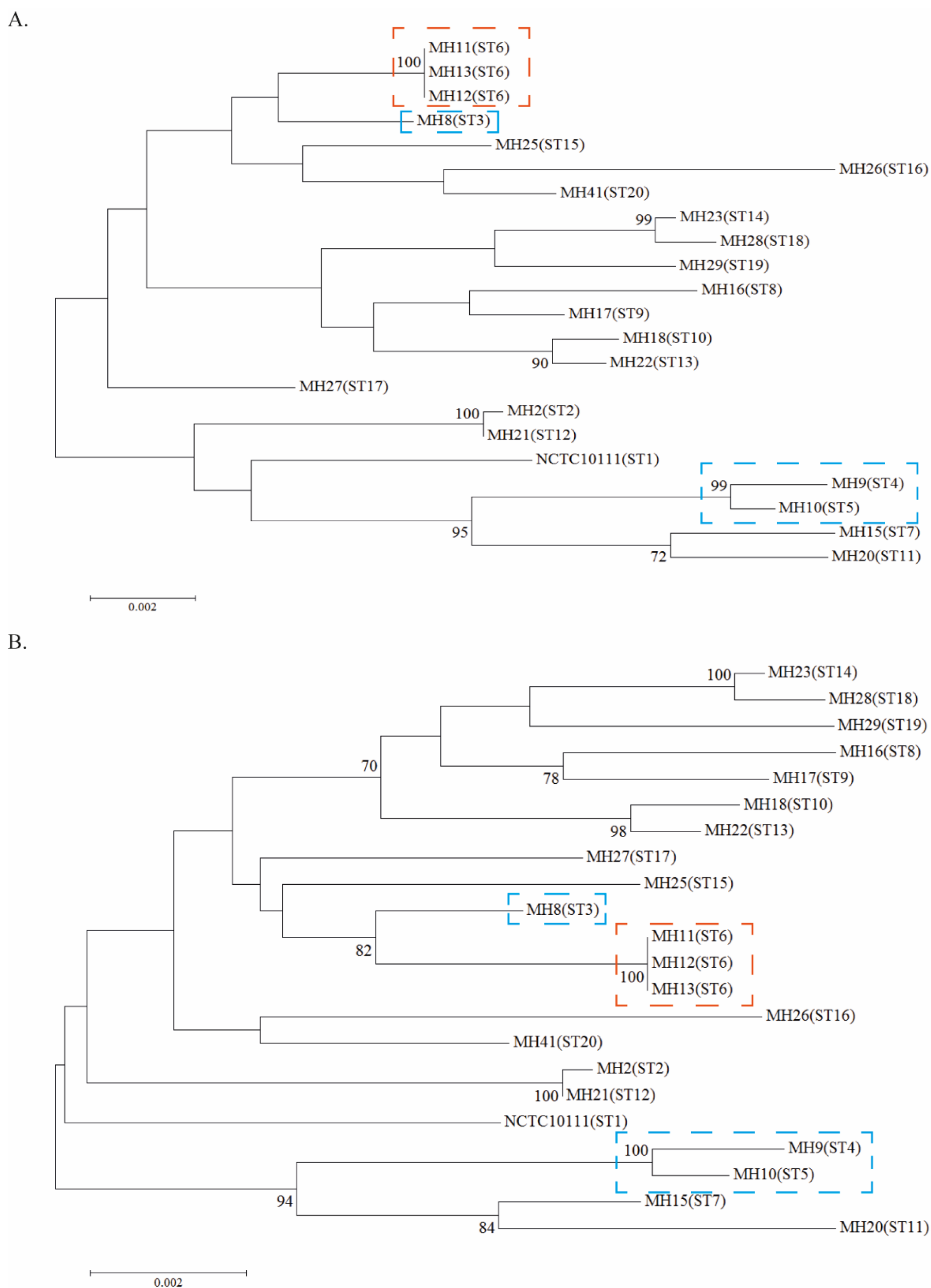
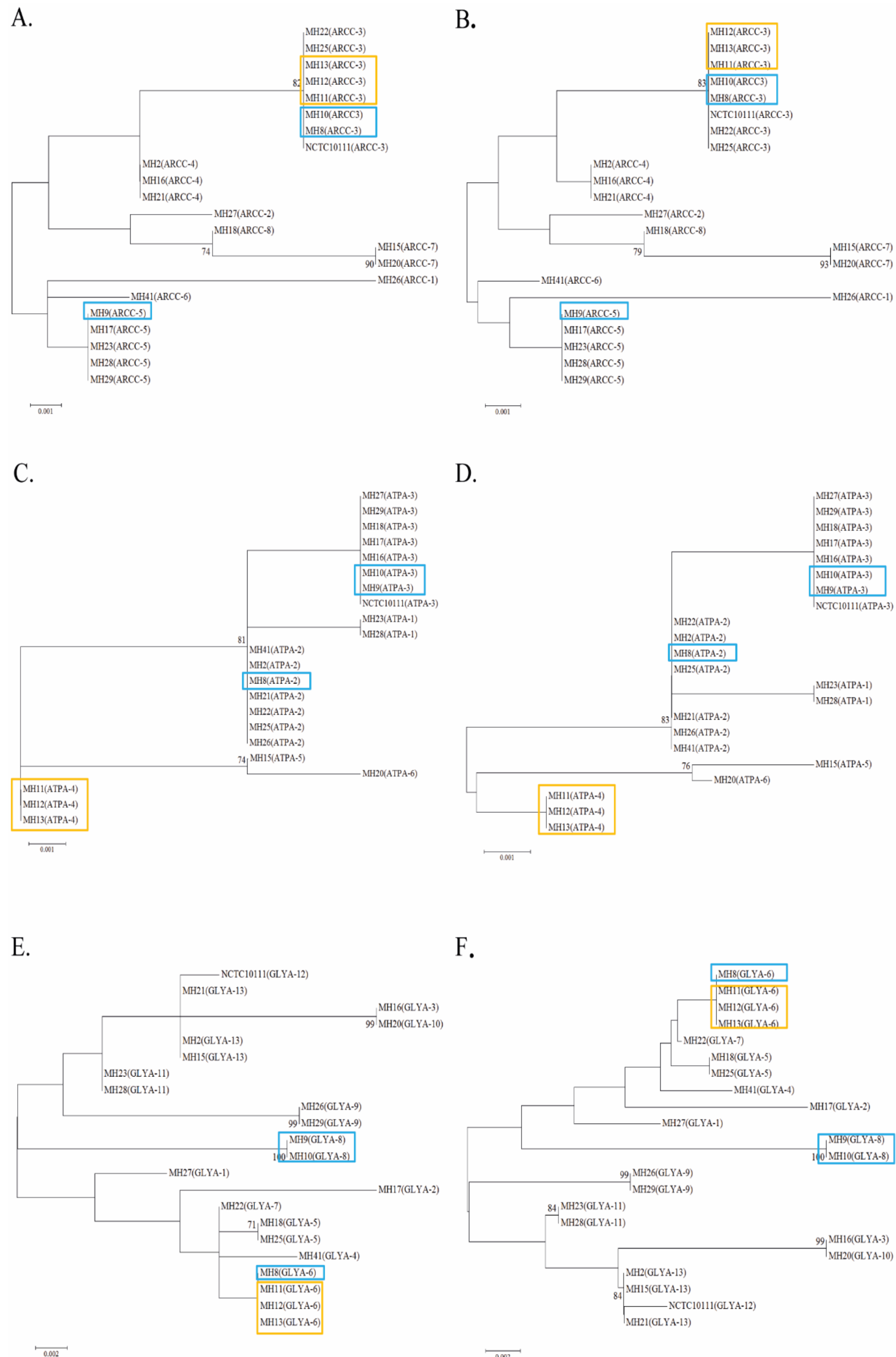
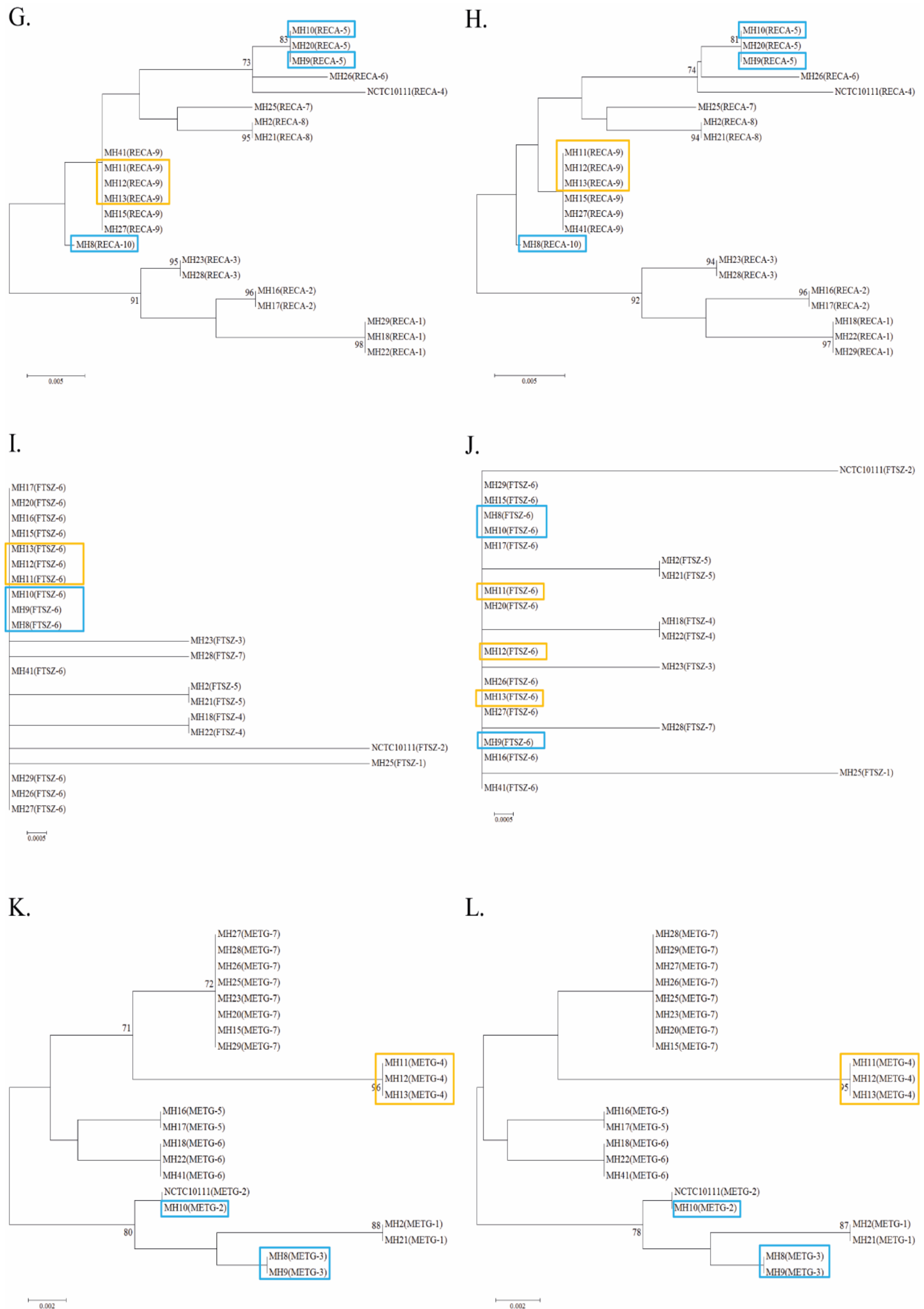


Figure 5.6. Phylogenetic trees based on concatenated sequences of eight SBT loci for *M. hominis*.

Phylogenetic trees were constructed based on concatenated sequences of eight housekeeping loci for 20 unique STs using maximum-likelihood (A) and neighbour joining (B) methods. Strains originating from the same patient sample are indicated by boxes. Bootstrap support values of over 70% are shown.


A.
MH22(ARCC-3)
MH25(ARCC-3)
MH13(ARCC-3)
MH12(ARCC-3)
MH11(ARCC-3)
MH10(ARCC3)
MH8(ARCC-3)
NCTC10111(ARCC-3)
82
MH2(ARCC-4)
MH16(ARCC-4)
MH21(ARCC-4)
MH27(ARCC-2)
MH18(ARCC-8)
74
MH15(ARCC-7)
90
MH20(ARCC-7)
MH26(ARCC-1)
MH41(ARCC-6)
MH9(ARCC-5)
MH17(ARCC-5)
MH23(ARCC-5)
MH28(ARCC-5)
MH29(ARCC-5)
0.001
B.
MH12(ARCC-3)
MH13(ARCC-3)
MH11(ARCC-3)
83
MH10(ARCC3)
MH8(ARCC-3)
NCTC10111(ARCC-3)
MH22(ARCC-3)
MH25(ARCC-3)
MH2(ARCC-4)
MH16(ARCC-4)
MH21(ARCC-4)
MH27(ARCC-2)
MH18(ARCC-8)
79
MH15(ARCC-7)
93
MH20(ARCC-7)
MH41(ARCC-6)
MH26(ARCC-1)
MH9(ARCC-5)
MH17(ARCC-5)
MH23(ARCC-5)
MH28(ARCC-5)
MH29(ARCC-5)
0.001
C.
MH27(ATPA-3)
MH29(ATPA-3)
MH18(ATPA-3)
MH17(ATPA-3)
MH16(ATPA-3)
MH10(ATPA-3)
MH9(ATPA-3)
NCTC10111(ATPA-3)
MH23(ATPA-1)
MH28(ATPA-1)
81
MH41(ATPA-2)
MH2(ATPA-2)
MH8(ATPA-2)
MH21(ATPA-2)
MH22(ATPA-2)
MH25(ATPA-2)
MH26(ATPA-2)
74
MH15(ATPA-5)
MH20(ATPA-6)
MH11(ATPA-4)
MH12(ATPA-4)
MH13(ATPA-4)
0.001
D.
MH27(ATPA-3)
MH29(ATPA-3)
MH18(ATPA-3)
MH17(ATPA-3)
MH16(ATPA-3)
MH10(ATPA-3)
MH9(ATPA-3)
NCTC10111(ATPA-3)
MH22(ATPA-2)
MH2(ATPA-2)
MH8(ATPA-2)
MH25(ATPA-2)
MH23(ATPA-1)
MH28(ATPA-1)
83
MH21(ATPA-2)
MH26(ATPA-2)
MH41(ATPA-2)
76
MH15(ATPA-5)
MH20(ATPA-6)
MH11(ATPA-4)
MH12(ATPA-4)
MH13(ATPA-4)
0.001
E.
NCTC10111(GLYA-12)
MH21(GLYA-13)
MH16(GLYA-3)
99
MH20(GLYA-10)
MH2(GLYA-13)
MH15(GLYA-13)
MH23(GLYA-11)
MH28(GLYA-11)
MH26(GLYA-9)
99
MH29(GLYA-9)
MH9(GLYA-8)
100
MH10(GLYA-8)
MH27(GLYA-1)
MH17(GLYA-2)
MH22(GLYA-7)
71
MH18(GLYA-5)
MH25(GLYA-5)
MH41(GLYA-4)
MH8(GLYA-6)
MH11(GLYA-6)
MH12(GLYA-6)
MH13(GLYA-6)
0.002
F.
MH8(GLYA-6)
MH11(GLYA-6)
MH12(GLYA-6)
MH13(GLYA-6)
MH22(GLYA-7)
MH18(GLYA-5)
MH25(GLYA-5)
MH41(GLYA-4)
MH17(GLYA-2)
MH27(GLYA-1)
MH9(GLYA-8)
100
MH10(GLYA-8)
99
MH26(GLYA-9)
MH29(GLYA-9)
84
MH23(GLYA-11)
MH28(GLYA-11)
99
MH16(GLYA-3)
MH20(GLYA-10)
MH2(GLYA-13)
MH15(GLYA-13)
84
NCTC10111(GLYA-12)
MH21(GLYA-13)
0.002



G.
MH10(RECA-5)
MH20(RECA-5)
MH9(RECA-5)
MH26(RECA-6)
NCTC10111(RECA-4)
MH25(RECA-7)
MH2(RECA-8)
MH21(RECA-8)
MH41(RECA-9)
MH11(RECA-9)
MH12(RECA-9)
MH13(RECA-9)
MH15(RECA-9)
MH27(RECA-9)
MH8(RECA-10)
MH23(RECA-3)
MH28(RECA-3)
MH16(RECA-2)
MH17(RECA-2)
MH29(RECA-1)
MH18(RECA-1)
MH22(RECA-1)
0.005
H.
MH10(RECA-5)
MH20(RECA-5)
MH9(RECA-5)
MH26(RECA-6)
NCTC10111(RECA-4)
MH25(RECA-7)
MH2(RECA-8)
MH21(RECA-8)
MH11(RECA-9)
MH12(RECA-9)
MH13(RECA-9)
MH15(RECA-9)
MH27(RECA-9)
MH41(RECA-9)
MH8(RECA-10)
MH23(RECA-3)
MH28(RECA-3)
MH16(RECA-2)
MH17(RECA-2)
MH18(RECA-1)
MH22(RECA-1)
MH29(RECA-1)
0.005
I.
MH17(FTSZ-6)
MH20(FTSZ-6)
MH16(FTSZ-6)
MH15(FTSZ-6)
MH13(FTSZ-6)
MH12(FTSZ-6)
MH11(FTSZ-6)
MH10(FTSZ-6)
MH9(FTSZ-6)
MH8(FTSZ-6)
MH23(FTSZ-3)
MH28(FTSZ-7)
MH41(FTSZ-6)
MH2(FTSZ-5)
MH21(FTSZ-5)
MH18(FTSZ-4)
MH22(FTSZ-4)
NCTC10111(FTSZ-2)
MH25(FTSZ-1)
MH29(FTSZ-6)
MH26(FTSZ-6)
MH27(FTSZ-6)
0.0005
J.
NCTC10111(FTSZ-2)
MH29(FTSZ-6)
MH15(FTSZ-6)
MH8(FTSZ-6)
MH10(FTSZ-6)
MH17(FTSZ-6)
MH2(FTSZ-5)
MH21(FTSZ-5)
MH11(FTSZ-6)
MH20(FTSZ-6)
MH18(FTSZ-4)
MH22(FTSZ-4)
MH12(FTSZ-6)
MH23(FTSZ-3)
MH26(FTSZ-6)
MH13(FTSZ-6)
MH27(FTSZ-6)
MH28(FTSZ-7)
MH9(FTSZ-6)
MH16(FTSZ-6)
MH25(FTSZ-1)
MH41(FTSZ-6)
0.0005
K.
MH27(METG-7)
MH28(METG-7)
MH26(METG-7)
MH25(METG-7)
MH23(METG-7)
MH20(METG-7)
MH15(METG-7)
MH29(METG-7)
MH11(METG-4)
MH12(METG-4)
MH13(METG-4)
MH16(METG-5)
MH17(METG-5)
MH18(METG-6)
MH22(METG-6)
MH41(METG-6)
NCTC10111(METG-2)
MH10(METG-2)
MH2(METG-1)
MH21(METG-1)
MH8(METG-3)
MH9(METG-3)
0.002
L.
MH28(METG-7)
MH29(METG-7)
MH27(METG-7)
MH26(METG-7)
MH25(METG-7)
MH23(METG-7)
MH20(METG-7)
MH15(METG-7)
MH11(METG-4)
MH12(METG-4)
MH13(METG-4)
MH16(METG-5)
MH17(METG-5)
MH18(METG-6)
MH22(METG-6)
MH41(METG-6)
NCTC10111(METG-2)
MH10(METG-2)
MH2(METG-1)
MH21(METG-1)
MH8(METG-3)
MH9(METG-3)
0.002

Figure 5.7. Phylogenetic trees for each of the eight SBT loci.
Phylogenetic trees were constructed for each of the eight SBT loci using maximum-likelihood (A, C, E, G, I, K, M, O) and neighbour-joining (B, D, F, H, J, L, N, P) methods. *arcC* = A+B, *atpA* = C+D, *glyA* = E+F, *recA* = G+H, *ftsZ* = I+J, *metG* = K+L, *lysS* = M+N, and *adk* = O+P. Strains originating from the same patient are indicated by coloured boxes. Bootstrap support values of over 70% are shown.

5.2.2.4 Determination of synonymous sequence changes in sequence based typing loci

Since SNPs may not result in altered amino acid sequence, the possibility of synonymous sequence changes (indicating a pressure to conserve amino acid sequence and protein structure) was investigated by comparing predicted translated sequence (ST based on amino acid sequence is included in Table 5.3). Analysis of deduced amino acid sequences of the eight loci for the 22 strains indicated that both synonymous and non-synonymous SNPs occurred. Non-synonymous SNPs are

highlighted in Figure 5.8. Amino acid sequence alignment analysis of RecA and AtpA yielded homologous sequences for all ATs, numbering ten and six ATs respectively. In comparison Adk analysis resulted in the largest number of non-synonymous changes in the amino acid sequence, with six changes in the sequence between 11 ATs. Generation of STs using the deduced amino acid sequences for each of the 22 *M. hominis* isolates resulted in 14 STs, with a discriminatory ability of 0.6 ST per isolate. Examination of the Hunter-Gaston DI indicated large diversity between ST generated from the amino acid sequences (DI: 0.944; 95% CI: 0.898-0.989); this is only slightly lower than the DI for STs generated from nucleotide sequences, indicating poor pressure for conservation even at the protein level. The greatest diversity was shown in Adk (DI: 0.866; 95% CI: 0.799-0.932) and the lowest diversity in both AtpA and RecA (DI: 0.000; 95% CI: 0.000-0.261).

A.

```
ARCC-3      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-4      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-7      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-8      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-1      IGPFFATEAEAIAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-2      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-5      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
ARCC-6      IGPFFATEAEAVAANPNSTIVEDAGRGYRKVVASPKPLNFVGINQIKKAIESGATVIVGG
            ***********:************************************************

ARCC-3      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDFVKVNYNKPDQKDL
ARCC-4      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDFVKVNYNKPDQKDL
ARCC-7      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDFVKVNYNKPDQKDL
ARCC-8      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDFVKVNYNKPDQKDL
ARCC-1      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDYVKVNYNKPDQKDL
ARCC-2      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDYVKVNYNKPDQKDL
ARCC-5      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDYVKVNYNKPDQKDL
ARCC-6      GGGIPTVEDENGFIKGVDGVIDKDFALAKMAALAKADYFIVLTAVDYVKVNYNKPDQKDL
            **********************************************:*************

ARCC-3      KHATKAE
ARCC-4      KHATKAE
ARCC-7      KHATKAE
ARCC-8      KHATKAE
ARCC-1      KHATKAE
ARCC-2      KHATKAE
ARCC-5      KHATKAE
ARCC-6      KHATKAE
            *******
```

B.

```
ATPA-1      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
ATPA-2      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
ATPA-3      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
ATPA-4      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
ATPA-5      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
ATPA-6      AAYIPTNVISITDGQIFTKEALFNSGQRPAIDIGYSVSRVGSAAQTKLTKKVVSSLKLEL
            ************************************************************

ATPA-1      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
ATPA-2      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
ATPA-3      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
ATPA-4      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
ATPA-5      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
ATPA-6      AQYNEMLAFAQFGSDLDQATRTVLDHGAKVYELLKQPQYSPYSSIEQI
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C.

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RECA-1      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-2      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-3      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA_4      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-5      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-6      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-7      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-8      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-9      AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
RECA-10     AALVPEAELNGEMKDQVVGAQARLMSKALRKITGSLSKNKTTVIFINQIREKIGVIFGNP
            ************************************************************

RECA-1      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-2      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-3      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA_4      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-5      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-6      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-7      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-8      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-9      ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
RECA-10     ETTTGGRALKFYSSIRLEVRKIQNVTNNGDITGNQIKCKVVKN
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D.

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GLYA-12       DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-1        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-2        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-3        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-4        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-5        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-6        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-7        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-9        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-10       DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-11       DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-13       DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
GLYA-8        DYDKILEIAKEVKPKMIICGYSAYSQIVDFAKFRKICDEVGAKLFADISHISGLIIAGLH
              ************************************************************

GLYA-12       PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLFHQIAAKAVS
GLYA-1        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-2        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-3        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-4        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-5        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-6        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-7        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-9        PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-10       PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-11       PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-13       PSPSGYADVIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
GLYA-8        PSPSGYADIIMTTTHKTLRGTRGAIILTNDEEIFKRVNSAVFPGVQGGPLLHQIAAKAVS
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GLYA-12       FREALQPEFIDYQKQL
GLYA-1        FREALQPEFIDYQKQL
GLYA-2        FREALQPEFIDYQKQL
GLYA-3        FREALQPEFIDYQKQL
GLYA-4        FREALQPEFIDYQKQL
GLYA-5        FREALQPEFIDYQKQL
GLYA-6        FREALQPEFIDYQKQL
GLYA-7        FREALQPEFIDYQKQL
GLYA-9        FREALQPEFIDYQKQL
GLYA-10       FREALQPEFIDYQKQL
GLYA-11       FREALQPEFIDYQKQL
GLYA-13       FREALQPEFIDYQKQL
GLYA-8        FREALQPEFIDYQKQL
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E.

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LYSS-5        DFGDILSVRGTLSKTNTDALVIRAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-1        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-2        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-4        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-6        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-7        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-8        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-9        DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-10       DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-11       DFGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
LYSS-3        DCGDILSVRGTLSKTNTDALVIKAQDIVLLTKSLKPLPDKFHGLVDPEERRRQRYVDLIM
              * ********************:*************************************

LYSS-5        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-1        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-2        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-4        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-6        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-7        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-8        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-9        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-10       NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-11       NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
LYSS-3        NEKTRNTFVMRTQIIKGIREYFDSLNYIEVDTPILNPILGGAAAKPFITFYNAL
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F.

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METG-1      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-2      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-3      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-5      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-6      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-7      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-8      YHIETIQKVFYLMEQKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
METG-4      YHIETIQKVFYLMERKGYIYKSKYEGLYSISDEEFLTKTQAISKDGKFYHPISGHELQEV
            **************:*********************************************

METG-1      KEESYFFNMSKFTPWI
METG-2      KEESYFFNMSKFTPWI
METG-3      KEESYFFNMSKFTPWI
METG-5      KEESYFFNMSKFTPWI
METG-6      KEESYFFNMSKFTPWI
METG-7      KEESYFFNMSKFTPWI
METG-8      KEESYFFNMSKFTPWI
METG-4      KEESYFFNMSKFTPWI
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G.

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ADK-1       ITNQLVEKKLTELIREKKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDEQIID
ADK-2       ITNQLVEKKLTELIREKKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDEQIID
ADK-10      ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-7       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-4       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDEQIID
ADK-3       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-5       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-6       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-8       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-9       ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
ADK-11      ITNQLVEKKLTELVRENKHFILDGYPRTLAQAKFLLSLANVGIKIDKVILLEITDKQIID
            *************:**:**************************************:****

ADK-1       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-2       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-10      RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPKVIKKRLSIYNEQTKCL
ADK-7       RLSKRRICLNCKIIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-4       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-3       RLSKRRICLNCKTIYHLQSFPPLSGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-5       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-6       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-8       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-9       RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
ADK-11      RLSKRRICLNCKTIYHLQSFPPLPGDLCIKCHEKVTKRPDDEPEVIKKRLSIYNEQTKCL
            ************ ********** *******************:****************

ADK-1       INFYQELGILLKINS
ADK-2       INFYQELGILLKINS
ADK-10      INFYQELGILLKINS
ADK-7       INFYQELGILLKINS
ADK-4       INFYQELGILLKINS
ADK-3       INFYQELGILLKINS
ADK-5       INFYQELGILLKINS
ADK-6       INFYQELGILLKINS
ADK-8       INFYQELGILLKINS
ADK-9       INFYQELGILLKINS
ADK-11      INFYQELGILLKINS
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H.
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FTSZ-1      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-3      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-4      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-5      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-6      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-7      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-8      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETVIG
FTSZ-2      NKLLQQYGNISFSDAFICANNVLKQTIRTIVDVIATPSIINLDFADLSTIIKNKGETLIG
            *********************************************************:**

FTSZ-1      IGQANGQDRAV
FTSZ-3      IGQANGQDRAV
FTSZ-4      IGQANGQDRAV
FTSZ-5      IGQANGQDRAV
FTSZ-6      IGQANGQDRAV
FTSZ-7      IGQANGQDRAV
FTSZ-8      IGQANGQDRAV
FTSZ-2      IGQANGQDRAV
            ***********
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Figure 5.8. Alignments of amino acid sequences of each allelic type at each of the eight SBT loci.
Amino acid sequence alignments were generated for each of the eight loci based on nucleotide sequence of each allelic type. Non-synonymous changes in amino acid sequence are highlighted in yellow. * (asterix) indicates positions which have a single, fully conserved residue; : (colon) indicates conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix.

5.2.2.5 Assessment of SBT loci stability

No previous classification scheme for mycoplasmas has confirmed that the ST classification is maintained following limited *in vitro* subculture. Therefore, the genetic stability of the eight SBT loci were examined by comparing sequences for each loci in six strains before and after ten subculture passages (Table 5.5). All ten strains co-located before and after ten passages to the same topological position within both maximum-likelihood and neighbour-joining trees (Figure 5.9). However, diversity between strains before and after ten passages was observed by SNP differences within the locus *glyA* (Table 5.5). The sequence alteration required the assignment of a new ST for one (MH17) of the six strains, where a SNP change was observed.

Examination of the Hunter-Gaston DI between the ten strains before and after ten passages indicated that strain MH17 had a diversity index indicative of complete

diversity (DI: 1.000; 95% CI: -0.522-1.000). However, the relationship between progenitor and daughter strains was apparent by clade relationship (co-localisation by phylogeny; Figure 5.9). Conversely, no diversity was seen in strains MH2, MH11, MH20, MH23 and MH26 after 10 passages (DI: 0.000; 95%CI: 0.000-0.266). In MH17 the only loci that displayed diversity after ten passages was *glyA* (DI: 1.000; 95% CI:-0.522-1.000). This indicates that there is differential strain variation over ten passages and that there is variable genetic fidelity of the genes included in this study.

Table 5.5. SNP differences between the original strain and following 10 passages in MM.
SNP differences and the position of the SNP are shown for each of the 8 loci. New assignment of a sequence types (STs) caused by the changes are indicated in bold.

Strain	ST	SNP difference number (position) per allele							
		adk	***arcC***	***atpA***	***glyA***	***recA***	***ftsZ***	***lysS***	***metG***
MH2	2	0	0	0	0	0	0	0	0
MH11	6	0	0	0	0	0	0	0	0
MH17	**21**	0	0	0	1 (349)	0	0	0	0
MH20	11	0	0	0	0	0	0	0	0
MH23	14	0	0	0	0	0	0	0	0
MH26	16	0	0	0	0	0	0	0	0

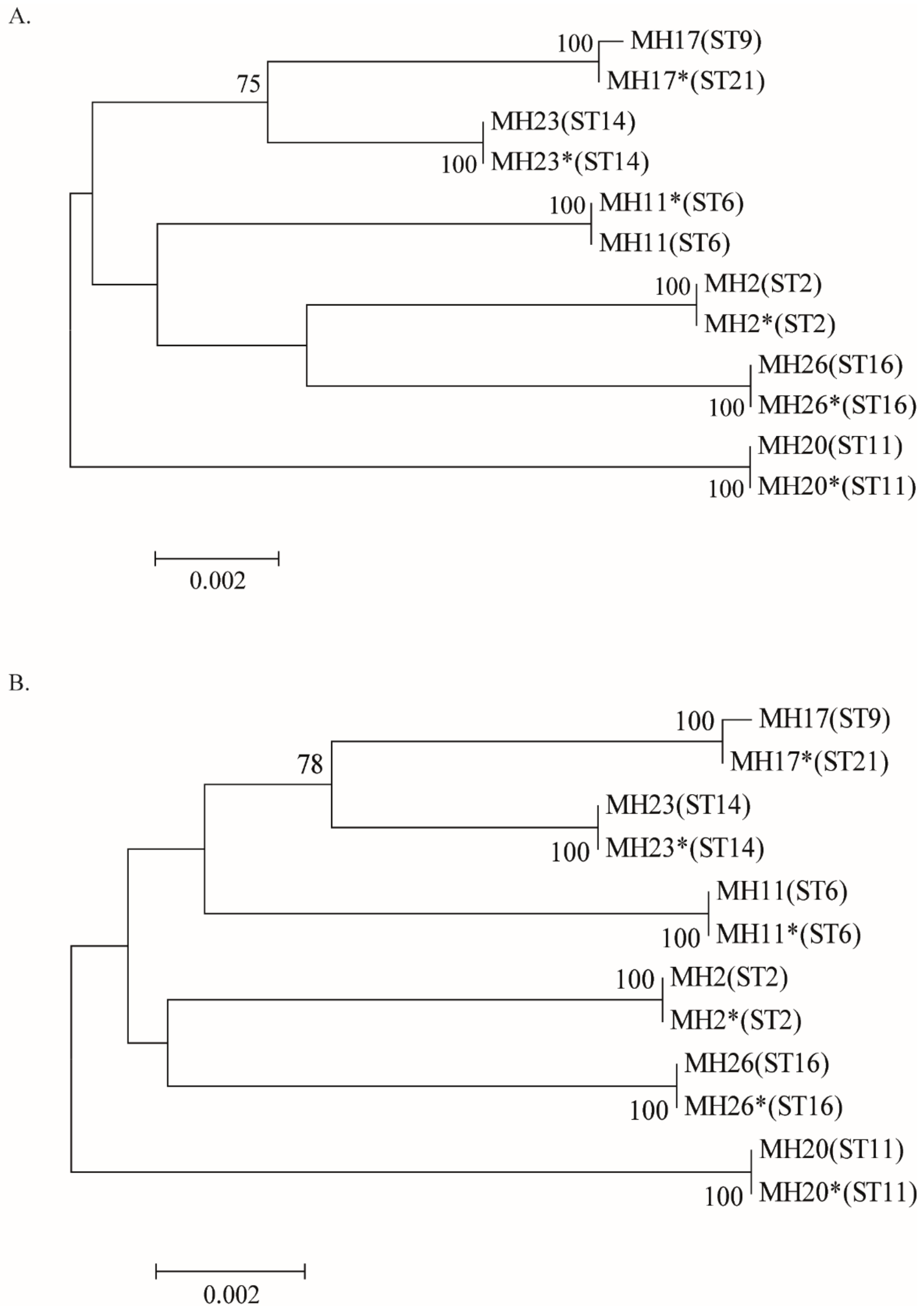

Figure 5.9. Phylogenetic trees based on concatenated sequences of eight SBT loci from the original strains tested and the strains after short-term passage in liquid culture.

Phylogenetic trees were constructed based on concatenated sequences of eight housekeeping loci for ten strains before and after ten passages in liquid culture using maximum-likelihood (A) and neighbour joining (B) methods. The strain after short-term passage are indicated by a * (asterix). Bootstrap values of over 70% are shown.

5.2.2.6 Genomic sequence analysis

The genomes of 16 *M. hominis* strains were sequenced and assembled using the PG21 reference genome as described in sections 2.6.2-2.6.5. Phylogenetic analysis based on SNP variant calling (section 2.6.8.1) of 18 *M. hominis* isolates and ATCC 27545 (NZ_CP009652.1) whole genome sequences (Figure 5.10) showed similarity in topology to the phylogenetic trees constructed from the concatenated SBT loci (Figure 5.6). Analysis of the two strains from a single wound swab (MH9 and MH10) confirmed observations from SBT, where MH9 and MH10 consistently collocated to the same clade in both SBT and genomic SNP analysis. MH11 and MH12, two isolates from a single peritoneal sample were found to have the same ST, collocating to the same clade after SNP analysis of partial whole genome sequencing and were found to be indistinguishable. Phylogenetic SNP analysis of the partial whole genome sequence of 19 of the *M. hominis* strains confirmed a high level of intra-species diversity, concordant with SBT Hunter-Gaston DI and phylogenetic analysis. Interestingly, an additional group of two unrelated strains were indistinguishable by SNP analysis, MH2 and MH21. MH2 and MH21 are strains from patients with extra-genital infections, and are considered invasive strains.

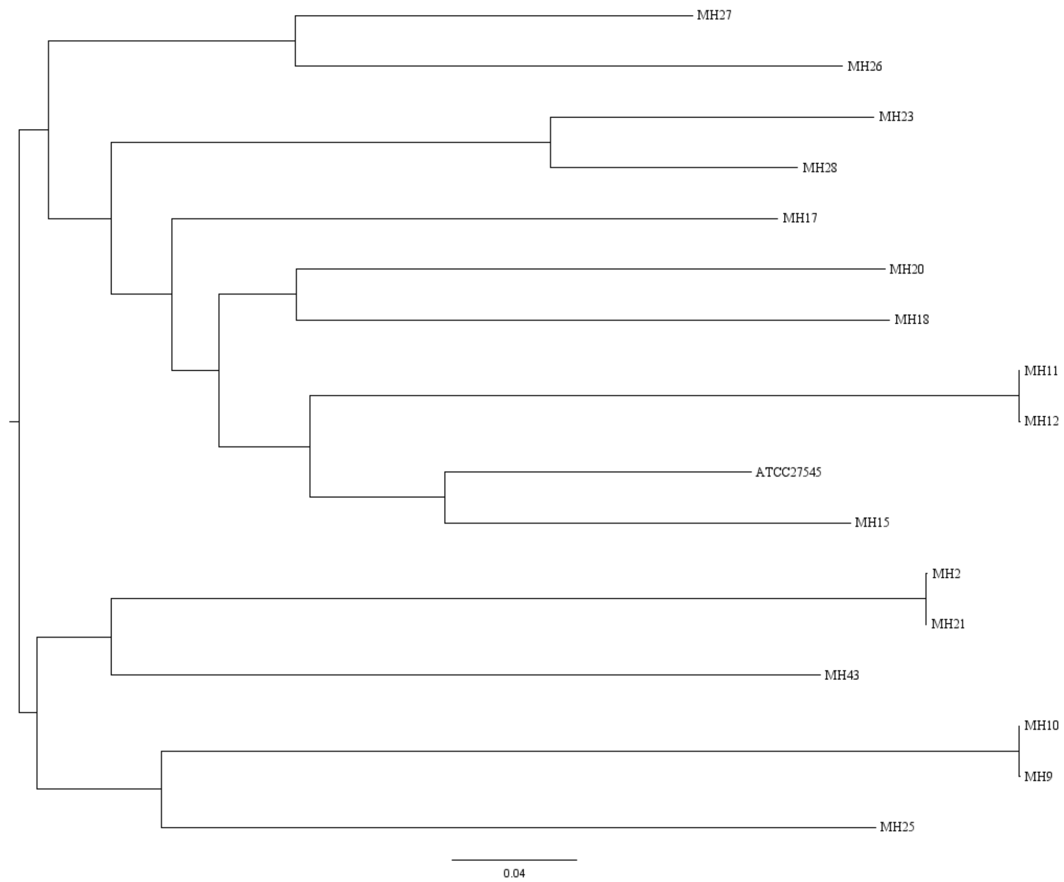

Figure 5.10. Phylogenetic tree based on the single nucleotide polymorphism profile of 19 *M. hominis* whole genome sequences.
The phylogenetic tree was constructed based on SNP variant calling for 16 *M. hominis* partial whole genome sequences and the prototype strain ATCC 27545 in comparison to PG21 using the maximum-likelihood method. Strains originating from the same patient sample are indicated by boxes.

5.2.3 Genomic sequence analysis and development of minimum MLST scheme

Bioinformatics analysis of *M. hominis* genomic sequence was used to derive the minimum number of genes required to accurately reflect genomic phylogeny and to identify possible minimal MLST (mMLST) schemes.

5.2.3.1 Pan-genome

Raw genomic sequence reads from 18 *M. hominis* clinical strains were assembled and scanned against a database of Hidden Markov Models (HMM) representing gene coding families constructed using the four complete genomes published on NCBI: ATCC 27545 (NZ_CP009652; 533 genes), PG21 (NC_013511; 497 genes), Sprott (NZ_CP011538; 524 genes) and AF1 (NZ_CP009677; 531 genes), as described in Section 2.6.6. On average, *M. hominis* pan-genome clustering was able to detect 550 (median 553) genes per sample, which is comparable to the mean number of genes found in the four reference genomes (521 genes). The *M. hominis* pan-genome contained a total of 777 genes (Figure 5.11) with 427 genes (54.95%; 95% CI: 51.44%-58.42%) present across all samples and the reference strain ATCC 27545 at least once. The shoulders in the pan-genome frequency distribution are likely to correspond to the genes found in the specific phylogenetic clades (Figure 5.11). The number of alleles per gene family has a bi-modal distribution (Figure 5.12); one peak at 1 allele per gene (representing sample-specific genes) and a second peak at 11 alleles per gene. The second peak is likely to contain a set of core genes, common to all samples, yet have unique alleles in each one. This is as expected given the relatively long phylogenetic distance from the reference genome in the whole genome SNP tree (Figure 5.10). No hyper-variant, large copy number genes, such as transposases and integrases, were detected in the pan-genome data analysis.

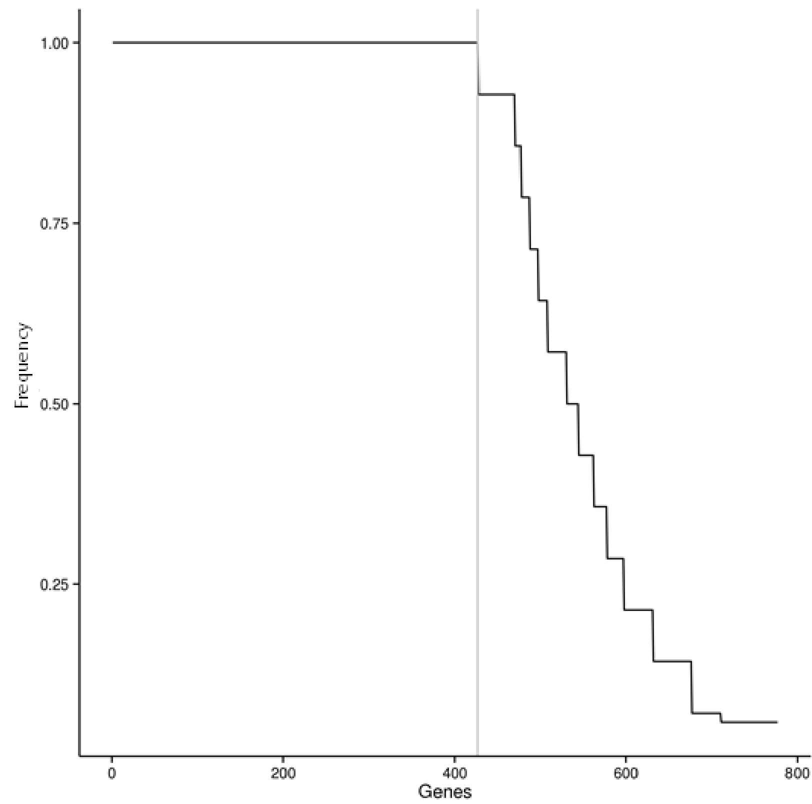

Figure 5.11. Frequency of genes in the *Mycoplasma hominis* pan-genome.
Grey line indicates the core number of genes appearing in all isolates and the reference strain ATCC 27545 at lease once (427 genes).

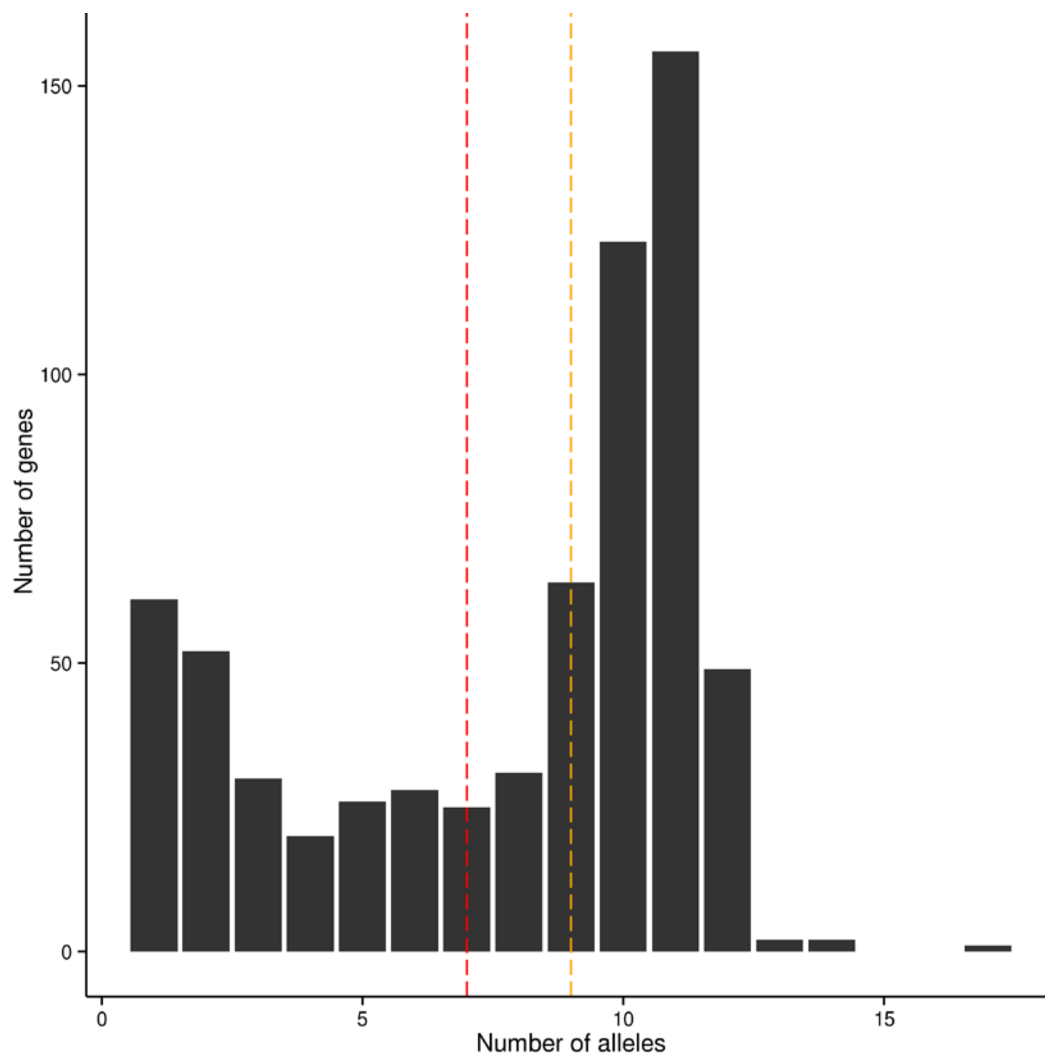

Figure 5.12. Bimodal distribution of alleles per gene in the *Mycoplasma hominis* pan-genome.
The median (red dotted line) and mean (orange dotted line) number of alleles per gene family in the pan-genome.

5.2.3.2 Core-genome MLST

A subset of genes found across all samples are likely to be highly conserved and carry little phylogenetic signal. Conversely, a small subset of genes could act as hotspots for the phylogenetic signal that drives tree topology. In order to identify genes that carry increased discriminatory potential, leave-one-out analysis was performed as described in section 2.6.9. This involved removing one gene at a time from the set of 427 core genes and constructing a phylogenetic tree using the remaining alleles from the 426 genes. The resulting phylogenetic tree was compared with the phylogeny derived using whole genome variants (considered the gold standard). Of the 427 genes, 379 genes (88.76%; 95% CI: 85.39-91.44) conferred the same phylogenetic topology as the whole genome tree whilst 48 genes (11.24%; 95% CI: 8.56-14.61) disrupted the phylogenetic relationship of the strains to a varying extent. These results suggest that these 48 genes are necessary for reconstructing the correct relationships between the strains. To assess the sufficiency of these genes to replicate the topology, a phylogenetic tree was constructed using only these 48 genes (Figure 5.13), confirming that these 48 genes were necessary and sufficient for tree reconstruction. These genes are considered the minimum gene set to construct a core-genome MLST scheme as they are present in all reference genomes and the 18 sequenced *M. hominis* strains, and are each required to reconstruct the whole genome topology.

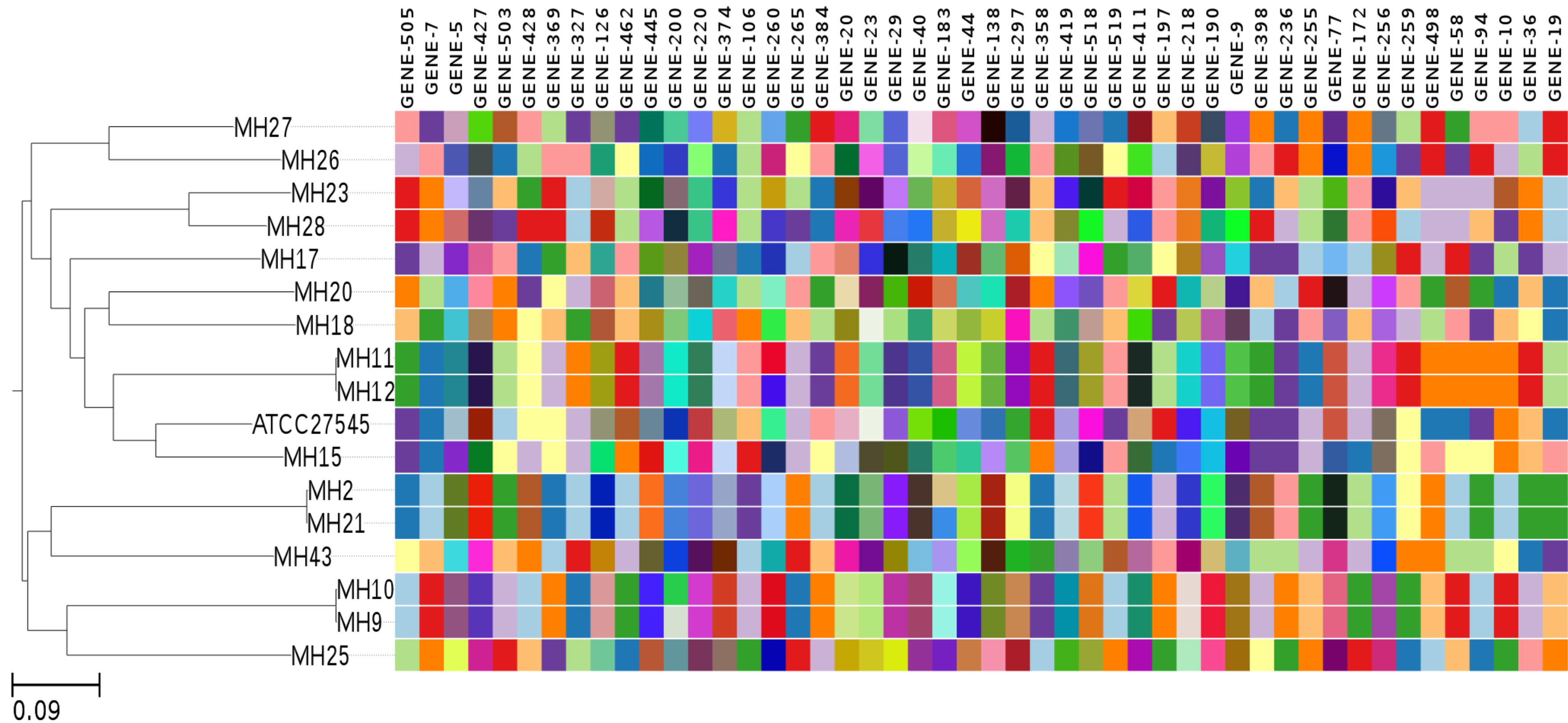

Figure 5.13. MLST mosaic of 48 core genes found after leave-one-out analysis.
The genes listed are necessary and sufficient for the reconstruction of the phylogenetic relationship of the strain observed using whole genome data. The coloured 'mosaic' tiles represent different alleles.

5.2.3.3 Seven-gene MLST (mMLST)

Whole genome sequencing is growing in ubiquity with decrease cost-per-sample however, culturing *M. hominis* to produce a high enough yield of DNA for whole genome sequencing is challenging and not routinely possible direct on clinical specimens. Routine whole genome sequencing for *M. hominis* is not currently performed therefore typing with a technique utilising PCR and Sanger sequencing, such as MLST and SBT, is preferred and more widely accessible. Although 48 genes are required to recapture the original phylogenetic relationships observed using whole genome SNP analysis, the aim of this section was to identify a reduced gene set that could be used to confer good discrimination between strains and could be of practical use in future studies direct on clinical specimens. To reduce the number of combinations of seven genes to search ($^{48}C_7$ = 73629072 total combinations), three sets of genes were selected: genes that caused the lowest overlap with the whole genome SNP tree in leave-one-out analysis (n = 10), manually selected genes for their biological functions (n = 9), and combination of the above two sets (n = 12). Two sets of *M. hominis* strains originating from the same patient samples, MH9 and MH10, and MH11 and MH12 were used to identify which genes sets conferred identical mMLST profiles for these strains. This reduction in genes resulted in 948 possible combinations of the three gene sets. All combinations were analysed for phylogenetic topology closest to the whole genome SNP tree (methodology described in section 2.6.9). Fifteen combinations (Table 5.6) were identified to produce the highest topological similarity among all tested combinations and three schemes were found to confer the highest similarity to the whole genome SNP tree. All 15 phylogenetic trees classified MH23 and MH28 into a different subtree compared to the original whole genome analysis. In addition some trees also classified MH17 into a different

topological position. Three selected schemes had overall very similar topology to the original whole genome SNP tree representing two general clades and correct bifurcations. The branch length of the whole genome SNP tree could not be reproduced as shorter sequences with a different number of SNPs were used. Overall the three schemes almost completely reproduced the phylogenetic relationship found using whole genome SNP data.

Table 5.6. Summary of *Mycoplasma hominis* mMLST schemes.
15 potential seven gene MLST schemes. All schemes were found to reproduce the desired topology of the whole genome sequence SNP tree. Genes that are important for distinguishing between MH2 and MH21 are in red. * indicates the schemes that produced a tree most similar to whole genome SNP tree.

mMLST Genes	Topological score ([0,1])
20,58,94,236,374,428,519	0.7692308
20,58,94,183,265,428,519	0.7692308
20,58,94,236,374,428,498	0.7692308
20,58,94,265,374,428,519	0.7692308
20,58,94,138,183,265,428*	0.7692308
20,58,94,172,265,428,519	0.7692308
20,58,94,138,183,236,519*	0.7692308
20,58,94,172,236,374,428	0.7692308
20,58,94,138,183,428,519	0.7692308
20,58,94,236,265,428,519*	0.7692308
20,58,94,138,183,236,374	0.7692308
20,58,94,183,374,428,519	0.7692308
20,58,94,172,183,265,519	0.7692308
20,58,183,236,374,428,519	0.7692308
20,58,94,172,183,236,265	0.7692308

5.2.3.4 Diversity of mMLST genes and determination of synonymous sequence changes

All three mMLST schemes revealed a discriminatory power of 0.97 ST per *M. hominis* strain. The Hunter-Gaston DI was calculated for each scheme (Table 5.7) and revealed that schemes 1 and 2 were equally discriminatory (DI: 0.97) in comparison to scheme 3 which was slightly less discriminatory (DI: 0.96). All three mMLST schemes are close together in terms of their diversity index indicating that there is no clear ideal combination of seven genes. Indeed, the schemes only differ by two genes (Table 5.7).

Genes under negative selective pressure are preferred for an MLST scheme as they are purifying and actively kept by the host. For each of the three proposed mMLST schemes the ratio of non-synonymous to synonymous (dN/dS) changes were calculated using the Nei and Gojobori method (Table 5.7). All genes in all three mMLST schemes have a dN/dS ratio of <1. Schemes 1 and 3 have two genes under moderate selective pressure (*oppA*: 0.23 [95% CI: 0.21-0.25] and GENE-58: 0.27 [95% CI: 0.25-0.30], GENE-58: 0.27 [95% CI: 0.25-0.30] and *dnaG*: 0.11 [95% CI: 0.08-0.13], respectively), and scheme 2 has three genes under moderate selective pressure (*oppA*: 0.23 [95% CI: 0.21-0.25], GENE-58: 0.27 [95% CI: 0.25-0.30], *dnaG*: 0.11 [95% CI: 0.06-0.13]). The remaining genes have a dN/dS ration below 0.1, indicating strong negative selection. Overall, scheme 3 appears to contain, on average, a more strongly selected gene set (average dN/dS ratio: 0.09), than the other two schemes (average dN/dS ratio: 0.11 and 0.13 for schemes 1 and 2, respectively).

Table 5.7. dN/dS ration and Hunter-Gaston Diversity Indices for the three proposed mMLST schemes

Gene name	Scheme 1	Scheme 2	Scheme 3	dN/dS (95% CI)	Hunter-Gaston Diversity Index (95% CI)
MHO_4840[a]	GENE-20	GENE-20	GENE-20	**0.09**(0.08-0.1)	**0.99** (0.97-1.00)
MHO_0720[a]	GENE-58	GENE-58	GENE-58	**0.27**(0.25-0.3)	**0.98** (0.95-1.00)
secD	GENE-94	GENE-94	GENE-94	**0.07**(0.04-0.09)	**0.95** (0.89-1.00)
oppA	GENE-138	GENE-138		**0.23**(0.21-0.25)	**0.98** (0.96-1.00)
argS	GENE-183	GENE-183		**0.07**(0.05-0.09)	**0.99** (0.97-1.00)
hisS		GENE-236	GENE-236	**0.09**(0.06-0.13)	**0.91** (0.8-1.00)
MHO_1160[a]	GENE-265		GENE-265	**0.02**(0.01-0.03)	**0.96** (0.91-1.00)
tyrS	GENE-428		GENE-428	**0.01**(0.01-0.02)	**0.97** (0.92-1.00)
dnaG		GENE-519	GENE-519	**0.11**(0.08-0.13)	**0.97** (0.92-1.00)
Mean dN/dS (95% CI)	**0.11** (0.01-0.2)	**0.13** (0.06-0.21)	**0.09** (0.01-0.17)		
Mean Diversity (95% CI)	**0.97** (0.96-0.99)	**0.97** (0.94-0.99)	**0.96** (0.94-0.99)		

[a] gene encoding a hypothetical protein. Gene name relates to annotation in ATCC 23114.

5.2.3.5 Seven gene mMLST stability

Stability of the genes for all three schemes was assessed in two *M. hominis* strains. Whole genome sequencing was performed following short-term passage (10 passages) in liquid culture and compared to the original sequence. All genes were completely stable in the two strains examined (Figure 5.14).

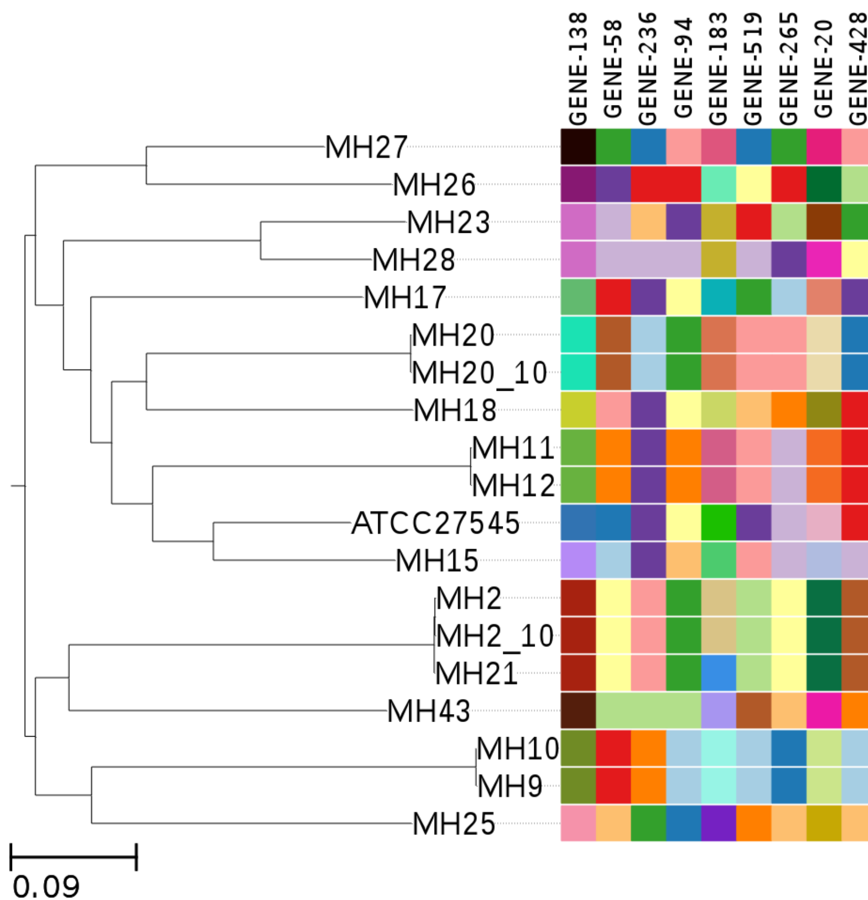

Figure 5.14. MLST mosaic indicating stability of the nine genes used in the three proposed mMLST schemes.
Strains that have undergone short-term passage are indicated by _10. The coloured 'mosaic' tiles indicate differing alleles. All genes are stable between original and passaged strains.

5.2.3.6 Recombination analysis

Genomic sequences of the *M. hominis* strains were assessed for predicted regions of variation arising from homologous recombination using Gubbins (section 2.6.10). Multiple potential areas of recombination were identified across all *M. hominis* strains (Figure 5.15). In particular, high levels of recombination were predicted in the phylogenetic clade containing the reference strain ATCC 27545, with multiple recombination events predicted at the same loci.

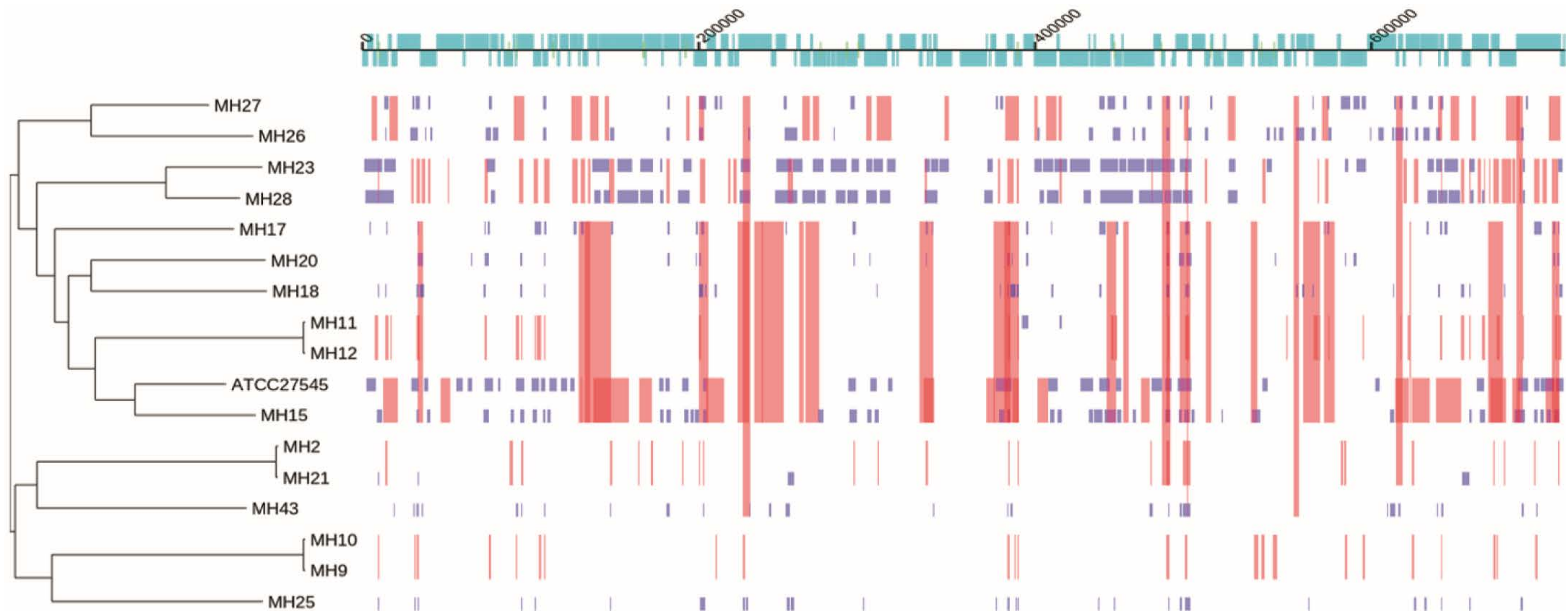

Figure 5.15. Prediction of recombination in the *M. hominis* isolates chromosomes.
Regions of variation in the genomes of the 18 clinical *M. hominis* isolates and the prototype strain ATCC 27545 which are predicted to have arisen by homologous recombination are shown in the panel on the right. Red blocks indicated recombination predicted to have occurred on internal nodes, blue indicates taxa-specific recombination, isolates are ordered according to the phylogenetic tree displayed on the left. The track along the top of the figure displays the PG21 chromosome and annotation, where protein coding sequences (CDS) are indicated in light blue.

5.3 Discussion

In this chapter, *M. hominis* has been characterised by: Vaa type, SBT of eight housekeeping genes (*adk*, *arcC*, *atpA*, *glyA*, *recA*, *ftsZ*, *lysS* and *metG*), genomic sequence SNP analysis, and bioinformatics analysis of genomic sequence to derive the minimum number of genes required to accurately reflect genomic phylogeny. These were used to examine the genetic diversity of *M. hominis* isolates (collected from 1986-2012), including triplicate isolates from two separate patients in the UK. Patients had a variety of urogenital and extra-genital clinical circumstances (detailed in Table 2.2).

5.3.1 Analysis of the variable adherence-associated antigen of *Mycoplasma hominis*

The Vaa antigen is a major adhesin of *M. hominis* displaying pronounced mutational variation in size, sequence and antigenic properties. High levels of sequence variation was observed in the 36 *M. hominis* isolates examined in this chapter, particularly in the C-terminal of the interchangeable cassette region of the protein. C-terminal modules appear to be hyper-variable in comparison to the other modules of the protein, indicating that there may be higher selective pressure to vary this region of the protein. This suggests that the C-terminus of Vaa may be more important to immune surveillance than more membrane-proximal modules. Antigenic variation is important to the expression of functionally conserved moieties within a clonal population that are antigenically distinct [28].

As a method of discriminating between *M. hominis* strains, Vaa categories provide a limited mechanism for characterisation. Of the isolates examined in this chapter, four Vaa categories were identified (0.11 categories per strain) with a Hunter-Gaston DI of 0.684. However, when examining nucleotide sequence, higher levels of

heterogeneity are observed (0.72 ATs per strain) and large diversity is observed between *vaa* sequences (Hunter-Gaston DI: 0.981).

The *vaa* locus has previously been described as a highly dynamic region of the *M. hominis* genome [135]. A divergent genetic islet encoding a large, putative membrane protein, has been identified within the *vaa* locus of some *M. hominis* isolates. The *vaa* gene in this case was found to be conserved 5' end and a highly variable region towards the 3' end containing the genetic islet. It has been proposed that the mechanism underlying variation in the *vaa* gene is intra-species recombination whereby variable regions of *vaa* are exchanged resulting in a variable and dynamic hot-spot in the *M. hominis* genome [135].

5.3.2 Analysis of SBT for *M. hominis*

The development of SBT schemes has been important both individually and epidemiologically for pathogenic bacteria. At the level of an individual patient, this approach allows discrimination between relapse or persistence and new infection. In this case *M. hominis* isolate SBT analysis was applied to three isolates from peritoneal fluid from a single transplant patient undergoing organ rejection. These isolates all had identical sequence in the eight genetic loci, co-locating by phylogenetic topology with zero detected SNP differences (ST6).

Analysis of three strains from a single wound swab (MH8-10), showed two closely related clones (MH9/10), and one strain that was distinctly different (MH8) in target gene sequence. The presence of single or multiple *M. hominis* isolates within one patient has been examined previously. Olson *et al*. [86] described multiple isolates of *M. hominis* obtained from the same joint of a septic arthritis patient over a six year period which exhibited differences in the expression of surface proteins. However,

these strains were indistinguishable by restriction endonuclease analysis. Conversely, Jensen *et al*. [412] demonstrated that *M. hominis* isolates from the same patient were identical when analysed with SDS-PAGE and PFGE, while isolates from different women exhibited a high degree of variation. It was originally suggested that strains isolated from the same anatomical region should reveal a higher degree of similarity than strains isolated from different anatomical sites [425]. Strains obtained from the same anatomical site or from patients with the same diagnosis have been reported by others to have different patterns by RFLP analysis [413] and comparison of 12 genital isolates with other strains from different origins demonstrated that the genital isolates varied as much from each other as strains of non-genital origin [84].

5.3.3 Fidelity of SBT loci

The accumulation of sequence changes in the *glyA* locus of MH17 after 10 subculture passages resulted in loss of SBT assignment. This indicates that this locus is unsuitable for use in the SBT scheme. The failure to amplify the genes *atpA* and *ftsZ* each for two strains using the original primer sets was caused by sequence alteration in the primer footprint itself and the same DNA sample was readily amplified by the seven other primer pairs. Thus, even though no changes were observed in the SBT locus sequence, SNPs were accumulated in the flanking regions required for amplification showing a high level of genetic variation in subsequent generations of this bacterial species.

While phylogenetic analysis of the strains before and after subculture showed clear association between progenitor and progeny strains, a question arises regarding how many cell divisions would be required to lose the ability to relate progenitor to progeny. Variation in strain and gene diversification rate was observed; MH17 acquired an additional SNP in the *glyA* locus; and two loci failed to amplify using the

original primers. These findings are in contrast to the complete conservation in ST's observed for eight MLST loci in ten strains of *M. pneumoniae* following ten passages (0) [424]. This indicates that there may be reduced replication fidelity in *M. hominis* compared to other *Mycoplasma* species. The relationship between genetic fidelity and pathogenicity, or degree of fidelity across the *M. hominis* genome requires further investigation.

This is the first study to examine genetic drift in *M. hominis* after a limited number of subculture passages and our primary finding demonstrates that the separation of clinical isolates into such a wide number of ST for more discriminative methods is directly related to the poor fidelity of the replicating genome. The reason for such varied strain genetic variation has not been identified and could be a reflection of recombination or integration of mobile genetic elements. In comparison to MLST schemes developed for *M. hyopneumoniae* and *M. agalactiae*, higher levels of variation and diversity can be observed at the SBT loci used for *M. hominis* [421, 423] and discriminatory power seen in this study is higher than for that of *Ureaplasma* (0.9 ST per *M. hominis* isolated versus 0.35 ST per *Ureaplasma* isolate) [426]; confirming that *M. hominis* is highly variable in comparison to other *Mycoplasma* species. While *M. hominis* was not included in the analysis, examination of four mycoplasma species; *M. pneumoniae*, *M. genitalium*, *M. pulmonis* and *U. urealyticum*, indicated a large number of repeats suggesting the existence of a large potential for recombination in these genomes [246]. These *Mycoplasma* species have high mutation rates and lack part of the SOS response and also a considerable number of the DNA repair proteins existing in *E. coli* and *Bacillus subtilis* [5]. The four genomes reveal the absence of MutSLH system for mismatch repair and therefore higher frequencies of recombination between divergent strains are predicted, contributing to

the accelerated evolution observed within the genus [246]. It would be interesting to determine if *M. hominis* also lacks these factors.

5.3.4 Application of SBT for *Mycoplasma hominis* infection

The potential for poor fidelity of *M. hominis* raises the question of the value of an SBT scheme, applied to *M. hominis* isolates, to identify sexual transmission of *M. hominis* infections or horizontal transmission between mother and baby. However, transformation of the target gene sequence into concatenated form and analysis for phylogenetic topological positioning association clearly showed the relationship between progenitor and progeny strains was retained after subculture and the capacity to use such analysis requires validation with serial samples from infected individuals to determine if this could be of use in typing this bacterial species. It is not known over how many generations such retention would be stable, nor is it known if *in vivo* pressure (e.g. symbiotic nutrient acquisition, innate/adaptive immunity, or other impacting factors) may limit the long term genetic drift. Genomic sequencing may eventually displace SBT based on multiple gene target PCR and sequencing. However, the international availability of genome sequence technology and the time required for processing enough high quality DNA and contig analysis with the specialised culture requirements of mycoplasmas and volume of biomass required to obtain sufficient high quality DNA for analysis is unlikely to ever lend whole genome sequencing analysis to provide practical clinical guidance for this bacterial species and other fastidious organisms. Other methods including MLVA typing of *M. hominis* isolates from two mother-neonate pairs resulted in the identification of identical MLVA types in each case studied, confirming mother-to-child transmission [133]. Additionally, PFGE results reported by Jensen *et al*. found that *M. hominis* isolates obtained from the cervices of pregnant women and from the ears or pharynges of their

new-born infants yielded the same genomic profile [412]. Similarly, identical arbitrarily primed-PCR patterns were observed in strains isolated within a mother-neonate pair [427]. It will be interesting to apply this SBT phylogenetic topological analysis of concatenated gene targets to similar mother-to-child transmission or between sexual contacts.

The major advantage of utilising SBT, relative to whole genome sequencing, is direct PCR amplification from clinical samples to provide rapid typing following detection in a clinical sample prior to culture. The advent and improvements of metagenomic processes and analysis may eventually supersede this; however, practical use on infections with mycoplasmas and mixed strains is not yet tested. Determination of SBT phylogenetic topological analysis with a mixed population of *M. hominis* strains would not be possible directly on a clinical sample, however, this may be feasible on infections originating from a clonal *M. hominis* infection.

At the population level, SBT assesses the genetic diversity of *M. hominis* strains. This study describes 20 SBT STs, revealing a genetic heterogeneity among this species. This finding is in agreement with the data obtained by studies using other molecular typing methods. MLVA typing of 210 *M. hominis* clinical isolates revealed high genetic heterogeneity within the species and this method was considered too discriminatory to be used for epidemiological studies at the population level [133]. Genetic heterogeneity of *M. hominis* was also detected by RAPD and confirmed by PFGE [415], describing changes in the genomes of *M. hominis* clinical isolates from patients with chronic infection. AFLP analysis provided further evidence of high level intra-species variability in *M. hominis*, by examining five randomly chosen clinical isolates [414]. RFLP analysis identified high heterogeneity among 20 *M. hominis* isolates obtained from colonised women and women with urogenital infections,

however, two strains sequentially isolated from one patient showed identical patterns after *Sma*I and *Mlu*I digestion [413]. Since the accumulation of SNP within the restriction enzyme recognition site is required to alter the less sensitive analysis methods, it is not surprising that these show the least amount of diversity. The mounting evidence suggests that lower resolution methods of typing are more successful for epidemiology and transmission studies for *M. hominis* for example, classification of the strain in this chapter by Vaa type (common practice for *M. pneumoniae* and *Ureaplasma* species) identified 13 category 1 strains, 12 category 2 strains, 1 category three strain, and five type 4 strains; however, allelic typing by *vaa* sequence results in 26 ATs for 36 strains.

5.3.5 The use of bioinformatics to develop minimum MLST schemes that represent genomic phylogeny

This chapter has successfully employed bioinformatics methodologies to develop seven gene mMLST schemes for the characterisation of *M. hominis*. The three potential mMLST schemes identified are representative of whole genome SNP phylogeny and therefore provide an accurate method for examining the relationship between strains.

Assessment of the pan-genome assembled for *M. hominis* revealed 427 core genes. Previous examination of *Mycoplasma* species pan-genome identified a core-genome of only 196 genes; however, this was an inter-species analysis and lower levels of conserved genes than observed in this chapter is to be expected. Of the entire pan-genome, the core genome represents 55% of all genes. This level of similarity between *M. hominis* strains is in stark contrast to *M. pneumoniae* where over 99% identity has been observed between genomes [17]. Congruence of the *M. hominis* pan-genome to other *Mycoplasma* species was assessed by comparison to *M. pneumoniae*

(strain M129) and *U. parvum* serovar 3 (strain AF222894). No significant hits to any of the genes in the *M. hominis* pan-genome were identified, suggesting that *M. hominis* is a distant relative to these *Mycoplasma* species, sharing little sequence similarity in the coding genes. This is concordant with *Mycoplasma* species phylogeny based on 16S rRNA sequences [6].

To determine the suitability of the proposed genes for the seven gene mMLST schemes, sequences were compared before and after short-term passage in two *M. hominis* strains. Genes found to be genetically variable after short-term passage would be unsuitable candidates for an mMLST scheme. From the set of 48 genes required to replicate whole genome sequence SNP phylogeny, one gene (GENE-220; *pcrA*) was found to have acquired mutations and was therefore not suitable for use in the mMLST schemes. As genomic stability was only assessed in two *M. hominis* strains, it is likely that further instability would be identified upon assessment of a larger number of strains. Sequence heterogeneity has been well documented for *M. hominis* [362] and therefore it is essential that the stability of the genes chosen for the three MLST schemes is further assessed.

The three mMLST schemes proposed in this chapter require further development to generate PCR-based schemes that can be widely utilised for the characterisation of *M. hominis*. Areas of the genes containing regions of variation responsible for the topology of the phylogenetic trees need to be identified; these can then be used as amplicons for a PCR-based method whilst retaining the conserved phylogeny. In addition, the ability to perform mMLST directly on clinical specimens would by-pass the need for specialised culture methodologies, allowing for a more rapid characterisation of *M. hominis*.

5.3.6 Recombination in *Mycoplasma hominis*

Recombination has previously been examined for *M. hominis*; however, only a limited number of genes were examined [362]. Nevertheless, analysis revealed inter- and intra-genic recombination in *M. hominis* and recombination was proposed as a method for the high intra-species variability of *M. hominis*. In addition, examination of *M. pneumoniae*, *M. genitalium*, *M. pulmonis* and *U. urealyticum* indicated a large number of repeats within the genomes of these organisms suggest the existence of a large potential for recombination [246]. In concordance with this, a large number of predicted recombination sites were identified in this chapter. This could be due to the diversity of the isolates from the reference used for mapping. The reference is on average 8500 SNPs from the isolates and each isolate is on average 9100 SNPs from each other isolate, indicating a large degree of diversity. This is, in part, due to low availability of previously sequenced M. hominis reference genomes. Therefore, when more fully sequenced and assembled genomes will become available the true extent of recombination in the species may be unravelled. Currently, with a potential presence of a large number of recombinations, any method that relies on phylogenetic tree has limitation. In this case, the phylogenetic tree is influenced to a greater extent by horizontal transfer of genetic material, rather than vertical inheritance from the parent strain.

5.3.7 Consequences of the genetic variability of *Mycoplasma hominis*

The ability of *M. hominis* to evade the immune system and persist in the human host may be related to the highly variable nature of the *M. hominis* genome as observed in this chapter. Genetic variation within genes studied in this chapter resulted in non-synonymous changes within the amino acid sequence and therefore changes in resulting expressed protein, providing a mechanism by which *M. hominis* can evade

the host defences. This could result in persistent infections and explain the carriage/commensal nature of *M. hominis* in humans. Assessment of the pathogenic potential of *M. hominis* is complicated by the high degree of genomic and antigenic heterogeneity observed within the species. Equally, as non-synonymous mutations in sequence is the basis for antibiotic resistance in many genes (for example, quinolone resistance determining regions of GyrA/B, ParC/E) [428], *M. hominis* may have an increased propensity to rapid development of antibiotic resistance mutations.

5.4 Summary

In this chapter, *M. hominis* has been characterised by the Vaa protein, SBT, whole genome sequence analysis and a novel method for developing MLST schemes. SBT phylogenetic topological analysis using either concatenated multi-gene sequences or partial whole genome sequences may enable meaningful epidemiological and transmission studies and may assist in increasing understanding of the characterisation and molecular typing of *M. hominis*. However, bioinformatics analysis has identified a minimal set of genes required to recapitulate the relationships observed in whole genome phylogeny constructed using SNP data. This chapter has identified three sets of seven genes that could be used to construct an mMLST typing scheme. The three mMLST schemes proposed in this chapter can be further developed for use in widely available PCR-based typing methods and ultimately MLST could be performed directly on clinical specimens where further information regarding potential strain transmission is warranted.

Chapter 6. Development of a multi-locus sequence typing scheme for the molecular typing of *Mycoplasma pneumoniae*

6.1 Introduction

Typing of clinical isolates by molecular methods is of importance for the understanding of the epidemiology of *M. pneumoniae* infection and for analysis of endemic outbreaks. Initial molecular typing targeted the gene encoding the major surface protein (P1) of *M. pneumoniae,* enabling the separation of isolates into two types, type 1 and 2 [128, 129, 222]. Recent studies have identified an additional six variants by utilising the repetitive regions, RepMp2/3 and RepMp4 [292, 429]. Multi-locus variable-number tandem-repeat (VNTR) analysis (MLVA) has also been used based on the variation in the copy number of tandem repeated sequences, called VNTRs, found at different loci across the genome. The variation of the copy number of these TRs depends on the isolate tested. Initially, 265 strains were grouped into twenty-six MLVA types, based on five VNTR loci (Mpn1, Mpn13-16) and an additional 18 novel types have since been reported [130, 132, 228]. However, locus Mpn1 is unstable in both clinical strains and in laboratory passages, and as most of the novel types came from variations in Mpn1 there is international consensus that this locus should be removed from the typing scheme [221].

Multi-locus sequence typing has also previously been attempted for the molecular typing of *M. pneumoniae.* However, due to the homogeneity of the *M. pneumoniae* species, very little polymorphism was found in the housekeeping genes examined and it has been concluded that the use of an MLST with housekeeping and structural genes is not useful for molecular typing [131]. In this study only three housekeeping genes were thoroughly examined for polymorphisms across 30 isolates of either P1 type 1, 2, or a variant strain. The other genes selected for analysis were examined against a single representative strain from each subtype.

This chapter describes an MLST scheme that has been developed with a high discriminatory ability to differentiate *M. pneumoniae* isolates based on sequence polymorphisms within eight housekeeping genes, improving on all published typing methods for *M. pneumoniae*.

6.2 Results

6.2.1 Determining MLST loci

Housekeeping genes that are considered to be conserved in other bacterial species under a low rate of selective pressure were chosen for analysis (Table 6.1). Locus sequences were selected using the available genome sequences of *M. pneumoniae* FH and M129 (FH: NC_017504.1; M129: NC_000912.1) and available whole genome sequences of 35 clinical isolates. Ten genes were included for initial analysis: recA protein (*recA*), inorganic phosphatase (*ppa*), phosphoglycerate mutase (*pgm*), DNA gyrase subunit B (*gyrB*), guanylate kinase (*gmk*), serine hydroxymethyltransferase (*glyA*), elongation factor P (*efp*), ATP synthase subunit α (*atpA*), carbamate kinase (*arcC*), and adenylate kinase (*adk*). Locus regions for PCR amplification were selected based on areas of the coding sequence (CDS) containing nucleotide polymorphisms. Initial examination of ten gene targets in the two type strains M129 and FH and genomic sequence from 35 *M. pneumoniae* clinical isolates identified variation, SNP differences, in eight out of the ten genes. Genes *recA* and *efp* were 100% conserved in all sequences analysed and were therefore excluded from the MLST scheme.

Table 6.1. MLST loci used in established bacterial MLST schemes also present in *M. pneumoniae*.

Bacterial Species	MLST Loci [a]									
	reca	***ppa***	***pgm***	***gyrb***	***gmk***	***glya***	***efp***	***atpa***	***arcc***	***adk***
Bacillus cereus					✓					
Chlamydia trachomatis						✓				
Campylobacter jejuni			✓			✓				
Escherichia coli	✓			✓						✓
Enterococcus faecium								✓		✓
Haemophilus influenzae	✓									✓
Helicobacter pylori		✓					✓	✓		
Moraxella catarrhalis		✓					✓			✓
Neisseria meningitidis			✓							✓
Staphylococcus aureus					✓				✓	
Staphylococcus epidermidis									✓	
Streptococcus suis	✓									
Vibrio vulnificus				✓						
Yersinia pseudotuberculosis										✓

[a] MLST loci were chosen based on the frequency of use in other bacterial MLST schemes (http://www.mlst.net/) and the presence of the gene in the published M129 and FH whole genomes.

6.2.2 MLST of *M. pneumoniae*

Genomic sequence analysis and additional PCR and sequencing (described in section 2.4.5) of a further 20 clinical isolates of all eight targets resolved a total of 12 STs (Table 6.2). The discriminatory typing ability for *M. pneumoniae* was 0.21 ST per isolate. The number of SNPs observed within each individual locus and the percentage of polymorphic sites are shown in Table 6.3, with *pgm* having the highest number of SNPs (10 SNPs) and the highest percentage of polymorphic sites corrected for sequence length (0.93%). The number of alleles per locus ranged from two (*ppa, gyrB,*

gmk and *arc*) to four (atpA) (Table 6.3). Examination of the Hunter-Gaston DI (which ranges from 0.0 = no diversity to 1.0 = complete diversity) indicated moderate diversity between the STs (DI: 0.784; 95% CI: 0.716-0.852) with the greatest individual diversity shown in *pgm* (DI: 0.620; 95% CI: 0.566-0.674) and the lowest diversity in *arcC* (DI: 0.069; 95% CI: 0.000-0.158).

Table 6.2. *Mycoplasma pneumoniae* strains, their sequence type (ST) and allelic profile, and their MLVA and P1 types.
Strains isolated from the same patient are indicated by grey shading.

Strain	ST	Allelic profile								MLVA type	P1 type
		ppa	*pgm*	*gyrB*	*gmk*	*glyA*	*atpA*	*arcC*	*adk*		
M129 (ATCC 29342)	1	1	2	1	1	1	3	2	1	4572	1
MPN135	1	1	2	1	1	1	3	2	1	4572	V1
FH (ATCC 15531)	2	2	3	2	2	2	4	1	1	3662	2
MPN007	2	2	3	2	2	2	4	1	1	NT[a]	2
MPN021	2	2	3	2	2	2	4	1	1	3662	NT[a]
MPN022	2	2	3	2	2	2	4	1	1	3562	2c
MPN023	2	2	3	2	2	2	4	1	1	3662	2
MPN101	2	2	3	2	2	2	4	1	1	3562	2
MPN102	2	2	3	2	2	2	4	1	1	3662	2
MPN107	2	2	3	2	2	2	4	1	1	3562	2
MPN114	2	2	3	2	2	2	4	1	1	3662	NT[a]
MPN117	2	2	3	2	2	2	4	1	1	3562	2
MPN119	2	2	3	2	2	2	4	1	1	3562	2
MPN121	2	2	3	2	2	2	4	1	1	3662	2c
MPN123	2	2	3	2	2	2	4	1	1	3662	2
MPN125	2	2	3	2	2	2	4	1	1	3562	2
MPN126	2	2	3	2	2	2	4	1	1	3662	2
MPN128	2	2	3	2	2	2	4	1	1	3662	NT[a]
MPN132	2	2	3	2	2	2	4	1	1	3562	2
MPN133	2	2	3	2	2	2	4	1	1	3662	2
MPN134	2	2	3	2	2	2	4	1	1	3662	2
MPN005	3	1	2	1	1	1	3	1	1	4572	1
MPN006	3	1	2	1	1	1	3	1	1	4572	NT[a]
MPN013	3	1	2	1	1	1	3	1	1	4572	1
MPN014	3	1	2	1	1	1	3	1	1	4572	1
MPN015	3	1	2	1	1	1	3	1	1	4572	1

Strain	ST	Allelic profile								MLVA type	P1 type
		ppa	*pgm*	*gyrB*	*gmk*	*glyA*	*atpA*	*arcC*	*adk*		
MPN016	3	1	2	1	1	1	3	1	1	4572	1
MPN017	3	1	2	1	1	1	3	1	1	4572	1
MPN020	3	1	2	1	1	1	3	1	1	4572	NT[a]
MPN103	3	1	2	1	1	1	3	1	1	4572	1
MPN105	3	1	2	1	1	1	3	1	1	4572	1
MPN108	3	1	2	1	1	1	3	1	1	4572	1
MPN109	3	1	2	1	1	1	3	1	1	4572	NT[a]
MPN113	3	1	2	1	1	1	3	1	1	4572	1
MPN116	3	1	2	1	1	1	3	1	1	4572	1
MPN118	3	1	2	1	1	1	3	1	1	4572	1
MPN120	3	1	2	1	1	1	3	1	1	4572	1
MPN122	3	1	2	1	1	1	3	1	1	4572	1
MPN136	3	1	2	1	1	1	3	1	1	4572	1
MPN004	4	2	1	2	2	2	4	1	1	3662	2
MPN104	4	2	1	2	2	2	4	1	1	3662	2
MPN106	4	2	1	2	2	2	4	1	1	3662	2
MPN110	4	2	1	2	2	2	4	1	1	3662	2
MPN124	4	2	1	2	2	2	4	1	1	3662	2
MPN131	4	2	1	2	2	2	4	1	1	3662	2
MPN111	5	1	2	1	1	1	2	1	1	4572	1
MPN011	6	2	3	2	2	2	1	1	1	3662	2
MPN112	6	2	3	2	2	2	1	1	1	3662	2
MPN127	7	2	3	2	2	2	4	1	2	3662	2
MPN129	8	2	3	2	2	2	4	1	3	3662	2
MPN130	9	1	2	1	1	1	3	1	4	4572	1
MPN008	10	2	1	2	2	2	4	1	2	3662	2
MPN018	10	2	1	2	2	2	4	1	2	3662	2
MPN010	11	1	2	1	1	3	3	1	1	3662	1
MPN003	11	1	2	1	1	3	3	1	1	4572	1

Strain	ST	Allelic profile								MLVA type	P1 type
		ppa	***pgm***	***gyrB***	***gmk***	***glyA***	***atpA***	***arcC***	***adk***		
MPN012	11	1	2	1	1	3	3	1	1	3562	NT[a]
MPN019	12	2	2	1	1	3	3	1	4	4572	1

[a]NT *M. pneumoniae* not classified by MLVA/P1 typing

Table 6.3. Variability of the *M. pnueomoniae* MLST loci

Name	No. of alleles	No. polymorphic sites	% polymorphic sites	Average G + C content (%)	Hunter-Gaston Diversity Index [a]	95% Confidence Interval
ppa	2	1	0.39	38.4	0.501	0.470-0.533
pgm	3	10	0.93	43.7	0.620	0.566-0.674
gyrB	2	2	0.47	39.9	0.505	0.482-0.528
gmk	2	1	0.25	40.1	0.505	0.482-0.528
glyA	3	2	0.30	45.6	0.560	0.493-0.627
atpA	4	3	0.38	44.8	0.557	0.502-0.612
arcC	2	1	0.18	45.5	0.069	0.000-0.158
adk	4	3	0.63	47.5	0.199	0.063-0.335

[a] Hunter-Gaston diversity index (DI, ranges from 0.0 indicates no diversity to 1.0 indicates complete diversity)

6.2.3 Phylogenetic analysis of *M. pneumoniae* sequence types

The construction of Neighbour-joining and maximum-likelihood trees from concatenated sequences of the eight loci for the 57 *M. pneumoniae* isolates (Figure 6.1; method described in section 2.4.8.1) illustrated two genetically distinct clusters which were confirmed by eBURST examination of relatedness (Figure 6.2; section 2.4.8.3). The two clusters, clonal complexes (CC) designated CC1 and CC2, contained ST1, ST3, ST5, ST9 and ST11, and ST2, ST4, ST6, ST7, ST8 and ST10, respectively. ST12 was located distal to the two main clusters; however, phylogenetic analysis revealed closer positioning to CC1. Neighbour-joining and maximum-likelihood trees were also constructed for the eight loci individually (Figure 6.3-Figure 6.10) and topology of both neighbour-joining and maximum-likelihood trees was consistent for all loci and concatenated sequences, confirming segregation into two distinct clusters.

Five homogenous strains (MPN13-MPN17) originating from nose and throat swabs from the same patient with Stevens-Johnson syndrome had identical STs (ST3). Additionally, two clinical isolates (MPN104 and MPN106) originating from separate sputum samples from a patient with bronchopneumonia taken four days apart also had identical STs (ST4). This indicates a single, clonal population responsible for infection in these cases. Phylogenetic positions of individual strains based on concatenated sequences of the eight MLST loci are shown in Figure 6.11.

A.

B.

Figure 6.1. Phylogenetic trees based on concatenated sequences of eight MLST loci.
Phylogenetic trees were constructed based on concatenated sequences of eight housekeeping loci for 12 unique STs using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown. STs are indicated by differential shading.

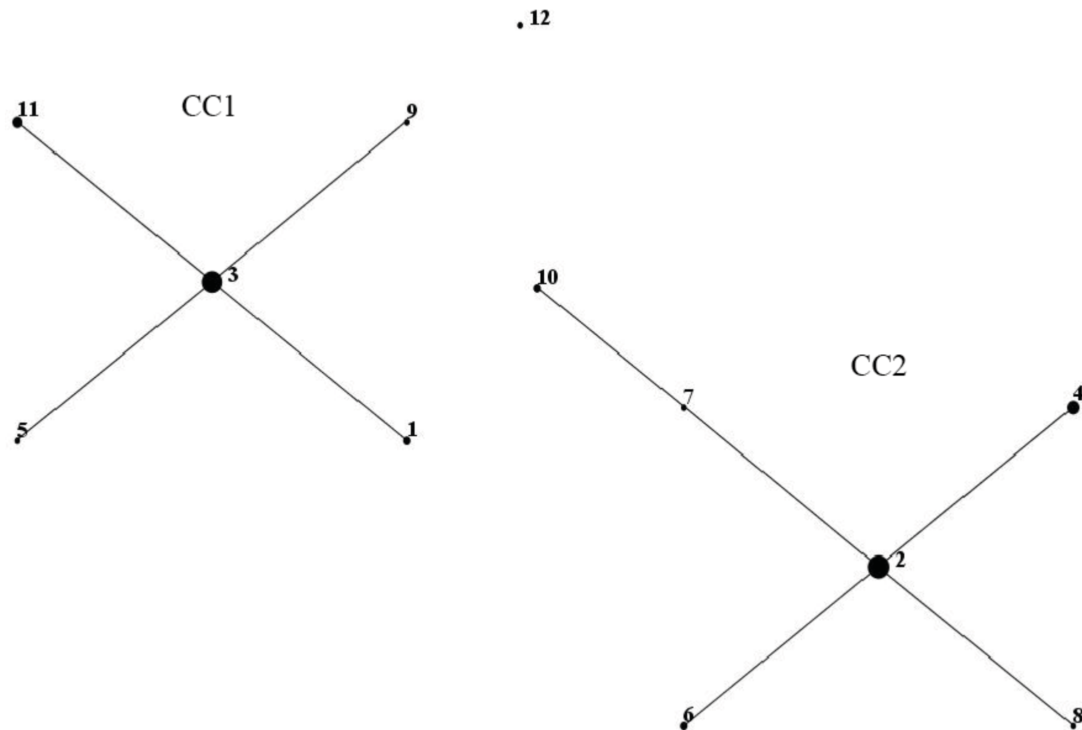

Figure 6.2. Analysis of *M. pneumoniae* using eBURST.
eBURST version 3 was used to analyse the 12 unique STs resolved for all 57 *M. pneumoniae* isolates. Two main clonal complexes (CC) were defined. The size of each dot is proportional to the number of isolates included in the analysis for each ST.

Figure 6.3. Phylogenetic trees based on the adenylate kinase locus for each individual strain.
Phylogentic trees were constructed based on the sequence of the *adk* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

Figure 6.4. Phylogenetic trees based on the carbamate kinase locus for each individual strain.

Phylogentic trees were constructed based on the sequence of the *arcC* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

Figure 6.5. Phylogenetic trees based on the ATP synthase subunit α locus for each individual strain.

Phylogentic trees were constructed based on the sequence of the *atpA* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

Figure 6.6. Phylogenetic trees based on the serine hydroxymethlytransferase locus for each individual strain.

Phylogentic trees were constructed based on the sequence of the *glyA* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

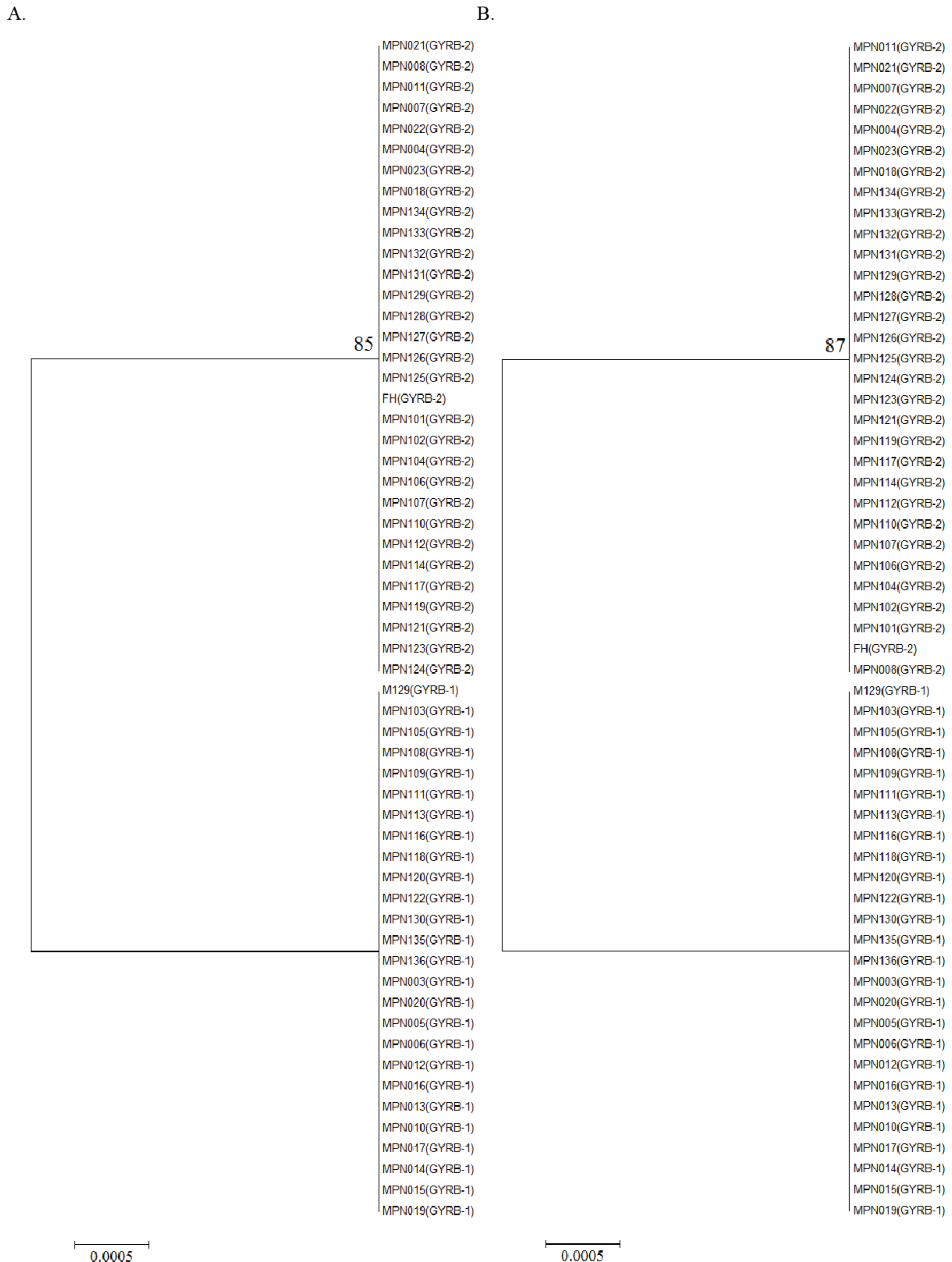

Figure 6.7. Phylogenetic trees based on the DNA gyrase subunit B locus for each individual strain.

Phylogentic trees were constructed based on the sequence of the *gyrB* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

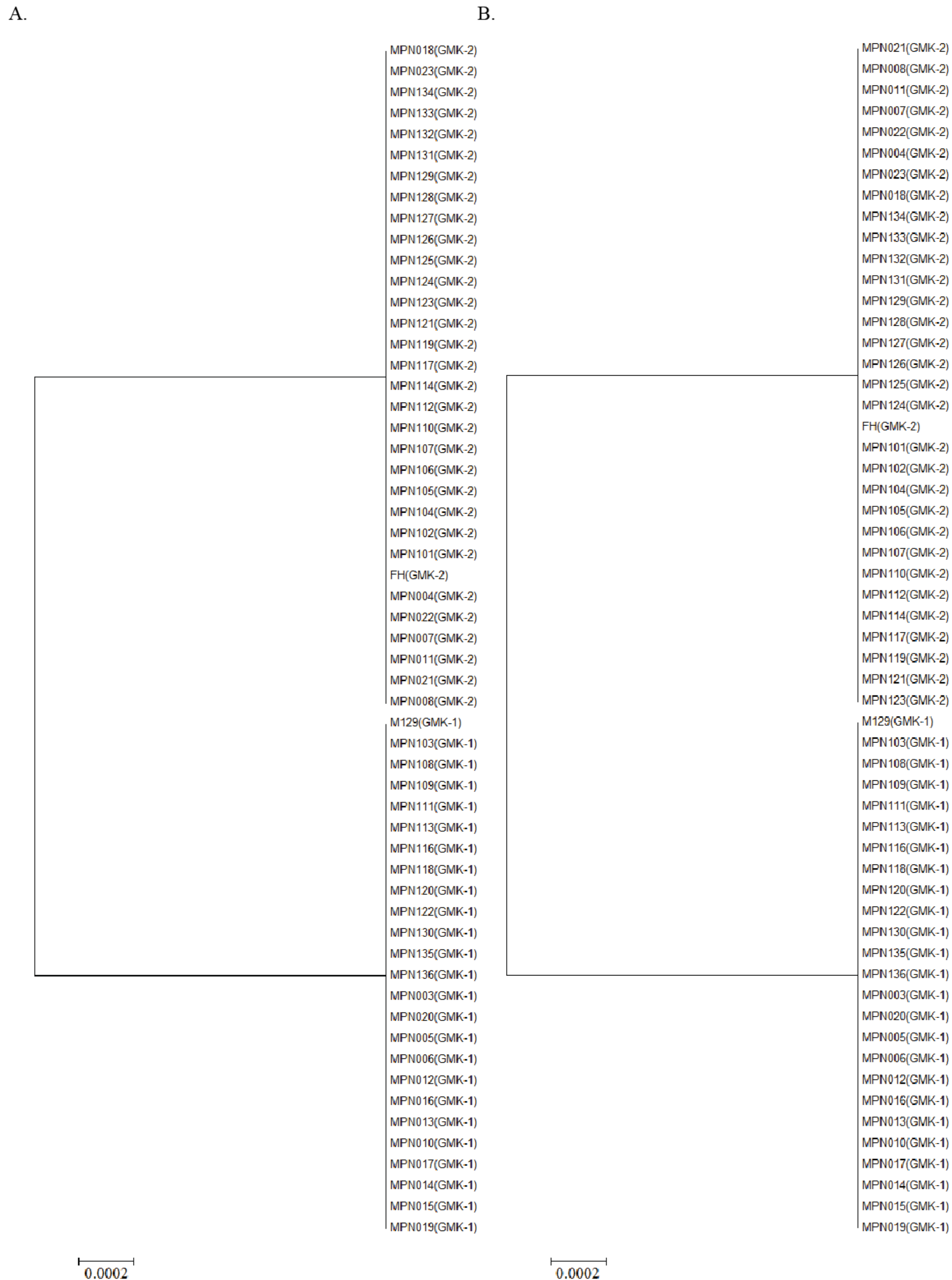

Figure 6.8. Phylogenetic trees based on the guanylate kinase locus for each individual strain.

Phylogenetic trees were constructed based on the sequence of the *gmk* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

Figure 6.9 Phylogenetic trees based on the phosphoglycerate mutase locus for each individual strain.

Phylogenetic trees were constructed based on the sequence of the *pgm* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

Figure 6.10. Phylogenetic trees based on the inorganic phosphatase locus for each individual strain.

Phylogenetic trees were constructed based on the sequence of the *ppa* locus for all 57 *M. pneumoniae* isolates using Maximum-likelihood (A) and Neighbour-joining (B) methods. Bootstrap support values of over 70% are shown.

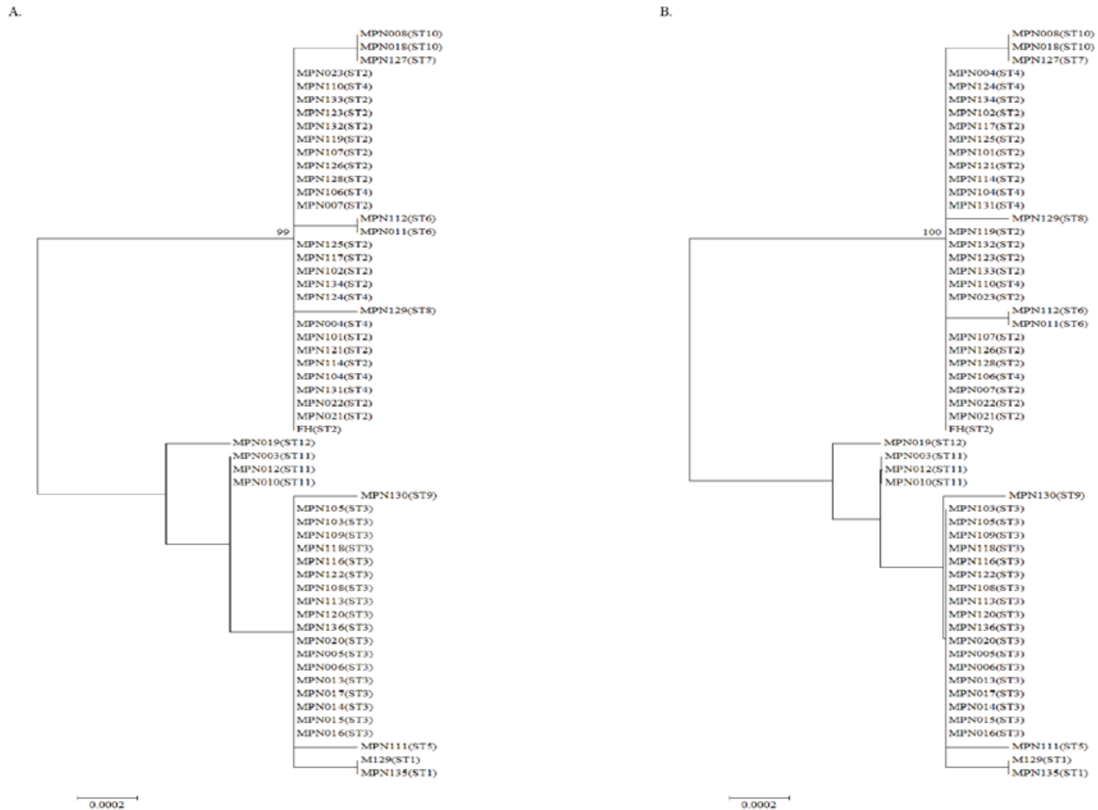

Figure 6.11. Phylogenetic trees based on concatenated sequences of eight MLST loci showing individual *M. pneumoniae* strain position.

Phylogenetic trees were constructed based on concatenated sequences of eight housekeeping loci for 12 unique STs using Maximum-likelihood (A) and Neighbour-joining (B) methods. Individual strains and STs can be identified in the traditional rectangular branch style. Bootstrap support values of over 70% are shown.

6.2.4 Determination of synonymous sequence changes in MLST loci

The possibility of synonymous sequence changes (indicating a pressure to conserve amino acid sequence and protein structure) was investigated by comparing predicted translated sequences for each locus. Analysis of deduced amino acid sequences of the eight loci for the 57 strains indicated that both synonymous and non-synonymous SNPs occurred, of which approximately 44% resulted in an amino acid change. Non-synonymous SNPs are highlighted in Figure 6.12. Amino acid sequences for ArcC, Gmk and GyrB yielded identical sequences for all ATs, numbering at two ATs for each locus. In comparison, Pgm analysis resulted in the largest number of non-synonymous changes in amino acid sequence, with four changes in the sequence between three ATs.

A.

```
ADK-4     VANTTGLFHLSTGDIFRTVMQEQGALSQTLAHYMNQGLYVPDELTNQTFWHFVTTHQNEL
ADK-3     VANTTGLFHLSTGDIFRSVMQEQGALSQTLAHYMNQGLYVPDELTNQTFWHFVTTHQNEL
ADK-1     VANTTGLFHLSTGDIFRSVMQEQGALSQTLAHYMNQGLYVPDELTNQTFWHFVTTHQNEL
ADK-2     VANTTGLFHLSTGDIFRSVMQEQGALSQTLAHYMNQGLYVPDELTNQTFWHFVTTHQNEL
          *****************:******************************************

ADK-4     HKGFILDGYPRTLNQLEFLQSKLQLDQVFHLKLSDPQVLVARILNRLVCPSCGSVYNKQS
ADK-3     HKGFILDGYPRTLNQLEFLQSKLQLDQVFHLKLSDPQVLVARILNRLVCPSCGSVYNKQS
ADK-1     HKGFILDGYPRTLNQLEFLQSKLQLDQVFHLKLSDPQVLVARILNRLVCPSCGSVYNKQS
ADK-2     HKGFILDGYPRTLNQLEFLQSKLQLDQVFHLKLSDPQVLVARILNRLVCPSCGSVYNKQS
          ************************************************************

ADK-4     KPPLKANQCDRCHATLQARNDDTEAVILKRLTLYEDT      157
ADK-3     KPPLKANQCDLCHATLQARNDDTEAVILKRLTLYEDT      157
ADK-1     KPPLKANQCDRCHATLQARNDDTEAVILKRLTLYEDT      157
ADK-2     KPPLKANQCDRCHAMLQARNDDTEAVILKRLTLYEDT      157
          ********** *** **********************
```

B.

```
ARCC-1     PHQAVYFLTQTLVEASDPAFQNPNKPVGPFYNTEETARSANPNSTVVEDAGRGWRKVVAS
ARCC-2     PHQAVYFLTQTLVEASDPAFQNPNKPVGPFYNTEETARSANPNSTVVEDAGRGWRKVVAS
           ************************************************************

ARCC-1     PKPVDVLGIDAIKSSFNQGNLVIVGGGGGVPTIKTKSGYATVDGVIDKDLALSEIAIKVE
ARCC-2     PKPVDVLGIDAIKSSFNQGNLVIVGGGGGVPTIKTKSGYATVDGVIDKDLALSEIAIKVE
           ************************************************************

ARCC-1     ADLFVILTAVDFVYINYGQPNEQKLTCINTKEAKTLMAANQFAKGSMLPKVEACLNFVQS
ARCC-2     ADLFVILTAVDFVYINYGQPNEQKLTCINTKEAKTLMAANQFAKGSMLPKVEACLNFVQS
           ************************************************************

ARCC-1     GTNKTAIIAQ 190
ARCC-2     GTNKTAIIAQ 190
           **********
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C.

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GLYA-1     ENYVSRDILEVTGSILTNKYAEGYPTRRFYEGCEVVDESESLAINTCKELFGAKWANVQP
GLYA-3     ENYVSRDILEVTGSILTNKYAEGYPTRRFYEGCEVVDESESLAINTCKELFGAKWANVQP
GLYA-2     ENYVSRDILEVTGSILTNKYAEGYPTRRFYEGCEVVDESESLAINTCKELFGAKWANVQP
           ************************************************************

GLYA-1     HSGSSANYAVYLALLKPGDAILGLDLNCGGHLTHGNKFNFSGKQYQPYSYTINPETEMLD
GLYA-3     HSGSSANYAVYLALLKPGDAILGLDLNCGGHLTHGNKFNFSGKQYQPYSYTINPETEMLD
GLYA-2     HSGSSANYAVYLALLKPGDAILGLDLNCGGHLTHGNKFNFSGKQYQPYSYTINPETEMLD
           ************************************************************

GLYA-1     YDEVLRVAREVKPKLIICGFSNYSRTVDFERFSAIAKEVGAYLLADIAHIAGLVAAGLHP
GLYA-3     YDEVLRVAREVKPKLIICGFSNYSRTVDFERFSAIAKEVGAYLLADIAHIAGLVAAGLHP
GLYA-2     YDEVLRVAREVKPKLIICGFSNYSRTVDFERFSAIAKEVGAYLLADIAHIAGLVAAGLHP
           ************************************************************

GLYA-1     NPLPYTDVVTSTTHKTLRGPRGGLIMSNNEAIIRKLDSGVFPGC      224
GLYA-3     NPLPYTDVVTSTTHKTLRGPRGGLIMSNNEAIIRKLDSGVFPGC      224
GLYA-2     NPLPYADVVTSTTHKTLRGPRGGLIMSNNEAIIRKLDSGVFPGC      224
           *****:**************************************
```

D.

```
ATPA-1      VADGIAKVSGLENALLNELIEFENNVQGIALNLEQNTVGVALFGDYSKIREGSTAKRTHN
ATPA-4      VADGIAKVSGLENALLNELIEFENNVQGIALNLEQNTVGVALFGDYSKIREGSTAKRTHN
ATPA-2      VADGIAKVSGLENALLNELIEFENNVQGIALNLEQNTVGVALFGDYSKIREGSTAKRTHN
ATPA-3      VADGIAKVSGLENALLNELIEFENNVQGIALNLEQNTVGVALFGDYSKIREGSTAKRTHN
            ************************************************************

ATPA-1      VMQTPVGDVMLGRIVNALGEPVDGRGPIKAEEFDQVEKIAPGVMTRKTVNQPLETGILTI
ATPA-4      VMQTPVGDVMLGRIVNALGEPVDGRGPIKAEEFDQVEKIAPGVMTRKTVNQPLETGILTI
ATPA-2      VMQTPVGDVMLGRIVNALGEPVDGRGPIKAEEFDQVEKIAPGVMTRKTVNQPLETGILTI
ATPA-3      VMQTPVGDVMLGRIVNALGEPVDGRGPIKAEEFDQVEKIAPGVMTRKTVNQPLETGILTI
            ************************************************************

ATPA-1      DALFPIGKGQRELIVGDRQTGKTSIAIDTIINQRGKDVYCVYVAMGQKNSSVAQIVHQLE
ATPA-4      DALFPIGKGQRELIVGDRQTGKTSIAIDTIINQRGKDVYCVYVAMGQKNSSVAQIVHQLE
ATPA-2      DALFPIGKGQRELIVGDRQTGKTSIAIDTIINQRGKDVYCVYVAMGQKNSSVAQIVHQLE
ATPA-3      DALFPIGKGQRELIVGDRQTGKTSIAIDTIINQRGKDVYCVYVAMGQKNSSVAQIVHQLE
            ************************************************************

ATPA-1      VTDSMKYTTVVCATASNPASMIYLTPFTGITIAEYWLKQGKDVLIVFDDLSKHAIAYRTL
ATPA-4      VTDSMKYTTVVCATASNPASMIYLTPFTGITIAEYWLKQGKDVLIVFDDLSKHAIAYRTL
ATPA-2      VTDSMKYTTVVCATASDPASMIYLTPFTGITIAEYWLKQGKDVLIVFDDLSKHAIAYRTL
ATPA-3      VTDSMKYTTVVCATASDPASMIYLTPFTGITIAEYWLKQGKDVLIVFDDLSKHAIAYRTL
            ****************:*******************************************

ATPA-1      SLLLRRPPGREAFPGDVFYLHSRLL 265
ATPA-4      SLLLRRPPGREAFPGDVFYLHSRLL 265
ATPA-2      SLLLRRPPGREAFPGDVFYLHSRLL 265
ATPA-3      SLLLRRPPGREAFPGDVFYLHSRLL 265
            *************************
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E.

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GMK-1       SGVGKSSLVRCLIDHFKDKLRYSISATTRKMRNSETEGVDYFFKDKAEFEKLIAADAFVE
GMK-2       SGVGKSSLVRCLIDHFKDKLRYSISATTRKMRNSETEGVDYFFKDKAEFEKLIAADAFVE
            ************************************************************

GMK-1       WAMYNDNYYGTLKSQAEQIIHNGGNLVLEIEYQGALQVKQKYPNDVVLIFIKPPSMEELL
GMK-2       WAMYNDNYYGTLKSQAEQIIHNGGNLVLEIEYQGALQVKQKYPNDVVLIFIKPPSMEELL
            ************************************************************

GMK-1       VRLKKRNDEDA 131
GMK-2       VRLKKRNDEDA 131
            ***********
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F.

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GYRB-1      VPDFTVMEKSDYKQTVIASRLQQLAFLNKGIQIDFVDERRQNPQSFSWKYDGGLVQYIHH
GYRB-2      VPDFTVMEKSDYKQTVIASRLQQLAFLNKGIQIDFVDERRQNPQSFSWKYDGGLVQYIHH
            ************************************************************

GYRB-1      LNNEKEPLFEDIIFGEKTDTVKSVSRDESYTIKVEVAFQYNKTYNQSIFSFCNNINTTEG
GYRB-2      LNNEKEPLFEDIIFGEKTDTVKSVSRDESYTIKVEVAFQYNKTYNQSIFSFCNNINTTEG
            ************************************************************

GYRB-1      GTHVEGFRNALVKIINRFAVEN    142
GYRB-2      GTHVEGFRNALVKIINRFAVEN    142
            **********************
```

G.

```
PGM-1      APCTMKSDLEAFMVFLKDYHNVIIGTLGGRYYGMDRDQRWDREEIAYNAILGNSKASFTD
PGM-2      APCTMKSDLEAFMVFLKDYHNVIIGTLGGRYYGMDRDQRWDREEIAYNAILGNSKASFTD
PGM-3      APCTMKSDLEAFMVFLKDYHNVIIGTLGGRYYGMDRDQRWDREEIAYNAILGNSKASFTD
           ************************************************************

PGM-1      PVAYVQSAYDQKVTDEFLYPAVNGNVDKEQFALKDHDSVIFFNFRPDRARQMSHMLFQTD
PGM-2      PVAYVQSAYDQKVTDEFLYPAVNGNVDKEQFALKDHDSVIFFNFRPDRARQMSHMLFQTD
PGM-3      PVAYVQSAYDQKVTDEFLYPAVNGNVDKEQFALKDHDSVIFFNFRPDRARQMSHMLFQTD
           ************************************************************

PGM-1      YYDYTPKAGRKHNLFFVTMMNYEGIKPSAVVFPPETIPNTFGEVIAHNKLKQLRIAETEK
PGM-2      YYDYTPKAGRKYNLFFVTMMNYEGIKPSAVVFPPETIPNTFGEVIAHNKLKQLRIAETEK
PGM-3      YYDYTPKAGRKHNLFFVTMMNYEGIKPSAVVFPPETIPNTFGEVIAHNKLKQLRIAETEK
           ***********:************************************************

PGM-1      YAHVTFFFDGGVEVDLPNETKCMVPSLKVATYDLAPEMACKGITDQLLNQINQFDLTVLN
PGM-2      YAHVTFFFDGGVEVDLPNETKCMVPSLKVATYDLAPEMACKGITDQLLNQINQFDLTVLN
PGM-3      YAHVTFFFDGGVEVDLPNETKCMVPSLKVATYDLAPEMACKGITDQLLNQINQFDLTVLN
           ************************************************************

PGM-1      FANPDMVGHTGNYAACVQGLEALDVQIQRIIDFCKANHITLFLTADHGNAEEMIDSNNNP
PGM-2      FANPDMVGHTGNYAACVQGLEALDVQIQRIIDFCKANHITLFLTADHGNAEEMIDSNNNP
PGM-3      FANPDMVGHTGNYAACVQGLEALDVQIQRIIDFCKANHITLFLTADHGNAEEMIDSNNNP
           ************************************************************

PGM-1      VTKHTVNKVPFVCTDTNIDLQQDSASLANIAPTILAYLGLKQPAEMTANSLLYKKV  356
PGM-2      VTKHTVNKVPFVCTDTNIDLQQDSASLANIAPTILAYLGLKQPAEMTANSLLISKK  356
PGM-3      VTKHTVNKVPFVCTDTNIDLQQDSASLANIAPTILAYLGLKQPAEMTANSLLISKK  356
           ****************************************************  .*
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H.

```
PPA-1      IFADQAFLPGVVVPTRIVGALEMVDDGELDTKLLGVIDCDPRYKEINSVNDLPKHRVDEI
PPA-2      IFADQAFLPGVVVPTRIVGALEMVDDGELDTKLLGVIDCDPRYKEINSVNDLPKHRVDEI
           ************************************************************

PPA-1      IGFLKTYKLLQKKEVIIKGVQSLEW  85
PPA-2      IGFFKTYKLLQKKEVIIKGVQSLEW  85
           ***:*********************
```

Figure 6.12. Alignments of amino acid sequences of each allelic type at each of the eight MLST loci.

Amino acid sequence alignments were generated for each of the eight loci based on nucleotide sequence of each allelic type. Non-synonymous changes in amino acid sequence are highlighted in yellow. * (asterix) indicates positions which have a single, fully conserved residue; : (colon) indicates conservation between groups of strongly similar properties – scoring > 0.5 in the Gonnet PAM 250 matrix; . (period) indicates conservation between groups of weakly similar properties – scoring < 0.5 in the Gonnet PAM 250 matrix; blank space indicates no similarity between amino acid residues.

6.2.5 Application of MLST scheme to published genomic sequences of *M. pneumoniae*

The MLST scheme was applied to the published complete genome sequences of *M. pneumoniae* available from NCBI: 309 (NC_016807.1), M129-B7 (CP003913.2), M29 (NZ_CP008895.1), PO1 (GCA_000319655.1), PI 1428 (GCA_000319675.1) and 19294 (GCA_000387745.1). These strains were determined as ST2, ST1, ST3, ST2, ST1 and ST7, respectively.

6.2.6 Assessment of MLST loci stability

The stability of each MLST locus was assessed in ten *M. pneumoniae* isolates. Isolates were re-typed following short-term passage (ten sequential sub-culture passages) in liquid medium. All loci were found to be completely stable, with no new SNPs detected in the short-term passage strains compared to the original isolates.

6.2.7 Genomic sequence analysis

Phylogenetic analysis of the whole genome sequence (method described in section 2.6.8.2) of 41 clinical *M. pneumoniae* isolates and the available published whole genomes sequences of eight additional strains revealed two distinct genomic clades (GC), GC1 and GC2, corresponding to P1 type 1 and type 2 , respectively (Figure 6.13). There was no strong clustering observed associated with the year when the strains were collected, the patient's age, sample origin or the patient's symptoms. This also includes non-exact clustering by STs; however this is not unexpected due to the high genome similarity and low clonality within the data set. Nevertheless, excellent parity was found in the phylogeny of both whole genome and concatenated MLST sequences, with all strains co-locating to the corresponding CC and GC.

Alignment of genomic sequence from 35 clinical isolates to M129 revealed two general areas of differences between GC1 and GC2 (Figure 6.14; section 2.6.8.2). The first area in which similarity is reduced to M129 is between 150,000-200,000 bp, where strains belonging to GC2 contain divergent sequences or the sequence is absent and strains belonging to GC1 contain sequences similar/identical to M129. The second area is around 550,000 bp., where the similarity between the two genomic clades is reduced.

Three regions of SNPs were predicted to have arisen by homologous recombination in the chromosomes of the 35 clinical *M. pneumoniae* isolates (Figure 6.15); one of which distinguished GC1 from GC2; and the other two which occur within GC1. Area one was predicted to occur in all strains in GC1 at 169,233-169,591 bp (M129 numbering) in a gene encoding a hypothetical protein (MPN130 [C09_orf140o]). Area two was predicted in ten strains within GC1 and occurs in an inter-genic region (position 570,767-570,790 bp in M129). Both these predicted recombination regions correspond to drops in similarity observed in the alignment of the strains to M129 (Figure 6.14). Area three is predicted in a single strain, MPN113, and occurs at position 322,651-322,692 bp (M129 numbering) in an inter-genic region. Following the removal of predicted areas of recombination, the two distinct genetic clades could still be identified (Figure 6.15).

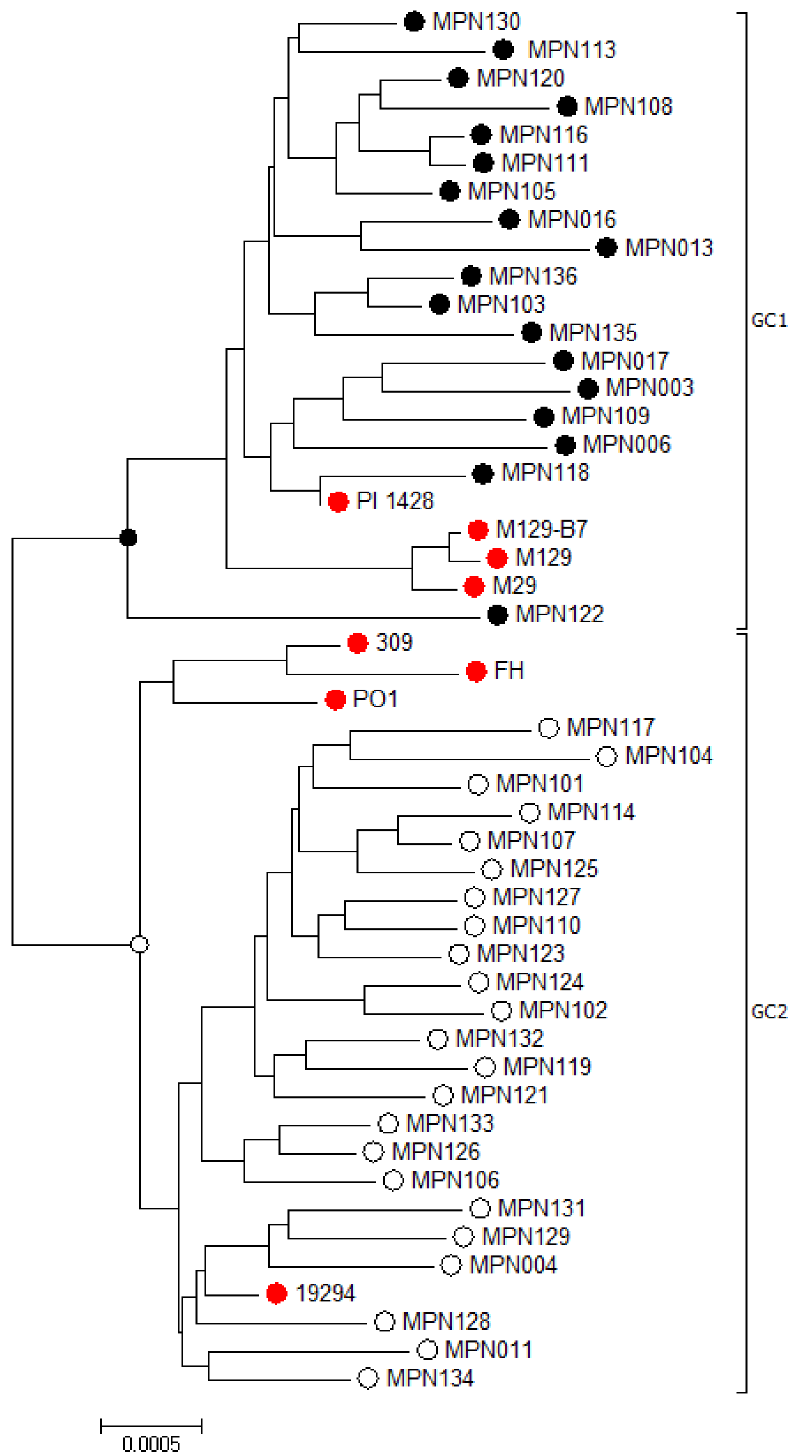

Figure 6.13. Phylogenetic tree based on whole genome alignments of 49 *M. pneumoniae* genomic sequences.

Phylogenetic tree was constructed based on whole genome alignments of 41 clinical *M. pneumoniae* strains and the eight available strains on NCBI using the Neighbour-joining method.

Figure 6.14. Overall sequence identity of the whole genome sequences of 35 *M. pneumoniae* clinical strains to the reference M129.

Strains were aligned to M129 (black) and G+C content is indicated in the middle. Strains belonging to GC1 are displayed in red and strains belonging to GC2 are displays in blue. Areas of differences to M129 are indicated by white shading in the strains. Each strain is represented by a separate circle. Genes are annotated based on M129. Location in the reference genome is indicated by numeration on the inside of the ring. GC content in the reference genome is indicated by the black bar graphs between the genomic coordinates and the coloured rings (bars pointing toward the outside of the circle indicate high GC content).

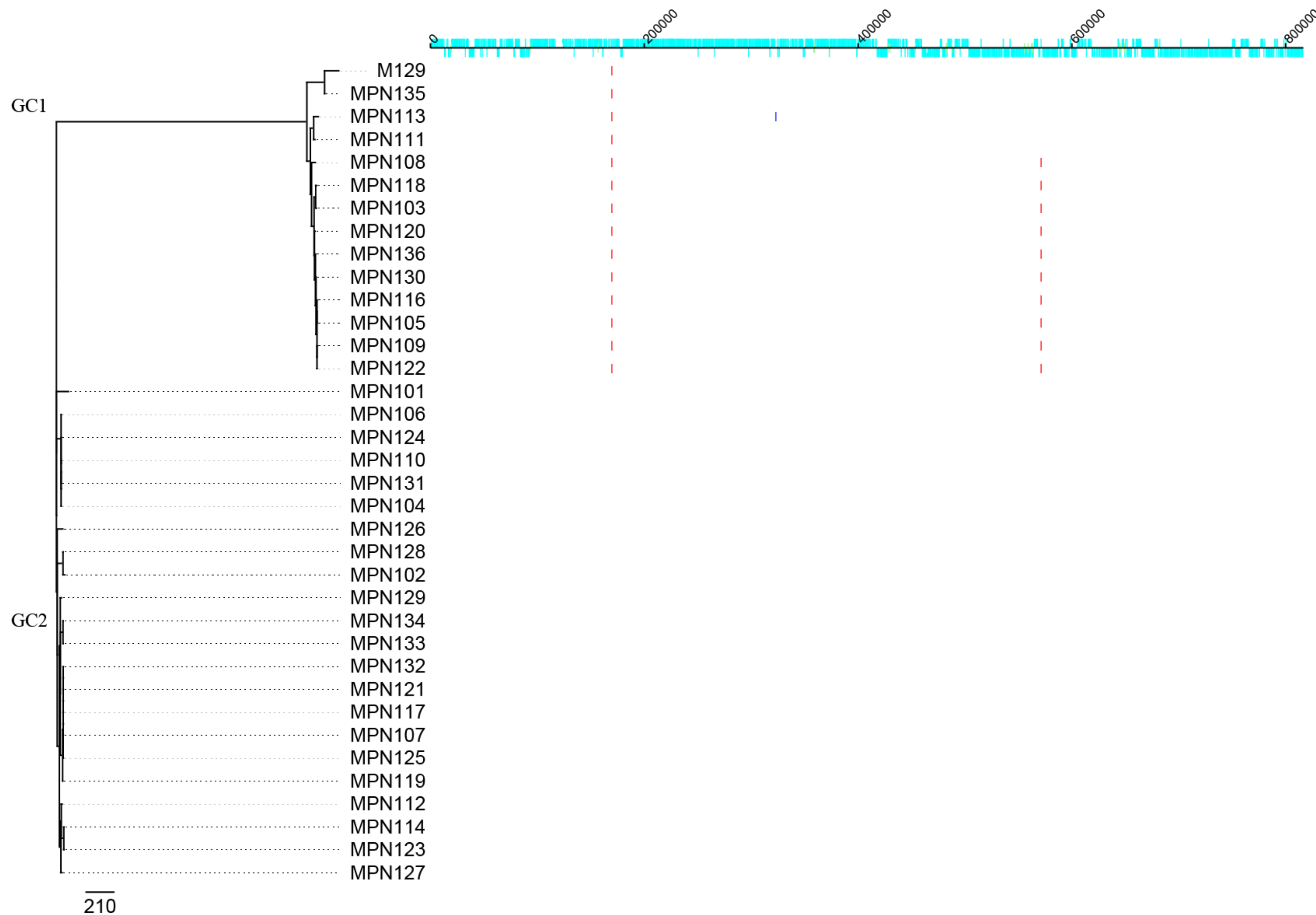

Figure 6.15. Prediction of recombination in the *M. pneumoniae* isolates chromosomes. Regions of variation in the genomes of the 35 clinical *M. pneumoniae* isolates and the type strain M129 which are predicted to have arisen by homologous recombination are shown in the panel on the right. Red blocks indicated recombination predicted to have occurred on internal nodes, blue indicates taxa-specific recombination. Isolates are ordered according to the phylogenetic tree displayed on the left. The track along the top of the figure displays the M129 chromosome and annotation, where protein coding sequences (CDS) are indicated in light blue.

6.2.8 Comparison to MLVA and P1 typing methods

The results have shown that MLST ST was related to P1 type (determined as described in section 2.4.4; Table 6.2), with the two most common STs, ST2 and ST3, containing strains that were P1 type 2 and P1 type 1, respectively. The MLST scheme was also comparable to MLVA typing (determined as described in section 2.4.7). The two major clusters observed, CC1 and CC2, could be directly linked to MLVA types; CC1 contained MLVA type 4572 whereas CC2 contained MLVA types 3662 and 3562. Each ST contained only one MLVA type with the exception of ST2 which contained both 3662 and 3562, and ST11 which contained 4572, 3662 and 3562 (Table 6.2). Distribution of MLVA type, P1 type and MLST ST can be observed in Figure 6.16, indicating that P1 type 1, MLST ST2 and MLVA types 3662 and 4572 were the most frequently occurring in the isolates tested.

For the *M. pneumoniae* isolates examined, MLST was deemed to be more discriminatory than both MLVA and P1 typing with the types per isolate being 0.21, 0.05 and 0.07, respectively. This was confirmed by examination of Hunter-Gaston DI, indicating larger discriminatory ability for the MLST scheme (DI: 0.784; 95% CI: 0.716-0.852) than the current MLVA scheme (DI: 0.633; 95% CI: 0.583-0.683) and P1 typing (DI: 0.567; 95% CI: 0.510-0.625).

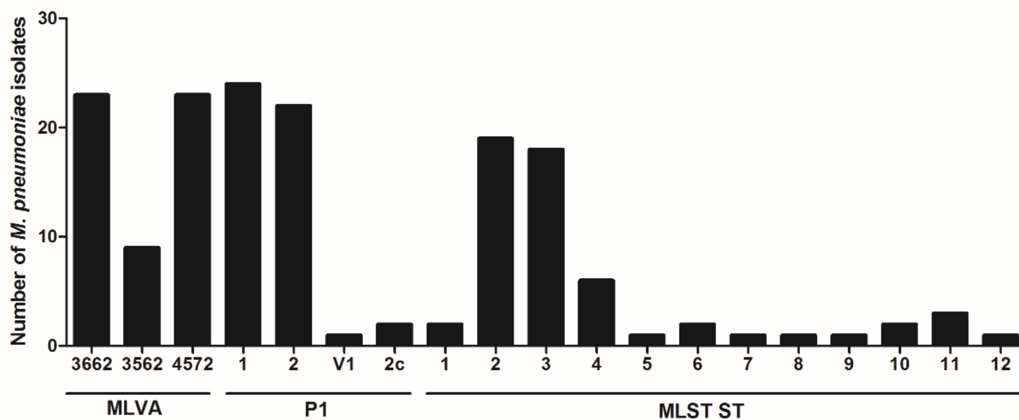

Figure 6.16. Distribution of MLVA, P1 type and MLST ST for 57 *M. pneumoniae* isolates.
The 57 isolates were separated independently for MLVA type, P1 type and MLST type (each group defined by line).

6.2.9 Molecular typing and epidemiology in the UK

There was no obvious link between the MLST ST and the year when the strains were collected, the patient's age and the sample origin; however, limited numbers of strains were available per year and for some years there were no strains. Indeed, multiple STs can be observed in a single year (Table 6.2). Typing data for strains were grouped into four-yearly intervals, representing the epidemic cycles of *M. pneumoniae* observed in the UK. Multiple MLST, MLVA and P1 types were observed in each four-yearly interval (Figure 6.17 - Figure 6.19); however a predominance of P1 type 1 can be seen for all intervals except 1981-1984 where equal numbers of P1 type 1 and type 2 strains were observed. Due to the low sample number in some four-yearly intervals and limited typing data, the variation in P1 types observed is likely to be an underestimate of the actual *M. pneumoniae* population present.

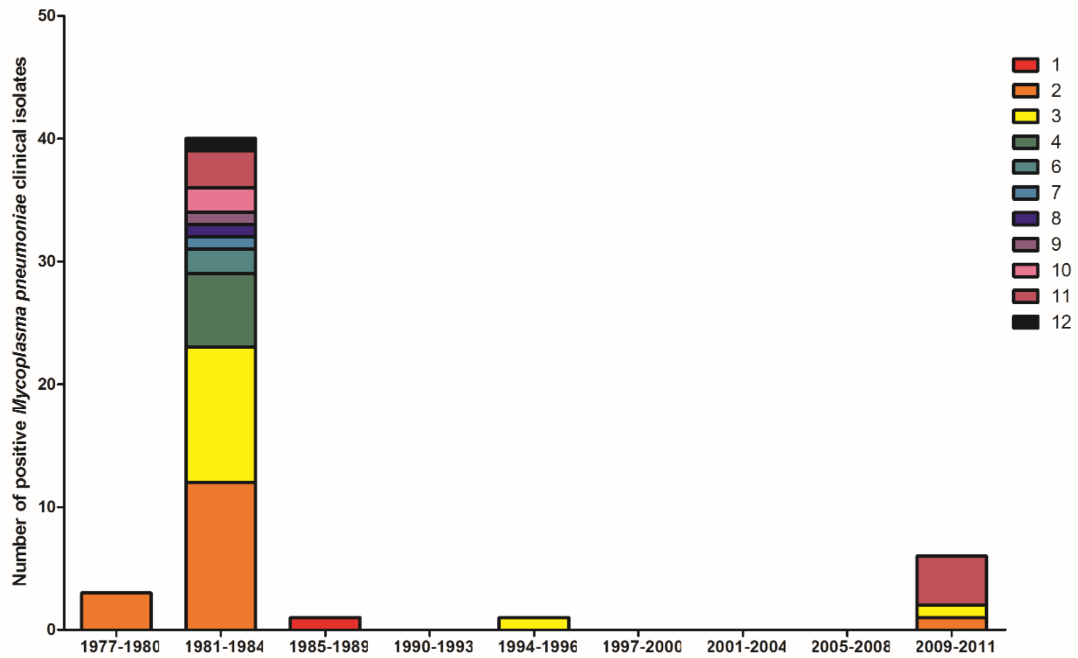

Figure 6.17. Distribution of MLST sequence types for 57 *M. pneumoniae* clinical isolates in the four-yearly epidemic cycles observed in the UK.

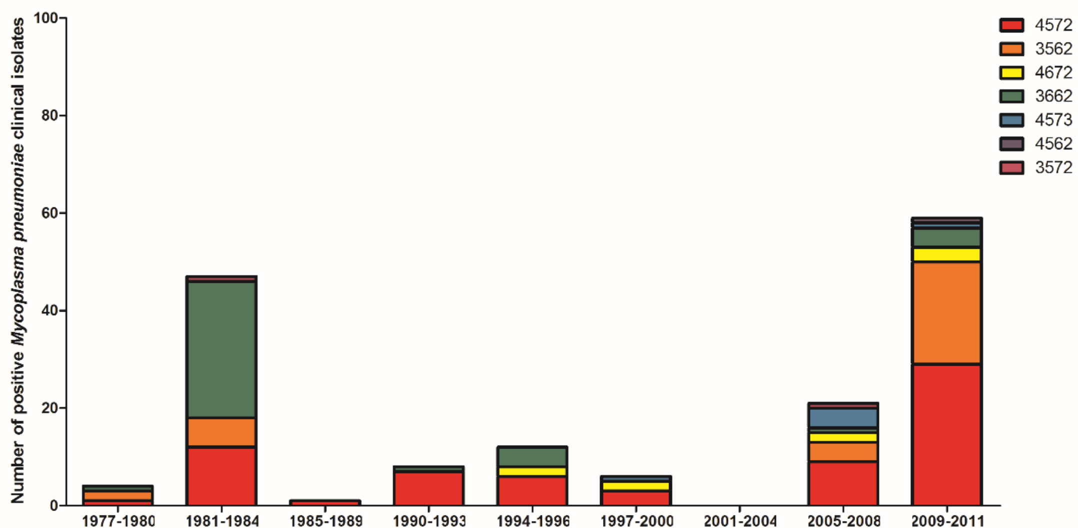

Figure 6.18. Distribution of MLVA types in positive *M. pneumoniae* clinical isolates collated into the four-yearly epidemic cycles observed in the UK.

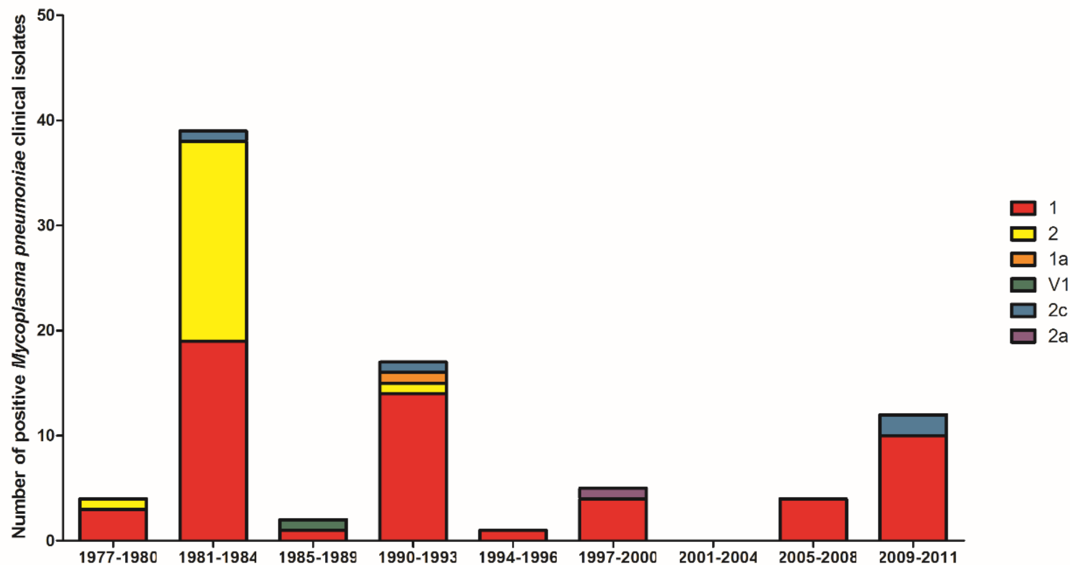

Figure 6.19. Distribution of P1 type in four-yearly epidemic cycles observed in the UK for positive *M. pneumoniae* clinical isolates.

6.2.10 Online database

A *M. pneumoniae* MLST online database has been created for both MLST allele and profile definitions and isolate data [297]; website http://pubmlst.org/mpneumoniae. This website combines two databases powered by the BIGSdb genomics platform [430]; the sequence definition database contains allele sequences and MLST profile definitions described in this chapter, whereas the isolate database contains provenance and epidemiological information. All 57 *M. pneumoniae* isolates included in this chapter have been added to the isolate database.

6.3 Discussion

MLST has been used to genotype several species of bacteria, including several *mycoplasma* species; *M. agalactiae, M. bovis* and *M. hyorhinis* [422, 431, 432]. This chapter has described the results of the successful development of a novel

M. pneumoniae MLST scheme to allow the characterisation of clinical isolates. This scheme was successfully used to discriminate between 55 clinical isolates of *M. pneumoniae* from respiratory and extra-pulmonary infection sites, the two type strains M129 and FH, and six additional strains with genomes published on NCBI.

6.3.1 Analysis of the MLST loci

Eight housekeeping genes were identified as suitable targets for the scheme and these were used to genotype *M. pneumoniae* isolates by either PCR followed by sequencing or whole genome sequence analysis.

gyrB contains a QRDR with documented *in vitro* mutations at amino acid positions 443, 464 and 483. Clinical use of quinolones may increase selective pressure *in vivo* resulting in a high mutation rate [289]. Nevertheless, the *gyrB* locus sequence amplified in this MLST scheme is in a different region of the gene from the QRDR and is therefore considered a suitable MLST target.

The stability of the eight loci was evaluated *in vitro* and was confirmed before and after ten repeated passages of ten strains in liquid medium. However, stability over a larger number of passages in liquid medium and evaluation of stability using *in vitro* tissue culture was not assessed.

6.3.2 Improved discrimination between *M. pneumoniae* isolates in comparison to MLVA and P1 typing

The discriminatory power of this MLST scheme with the eight loci was 0.784 for the collection of 57 isolates. In comparison, the Hunter-Gaston DI of the P1-typing method for the 57 isolates was 0.567 and the DI of the MLVA scheme was 0.633. This MLST scheme was therefore more discriminatory for the isolates tested. It has

previously been shown that the established MLVA method is more discriminatory than P1 typing [132], which has been confirmed in this study.

The allelic diversity of each of the MLST loci varied significantly at each locus, with the *pgm*, *glyA*, *atpA*, *gyrB*, *gmk* and *ppa* loci being more discriminatory than the *adk* and *arcC* loci. The association of this set of markers with variable Hunter-Gaston DIs makes this MLST, in theory, more optimal for epidemiological studies than other existing methodologies.

6.3.3 Analysis of *M. pneumoniae* infection within an individual patient

Analysis of *M. pneumoniae* infection at an individual patient level was possible using this scheme. Multiple clinical isolates were available from two of 50 patients: five from a patient with Stevens-Johnson syndrome (MPN013-MPN017), and two from a patient with bronchopneumonia clinical specimens taken four days apart. In both cases the MLST ST, MLVA type and P1 type remained the same, indicating a single clonal isolate was responsible for infection. Additionally, genomic sequences of the strains from a patient with bronchopneumonia co-locate phylogenetically.

Recurring or re-infection of *M. pneumoniae* could be determined using this scheme. Recurring infection would have the same ST as the original infection whereas re-infection with *M. pneumoniae* would likely be a different ST. Genetic MLST instability in isolates could occur but this was not seen over ten passages in this study.

6.3.4 Population modelling using MLST sequence types

The eBURST analysis illustrates the relationship of STs on the basis on the number of MLST loci that differ between two STs. Analysis of this population modelling indicates that the two clusters, CC1 and CC2, differed by more than one

locus, but within each cluster the STs did not differ by more than one locus. Within a cluster, this highlights the homogenous nature of the *M. pneumoniae* species, although a definitive split can be observed between the two clusters in both MLST ST and MLVA type. A possible divergent clade with ST12 from CC1 is also apparent but more isolates would need to be typed by this method to confirm this observation.

Few typing methods have previously been able to detect significant differences between strains, including one previous attempt to subtype *M. pneumoniae* by MLST with housekeeping and structural genes [129, 131, 292]. The previous MLST was determined to be not sufficiently discriminatory to be used for epidemiological purposes. However, results for the MLST scheme presented in this chapter illustrates a scheme that is able to discriminate between *M. pneumoniae* isolates, resulting in two genetically distinct clusters, indicating significant differences between strains.

6.3.5 Genomic sequence comparison

Whole genome sequencing has greatly facilitated the understanding of *M. pneumoniae* and currently there are nineteen distinct *M. pneumoniae* strains completely sequenced, excluding M129-B7 which is a chimera sequence generated by combining the re-sequenced M129 genome (by next generation sequencing) and the first Sanger-based M129 genome. The genome of the GC1 strain, M129, was the first *M. pneumoniae* genome sequenced and was reported in 1996 and subsequently re-annotated in 2000 as having 816,394 bp, 730 genes, and an average GC content of 40% [16, 433]. The genome sequence of the GC2 strains, FH and 309 were completed using next generation sequencing methods [434, 435]. An additional strain, M29 has also been sequenced but at this time has not yet been annotated. Preliminary

comparison of the three genomes M129, FH and 309 indicated that they are genetically very similar, except for variation in a 6-kb insertion region coding lipoproteins [435].

A comparative genomic study of 15 *M. pneumoniae* genomes indicated that SNP and indel variants occur between type 1 and type 2 strains but that overall there was a high degree of sequence homology between the strains [17]. Overall genomes were described as being 99->99% identical with less than 0.1% difference between strains belonging to the subgroups (i.e. type 1 and type 2). One distinct region was identified where sequence identity dropped to ~95% in type 2 strains in comparison to the type 1 strain M129 which corresponded with the *p1* gene. Additionally two subtype-specific insertions were observed; type 1-specific insertion at 557,178-560,601 bp (M129 numbering) and a type 2-specific insertion at 713,023-713,984 bp (M129 numbering). The type-1 specific insertion corresponds with the reduction in sequence identity observed between the 35 *M. pneumoniae* genomes described in this chapter along with positioning next to the predicted recombination region Area two. In comparison, the type 2-specific insertion observed by Xiao *et al.* [17] was not identified in the GC2 strains examined in this chapter. This type 2 insertion event was initially only described in strain 309 [436] but it was also found in all sequenced type 2 strains and FH by Xiao *et al.* [17]. It was therefore proposed that this insertion was a signature of type 2 strains rather than a unique feature of strain 309 but data in this chapter suggests that this is not the case. The presence of two different phylogenetic lineages observed by Xiao *et al.* [17] supports the findings in this chapter and confirms that clade-specific variation occurs.

Analysis of the 15 *M. pneumoniae* strains, performed by Xiao *et al.* revealed 182 genes with no variants (using M129 as a reference), including the genes *recA* and

efp [17], which were excluded from the MLST scheme in this chapter due to 100% sequence identity. Additionally, all other MLST targets were found to contain variants, supporting the use of these gene loci in an MLST scheme for *M. pneumoniae*.

Comparison between genomic sequence analysis after the removal of predicated areas of recombination and phylogenetic analysis of concatenated MLST sequences showed similar topology and the same distinct genetic clustering. This indicates that this MLST scheme is representative of the genome and confirms *M. pneumoniae* can be subdivided into two distinct genetic lineages.

Strains belonging to GC1 and GC2 appear to be clonal as seen for some other bacterial species such as *Mycobacterium tuberculosis* [437]. It is thought that strains of *M. pneumoniae* may have only recently diverged, despite geographical separation, as the differences between type 1 and type 2 strains are concentrated to specific areas of the genome, rather than being evenly distributed across the genome. It is thought that this may suggest the existence of positive selective pressure for particular variants, as might be expected for genes encoding proteins that interact with host cells, such as the P1 adhesin [17]. In comparison, it has been estimated based primarily on rRNA analysis that mycoplasmas are evolving more rapidly than most bacteria and that *M. pneumoniae* and closely related species are evolving more rapidly than most other mycoplasmas, suggesting a high mutation rate [15, 438]. Nevertheless, conservation of the majority of genes within the two subtypes, including the CARDS toxin gene, a major virulence factor, indicates a slow rate of evolution [17].

6.3.6 Clinical relevance and use in epidemiology

Typing of clinical *M. pneumoniae* isolates is becoming increasingly important due to the changing epidemiology of *M. pneumoniae* infections and the increase in macrolide-resistant strains [439, 440]. This scheme provides a more discriminatory method than both the MLVA and P1 typing methods currently in use, allowing further and more detailed analysis of observed epidemic peaks of *M. pneumoniae* infection.

Community outbreaks of pneumonia caused by *M. pneumoniae* have been described worldwide [27, 441, 442], and it would be interesting to evaluate this MLST scheme in such epidemic situations. The level of discrimination of this typing method and usefulness in epidemic analysis chould be confirmed by comparing outbreak-related strains to a set of control strains that were isolated from a similar time period and geographical area but that are not epidemiologically related.

More severe or adverse infections with *M. pneumoniae* are seen in some patients. The reason for this is not clear however, it can be postulated that this is due to specific microbe pathogenicity (identified through genetic markers) or variance in host susceptibility. This method could assist in determining if this is a strain specific phenomenon.

One advantage of MLST is that it is PCR based and does not require the growth of bacteria, which can be a lengthy process for *M. pneumoniae,* and it does not limit investigation through the requirement of specialist methodology. However, there is a large amount of sequencing required for this method which can be laborious and expensive; therefore, adaptation for wide-spread use directly on clinical specimens would be beneficial.

6.3.7 Review of current molecular typing methods for *M. pneumoniae*

Traditionally, molecular typing is used to characterise epidemic outbreaks of *M. pneumoniae*; however it is generally considered that molecular typing of *M. pneumoniae* is hampered by the fact that the pathogen is a genetically homologous species [128]. Despite this, molecular typing methods have been developed for this organism including: RFLP of the major surface adhesin P1 [128], MLVA [132], and MLST [424] and the SNaPshot™ mini-sequencing assay [443]. The mechanisms driving fluctuations in incidence of *M. pneumoniae* infections have not been defined. It has been postulated that shifts in the proportion of individual strains with specific P1 type or concurrent increased incidence of several strains may result in epidemics or immunity. Additionally, it is believed that the genotype of *M. pneumoniae* may be changing, generating diverse genetic material in each epidemic with a study reporting the detection of polyclonal strains in a single epidemic [444].

Speculation that a shift in P1 adhesin type may be the cause of epidemics has been disputed with evidence indicating the presence of multiple P1 adhesin types in observed increases of infection [132, 222, 444]. It was hypothesised that a decline in immunity or an increase of the immunologically naïve population may result in the four-year cycle of epidemic periods [134]. In other geographical locations, it has also been observed that multiple P1 types can be detected during outbreaks, and it has been suggested that although immunological pressure may favour shifts of P1 type, a co-circulation of P1 types appears to be common [445].

MLVA has been increasingly used internationally for strain characterisation and was documented to be more discriminatory for *M. pneumoniae* strains than P1 typing, providing an additional level of classification for transmission studies.

However, reports of observed instability in the Mpn1 locus has called into question the reliability of the marker. Additionally, inconsistency in nomenclature and identification of repeat regions has led to international standardisation of the MLVA with the removal of Mpn1 as a locus [221]. Analysis of the 2010/2011 epidemic in the UK revealed a total of 11 distinct MLVA types present using the original typing method [134] but reanalysis using international guidelines reduces the MLVA types detected to five distinct types. The discriminatory power of the MLVA for characterisation of *M. pneumoniae* strains has been reduced with the removal of the Mpn1 locus, necessitating either the identification of new loci or alternative typing methods.

Development of a SNaPshot^{TM} mini-sequencing assay has resulted in identification of 9 SNP types [443]. This method is rapid and appears to have greater discriminatory ability than MLVA and P1 typing. A direct comparison of MLST and SNaPshot^{TM} mini-sequencing assay has not been undertaken and both methods may have similar discriminatory abilities. However, MLST resulted in a larger number of defined sequence types.

These typing methods are all PCR based and do not necessarily require the growth of bacteria. P1 typing, MLVA and MLST do not limit investigation through the requirement of specialist methodology. However, MLST can be laborious and expensive and with the cost of genomic sequencing reducing it is becoming a more attractive option for genetic analysis of strains. Genomic sequencing may allow the concurrent identification of P1 type, MLVA profile and MLST sequence type directly from the genomic sequence as well as providing additional information such as the presence of antibiotic-resistance and toxin markers. Improvements in sequencing

technology and the development of methodologies to produce longer sequence reads with an increase in the reliable sequence, means that the determination of repeated DNA is possible [446]. This is important for species such as *Mycoplasma*, in which large tracts of repeated sections within AT rich genomes are common. For genetically homologous species such as *M. pneumoniae,* the use of genomic sequencing to analyse phylogeny inferred from single nucleotide polymorphism analysis will improve the ability to accurately segregate this species into distinct lineages allowing in depth epidemiology studies. Due to the fastidious nature of *M. pneumoniae* and other human Mollicutes such as *Mycoplasma amphoriforme* the use of metagenomic approaches to identify pathogens in studies of human infections [447] will no doubt improve detection of infections caused by Mollicutes whilst obviating the need for expensive and laborious culture and typing methods and simultaneously providing additional data such as the detection of mutations known to confer resistance. But, genomic sequencing is not currently widely available, therefore methodologies, like MLST, that can be widely accessible remain necessary for the characterisation of *M. pneumoniae.*

6.4 Summary

This chapter presents a robust MLST scheme that has proven discriminatory for *M. pneumoniae*, providing isolate characterisation and a higher level of discrimination than MLVA and P1-typing methods. In addition, phylogenetic analysis of both MLST STs and whole genome sequence data revealed two genetically distinct clusters. Crucially, this scheme for *M. pneumoniae* is also supported by a public web-based database (http://pubmlst.org/mpneumoniae).

Chapter 7. General discussion

This thesis has addressed and answered a diverse range of novel research questions with the central theme being bacteria from the genus *Mycoplasma*. The first two results chapters described the validation of a multiplex real-time PCR assay that can simultaneously detect *M. hominis*, *U. parvum* and *U. urealyticum* in clinical specimens and the application of this assay as a tool for monitoring bacterial response to antibiotics. The third results chapter examined the genetic heterogeneity of *M. hominis* through the development of MLST schemes using novel bioinformatics techniques. Finally, this thesis addressed the molecular typing of *M. pneumoniae* with the development of an MLST scheme. In addition, analysis of *M. pneumoniae* genomic sequence was also performed.

7.1 Diagnosis of *mycoplasma* infections

Mycoplasma hominis and *M. pneumoniae* have been linked to a diverse range of diseases with varying prevalence, severity and pathogenicity. Effective and rapid diagnosis of infection is vital when considering particular patient cohorts, such as preterm neonates. The detection of *M. hominis* in neonates is often coupled with the detection of *Ureaplasma* species; both are genital mycoplasmas that can cause disease in neonates. *Ureaplasma* species have been strongly linked to an infectious cause of premature birth where the organism can additionally be transmitted from mother to child either *in utero*, as a result of ascending infection from the mother's urogenital tract, or acquisition during birth.

Culture is considered to be the reference standard for detection of *M. hominis* and *Ureaplasma* species; indeed culture is required to identify active infection. However, culture is expensive and requires specialised media and expertise that are not widely available outside of larger medical centres or mycoplasma research and reference laboratories. In addition, upon specimen receipt, confirmed culture results

are usually only available after 2-5 days [3]. Diagnostic focus has shifted towards nucleic acid amplification and the development of PCR assays for rapid detection of mycoplasmas in clinical specimens. The literature now describes multiple PCR assays for the detection of *M. hominis* and *Ureaplasma* species, most commonly associated with other urogenital pathogens forming STI and urethritis screening tests. This thesis describes an assay specifically developed for detection of *M. hominis* and *Ureaplasma* species in neonatal respiratory samples (Chapter 3). The notorious genetic heterogeneity of *M. hominis* has added challenges to developing reliable PCR targets for sensitive and specific diagnosis. Many of the published PCR assays utilise the 16S rRNA of *M. hominis* as the target for detection. Minimal genetic heterogeneity exists in this target but other targets, such as the *gap* gene, exhibit high levels of sequence intra-species heterogeneity and regions of recombination [315, 362]. The *yidC* target was used in this thesis. The *M. hominis* strains examined in this thesis along with strains examined by Ferandon *et al.* [125] indicated that although intra-species heterogeneity is observed within the gene, this is at a much lower level than other utilised PCR targets and lies outside of the primer and probe binding sites utilised in the assay. In order to improve the reliability of PCR assays for the detection of *M. hominis,* further robust targets need to be identified with low levels of intra-species heterogeneity. Multiple genomic targets have been utilised for the detection of other bacterial species, such as *Francisella tularensis*, in order to enhance the specificity of diagnostic tests [448]. The use of multiple targets in a single assay for *M. hominis* detection may alleviate detection failure in clinical specimens and increase the specificity of PCR assays.

The improvement of diagnostic techniques for mycoplasmas is beneficial to patients and public health. As well as *M. hominis*, culture is a definitive method for

diagnosis of *M. pneumoniae* infection. Culture is essential for the ongoing maintenance of a collection of clinical isolates which is critical for monitoring trends in epidemiology, antimicrobial resistance typing and for further characterisation of these species. However, culture is slow and the recovery of isolates is highly variable and may be low, even in specialised laboratories and is therefore not practical for rapid detection of acute infection [449, 450].

7.1.1 Importance of diagnosis in neonates and pregnancy

Genital mycoplasmas as neonatal pathogens are becoming of increasing importance, with *Ureaplasma* species in particular having links with lung disease in neonates. The rates of lung colonisation by *Ureaplasma* species has, on numerous occasions, been shown to be inversely proportional to gestational age [451, 452] and vertical transmission of *Ureaplasma* species from mother to baby has been reported from 18% to 88% [3]. The rate of *M. hominis* colonisation is lower than that for *Ureaplasma* species (Chapter 4); however, it has been reported that approximately 30% of exposed infants are colonised with *M. hominis* [453]. It has been documented that *in utero* infection of the foetus is common, but neonates may also be colonised initially at the time of delivery [310]. Data from a cohort of 45 intubated neonates confirmed that neonates were statically more likely to be colonised with either *Ureaplasma* species or *M. hominis* when born earlier than 26 weeks gestation than those born closer to full term (Chapter 4).

Due to the residence of these bacteria in the normal vaginal flora, controversies exist regarding their true role in pregnancy and therefore the need to screen for and treat these organisms. There is growing evidence for the association and implication of *Ureaplasma* species and *M. hominis* in adverse pregnancy outcomes; suggesting a

role of cervicovaginal colonisation as well as amniotic fluid infection with both pathogens in adverse pregnancy outcomes [454].

Given the large amount of evidence that links subclinical maternal infection with preterm labour, it has been postulated that the prophylactic use of antibiotics in pregnancy or adjunctive use of antibiotics for preterm labour should result in improved perinatal outcome. The results of published studies addressing the issue of antibiotic intervention to prolong gestation vary. In a recent review of 15 trials [455], the authors concluded that, despite eradication of bacterial vaginosis in pregnancy, antibiotic treatment did not reduce the risk of preterm delivery. Meta-analysis of randomised clinical trials showed that treatment with macrolides and clindamycin during the second trimester of pregnancy was associated with a lower rate or preterm birth [456]. In addition, further analysis confirmed that infection screening and treatment programs in pregnant women may reduce preterm birth and preterm low birth weights [457]. Experimental data in the rhesus monkey supports the use of antibiotic treatment of pregnant women, as it was shown that preterm birth can be delayed in this non-human primate model by the administration of specific antibiotics such as azithromycin combined with anti-inflammatory agents. Maternally administered azithromycin was shown to accumulate in the amniotic fluid and resulted in eradication of detectable *U. parvum* infection within four days. Eradication of *U. parvum* from foetal tissues using this treatment also reduced foetal lung injury [184]. Diagnosis of maternal ureaplasma and mycoplasma infection would allow targeted antibiotic treatment of these infections, potentially reducing mother-to-infant transmission and adverse pregnancy outcomes.

Accurate diagnosis of the causative agent of infection allows targeted treatment. This reduces unnecessary and prolonged broad-spectrum antibiotic usage

and concurrent development of antibiotic resistance. Limited data is available on the clinical and microbiological efficacy of antibiotics for treating clinically significant systemic infections caused by *Ureaplasma* species and *M. hominis* such as pneumonia, bacteraemia and meningitis in neonates, and what is known originates from reports of individual case studies. Erythromycin has been the most commonly used antibiotic to treat *Ureaplasma* species infection in neonates but varying rates of organism eradication from the respiratory tract have been reported [3] and this antimicrobial agent is not effective against *M. hominis*. Results presented in this thesis have shown successful clearance of *Ureaplasma* species from respiratory specimens collected from intubated neonates following clarithromycin treatment (Chapter 4). Clindamycin has been used to successfully treat neonatal infections due to *M. hominis* [310]. Tetracyclines have been used to successfully treat neonates with meningitis caused by *M. hominis* and *Ureaplasma* species, but they are rarely used due bone toxicity effects when given to infants. Lower rates of *M. hominis* detection than *Ureaplasma* species in neonates could be due to their susceptibility to gentamicin (Chapter 4) compared to the intrinsically high MICs of gentamicin for *Ureaplasma* species [375]. Neonates presenting with respiratory symptoms or pneumonia are commonly given ampicillin and gentamicin as a first-line treatment option [458]; so, empiric treatment with gentamicin will theoretically affect *M. hominis* infection, potentially prior to subsequent isolation, detection and identification of the aetiology of infection.

The use of antibiotics in premature neonates is often necessary and indeed it is common practice for antibiotics to be prescribed shortly after birth. In the majority of very low birth rate infants, antibiotics are usually administered immediately after birth with the rationale that many babies have respiratory distress, which is difficult to distinguish from pneumonia [459]. Additionally, routine antibiotic treatment

(penicillin and gentamicin) is given to premature neonates due to the possibility of maternal infection-induced pre-term labour; *Ureaplasma* species have been documented to have a causal role in premature labour [310]. The prolonged use of antibiotics in neonates has been associated with an increased odds ratio of the development of NEC, an acute inflammatory disease of the gastrointestinal tract characterised by variable damage to the intestinal tract ranging from mucosal injury to full-thickness necrosis and perforation [460]. The pathophysiology of NEC is poorly understood but it is considered that a relationship between nutrition, the intestinal microbes, and the interaction of the host with these factors is likely to have a major role in the pathogenesis of the disease [459]. Treatment with antibiotics from birth can lead to a dysbiosis of the developing intestinal microflora of a neonate and is likely to contribute to the development of NEC [459]. Implications associated with antibiotic usage in premature neonates' cements the need for improved and rapid diagnosis of bacterial infections to prevent unnecessary antibiotic usage and allow targeted treatment.

7.2 Genetic characterisation of *Mycoplasma hominis* and *Mycoplasma pneumoniae*

Beyond the use of molecular methods for detection of *M. hominis* and *M. pneumoniae* in clinical specimens, numerous molecular methods have been developed that exploit the limited genomic diversity of *M. pneumoniae* isolates in order to characterise isolates for epidemiological purposes, although no clear correlation of strain type with clinical presentation, disease severity, or patient outcome has been observed to date [450].

7.2.1 The usefulness of molecular typing for *Mycoplasma* epidemiology

Localised outbreaks of *M. pneumoniae* have been frequently reported, especially in closed settings, and transmission between household contacts is known to occur [461-465]. The long incubation period of up to three weeks and prolonged shedding after infection allows outbreaks of *M. pneumoniae* to often go unnoticed and extend for long periods of time [11, 144, 461, 462]. In England and Wales, seasonal peaks of infection are detected from December to February each year with epidemics at approximately four-yearly intervals [146, 221]. Cyclical patterns are also seen in Denmark, Sweden, Norway, Finland, Korea and Japan [466-471]. Epidemic peaks have recently been attributed to minor variations in the duration of immunity at the population level [472]. A con-current increase in reported *M. pneumoniae* cases was documented in several European countries in 2011 [473] and, in England and Wales, the most recent increase has been noted in 2015 [220]. The recent global increase in macrolide resistance observed in cases of *M. pneumoniae* infection is of increasing concern and importance to the international community [474], making it all the more important to monitor the epidemiology of *M. pneumoniae* infection.

Mycoplasma pneumoniae is considered a highly genetically conserved species, with genomic comparisons indicating >99% sequence similarity between strains [17]. Nevertheless, methods have been developed to characterise *M. pneumoniae* strains based on genetic variation, allowing for the classification of *M. pneumoniae* for epidemiological purposes. In addition to the well documented P1 and MLVA typing methods established for *M. pneumoniae*, this thesis has established a robust MLST scheme for *M. pneumoniae* with a higher discriminatory ability than both P1 typing and MLVA. The use of whole genome sequencing aided the identification of areas

within the genome containing variation that could be targeted to achieve an MLST scheme with greater discriminatory power.

Effective infection control is achieved through active surveillance of infection and recognition of burgeoning disease outbreaks in addition to preventative measures [475]. Molecular epidemiological investigations have become increasingly important in maintaining infection control and rely on methods that can assess the genetic relatedness between strains [475-477]. The main role of microbial molecular typing is to assess the relationships between microbial isolates. Understanding clonal relatedness between bacterial strains is essential for the determination of the source and routes of infections, confirmation of outbreaks, trace cross-transmission of healthcare-associated pathogens, the recognition of virulent strains, and to evaluate the effectiveness of infection control measures [478].

For large epidemiological studies, molecular typing appears to hold little value. Analysis of *M. pneumomniae* epidemics in 0, revealed multiple MLST, MLVA and P1 types present within epidemic cycles; however, a predominance of a particular P1 type can be observed (Figure 6.19). In addition, no obvious link was observed between molecular ST and the year when the strain was collected, the patient's age, and the sample origin. However, molecular typing within community and localised outbreaks has the ability to trace transmission and confirm the presence of an outbreak of infection; for example localised clusters of particular MLVA types have been observed in Hong Kong, China [479].

Population modelling of *M. pneumoniae* MLST STs revealed two clonal clusters (Figure 6.2) correlating to P1 typing and MLVA types. Furthermore, genomic sequence analysis of *M. pneumoniae* strains revealed two genomic complexes, with

the same distinct split as observed for MLST ST. The development of typing schemes from genomic sequences provides the ability to design a scheme that mirrors genomic phylogeny, thereby accurately representing the whole genome sequence. This thesis reports the first use of bioinformatics analysis to develop such an MLST scheme (Section 5.2.3). Three mMLST schemes have been proposed for the typing of *M. hominis* strains in this thesis, all of which reflect the relationships observed in genomic phylogeny. Due to the commensal nature of *M. hominis*, detailed epidemiological studies of this bacteria have not been undertaken. The application of typing schemes for *M. hominis* have not been examined for large epidemiological studies, however transmission trails between mother and baby and in nosocomial infections have been examined [133]. In the three mMLST schemes proposed, strains originating from the same patient sample were assigned the same ST (Section 5.2.3.5). Unfortunately, no two strains originating from documented mother to baby transmission were available to study. It would have been interesting to assess the relatedness of epidemiologically linked strains by this method. The use of MLST for both *M. hominis* and *M. pneumoniae* would allow the differentiation between recurring infection and re-infection of a patient with a differing ST.

7.2.2 Is genomic sequencing the way forward?

Whole genome sequencing has the potential to permanently change the field of *Mycoplasma* biology and epidemiology by allowing improved characterisation of strains and better discriminatory power compared to any previous typing method. Within this thesis the genomes of 18 *M. hominis* and 41 *M. pneumoniae* strains were sequenced and these sequences were used to inform MLST assay design in chapters Chapter 5 and 0, respectively. Further to this, whole genome sequencing of *M. pneumoniae* was also used to develop a SNaPshot mini-sequencing assay for typing

of *M. pneumoniae* [443]. Eventually, whole genome sequencing directly on clinical specimens may become the standard methodology for determining the aetiology of respiratory infections; however, whilst the cost of bacterial genomic sequencing has reduced, sequencing on the whole is still only performed on bacterial isolates. Nevertheless, genomic sequencing directly on clinical specimens has already been demonstrated for the detection of respiratory viruses [480] and *M. tuberculosis* [481]. Further development of genomic sequencing techniques will progress the field of diagnostics with the potential for direct metagenomics analysis of a patient specimens. There is established proof-of-principle describing the use of metagenomics to not only detect, but also to characterise bacterial pathogens; allowing typing data and phylogenetic profiles to be determined [482, 483]. This technique would also allow the entire microbial flora within the specimen to be identified, providing data for detailed epidemiological tracking of temporal and geographical trends in strain circulation. The data created in this thesis will serve as a bank to inform future metagenomics analysis for *M. hominis* and *M. pneumoniae.*

7.3 Scope for future lines of investigation

The data presented in this thesis has answered multiple questions, but has, at the same time, produced many more.

7.3.1 Multiplex real-time PCR

With regards to the development of the multiplex real-time PCR for the simultaneous detection of *M. hominis* and *Ureaplasma* species, further validation of the assay would be beneficial. Even though mixed infections of *M. hominis* and *Ureaplasma* species were identified in clinical specimens using the multiplex real-time PCR, investigation of possible inhibition in patients' specimens should be assessed. The ability of the assay to detect low levels of *M. hominis* in the presence of high concentrations of

either *U. parvum* or *U. urealyticum* and vice versa will determine if there is any inhibition of the primers and probes for the organism present at the lower concentration. Furthermore, spiking of clinical specimens with low concentrations of either *M. hominis*, *U. parvum*, or *U. urealyticum* will determine if inhibition is present in the patient specimens and the efficiency of the PCR in clinical specimens (Figure 7.1 A). In the validation of the reproducibility of the multiplex real-time PCR, the CV of the concentration was markedly increased for concentrations of $<10^2$ copies/µL (Chapter 3, Table 3.2). This increased variation for low copy number was most likely due to the logarithmic scale and natural random variation; however, further analysis of the control plasmid stability is warranted (Figure 7.1 A).

Comparison of the multiplex real-time PCR to commercially available assays that detect *M. hominis* and *Ureaplasma* species would provide a useful comparator to assess the sensitivity and specificity of the multiplex assay. Furthermore, direct comparison between the multiplex real-time PCR and the singleplex assays from which the primers and probes originates would be of interest [125, 290] (Figure 7.1 B). Clinical specimens would be tested by both the multiplex real-time PCR and the singleplex assays for both *M. hominis* and *Ureaplasma* species, allowing statistical analysis (Fisher's exact test) to determine if the multiplex has higher or lower clinical sensitivities and specificities that the singleplex assays. In addition, DNA concentration detected in clinical specimens could be compared between the multiplex real-time PCR and singleplex assay and correlation between the two methods could be assessed. The logarithm of the DNA concentrations determined by the multiplex real-time PCR and the singleplex assays could be compared by linear regression analysis using log concentration of the singleplex assays as the dependent variable.

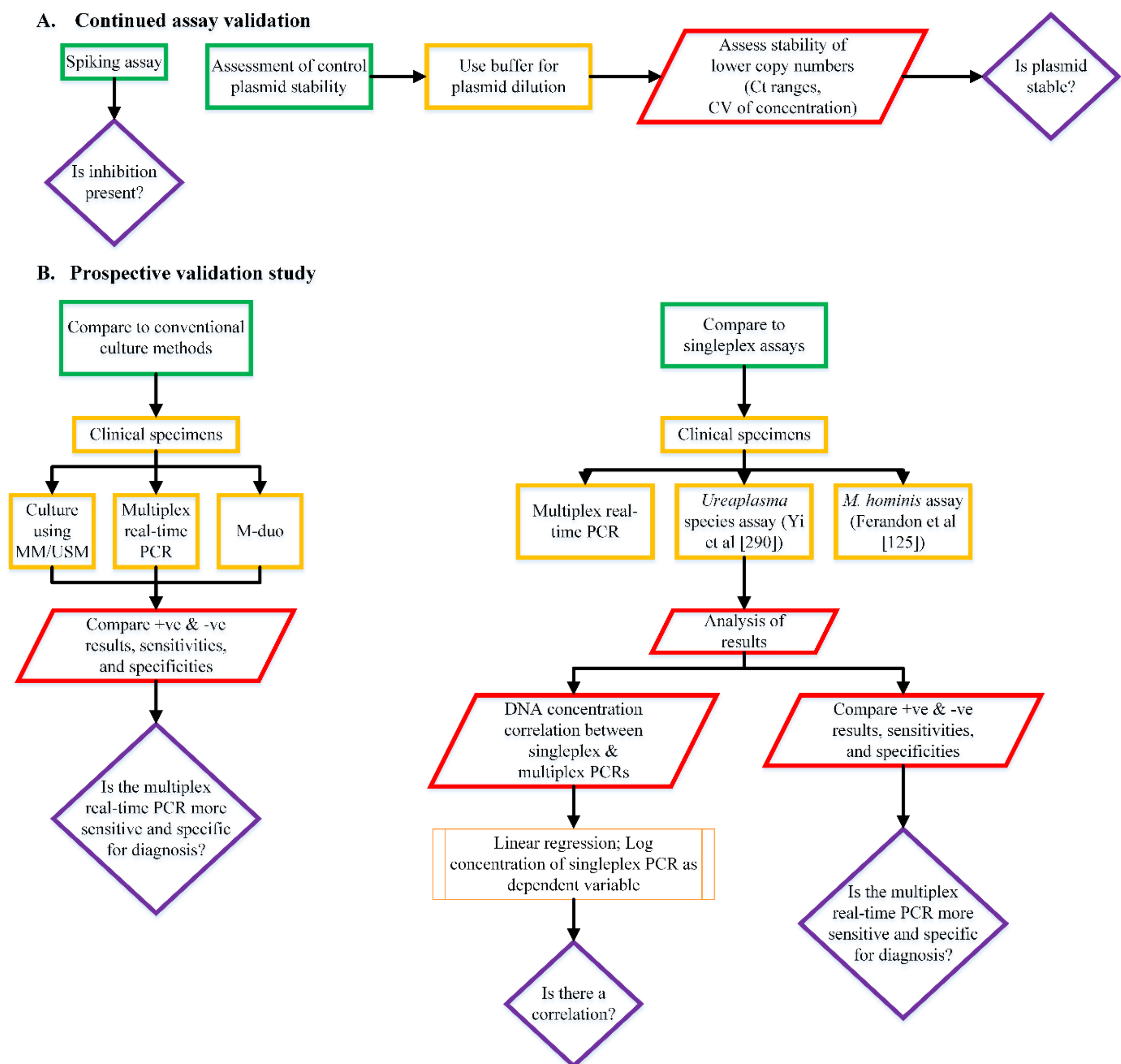

Figure 7.1. Work-flows for future validation of the multiplex real-time PCR assay for the detection of *M. hominis* and *Ureaplasma* species. A) Continued validation of the assay incorporating a spiking assay and further analysis of control plasmid stability. B) Prospective validation study using clinical specimens to compare to conventional culture and singleplex PCR assays.

The availability of multiple-pathogen detection technologies has the potential to improve the identification and aetiology in a variety of infectious disease syndromes, ultimately resulting in a more comprehensive clinical evaluation. In recent years, a number of multiple-pathogen detection systems have been developed and evaluated on respiratory clinical specimens. These systems use multiplex PCR assays combined with various detection systems, including mass spectroscopy [484], bead sorting

[485], dye-labelled probes [486], and microarray technology [487]. However, these systems require extensive validation and the inclusion of new primers and probes or changes to existing primers and probes requires revalidation of the entire multiple-pathogen detection assay. Further technology developments have led to the increased use of microfluidic-technology for infectious pathogen detection. TaqMan low-density array cards utilise such technology and is based on singleplex qPCR assays [488]. This technology has previously been described for research in neonatal and respiratory screening [488, 489] and is currently used routinely for clinical diagnosis of hepatitis and respiratory screening by PHE. Further development of a neonatal multi-pathogen screening test that includes the primers and probes described in Chapter 3 would provide a useful tool for rapid determination of the aetiology of infection in this vulnerable patient cohort and prevent unnecessary antibiotic usage. The current published neonatal screening array [489] includes *Ureaplasma* species as a target but utilises the *ureaseA* gene and is unable to differentiate between the two species. Furthermore, *M. hominis* is not included as a target pathogen.

7.3.2 *Mycoplasma hominis* antibiotic susceptibilities

This project has only examined the susceptibility of *M. hominis* strains to two antibiotics, gentamicin and tetracycline. Follow on work could assess the susceptibility of *M. hominis* to additional antibiotics such as clindamycin, moxifloxacin and levofloxacin, using all three methods described in Chapter 3. Comparison to published quality-control ranges [235] would allow verification of the use of qPCR to quantify bacterial load at varying concentrations of antibiotics, thereby determining the break-point at which the concentration of antibiotic prevents bacterial growth. *M. hominis* strains from diverse origins and clinical presentations were used

throughout this thesis and it would be interesting to assess if any antibiotic resistance coincides with severe or invasive clinical presentation.

If more time had been available then work would have been carried out upon understanding the failure of the *tetM* gene in MH111 to confer a resistant phenotype. Sequencing of the entire *tetM* and comparing to the tetracycline resistance strain Sprott (ATCC 33131) [490] would enable the identification of mutations within the coding region of the *tetM* and could account for the inability of the resultant protein to mediate tetracycline resistance. If any mutations were identified, they should be assessed as to whether they confer changes in amino acid sequence. The endogenous *tetM* gene promoter should also be assessed for alterations that may stop TetM protein expression. Additional genetic comparison should be undertaken with IS*Mhom1*, an insertion element detected in a single tetracycline-susceptible *tetM* carrying *M. hominis* strain, whereby an insertion of 1,260 bp in the leader peptide sequence upstream of *tetM* was proposed to be involved in the lack of transcription of *tetM* [403]. The questions of successful transcription of *tetM* in MH111 should be assessed. By isolating mRNA from actively growing MH111, cDNA could be produced by reverse transcription to identify if *tetM* was transcribed. Furthermore, Dégrange *et al.* [403] successfully induced resistance conferred by *tetM* via sequential short-term passage in media containing doxycycline. It would be interesting to see if resistance could be induced in MH111 using this method.

7.3.3 mMLST

This project has piloted the use of bioinformatics analysis to develop an MLST scheme that is representative of the whole genome sequence and accurately reflects genomic sequence phylogeny (mMLST; Chapter 5). The three potential schemes identified require development into a PCR-based method that can be utilised by labs

without the need for whole genome sequencing (Figure 7.2). Ideally, MLST loci of 400-700 bp need to be identified for each gene in each scheme that can be reliably amplified by PCR and sequenced. The gene targets will be examined *in silico* to identify the regions that contain variability and these will be utilised as regions for PCR amplification. As MLST loci used in PCR-based methods target only a region of the gene, the number of SNPs used to generate phylogeny will be reduced. In order to ensure that genomic phylogeny is still represented, gene areas for PCR will have to be assessed individually and in combination to see the effect of the loci size reduction on the overall phylogeny. It is assumed that phylogeny produced using a PCR-based method will reflect the phylogeny of the genomic sequence SNP tree with reduced branch length, as shorter sequences with a different number of SNPs will be used (as seen for the mMLST).

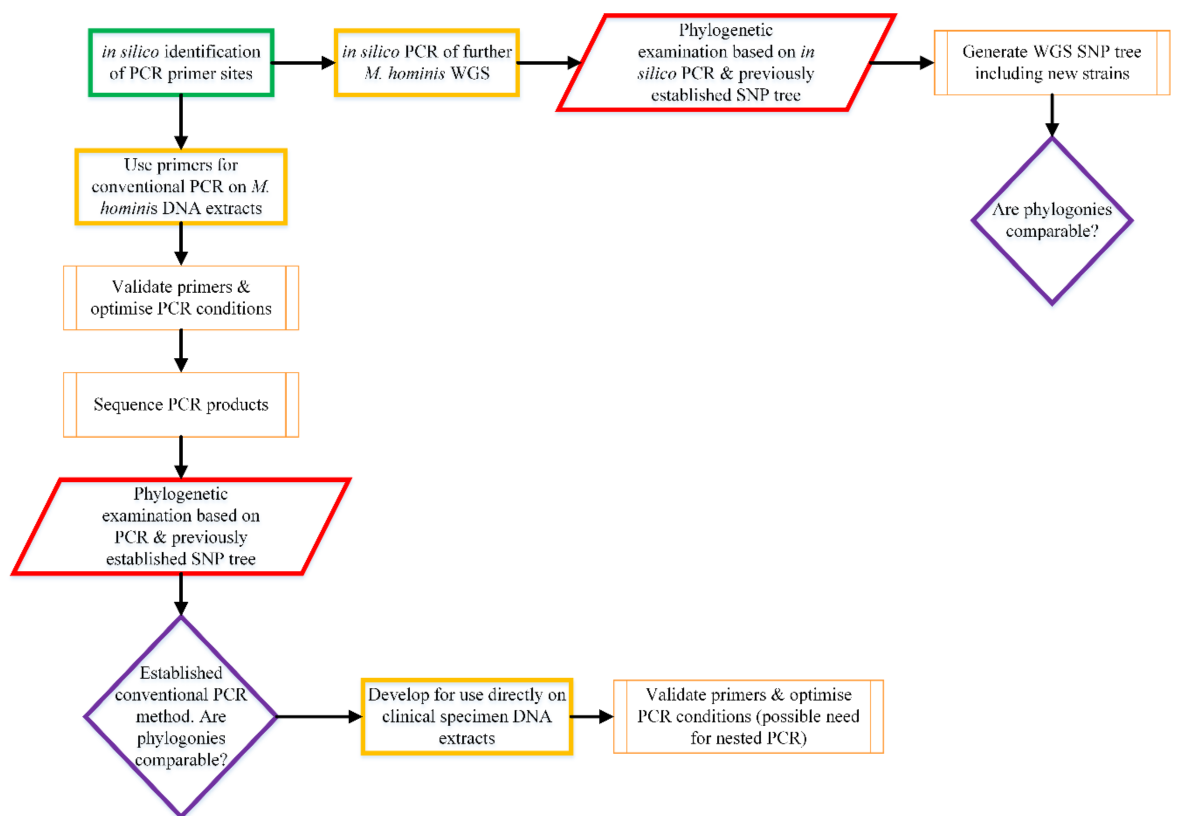

Figure 7.2. Work-flows for the development of mMLST by conventional PCR methods and expansion to additional *M. hominis* strains.

Epidemiological knowledge of *M. hominis* is limited and most cases of *M. hominis* infection have been reported as individual case studies. The genotypic diversity of *M. hominis* has previously been assessed across a wide range of *M. hominis* clinical isolates from both genital and extra-genital infections using MLVA [133], revealing high levels of genetic heterogeneity at a population level. Application of the mMLST to a large cohort of *M. hominis* isolates will provide further information on the genetic heterogeneity of *M. hominis* (Figure 7.2). Furthermore, as mMLST is representative of genomic phylogeny, relationships between strains and clinical presentation, year isolated and geographical location could be examined in detail.

At the level of an individual patient, it would be interesting to apply mMLST for *M. hominis* to allow discrimination between relapse and persistent, and new infections. In the first case, the mMLST type would remain the same, whereas in the second case a different mMLST type would be expected. Application of mMLST to mother-neonate pairs would allow confirmation of mother-to-child transmission if mMLST STs were identical. This has been previously documented for *M. hominis* using MLVA [133], PFGE [412] and AP-PCR [427]. *M. hominis* has been isolated from surgical wounds and has been noted as the causative agent of post-operative infections. Testing the mMLST method on a cohort of isolates from surgical sites of infection within hospitals would allow identification of transmission chains and identification of nosocomial infections, aiding infection prevention methods. As *M. hominis* infection is rarely identified, acquisition of a suitable number of isolates to extensively examine the use of mMLST in this scenario could prove challenging and would be limited to presentation of a suitable opportunity.

The bioinformatics technique used to identify the three mMLST schemes for *M. hominis* has a wider application. The methodology could be applied to any

bacterial species where there is interest in developing an MLST scheme that accurately represents genomic phylogeny. Utilisation of the 35 *M. pneumoniae* genomic sequences described in 0 and the 19 published sequences would allow for further validation of this methodology using a minimal genome. Furthermore, it would be interesting to compare any mMLST developed for *M. pneumoniae* with the MLST scheme developed in 0. Although the *M. pneumoniae* MLST is considered representative of the whole genome via phylogenetic positioning into the two genomic clades, an mMLST scheme will provide further evidence of relatedness between strains without the requirement for whole genome sequencing.

7.3.4 *Mycoplasma pneumoniae* MLST

Further interesting lines of research would be to validate the *M. pneumoniae* MLST for use directly on clinical specimens without the need for isolation of *M. pneumoniae*. The PCR primers detailed in Section 2.4.5 can initially be used to see if *M. pneumoniae* DNA can be amplified directly from clinical specimens with adequate DNA concentration for sequencing. If adequate DNA is obtained, then MLST can be performed on clinical specimens as for isolates of *M. hominis*. However, in the absence of sufficient DNA for sequencing or amplification failed directly on clinical specimens, nested PCR could be developed for the MLST loci as described for P1-typing [292]. This work is currently underway at PHE.

The epidemiology of *M. pneumoniae* is changing. In England and Wales, epidemics occur approximately every four years and globally epidemics are observed every 4-7 years. However, in some countries, such as Israel, a persistent and ongoing epidemic has been observed since 2010 [229], where cyclical epidemics used to be the norm. These changes in epidemic patterns emphasise the need to understand the epidemiology and pathogenesis of epidemics of *M. pneumoniae* infection better.

MLST can be used to characterise epidemics of *M. pneumoniae* infections. This was undertaken to a limited extent in 0; however, sample numbers were low, particularly in recent years. Examination of the most recent epidemics, notably the 2010-2011 and 2014-2015 epidemics in England and Wales, using MLST would provide discriminatory information to the clonality of the epidemics in England and Wales. The expectation is for the identification of a polyclonal population, as has been observed in both France and Israel using MLVA typing. However, MLST is representative of the genomic sequence phylogeny, and as it is more discriminatory than MLVA, it could shed more light on the phylogenetic relationship between *M. pneumoniae* strains in an epidemic period.

Finally, the incidence of macrolide-resistant *M. pneumoniae* is increasing globally and is becoming a major concern. Macrolide resistance-conferring mutations are identified in *M. pneumoniae* via the sequencing of a region of the 23S rRNA [275]. This could easily be added as an additional loci to the MLST scheme, providing clinically relevant information alongside epidemiological knowledge.

7.4 Concluding statement

Mycoplasma hominis and *M. pneumoniae* are human pathogens with differing epidemiology, clinical presentation and genetic heterogeneity. This thesis has contributed to the rapid diagnosis and characterisation of these bacterial species, which is becoming of increasing importance, particularly in preterm neonates and with the emergence of increasing antibiotic resistance. The genetic characteristics of these two *Mycoplasmas* are markedly different with implications for the development of reproducible and representative typing schemes for these organisms.

Appendices

Table A.1. Reference strains used for specificity testing of multiplex assay. Bacteria and virus species are listed, along with their collection ID or source, that were used to test the multiplex assay for specificity against *U. parvum*, *U. urealyticum* and *M. hominis*.

Species	Collection ID or Source
Acholeplasma laidlawii	NCTC 10116
Acinetobacter baumannii	ATCC 19606
Acinetobacter calcoaceticus	NCTC 23055
Acinetobacter haemolyticus	ATCC 17906
Acinetobacter johnsonii	ATCC 17909
Acinetobacter junii	ATCC 17908
Acinetobacter iwoffi	NCTC 5866
Bordetella pertussis	NCTC 8467
Chlamydia trachomatis (and serotypes F,G,H,I,J)	ATCC VR 902B
Chlamydophila pneumoniae	ATCC VR 1360
Chlamydophila psittaci	ATCC VR 125
Corynebacterium diphtheria	NCTC 10356
Corynebacterium ulcerans	PHE Colindale
Enterococcus faecalis	NCTC 775
Enterococcus faecium	NCTC 7171
Escherichia coli	NCTC 9001
Fusobacterium necroforum	NCTC 10575
Haemophilus ducreyi	PHE Colindale
Haemophilus influenzae non-capsulated	NCTC 12699
Haemophilus influenzae type a	ATCC 9006
Haemophilus influenzae type b	ATCC 10211
Haemophilus influenzae type c	ATCC 9007
Haemophilus influenzae type d	ATCC 9008
Haemophilus influenzae type e	ATCC 8142

Species	Collection ID or Source
Haemophilus influenzae type f	ATCC 9833
Haemophilus parainfluenzae	ATCC 33392
Herpes simplex virus	PHE Colindale
Legionella pneumophila	Cambio (Cambridge, UK; Cat. No. 52-0101)
Mobiluncus mulieris	NCTC 11658
Mycobacterium tuberculosis H37Rv	NCTC 7416
Mycoplasma amphoriforme	NCTC 11740
Mycoplasma buccale	NCTC 10136
Mycoplasma faucium	NCTC 10174
Mycoplasma fermentans	NCTC 10117
Mycoplasma genitalium	NCTC 10195
Mycoplasma lipophilum	NCTC 10173
Mycoplasma orale	NCTC 10112
Mycoplasma penetrans	ATCC 55252
Mycoplasma pirum	NCTC 11702
Mycoplasma pneumoniae	NCTC 10119
Mycoplasma primatum	NCTC 10163
Mycoplasma salivarium	NCTC 10113
Mycoplasma spermatophilum	NCTC 11720
Neisseria gonorrhoea	PHE Colindale
Prevotella bivia	NCTC 11156
Proteus mirabilis	NCTC 2896
Pseudomonas aeruginosa	NCTC 10332
Pseudomonas fluorescens	NCTC 10038
Pseudomonas putida	NCTC 10936
Pseudomonas stutzeri	CIP 103022
Staphylococcus aureus (MRSA)	NCTC 13435
Staphylococcus aureus (MSSA)	NCTC 13434
Staphylococcus auricularis	NCTC 12101
Staphylococcus capitis	NCTC 11045
Staphylococcus cohnii	NCTC 11041

Species	**Collection ID or Source**
Staphylococcus epidermidis	NCTC 11047
Staphylococcus haemoliticus	NCTC 11042
Staphylococcus hominis	NCTC 11320
Staphylococcus intermedius	NCTC 11048
Staphylococcus lugdunensis	NCTC 12217
Staphylococcus saccharolyticus	NCTC 11807
Staphylococcus saprophyticus	NCTC 7292
Staphylococcus schleiferi	NCTC 12218
Staphylococcus simulans	NCTC 11046
Staphylococcus warneri	NCTC 11044
Staphylococcus xylosus	NCTC 11043
Streptococcus (Group C)	PHE Colindale
Streptococcus (Group G)	NCTC 9603
Streptococcus agalactiae (Group B)	NCTC 11360
Streptococcus pyogenes (Group A)	NCTC 12696
Treponema pallidum	PHE Colindale
Yersinia enterocolitica	NCTC 12982

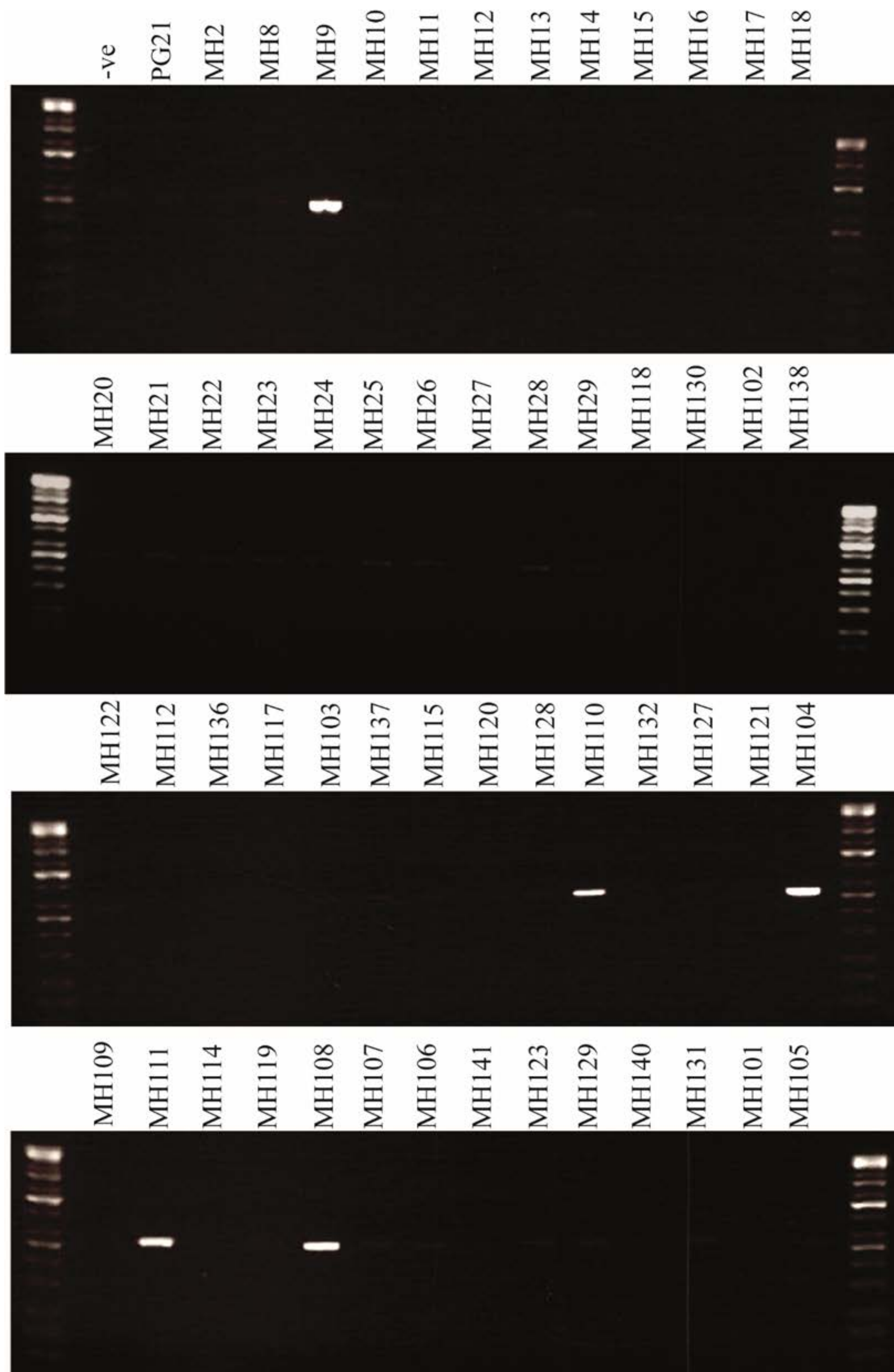

Figure A.1. Agarose gel showing the results of a PCR for the presence of *tetM* in *Mycoplasma hominis* strains.
The PCR detected a 397 bp fragment of the trasnferable element *tetM* in *M. hominis* strains MH9, MH110, MH104, MH111 and MH108.

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Mycoplasma hominis Variable Adherence-Associated Antigen: A Major Adhesin and Highly Variable Surface Membrane Protein

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Abstract

Mycoplasma hominis **is a member of the genus** ***mycoplasma*** **and has only been isolated from humans. It is most frequently isolated from the urogenital tract in the absence of symptoms, but has been isolated from wounds, brain abscess, inflamed joints, blood and placenta from pregnancy with adverse outcomes (especially preterm birth and occasionally term stillbirth). Controversy surrounds whether this organism is a commensal or a pathogen; however,** ***Mycoplasma hominis*** **has been shown to induce preterm birth and foetal lung injury in an experimental primate model as a sole pathogen. These bacteria are known to exist as a parasitic infection, due to a number of missing synthetic and metabolism pathway enzymes from their minimal genome; therefore, the ability to adhere to host cells is important. Here we provide a review that clarifies the different nomenclature (variable adherence-associated antigen and P50) that has been used to investigate the major surface adhesin for this organism, as well as reported mechanisms responsible for turning off its expression. Variation in the structure of this protein can be used to separate strains into six categories, a method that we were able to use to distinguish and characterise 12 UK strains isolated from between 1983 and 2012. We propose that the Vaa should be used in further investigations to determine if commensal populations and those that are associated with disease utilise different forms of this adhesin, as this is under-studied and identification of pathogenic determinants is overdue for this organism.**

Keywords

Mycoplasma hominis**, Variable-Adherence Associated Antigen, Host-Pathogen Interaction, Surface**

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Expressed Proteins

1. Introduction

Mycoplasmas are characterised by vastly reduced genomes and are among the smallest of the free-living organisms. Furthermore, *mycoplasmas* are diverse in terms of host environment, phenotypic features, as well as genomic characteristics [1]. They feature a reduced number of DNA repair proteins and exhibit high mutation rates, which contributes to the accelerated evolution observed within the genus [2].

Most *mycoplasmas* are parasites, usually exhibiting strict host and tissue specificities. These organisms have evolved molecular mechanisms needed to deal with the host immune response including: mimicry of host antigens, survival within phagocytic and non-phagocytic cells and generation of phenotype plasticity. *Mycoplasma hominis* is an opportunistic human pathogen and resides as a commensal on the mucosal surfaces of the cervix or vagina of 21% to 53% of sexually mature, asymptomatic women; this is somewhat lower in the urethra of males [3]. However, *M. hominis* has also been associated with clinically diverse diseases including; urogenital diseases [4] [5], postpartum fever [6], pneumonia [7], meningitis [8] [9] and septic arthritis [10] [11]. Although this organism has only been isolated from humans, it was found that *in utero* administration of *M. hominis* to pregnant macaque monkeys led to preterm labour and foetal lung injury [12].

Mutation-based phenotypic and genetic variation is a strategy utilised by many pathogenic bacteria and protozoa to adapt to divergent host environments [13] [14]. These mutations often affect the surface structures of a pathogen and may therefore change functional aspects of the organism such as adherence, colonisation of the host, or immune evasion. High-frequency mutations, that are distinct from classical regulation of gene expression, are an adaptive tactic that can affect the expression or structure of selected gene products, creating and maintaining repertoires of functionally variant organisms within a population. This diversity may contribute in many ways to the survival, propagation, transmission and pathogenic properties of an infectious agent.

Mycoplasmas are heterogeneous organisms: many display antigenic variation and pronounced variation of surface proteins. This is thought to be an important way of evading the host immune response, particularly the humoral immune response, resulting in the chronic infection characteristic of many *mycoplasma* infections [15]. Previous studies have indicated that the surface antigenic profiles of *M. hominis* strains are highly heterogeneous expressing both size and phase variants of surface exposed membrane proteins [16]-[19]. Several *M. hominis* surface proteins have been characterised including P120, P135 and P50, however the molecular basis of variation in *M. hominis* has only been elucidated in some cases. The mechanisms involved in the diversification of *mycoplasma* surface proteins are highly complex and include: size variation caused by gain or loss of intragenic repetitive sequences; phase switching by deletion/insertion mutations or DNA inversion affecting promoter activity; and presence of multigene families or multiple copies of partial genes in the mycoplasmal chromosome [1].

The presence of a variable adherence-associated surface protein of *M. hominis* was initially identified over 20 years ago as a potential adhesin of *M. hominis* by using specific monoclonal antibodies to inhibit *mycoplasma* adherence to cultured cells [17] [20]. This protein was first identified; as a 49 kDa surface protein in *M. hominis* strain PG21 [21]; as an adherence-associated, multiple-banding membrane lipoprotein in strain 1620 [17]; and as a 50 kDa adhesin in strain FBG, known as P50 [20]. While these groups used different nomenclature (Vaa versus P50), the surface lipoprotein in question is the same protein. We will use the original terminology, variable adherence-associated (Vaa) antigen, for the rest of this review as this lipoprotein often has varying molecular masses with conserved regions; therefore P50 is a less accurate descriptor. Variation in the composition and size of the Vaa proteins results from allelic variant forms of the single copy *vaa* gene in *M. hominis* [22] [23]. Despite discovery for more than two decades no comprehensive review of Vaa regulation and expression has been previously undertaken, the importance of Vaa in the pathogenesis and immune evasion of *M. hominis* infections is still not fully elucidated.

2. Phase Variation of the *vaa* Gene

Tandem repeats (TRs) are nucleotide sequences that are directly repeated in a head-to-tail manner. According to

the conservation of the repeated sequences, TRs are classified as identical/perfect TRs or degenerated/imperfect TRs. Furthermore, TRs can be classified into three categories according to the size of the repeated unit: microsatellites, minisatellites and macrosatellites [24] [25]. The term "satellite DNA" originally refers to the very large arrays of tandemly repeated non-coding DNA that are characteristic of eukaryotic genomes however in the context of bacterial genomes the term is also used to include small and intragenic TRs [26]. In comparison to other bacterial species, several *mycoplasma* species, including *M. genitalium*, *M. gallisepticum*, and *M. hyopneumoniae*, contain long trinucleotide repeats in their genomes at a higher prevalence than is observed in other bacterial species. These repeat regions occur mainly in intragenic regions in the two former species and within coding regions of the latter [27]. In *M. hyopneumoniae* the trinucleotide repeats are located within hypothetical open reading frames or defined adhesins in which the gain or loss of these repeats results in variability of amino acid sequence. These changes in protein size and structure are speculated to influence protein-protein interactions and adhesion [27].

There is abundant evidence that intragenic and intergenic TRs can promote phase variation. However, the underlying mechanisms are dependent on the nature of the TR. For example, if the TR unit size is not a multiple of three, rearrangements are able to induce frame-shift mutations causing ON/OFF phase variation of down-stream sequence (truncation) [26]. Phase variation refers to reversible molecular switches encoding ON/OFF gene expression resulting in variation in expression of one or more open-reading frames between individual cells of a clonal population. The frequency of phase variation is characteristic for the gene, the bacterial species, and the regulatory mechanism resulting in modulation of the switching frequency. Phase variation results in phenotypic variation within bacterial species [28]. Differences in the expression of Vaa between strains of *M. hominis* have been observed and Vaa has been shown to undergo high-frequency phase variation resulting in ON/OFF expression [17] [22]. Sequence differences have been observed between Vaa positive (Vaa^+) and Vaa negative (Vaa^-) variants derived from a single clonal lineage, with a single nucleotide deletion observed in a short tract of adenine residues (poly-A tract) located 166 nucleotides downstream of the ATG start codon. This tract contains eight alanine residues in cells expressing the full-length Vaa protein [23]. The deletion observed in Vaa^- variants creates a frame-shift resulting in an in-frame UAG stop codon downstream of the poly-A tract.This mutation causes premature termination of translation and prevents Vaa expression [22]. Correction of this mutation has been observed in Vaa^+ variants derived from a Vaa^- clonal population, restoring the eight A residues in the poly-A tract, and resulting in the expression of the full-length Vaa [22]. Thus phase variation of Vaa is controlled at translation, not as a consequence of transcriptional events due to promoter sequence divergence unlike transcriptional modulation of frame-shift mutations seen in other bacteria such as *Necesseria gonorrhoeae* and *Ureaplasma* sp. [22] [29]-[31].

3. Size and Antigenic Variation of *vaa*

Size variation has been observed in Vaa using the monoclonal antibody H3 that was used to initially identify this protein, can inhibit the growth of *M. hominis*, and blocks attachment to host cells [17]. The size of Vaa observed in different isolates ranged from 28 kDa to 72 kDa and resulted from the gain or loss of intragenic repetitive sequences. Size variation of Vaa was initially examined in clonal lineages generated from *M. hominis* strain 1620, wherein three size variants of the Vaa antigen were identified and designated Vaa-2, Vaa-3, and Vaa-4. Sequence analysis showed that this *vaa* gene length variation corresponded to the number of 363 bp intragenic TR elements and the number of repeats was then used in the nomenclature of the different clonal lineages: Vaa-2 (two repeats), Vaa-3 (three repeats), and Vaa-4 (four repeats) [23]. These repeats form the basis of "modules" which provide a platform for further separation of Vaa types into categories (**Figure 1**). This 363 bp repeat became the prototype for what is now referred to as module III.

As shown in **Figure 1**, the first 240 amino acids of the FBG (P50) and 1620 Vaa proteins are highly homologous (96% amino acid identity), both of which contain a module III, however, divergence of the downstream sequence required the classification of further modules [32]. Sequence homology for each module type was initially restricted to 82% between strains [23] [32]. All *vaa* genes described thus far all start with highly homologous modules I and II, followed by either module II' or II". All reported combinations of identified module types analysed in over 100 clinical isolates [32] [33] has resulted in six possible categories (**Figure 1**); however, the number of module III in category 4 have been found to vary between two and four repeats.

Module I contains 27 amino acids that encode the putative prolipoprotein signal peptide of the precursor protein [34]. This signal peptide is cleaved off in the mature Vaa, and the resulting N-terminal cysteine is lipid-

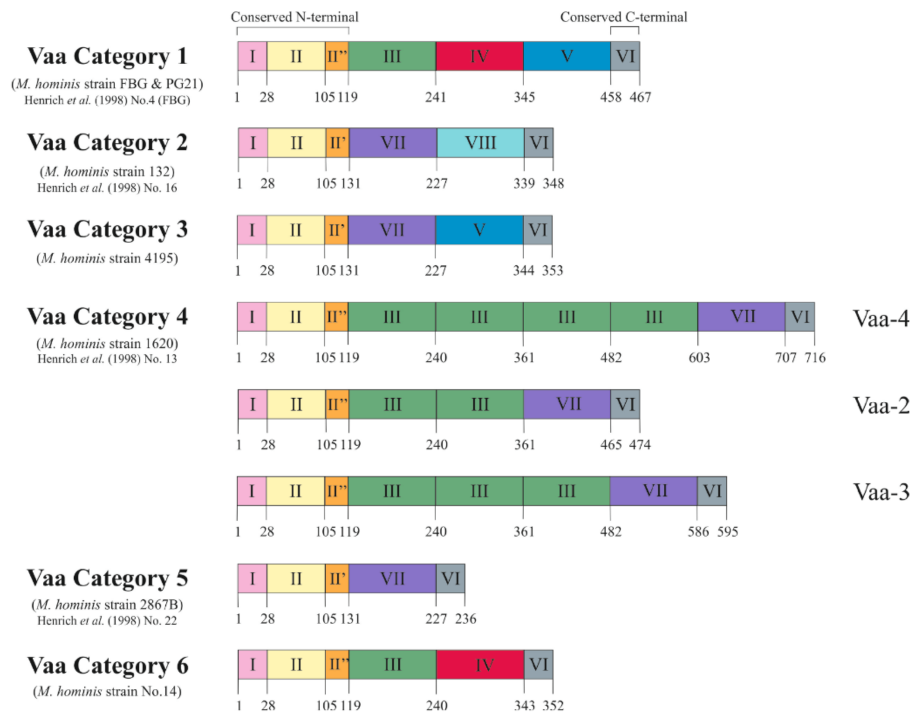

Figure 1. Schematic representation of the deduced amino acid sequences of the six *vaa* gene types. The proteins show a modular composition with homologous modules showing more than 82% amino acid identity. Modules I, II and I'/II" form the conserved N-terminal of the protein and Module VI represents the 10 amino acids conserved at the C-terminal. Modules III, IV, V, VII and VIII form the interchangeable cassettes. Prototype *M. hominis* strains for each Vaa category are stated along with corresponding strains from Henrich *et al.* (1998) [33]. Figure modified from Boesen *et al.* (1998) [32].

modified, allowing the protein to be anchored to the bacterial membrane [35]. Module II encodes the conserved N-terminal end of the mature protein. Due to low sequence homology in the C-terminal end of this module, the amino acid region 105 - 118 has been split into two further modules, module II' and module II". Module VI encodes the conserved 10 amino acids at C-terminal of all reported Vaa proteins.

Modules III, IV, V, VII and VIII form an interchangeable set of sequences that provide the size variation observed in Vaa variants. Inter-module homology (38% - 78%) suggests a common ancestral sequence [32]. A stable, repeated motif of four amino acids (SFKE) was observed in module II, a constant part of the gene. This motif was extended to ELESFKE in almost all of the interchangeable cassettes (identified by arrowed regions in **Figure 2** and **Figure 3**). Three highly conserved tryptophan residues were also identified in distinct positions in a 16 amino acid region situated in the C-terminal part of the cassette sequence (identified by triangles in **Figure 2** and **Figure 3**) [32].

Using the previously proposed method of characterising Vaa type based on module composition as in **Figure 1**, we analysed 12 UK *M. hominis* strains that were collected between 1983 and 2012. The entire *vaa* gene was sequenced and the amino acid composition predicted from the open-reading frame (methods given in supplementary appendix). Eight of the UK strains belonged to category 1 and the remaining 4 were determined to belong to category 2 (alignments shown in **Figure 2** and **Figure 3**). Aligning UK and prototype strains for category 1 Vaa showed a very high homology for modules I, II, and II". The UK2012c strain had five unique polymorphisms through this region compared to the other strains, and it is interesting to note that this colony-

purified strain originated from the same patient sample as UK2012b. This suggests that while Vaa type may be conserved within isolates from the same sample, micro-polymorphisms can exist within the same isolate. Of further note, nucleotide homology between these two strains for ten other essential genes showed 100% identity (data not shown), suggesting that Vaa may be more prone to mutation. In all strains analysed, the SFKE and ELESFKE motifs were conserved in module II and in the interchangeable cassettes, respectively. The conserved tryptophan residues can also be observed in the interchangeable cassettes. In the strains analysed, the modules that contain the highest levels of variation occur at the C-terminal end of the interchangeable cassette region, module V and module VII in Vaa category 1 and Vaa category 2, respectively. In fact, module V fails to maintain the 82% homology between isolates suggested by Boesen *et al.* (1998) to be required for inclusion in a particular module [32]. C-terminal modules appear to be hyper-variable in comparison to the other modules of the protein, indicating that there may be higher selective pressure to vary this region of the protein, suggesting that the C-terminus of Vaa may be more important to immune surveillance than more membrane-proximal modules. An-

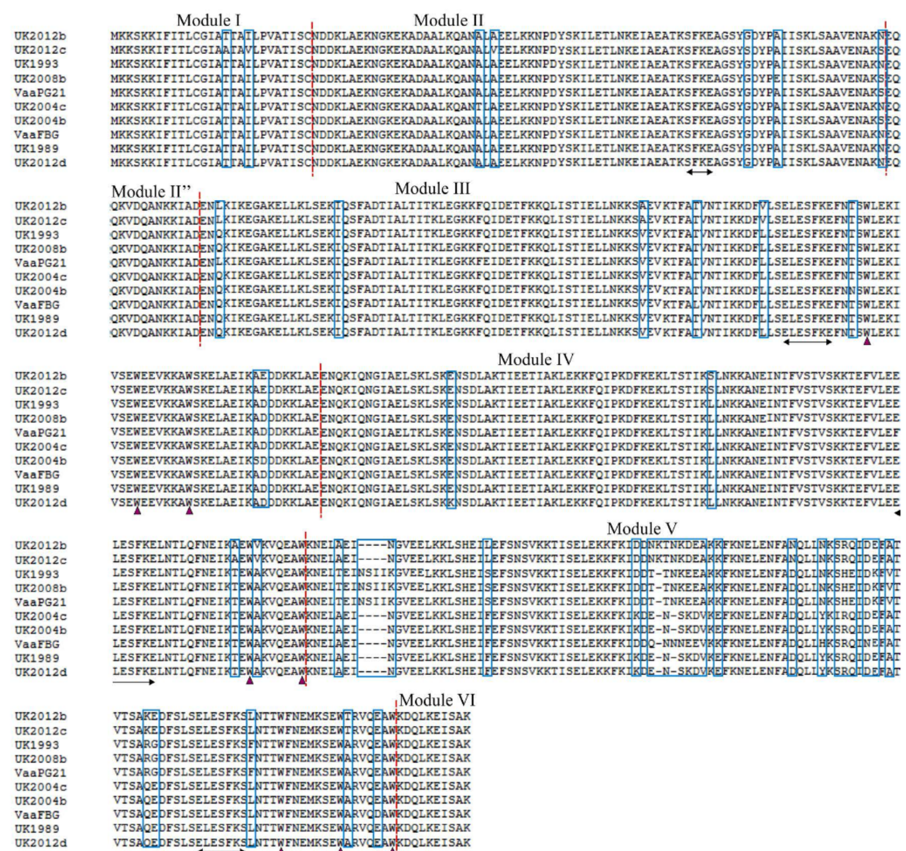

Figure 2. Alignment of the deduced amino acid sequence of Vaa category 1. Amino acid sequences of eight UK *M. hominis* strains and two prototype *M. hominis* strains (FBG and PG21) are shown. The modular composition of the protein is indicated and polymorphisms are highlighted by a blue box. The conserved SFKE and ELESFKE motifs (arrowed region) are observed in all proteins. Three tryptophan residues (triangle) are also conserved in the interchangeable cassette sequences.

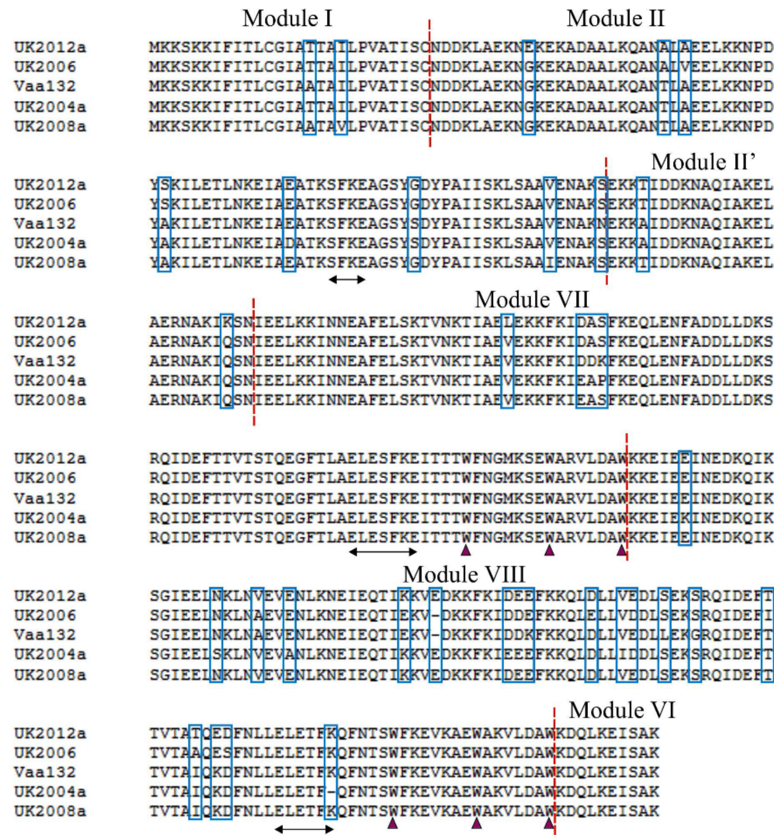

Figure 3. Alignment of the deduced amino acid sequence of Vaa category 2. Amino acid sequences of four UK *M. hominis* strains and a prototype *M. hominis* strain (132) are shown. The modular composition of the protein is indicated and polymorphisms are highlighted by a blue box. The conserved SFKE and ELESFKE motifs (arrowed region) are observed in all proteins. Three tryptophan residues (triangle) are also conserved in the interchangeable cassette sequences.

tigenic variation is important to the expression of functionally conserved moieties within a clonal population that are antigenically distinct [28].

The presence of these highly homologous interchangeable cassettes in the *vaa* gene suggests a mechanism of variation in which homologous recombination provides insertions or deletions of whole cassettes. This is most obvious in the Vaa-2, Vaa-3, and Vaa-4 variants isolated from a common ancestor (strain 1620). Homologous recombination can bring mutations arising in different genomes together and has a strong impact on pathogenic adaptation [36]. Homologous recombination was found in the penicillin-binding-proteins (PBPs) of *Streptococcus pneumoniae*, *N. gonorrhoeae* and *N. meningitides* and resulted in a mosaic gene structure [37]-[39]. Sequence blocks in the class A genes of resistant strains of *S. pneumoniae* confer decreased affinity to penicillin however these sequence blocks also contain mosaics of sequence similar to the sensitive strains. These blocks were thought to arise by interspecies horizontal genetic transfers followed by homologous recombination [40]. The class I outer membrane protein of *N. meningitidis* displays evidence of homologous recombination follow-

ing intraspecies horizontal gene transfer resulting in the exchange of variable domains giving rise to antigenic variation of this protein [41]. The absence of sequences homologous to the *vaa* gene in other members of the *mycoplasma* family or other bacterial species suggests that intraspecies genetic transfer is responsible for the current array of Vaa categories.

4. Secondary and Tertiary Structure of Vaa

Sequence analysis and modelling of the Vaa protein indicates that Vaa belongs to the group of monomeric microbial surface-exposed coiled-coil proteins similar to Protein A of *Staphylococci* [35] [42] [43]. Vaa axial shape ratios indicate that the C-terminal region of the protein is elongated whereas the N-terminal region is globular. The secondary structure of Vaa examined by circular dichroism spectra and Jpred2 analysis indicated a primarily α helical structure with a predicted N-terminal region containing three α-helices interrupted by short breaks in helicity. Jpred2 analysis of the cassette region of the protein predicted two α-helices followed by two β-sheets and an α-helix. The secondary structure prediction of the C-terminal region of the protein implies the presence of two α-helices separated by a β-sheet [35].

A hypothetical model, **Figure 4**, of the topology of Vaa shows a bacterial membrane lipid anchor that is typical of prokaryotic lipoproteins attached to the N-terminal cysteine residue of the mature Vaa with the conserved N-terminal in a triple-helix bundle, extending into an elongated helix. Two β sheets then form a loop region and a C-terminal helix folds back on the elongated helix. This model indicates that Vaa is composed of an N-terminal base domain in close proximity to the membrane and a C-terminal spike cassette domain projecting out from the surface of *M. hominis* [35]. The Vaa protein is characterised by its modular structure with different numbers

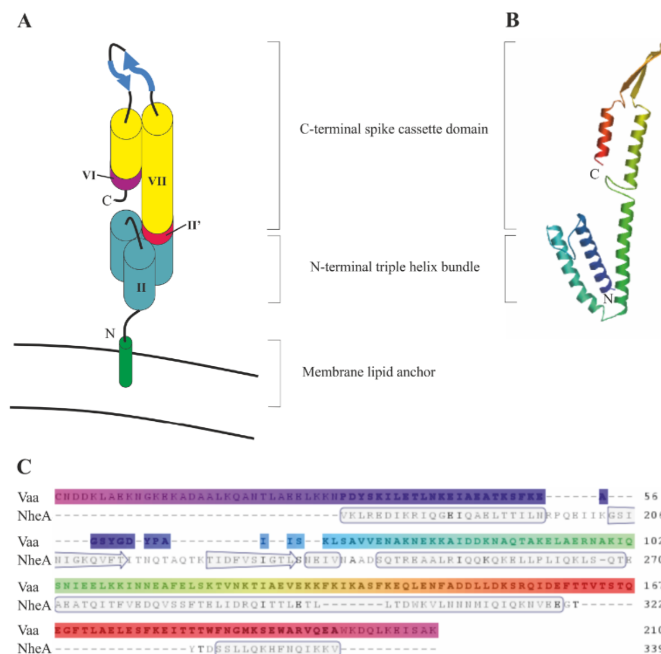

Figure 4. Model of the predicted protein structure for Vaa category 5. (A) Schematic representation of Vaa category 5 modified from Boesen *et al.* (2001) [35]. The modules are numbered as outlined in **Figure 1**. (B) Protein homology model of Vaa category 5 strain 2867B. Model was created using www.swissmodel.expasy.org and uses NheA protein as a template. (C) Alignment of Vaa category 5 strain 2867B amino acid sequence with the NheA protein template. Secondary structure is indicated in the template.

of interchangeable cassette sequences. It was originally proposed that the addition of a cassette could create a more elongated protein. However, analysis of a Vaa category 3 protein and a Vaa category 5 protein has revealed that the axial shape ratios of these two proteins, determined by circular dichroism, are almost identical, indicating that the interchangeable cassettes were arranged in parallel and not end-to-end [23] [35].

5. Role of Vaa in Cyto-Adherence

Mycoplasmas have small genomes and limited biosynthetic capabilities, restricting them to a parasitic existence in association with eukaryotic cells of their host [3] [44]. The ability of *mycoplasmas* to adhere to host epithelial cells on mucosal surfaces, in the case of *M. hominis* the urogenital tract, is an essential stage to establish successful colonisation. Several *mycoplasmas*, such as *M. pneumoniae* and *M. genitalium*, have adhesin proteins associated with adhesion concentrated at specific tip structures [45]. In comparison, *M. hominis*, along with other *mycoplasma* species, lack this attachment organelle. Many surface antigens have been identified in *M. hominis* and some of these play a role in cyto-adherence as shown by monoclonal antibody inhibition assays [17] [20].

Vaa has been shown to be involved in the adherence of *M. hominis* to host cells. Vaa was identified as a potential adhesion of *M. hominis* using monoclonal antibody inhibition, with the masking of Vaa (P50) showing prominent difference in the ability of *M. hominis* to adhere to HeLa cells [17] [20]. The role of Vaa as an adhesin of *M. hominis* was further investigated by determining which region of a category 1 Vaa protein was involved in adhesion to host cells [46]. Adhesion to glutaraldehyde-fixed HeLa cells with module III, modules III + IV, modules III + V (truncated proteins expressed in *Escherichia coli*) indicated the adherent property was distributed over the entire molecule, not localised to a specific region. However, adherence was increased when examining multiple modules [20] [46].

This is further supported by the markedly reduced cyto-adherence of truncated Vaa proteins to cultured HeLa cells compared to the complete protein. Phase variation of the Vaa gene in a clonal lineage of *M. hominis* 1620 results in a mutation in the N-terminal of the gene and the production of a truncated form of the Vaa protein (Vaa^-). This Vaa^- variant showed a >70% reduction in adherence to HeLa cells compared to a variant expressing the full length protein [22]. Examination of the membrane protein profiles of both the Vaa^- and Vaa^+ variants revealed that the only detectable difference was the presence or absence of the Vaa protein indicating that the difference in adhesion was directly attributed to the expression of this protein [22]. Low residual adhesion of the Vaa^- variant could be attributed to non-specific interactions between *M. hominis* and HeLa cells or from additional, unidentified adhesins [22]. However, adherence of a recombinant peptide containing the N-terminal region of category 1 Vaa has been demonstrated indicating that even the Vaa^- variant may retain the ability to adhere through the N-terminal region of the peptide [46].

6. Conclusion

The ability for *M. hominis* to adhere to host cells is a crucial step towards colonisation of a host. The Vaa antigen is a major adhesin of *M. hominis* and displays pronounced mutational variation in size as well as sequence and antigenic variation. To date the only mechanism described of altering Vaa expression relates to a truncation mechanism mediated by a poly-A (alanine-encoding) tract 161 bp down-stream of the ATG start codon, rather than a recombinase-mediated gene rearrangement such as described for phase variation in other *mycoplasmas*. Vaa truncation does, however, directly relate to the ability of *M. hominis* to adhere to host cells. Vaa displays a mosaic gene structure formed from interchangeable cassette sequences. Recombination of these cassette sequences results in different gene types thereby generating and maintaining functional diversity in *M. hominis*. Furthermore, methods of sequencing and characterising these distinct groups should be employed to determine if different Vaa modules are associated with isolation from different patient groups or sample sites. A method that begins the process of investigating differences between commensal populations and those that are associated with disease is overdue for this organism.

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Supplementary Appendix

Materials and Methods

Isolates, Growth Media, and DNA

M. hominis isolates UK2012a, Uk2004a, UK2006, UK2008a, UK2012b, UK2012c, UK2004b, UK1993, UK2008b, UK2004c, UK1989 and UK2012d were submitted to Public Health England, U.K. for clinical diagnostic purposes. *M. hominis* isolates were grown in Mycoplasma Liquid Medium (Mycoplasma Experience, UK). Bacterial DNA from a 500 µl 48 hour culture was released by boiling lysis (95˚C for 10 minutes) following centrifugation at 13,000 x g for 10 minutes, removal of all MLM, and resuspended in 50 µl sterile water.

PCR Amplification

PCR was performed using the published primers of Zhang and Wise (1996) and the PCR conditions were performed as detailed by Boesen *et al.* (1998) [23] [32]. All the oligonucleotide primers were synthesised by invitrogen™ (UK) and the sequences of these primers are detailed in **Table 1**. All PCR amplifications were performed in a DNA thermocycler (Techne Prime) in a volume of 50 µl containing: 1 x GoTaq® Flexi Buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 pM of each primer, 1.56 units of GoTaq® DNA Polymerase (Promega), and 2.5 µl DNA. The PCR products were analysed on 1.5% agarose gels with ethidium bromide visualisation. All PCR reactions were repeated twice.

Table 1. Primer sequences used for amplification of the *vaa* open-reading frame.

Primer name	Sequence	Target	Reference
VaaF1	5'-CCCCGGAGATTATTAAGTCT-3'	Flank the entire open-reading frame encoded by the *vaa* gene	Zhang and Wise (1996) [23]
VaaR1	5'-GTGCCCATTAGTAGCACTAT-3'		

DNA Sequencing

PCR amplicons were purified using a Qiagen Mini Prep kit (Qiagen) as per manufacturer's instructions and sequenced using the amplification primers, as performed by MWG Eurofins (Germany).

Sequence Analysis

Open-reading frame amino acid sequence was identified using Expasy translation tool (*mycoplasma* setting; web.expasy.org/translate/). Sequences were aligned with CLUSTAL omega (www.ebi.ac.uk/tools/msa/clustalo/) and deduced amino acid sequences were compared to published Vaa amino acid sequences. Module composition was based on published sequence data by Boesen *et al.* (1998) [32].

Development of a Multilocus Sequence Typing Scheme for Molecular Typing of *Mycoplasma pneumoniae*

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Mycoplasma pneumoniae **is a major human respiratory pathogen causing both upper and lower respiratory disease in humans of all ages, and it can also result in other serious extrapulmonary sequelae. A multilocus sequence typing (MLST) scheme for *M. pneumoniae* was developed based on the sequences of eight housekeeping genes (*ppa*, *pgm*, *gyrB*, *gmk*, *glyA*, *atpA*, *arcC*, and *adk*) and applied to 55 *M. pneumoniae* clinical isolates and the two type strains M129 and FH. A total of 12 sequence types (STs) resulted for 57 *M. pneumoniae* isolates tested, with a discriminatory index of 0.21 STs per isolate. The MLST loci used in this scheme were shown to be stable in 10 strains following 10 sequential subculture passages. Phylogenetic analysis of concatenated sequences of the eight loci indicated two distinct genetic clusters that were directly linked to multilocus variable-number tandem repeat analysis (MLVA) type. Genetic MLST clustering was confirmed by genomic sequence analysis, indicating that the MLST scheme developed in this study is representative of the genome. Furthermore, this MLST scheme was shown to be more discriminatory than both MLVA and P1 typing for the *M. pneumoniae* isolates examined, providing a method for further and more detailed analysis of observed epidemic peaks of *M. pneumoniae* infection. This scheme is supported by a public Web-based database (http://pubmlst.org/mpneumoniae).**

Mycoplasma pneumoniae is a common cause of community-acquired pneumonia (CAP) transmitted by aerosol or close contact (1). *M. pneumoniae* may cause other serious extrapulmonary sequelae, such as encephalitis (2). The pathogen is found in all age groups, with a higher prevalence in children age 5 to 14 years (3, 4). Admissions to a United Kingdom hospital in patients with CAP that were attributed to *M. pneumoniae* were estimated at 18% in 1982 and 4% in 1999 (5). Major increases and decreases in *M. pneumoniae* infection have occurred periodically in the United Kingdom; historically, epidemics have occurred at approximately 4-year intervals and have lasted 12 to 15 months, concurrent with sporadic infection at a lower level and seasonal peaks from December to February (4, 6). However, globally, peaks of infection have been observed in either summer or autumn, with no obvious explanation for this seasonal variation (7–10).

Typing of clinical isolates by molecular methods is of importance for the understanding of the epidemiology of *M. pneumoniae* infection and for an analysis of endemic outbreaks. It is generally considered that molecular typing of *M. pneumoniae* is hampered by the fact that the pathogen is a genetically homologous species (11). Initial molecular typing targeted the gene encoding the major surface protein (P1) of *M. pneumoniae*. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the P1 gene, which encodes a major adhesion, is the most common genotyping method. This enables the separation of isolates into two types, 1 and 2 (11–13). More recent studies utilize the repetitive regions, RepMp2/3 and RepMp4, which can be found in the P1 gene, for molecular typing and have resulted in the identification of an additional subtype and three variants of these subtypes (14, 15). Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has also been used, which is based on the variation in the copy number of tandem repeated sequences (VNTRs) found at different loci across the genome. The variation in the copy number of these tandem repeats (TRs) depends on the isolate tested. Initially, 265 strains were grouped into 26 MLVA types based on five VNTR loci (Mpn1 and Mpn13 to Mpn16), and an additional 18 novel types have since been reported (16–18). However, the Mpn1 locus is unstable in both clinical strains and in laboratory passages, and most of the novel types came from variations in Mpn1; therefore, there is international consensus that this locus should be removed from the typing scheme (19).

Multilocus sequence typing (MLST) was previously attempted for the molecular typing of *M. pneumoniae*; however, due to the homogeneity of the *M. pneumoniae* species, very little polymorphisms were found in the housekeeping genes examined, and it was previously concluded that MLST with housekeeping and structural genes was not useful for molecular typing (22). Only three housekeeping genes were thoroughly examined for polymorphisms across 30 isolates of either P1 type 1, 2, or a variant strain. The other genes selected for analysis were examined against a single representative strain from each subtype. In this study, an MLST scheme was developed with a high discriminatory ability to differentiate *M. pneumoniae* isolates based on sequence polymor-

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phisms within eight housekeeping genes, improving on all published typing methods for *M. pneumoniae*.

MATERIALS AND METHODS

***M. pneumoniae* strains, culture conditions, and sample preparation.** The strains analyzed in this study are listed in Table 1. Fifty-five *M. pneumoniae* strains were submitted to Public Health England, United Kingdom, for clinical diagnostic purposes, and the two *M. pneumoniae* type strains, FH (NCTC 10119, ATCC 15531) and M129 (ATCC 29342), were obtained from National Collection of Type Cultures (NCTC) (held by Public Health England). All strains were triple cloned on *Mycoplasma* agar (Mycoplasma Experience, Surrey, United Kingdom) and confirmed to be *M. pneumoniae* by amplification of the *p1* gene (23).

All strains were subsequently cultured in *Mycoplasma* liquid medium (MLM) (Mycoplasma Experience, Surrey, United Kingdom). For genomic sequencing, strains were grown in 100 ml of broth culture, and genomic DNA was extracted using the GenElute bacterial genomic DNA kit (Sigma, Dorset, United Kingdom). PCR amplification was performed on bacterial DNA from a 500-μl 4-day culture that was released by boiling lysis (95°C for 10 min) following centrifugation at 17,000 × *g* for 10 min, removal of all MLM, and resuspension in 50 μl of sterile water.

Multilocus sequence typing. Housekeeping genes considered to be conserved in other bacterial species under a low rate of selective pressure were chosen for analysis (Table 2). The locus sequences were selected using the available genome sequences of *M. pneumoniae* FH and M129 (FH GenBank accession no. NC_017504.1, and M129 GenBank accession no. NC_000912.1) and the available whole-genome sequences of 35 clinical isolates. Ten genes were included for initial analysis: RecA protein (*recA*), inorganic phosphatase (*ppa*), phosphoglycerate mutase (*pgm*), DNA gyrase subunit B (*gyrB*), guanylate kinase (*gmk*), serine hydroxymethyltransferase (*glyA*), elongation factor P (*efp*), ATP synthase subunit α (*atpA*), carbamate kinase (*arcC*), and adenylate kinase (*adk*); however, *recA* and *efp* were excluded from the resulting MLST scheme. The locus regions for PCR amplification were selected based on areas of the protein-coding sequences (CDS) containing nucleotide polymorphisms.

PCR utilizing the primers listed in Table 3 was used to amplify the target genes from a further 20 *M. pneumoniae* clinical isolates. Amplification of each of the locus sequences was performed in a DNA thermocycler (Techne Prime, Stone, United Kingdom) in 50-μl reaction mixtures containing 1× GoTaq Flexi buffer (Promega, Southampton, United Kingdom), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.5 pmol/μl each primer, 1.56 units of GoTaq DNA polymerase (Promega), and 2.5 μl of template DNA. PCRs consisted of an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 60°C, and 60 s at 72°C. A final extension step was maintained for 10 min at 72°C. The primer sequences and PCR product sizes are shown in Table 3. The PCR products were analyzed on 1.5% agarose gels with ethidium bromide visualization. All PCRs were performed in duplicate.

PCR amplicons were purified using a Qiagen miniprep kit (Qiagen, Inc., Hilden, Germany), as per the manufacturer's instructions, and sequenced using the amplification primers, which was performed by MWG Eurofins (Ebersberg, Germany). The sequences obtained from each corresponding forward and reverse primer were assembled and trimmed for double-stranded high-quality sequences. All the sequences obtained for each locus were aligned using Clustal W (Vector NTI, Paisley, United Kingdom), and different allelic types (ATs) (sequences with at least a 1-nucleotide difference) were assigned sequential numbers. The combination of the eight alleles determined the allelic profile of a strain, and each unique allelic profile was designated a unique sequence type (ST). Open reading frame amino acid sequences were identified using the ExPASy translation tool (*Mycoplasma* setting [web.expasy.org/translate/]) for each AT. The deduced amino acid sequences were aligned using Clustal W (Vector NTI) for each locus, and synonymous changes were identified.

MLVA and P1 typing. MLVA types were determined as described by Dégrange et al. (16), excluding the VNTR locus Mpn1 and using international nomenclature consensus (19). P1 types were determined as described by Dumke et al. (15).

Genomic sequencing. Genomic sequence data for 35 isolates were obtained using the Illumina Nextera XT sample prep kit (Illumina, Cambridge, United Kingdom) and sequenced on an Illumina HiSeq 2500 platform with TruSeq rapid SBS kits (200 cycles; Illumina) and cBOT for cluster generation (Illumina). Fastq reads were trimmed using Trimmomatic 0.32, with the following parameters: leading, 30; trailing, 30; slidingwindow, 10:30; and minlen, 50 (20). The Illumina reads were assembled to the M129 type strain (GenBank accession no. NC_000912.1) using SPAdes version 2.5.0 (21) and mapped to M129 using Geneious version 8.0.4. Sequencing yielded at least one contig of between 99,047 bp and 324,397 bp with homology to the M129 type strain (GenBank accession no. NC_000912.1), passing quality and coverage checks. Identification as *M. pneumoniae* from the genomic data was confirmed with 16S rRNA sequence analysis. The Illumina reads for all isolates were mapped against the reference chromosome of M129 (EMBL accession no. U00089) using SMALT (http://www.sanger.ac.uk/resources/software/smalt/) to identify single-nucleotide polymorphisms (SNPs), as previously described (39). Regions of recombination in the whole chromosomes of the isolates were analyzed using Genealogies Unbiased By recomBinations In Nucleotide Sequences (GUBBINS) (40).

Phylogenetic analysis. The locus sequences corresponding to each strain were concatenated head-to-tail for diversity analysis. Sequence analyses and tree construction were performed using MEGA 6.0. Neighbor-joining trees were constructed for each individual locus and concatenated sequences using Kimura's two-parameter model (24, 25). Maximum-likelihood trees were constructed for each individual locus using the Jukes-Cantor model of sequence evolution (26). Maximum-likelihood trees were constructed from concatenated sequences of the eight MLST loci using the generalized time-reversible (GTR) model of sequence evolution, with uniform rates of variation (27). Bootstrap analyses with 1,000 replicates were performed for every phylogenetic tree (28). Relatedness between STs was analyzed based on allelic profiles using eBURST version3. Maximum-likelihood trees were constructed from genomic sequences after the removal of areas of recombination. In total, 1,854 SNP sites were identified in comparison to the M129 reference chromosome. Three regions were predicted to contain SNP sites that had arisen by recombination, and these contained 28 SNP sites.

RESULTS

MLST of *M. pneumoniae*. An initial examination of 10 gene targets in the two type strains M129 and FH and the genomic sequences of 35 *M. pneumoniae* clinical isolates identified variation, in the form of SNP differences, in 8 out of the 10 genes. The *recA* and *efp* genes were 100% conserved in all sequences analyzed and were therefore excluded from the MLST scheme. Genomic sequence analysis and additional PCR and sequencing of all eight targets in a further 20 clinical isolates resolved a total of 12 STs. The discriminatory typing ability for *M. pneumoniae* was 0.21 ST per isolate. The number of SNPs observed within each individual locus and the percentage of polymorphic sites are indicated in Table 3, with *pgm* having the highest number of SNPs (10 SNPs) and the highest percentage of polymorphic sites corrected for sequence length (0.93%). The number of alleles per locus ranged from two (*ppa*, *gyrB*, *gmk*, and *arc*) to four (*atpA*) (Table 3). Examination of the Hunter-Gaston diversity index (DI) (which ranges from 0.0 for no diversity to 1.0 for complete diversity) indicated moderate diversity between the STs (DI, 0.784; 95% confidence interval [CI], 0.716 to 0.852), with the greatest individual diversity shown in *pgm* (DI, 0.620; 95% CI, 0.566 to 0.674) and the lowest diversity shown in *arcC* (DI, 0.069; 95% CI, 0.000 to 0.158).

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TABLE 1 Description of *M. pneumoniae* strains used in this study, their sequence type, allelic profile, and MLVA and P1 types

Strain	Yr of isolation	Country of isolation	Isolation site	ST	Allelic profile								MLVA type	P1 type[a]
					ppa	*pgm*	*gyrB*	*gmk*	*glyA*	*atpA*	*arcC*	*adk*		
M129 (ATCC 29342)	1969	USA	Unknown	1	1	2	1	1	1	3	2	1	4572	1
MPN135	1986	USA	Unknown	1	1	2	1	1	1	3	2	1	4572	V1
FH (ATCC 15531)	1944	USA	Sputum	2	2	3	2	2	2	4	1	1	3662	2
MPN007	1978	UK	Throat swab	2	2	3	2	2	2	4	1	1	NT[a]	2
MPN021	1983	UK	Unknown	2	2	3	2	2	2	4	1	1	3662	NT
MPN022	2010	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2c
MPN023	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	2
MPN101	1978	UK	Unknown	2	2	3	2	2	2	4	1	1	3562	2
MPN102	1981	UK	Brain frontal lobe	2	2	3	2	2	2	4	1	1	3662	2
MPN107	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2
MPN114	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	NT
MPN117	1982	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2
MPN119	1982	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2
MPN121	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	2c
MPN123	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	2
MPN125	1983	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2
MPN126	1979	UK	Unknown	2	2	3	2	2	2	4	1	1	3662	2
MPN128	1976	USA	Unknown	2	2	3	2	2	2	4	1	1	3662	NT
MPN132	1982	UK	Sputum	2	2	3	2	2	2	4	1	1	3562	2
MPN133	1982	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	2
MPN134	1982	UK	Sputum	2	2	3	2	2	2	4	1	1	3662	2
MPN005	1983	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN006	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	NT
MPN013[b]	2009	UK	Nose and throat swabs	3	1	2	1	1	1	3	1	1	4572	1
MPN014[b]	2009	UK	Nose and throat swabs	3	1	2	1	1	1	3	1	1	4572	1
MPN015[b]	2009	UK	Nose and throat swabs	3	1	2	1	1	1	3	1	1	4572	1
MPN016[b]	2009	UK	Nose and throat swabs	3	1	2	1	1	1	3	1	1	4572	1
MPN017[b]	2009	UK	Nose and throat swabs	3	1	2	1	1	1	3	1	1	4572	1
MPN020	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	NT
MPN103	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN105	1983	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN108	1983	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN109	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	NT
MPN113	1967	UK	Unknown	3	1	2	1	1	1	3	1	1	4572	1
MPN116	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN118	1996	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN120	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN122	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN136	1982	UK	Sputum	3	1	2	1	1	1	3	1	1	4572	1
MPN004	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN104[c]	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN106[c]	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN110[c]	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN124	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN131	1981	UK	Sputum	4	2	1	2	2	2	4	1	1	3662	2
MPN111	1968	UK	Unknown	5	1	2	1	1	1	2	1	1	4572	1
MPN011	1983	UK	Sputum	6	2	3	2	2	2	1	1	1	3662	1
MPN112	1983	UK	Sputum	6	2	3	2	2	2	1	1	1	3662	1
MPN127	1982	UK	Sputum	7	2	3	2	2	2	4	1	2	3662	2
MPN129	1983	UK	Sputum	8	2	3	2	2	2	4	1	3	3662	2
MPN130	1983	UK	Sputum	9	1	2	1	1	1	3	1	4	4572	1
MPN008	1981	UK	Sputum	10	2	1	2	2	2	4	1	2	3662	2
MPN018	1981	UK	Sputum	10	2	1	2	2	2	4	1	2	3662	2
MPN010	1983	UK	Sputum	11	1	2	1	1	3	3	1	1	3662	1
MPN003	1983	UK	Sputum	11	1	2	1	1	3	3	1	1	4572	1
MPN012	1981	UK	Brain cyst	11	1	2	1	1	3	3	1	1	3562	NT
MPN019	1983	UK	Sputum	12	2	2	1	1	3	3	1	4	4572	1

[a] NT, *M. pneumoniae* strain not classified by MLVA/P1 typing.
[b] Strains isolated from the same patient.
[c] Strains isolated from the same patient.

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TABLE 2 MLST loci used in established bacterial MLST schemes also present in *M. pneumoniae*

	Presence of MLST locus[a]									
Bacterial species	*recA*	*ppa*	*pgm*	*gyrB*	*gmk*	*glyA*	*efp*	*atpA*	*arcC*	*adk*
Bacillus cereus					✓					
Chlamydia trachomatis						✓				
Campylobacter jejuni			✓			✓				
Escherichia coli	✓			✓						✓
Enterococcus faecium								✓		✓
Haemophilus influenzae	✓									✓
Helicobacter pylori		✓					✓	✓		
Moraxella catarrhalis		✓					✓			✓
Neisseria meningitidis			✓							✓
Staphylococcus aureus					✓				✓	
Staphylococcus epidermidis									✓	
Streptococcus suis	✓									
Vibrio vulnificus				✓						
Yersinia pseudotuberculosis										✓

[a] MLST loci were chosen based on the frequency of use in other bacterial MLST schemes (http://www.mlst.net/) and the presence of the gene in the published M129 and FH whole genomes.

Neighbor-joining and maximum-likelihood trees constructed from concatenated sequences of the eight loci for the 57 *M. pneumoniae* isolates (Fig. 1) illustrated two genetically distinct clusters, which were confirmed by eBURST examination of relatedness (Fig. 2). The two clusters, designated clonal complex 1 (CC1) and CC2, contained ST1, ST3, ST5, ST9, and ST11, and ST2, ST4, ST6, ST7, ST8, and ST10, respectively. ST12 located distal to the two main clusters; however, phylogenetic analysis revealed closer positioning to CC1. Neighbor-joining and maximum-likelihood trees were constructed for the eight loci individually (data not shown), and the topology of both neighbor-joining and maximum-likelihood trees was consistent for all loci and concatenated sequences.

Five homogenous strains (*M. pneumoniae* MPN13 to MPN17) originating from nose and throat swabs from the same patient with Stevens-Johnson syndrome had identical STs (ST3). Additionally, two clinical isolates (*M. pneumoniae* MPN104 and MPN106) originating from separate sputum samples from a patient with bronchopneumonia taken 4 days apart also had identical STs (ST4). This indicates a single clonal population responsible for infection in these cases.

The possibility of synonymous sequence changes (indicating a pressure to conserve amino acid sequence and protein structure) was investigated by comparing the predicted translated sequences for each locus. Analysis of the deduced amino acid sequences of the eight loci for the 57 strains indicated that both synonymous and nonsynonymous SNPs occurred, of which approximately 44% resulted in an amino acid change. Nonsynonymous SNPs are highlighted in Fig. SA2 in the supplemental material. The amino acid sequences for ArcC, Gmk, and GyrB yielded homologous sequences for all ATs, numbering at two ATs for each locus. In comparison, Pgm analysis resulted in the largest number of non-

TABLE 3 Primer pairs developed in this study and variability of the different loci

Name	Direction[a]	Primer sequence (5′–3′)	Amplicon size (bp)	MLST locus location	No. of alleles	No. of polymorphic sites	% polymorphic sites	Avg G+C content (%)	Hunter-Gaston diversity index[b]	95% confidence interval
ppa	F	CGCTGACCAAGCCTTTCTAC	256	192–440	2	1	0.39	38.4	0.501	0.470–0.533
	R	CACTCCAAACTTTGCACTCCC								
pgm	F	AGCACCTTGCACGATGAAGA	1,072	456–1652	3	10	0.93	43.7	0.620	0.566–0.674
	R	CCTGCGCCTTCGTTAATTGG								
gyrB	F	TTGTCCCGGACTTTACCGTG	429	524–952	2	2	0.47	39.9	0.505	0.482–0.528
	R	TGTTTTCGACAGCAAAGCGG								
gmk	F	GAGCGGTGTTGGCAAAAGTA	394	189–582	2	1	0.25	40.1	0.505	0.482–0.528
	R	TGCATCCTCGTCATTACGCTT								
glyA	F	CAGAGAACTATGTGAGTAGGGACA	676	74–749	3	2	0.30	45.6	0.560	0.493–0.627
	R	TGACAACCCGGAAAGACACC								
atpA	F	GTCGCTGATGGCATTGCTAAG	796	100–895	4	3	0.38	44.8	0.557	0.502–0.612
	R	CCAGTAAACGCGAGTGCAAG								
arcC	F	CCCCATCAAGCCGTGTACTT	570	304–873	2	1	0.18	45.5	0.069	0.000–0.158
	R	TTGGGCAATAATGGCCGTCT								
adk	F	GTAGCCAACACCACCGGATT	473	70–542	4	3	0.63	47.5	0.199	0.063–0.335
	R	ACGGTGTCTTCGTAAAGCGT								

[a] F, forward; R, reverse.

[b] Hunter-Gaston diversity index (DI) ranges from 0.0 for no diversity to 1.0 for complete diversity.

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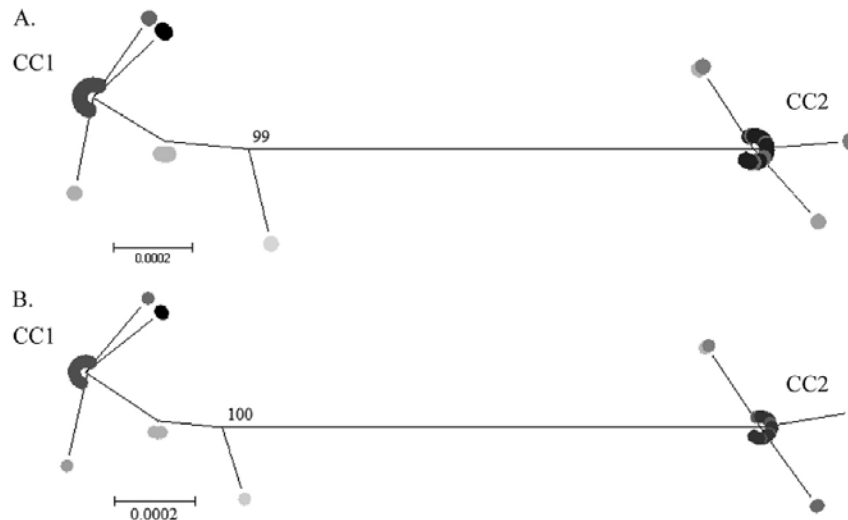

FIG 1 Phylogenetic trees were constructed based on concatenated sequences of eight housekeeping loci for 12 unique STs using maximum-likelihood (A) and neighbor-joining (B) methods. Bootstrap support values of >70% are shown. STs are indicated by differential shading.

synonymous changes in the amino acid sequence, with four changes in the sequence between three ATs.

The MLST scheme was applied to the published complete genome sequences of *M. pneumoniae* available from NCBI for strains 309 (GenBank accession no. NC_016807.1), M129-B7 (GenBank accession no. CP003913.2), and M29 (GenBank accession no. NZ_CP008895.1) and to assembled contigs available from NCBI for strains PO1 (GCA_000319655.1), PI 1428 (GCA_000319675.1), and 19294 (GCA_000387745.1). These strains were determined to belong to ST2, ST1, ST3, ST2, ST1, and ST7, respectively.

The stability of each MLST locus was assessed in 10 *M. pneumoniae* isolates. Isolates were retyped following short-term passage (10 sequential subculture passages) in liquid medium. All loci were found to be completely stable, with no SNPs in comparison to the original isolate.

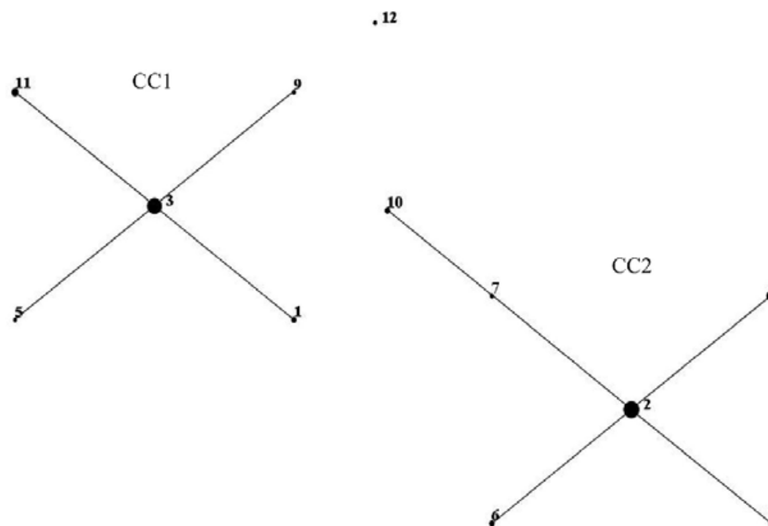

FIG 2 eBURST version 3 was used to analyze the 12 unique STs resolved for all 57 *M. pneumoniae* isolates. Two main clonal complexes (CC) were defined. The size of each dot is proportional to the number of isolates included in the analysis for each ST.

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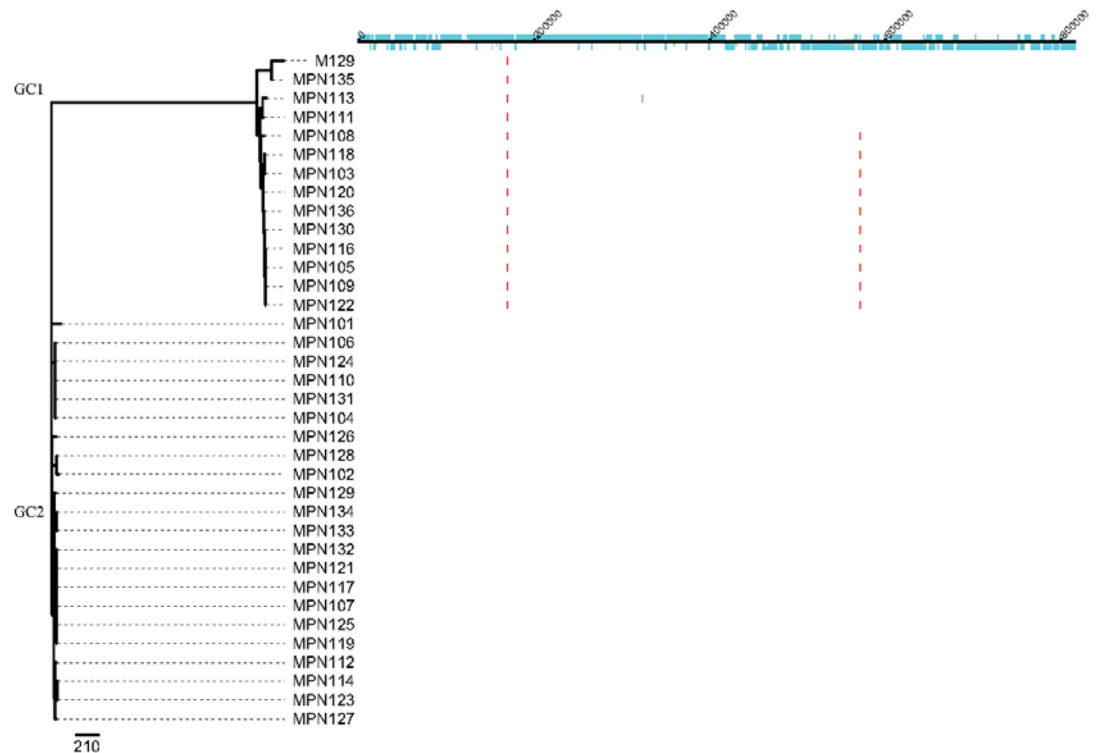

FIG 3 Prediction of recombination in the *M. pneumoniae* isolate chromosomes. Regions of variation in the genomes of the 35 clinical *M. pneumoniae* isolates and the type strain M129, which are predicted to have arisen by homologous recombination, are shown on the right. Red blocks indicate recombination predicted to have occurred on internal nodes, and blue indicates taxon-specific recombination. Isolates are ordered according to the phylogenetic tree displayed on the left. The track along the top of the figure displays the M129 chromosome and annotation, in which protein-coding sequences (CDS) are indicated in light blue.

Genomic sequence analysis. Three regions of SNPs were predicted to have arisen by homologous recombination in the chromosomes of the 35 clinical *M. pneumoniae* isolates (Fig. 3), one of which distinguished genomic clade 1 (GC1) from GC2, and the other two occurred within GC1. Area 1 was predicted to occur in all strains in GC1, area 2 in 10 strains, and a single strain, MPN113, had a single additional predicted area of recombination, area 3. Following removal of the predicted areas of recombination, two distinct genetic clades were identified, GC1 and GC2 (Fig. 3). Excellent parity was found using this method and concatenated MLST sequences, with all strains colocating to the corresponding CC and GC.

Comparison to other typing methods. There was no obvious link between the ST and the year when the strains were collected, the patient's age, and the sample origin; however, limited numbers of strains were available per year, and for some years, there were no strains. Indeed, multiple STs can be observed in a single year. Furthermore, ST was related to P1 type (Table 1), with the two most common STs, ST2 and ST3, containing strains that were P1 type 2 and P1 type 1, respectively. Additionally, this MLST scheme was also comparable to MLVA typing. The two major clusters observed, CC1 and CC2, were directly linked to MLVA type: CC1 contained MLVA type 4572, whereas CC2 contained MLVA types 3662 and 3562. Each ST contained only one MLVA type, with the exception of ST2, which contained both 3662 and 3562, and ST11, which contained 4572, 3662, and 3562 (Table 1). Distributions of MLVA type, P1 type, and ST can be observed in Fig. 4, indicating that P1 type 1, MLST ST2, and MLVA types 3662 and 4572 occurred most frequently in the isolates tested.

In the isolates tested in this scheme, MLST was deemed to be more discriminatory than both MLVA typing and P1 typing, resulting in 0.21, 0.05, and 0.07 types per isolate, respectively. This was confirmed by examination of the Hunter-Gaston DI, which indicated higher discriminatory ability for the MLST scheme (DI, 0.784; 95% CI, 0.716 to 0.852) than that of the current MLVA scheme (DI, 0.633; 95% CI, 0.583 to 0.683) and P1 typing (DI, 0.567; 95% CI, 0.510 to 0.625).

Online database. An *M. pneumoniae* MLST online database was created for both MLST alleles, profile definitions, and isolate data (29) (http://pubmlst.org/mpneumoniae).

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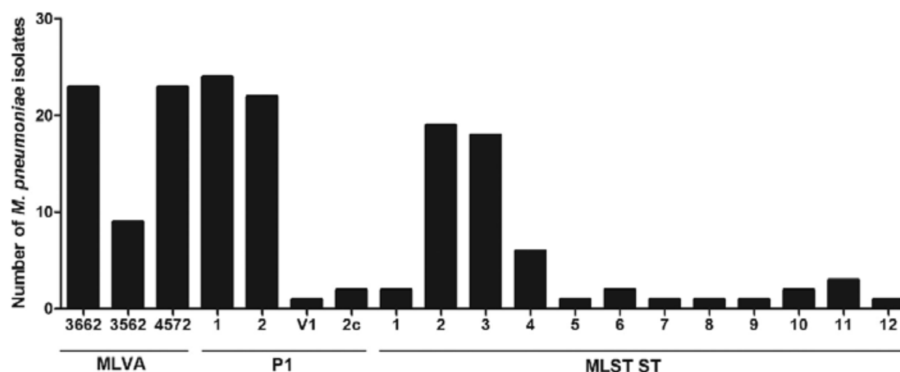

FIG 4 Distribution of MLVA, P1 type, and ST for 57 *M. pneumoniae* isolates (each group defined by lines below).

DISCUSSION

MLST has been used to genotype several species of bacteria, including several *Mycoplasma* species: *Mycoplasma agalactiae*, *Mycoplasma bovis*, and *Mycoplasma hyorhinis* (30–32). This study has described the successful development of a novel *M. pneumoniae* MLST scheme to allow the characterization of clinical isolates. This scheme was successfully used to discriminate 55 clinical isolates of *M. pneumoniae* from British patients (with the exception of two U.S. isolates) within the reference laboratory collection, from respiratory and extrapulmonary sites, and the two type strains M129 and FH. Eight housekeeping genes were identified as suitable targets for the scheme, and these were used to genotype *M. pneumoniae* isolates by either PCR followed by sequencing or whole-genome sequence analysis. *gyrB* contains a quinolone resistance-determining region (QRDR) with documented *in vitro* mutations at amino acid positions 443, 464, and 483. The clinical use of quinolones may increase selective pressure *in vivo*, resulting in a high mutation rate (33). However, the *gyrB* locus sequence amplified in this MLST scheme is in a different region of the gene from the QRDR and is therefore considered a suitable MLST target. The stability of the eight loci was evaluated *in vitro* and was confirmed before and after 10 repeated passages of 10 strains in liquid medium. However, stability over a larger number of passages in liquid medium and an evaluation of stability using *in vitro* tissue culture were not assessed.

The discriminatory power of this MLST scheme with the eight loci was 0.784 for the collection of 57 isolates. In comparison, the Hunter-Gaston DI of the P1 typing method for the 57 isolates was 0.567, and the DI of the MLVA scheme was 0.633; therefore, this MLST scheme was more discriminatory for the isolates tested. However, it has been shown that the established MLVA method is more discriminatory than P1 typing (16), which was confirmed in this study. The allelic diversity of each of the MLST loci varied significantly at each locus, with the *pgm*, *glyA*, *atpA*, *gyrB*, *gmk*, and *ppa* loci being more discriminatory than the *adk* and *arcC* loci. The association of this set of markers with varied Hunter-Gaston DIs makes this MLST, in theory, more optimal for epidemiological studies than other existing methodologies.

Analysis of *M. pneumoniae* infection at the individual patient level was possible using this scheme. Multiple clinical isolates were available from two of 50 patients: five were from a patient with Stevens-Johnson syndrome (MPN013 to MPN017), and two were from a patient with bronchopneumonia, taken 4 days apart. In both cases, the ST, MLVA type, and P1 type remained the same, indicating that a single clonal isolate was responsible for infection. Recurring infection or reinfection of *M. pneumoniae* can be determined using this scheme. Recurring infection would have the same ST as the original infection, whereas reinfection with *M. pneumoniae* would likely be a different ST. Genetic MLST instability in isolates might occur; however, in this study, this was not seen over 10 passages.

The eBURST analysis illustrates the relationship of STs on the basis of the number of MLST loci that differ between two STs. Analysis of this population modeling indicates that the two clusters, CC1 and CC2, differed by more than one locus, but within each cluster, the STs did not differ by more than one locus. Within a cluster, this highlights the homogenous nature of the *M. pneumoniae* species; however, a definitive split can be observed between the two clusters in both ST and MLVA type. A possible divergent clade with ST12 from CC1 is also apparent; however, more isolates need to be typed by this method to confirm this observation. Few typing methods have been able to detect significant differences between strains, including one previous attempt to subtype *M. pneumoniae* by MLST with housekeeping and structural genes (12, 15, 22). The previous MLST was determined to be not sufficiently discriminatory to be used for epidemiological purposes. However, the MLST scheme developed in this study was able to discriminate between *M. pneumoniae* isolates and resulted in two genetically distinct clusters, indicating significant differences between strains.

A comparison between genomic sequence analysis after the removal of predicted areas of recombination and phylogenetic analysis of concatenated MLST sequences showed similar topology and the same distinct genetic clustering. This indicates that this MLST scheme is representative of the genome and confirms that *M. pneumoniae* can be subdivided into two distinct genetic lineages.

Typing of clinical *M. pneumoniae* isolates is becoming increasingly important due to the global increase in *M. pneumoniae* infections and the increase in macrolide-resistant strains (34, 35). This scheme provides a more discriminatory method than both the MLVA and P1 typing methods currently in use, allow-

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ing further and more detailed analysis of observed epidemic peaks of *M. pneumoniae* infection. Community outbreaks of pneumonia caused by *M. pneumoniae* have been described worldwide (36–38), and it would be interesting to evaluate this MLST scheme in such epidemic situations. The level of discrimination of this typing method and its usefulness in epidemic analysis should be confirmed by comparing outbreak-related strains to a set of control strains that were isolated from a similar time period and geographical area but that are not epidemiologically related. More severe or adverse infections with *M. pneumoniae* are seen in some patients. The reason for this is not clear; however, it can be postulated that this is due to specific microbe pathogenicity (identified through genetic markers) or variance in host susceptibility. This method could assist in determining if this is a strain-specific phenomenon. One advantage of MLST is that it is PCR based and does not require the growth of bacteria, which can be a lengthy process for *M. pneumoniae*. However, there is a large amount of sequencing required for this method, which can be laborious and expensive; therefore, its adaptation for widespread use directly on clinical specimens would be beneficial.

In conclusion, this study presents a robust MLST scheme that has proven discriminatory for *M. pneumoniae*, providing isolate characterization and a higher level of discrimination than MLVA and P1 typing methods. In addition, phylogenetic analysis of both STs and whole-genome sequence data revealed two genetically distinct clusters. Crucially, this scheme for *M. pneumoniae* is also supported by a public Web-based database (http://pubmlst.org/mpneumoniae).

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We declare no conflicts of interest.

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EDITORIAL

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Molecular typing of *Mycoplasma pneumoniae*: where do we stand?

"Mycoplasma pneumoniae is a respiratory bacterial pathogen causing upper and lower respiratory disease in humans of all ages."

Rebecca J Brown[1,2], Brad O Spiller[2] & Victoria J Chalker*[,1]

KEYWORDS
• CAP • MLST • MLVA • *Mycoplasma pneumoniae* • P1 typing • WGS

Mycoplasma pneumoniae is a respiratory bacterial pathogen causing upper and lower respiratory disease in humans of all ages. It is considered a major cause of pneumonia, especially in children of school age and in some cases can result in serious extrapulmonary sequelae. A large increase in reported *M. pneumoniae* cases was documented in several European countries in 2011 [1]. In England and Wales, seasonal peaks of infection are detected from December to February each year with epidemics at approximately four yearly intervals, lasting 12–15 months [2]. Epidemics are not concurrent worldwide, however, differing countries also report cyclical patterns, as observed in England and Wales, such as Denmark, Sweden, Norway, Finland, Korea and Japan [3,4]. Additionally, in differing countries, seasonal peaks of infection have been observed in either summer or autumn and no definitive factor has been proven to account for seasonal variation or the formation of epidemic peaks.

Traditionally, molecular typing was used to characterize epidemic outbreaks of *M. pneumoniae*, however, it has been postulated that molecular typing of *M. pneumoniae* is hampered by the genetically homologous nature of the species [5]. Despite this, molecular typing methods have been developed for this organism including: PCR-restriction length polymorphism (RFLP) of the major surface adhesin P1 [5], multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) [6], multilocus sequence typing (MLST) [7] and the recent SNaPshot™ minisequencing assay [8]. The mechanisms driving fluctuations in incidence of *M. pneumoniae* infections have not been defined. It has been postulated that shifts in proportion of individual strains with specific P1 type or concurrent increased incidence of several strains may result in epidemics or immunity. Additionally, it is believed that the genotype of *M. pneumoniae* may be changing, generating diverse genetic material in each epidemic with a recent study reporting the detection of polyclonal strains in a single epidemic [9].

The initial molecular typing procedure targeted the gene encoding of the major surface adhesin, P1, of *M. pneumoniae*.

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RFLP analysis of the *p1* gene was the most common genotyping method, enabling separation of *M. pneumoniae* isolates into two types, type 1 and 2 [5,10]. Studies utilizing the repetitive regions, RepMp2/3 and RepM4 in the *p1* gene resulted in the identification of an additional six variants [11–13]. Speculation that a shift in P1 adhesin type may be the cause of epidemics has been disputed with evidence indicating the presence of multiple P1 adhesin types in observed increases of infection [6,9,10]. It was hypothesized that a decline in immunity or an increase of the immunologically naive population may result in the 4-year cycle of epidemic periods [14]. In other geographical locations, it has also been observed that multiple P1 types can be detected during outbreaks, and it has been suggested that although immunological pressure may favor shifts of P1 type, a co-circulation of P1 types appears to be common [15].

"Traditionally, molecular typing was used to characterize epidemic outbreaks of *M. pneumonia*…"

MLVA has been increasingly used internationally for strain characterization and is based on variation in the copy number of tandem repeated sequences, called VNTRs, found at different loci across the genome. The variation of the copy number of these tandem repeats depends on the isolate tested. Initially, 265 strains were grouped into 26 MLVA types, based on five VNTR loci (Mpn1, Mpn13–16) and additional novel types have since been reported [6,16]. MLVA was documented to be more discriminatory for *M. pneumoniae* strains than P1 typing, providing an additional level of classification for transmission studies. However, reports of observed instability in the Mpn1 locus has called into question the reliability of the marker. Additionally, inconsistency in nomenclature and identification of repeat regions has led to international standardization of the MLVA and the removal of Mpn1 as a locus [17]. Analysis of the 2010/2011 epidemic in the UK revealed a total of 11 distinct MLVA types present using the original typing method [14], however, reanalysis using international guidelines reduces the MLVA types detected to five distinct types [Unpublished Data]. The discriminatory power of the MLVA method for characterization of *M. pneumoniae* strains has reduced with the removal of the Mpn1 locus, necessitating either the identification of new loci or alternative typing methods.

Initial attempts at developing an MLST scheme for *M. pneumoniae* were unsuccessful due to low levels of polymorphisms found in the housekeeping genes examined, suggested to be because of the homogeneity of the *M. pneumoniae* species, and it was concluded that the use of an MLST scheme with housekeeping and structural genes was not useful for molecular typing. However, three housekeeping genes were examined for polymorphisms across 30 isolates of either P1 type 1, 2 or a variant strain and the other genes selected for analysis were examined against a single representative strain from each P1 type [18]. Recently, an MLST scheme was successfully developed to differentiate *M. pneumoniae* isolates based on sequence polymorphisms in eight housekeeping genes, which improved on existing typing methods for *M. pneumoniae* [7]. This MLST scheme discriminated between 57 *M. pneumoniae* isolates with a higher level than both MLVA (with the removal of Mpn1) and P1 typing and it may prove more optimal for epidemiological studies than other existing methods. Population modeling and phylogenetic analysis of concatenated MLST profiles revealed two distinct genetic clades of *M. pneumoinae,* showing similar topology to phylogenetic data and distinct genetic clustering obtained using genomic sequence analysis. The typing profiles obtained using the MLST method infers representation of the genetic phylogeny, reflecting that *M. pneumoniae* can be subdivided into two distinct genetic lineages [7]. Nevertheless, this MLST scheme has not yet been applied to localized outbreak or epidemic strain analysis or has not been demonstrated direct on clinical specimens. Recent development of a SNaPshot™ mini sequencing assay has resulted in identification of nine SNP types [8]. This method is rapid and appears to have greater discriminatory ability than MLVA and P1 typing. A direct comparison of MLST and SNaPshot™ minisequencing assay has not been undertaken and both methods may have similar discriminatory abilities. However, MLST resulted in a larger number of defined sequence types.

These methods are all PCR-based and do not necessarily require the growth of bacteria, which can be a lengthy process for *M. pneumoniae.* P1 typing, MLVA and MLST do not limit investigation through the requirement of specialist methodology. However, MLST can be laborious and expensive, with the cost of genomic sequencing reducing and becoming a more attractive option for genetic analysis of strains. Genomic sequencing may allow the concurrent identification of P1 type, MLVA profile and MLST sequence type

directly from the genomic sequence as well as providing additional information, such as the presence of antibiotic resistance and toxin markers. Improvements in sequencing technology and the development of methodologies to produce longer sequence reads enables the reliable determination of repeated DNA sequences [19]. This is of importance for species such as *Mycoplasma*, in which large tracts of repeated sections within AT-rich genomes are common. For genetically homologous species such as *M. pneumoniae,* the use of genomic sequencing to analyze phylogeny inferred from single nucleotide polymorphism analysis will improve the ability to accurately segregate this species into distinct lineages allowing in-depth epidemiology studies. Due to the fastidious nature of *M. pneumoniae* and other human Mollicutes, such as *Mycoplasma amphoriforme*, the use of metagenomic approaches to identify pathogens in studies of human infections [20] will no doubt improve detection of infections caused by Mollicutes, while obviating the need for expensive and laborious culture and typing methods, simultaneously providing additional data such as the detection of mutations known to confer resistance.

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RAPID COMMUNICATIONS

Detection of macrolide resistant *Mycoplasma pneumoniae* in England, September 2014 to September 2015

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Mycoplasma pneumoniae infection can cause pneumonia, particularly in children. Global increase in macrolide-resistant *M. pneumoniae* is of concern due to limited therapeutic options. We describe the detection of macrolide resistance-conferring mutations in 9.3% of 43 clinical specimens where *M. pneumoniae* was detected in England and Wales from September 2014-September 2015. This study aims to impact by highlighting the presence of macrolide resistance in *M. pneumoniae* positive patients, promoting increased clinical vigilance.

Here we report the detection of mutations associated with macrolide resistance in *M. pneumoniae*-positive specimens from four patients with pneumonia in Englandin the periodSeptember 2014 to September 2015. Prior to 2014, in the United Kingdom seven cases of macrolide-resistant *M. pneumoniae* infections were reported between 2008 and 2011, mainly from Scotland [1,2].

Macrolide resistance determination

The Bacteriology Reference Department, Public Health England (PHE), London, receives specimens from England and Wales for *M. pneumoniae* testing and confirmatory testing. Here we detected *M. pneumoniae* by qPCR in 60 clinical specimens from 60 patients (Cambridge, Leeds, London, Manchester, Nottingham and Oxford) that were submitted to PHE between 1 September 2014 and 1 September 2015. DNA extractions from specimens, where *M. pneumoniae* was detected, were screened for point mutations known to confer macrolide resistance. Mutations in domain V of the 23S rRNA were detected by a modified version of the method described by Li et al., 2009 [3], wherein the entire region of interest is amplified and sequenced as one product. Primers used were as follows: forward primer 5'-ATCTCTTGACTGTCTCGGC-3' and reverse primer 5'-TACAACTGGAGCATAAGAGGTG-3'.

Of the 60 specimens, 17 (28.3%; 95% confidence interval (CI): 18.4--40.8) contained insufficient DNA to determine macrolide resistance-conferring mutations. Of the remaining 43 specimens mutations in the 23S rRNA known to confer macrolide resistance were found in four (9.3%; 95% CI: 3.1–22.2). Of these 43 specimens, 32 were from a single city in England, Leeds, and a single specimen among these was positive for the mutation, 3.1% (95% CI: 0.01–17.1). The cases identified with point mutations known to confer macrolide-resistant *M. pneumoniae* were in two women and two men, respectively, aged > 15 to <65 years old. Three were hospitalised with pneumonia (Table) with no known connection between patients.

Interestingly, two of the macrolide-resistant cases were patients that had recently arrived from the United States (exact timeline unknown); of which one had received clarithromycin whilst undergoing treatment in the UK. The origins of the infecting *M. pneumoniae* strains in these two cases may have been external to England and Wales. The other two cases were from separate cities in England. All macrolide resistance-conferring mutations were A2058G (*Escherichia coli* numbering) point mutation in the 23S rRNA.

Background

Mycoplasma pneumoniae can be isolated from patients with lower respiratory tract infection, including pneumonia, and has also been associated with prolonged persistent cough and exacerbation of asthma [4]. *M. pneumoniae* infections may manifest infrequently as extra-pulmonary sequelae after the onset of or even in the absence of respiratory illness [5]; including encephalitis [6], dermatological manifestations such

Table

Details of patients with macrolide-resistant *Mycoplasma pneumoniae*-positive clinical specimens, England and Wales, September 2014–September 2015 (n=4)

Case	Age group (years)	Sample type	Pneumonia	Hospitalised	Macrolide before sampling
1	45–65	TS	Yes	Yes	Unknown
2	15–25	BAL	Yes	Yes	Yes
3	45–65	BAL	Yes	Yes	Unknown; Antibiotics class unknown administered before admission
4	15–25	TS	Yes	Unknown	Unknown

BAL: bronchoalveolar lavage; TS: throat swab.

as Stevens-Johnson syndrome [7], and haemolytic anaemia [8]. Asymptomatic carriage of *M. pneumoniae* has been documented in nasopharyngeal swabs at low levels in England, e.g. at 0.25% based on PCR in a 2001 carriage study [9], however, a study from the Netherlands reported a much higher carriage rate (21.2%) [10]. In England and Wales, *M. pneumoniae* infection can be found in all age groups, with a higher prevalence in children of school age [9]. In England and Wales, seasonal peaks of infection are detected from December to February each year with epidemics at approximately four-yearly intervals, lasting 12 to 15 months [9]. A large increase in reported *M. pneumoniae* cases was documented in several European countries, including England and Wales, in 2011 [11].

Discussion

In the past 15 years, a significant increase in macrolide-resistant *M. pneumoniae* has been reported globally, of increasing concern and importance to the international community [12]. In Asia, resistance rates of over 90% have been reported [13], particularly in China, whereas in Europe and North America resistance rates of up to 25% have been documented [14,15]. Macrolide-resistant strains of *M. pneumoniae* have not been documented to show cross-resistance to other classes of antibiotics i.e. tetracyclines and fluoroquinolones [16].

Prior to 2014, in the United Kingdom, seven cases of macrolide-resistant *M. pneumoniae* infections were reported between 2008 and 2011, one case in England and Wales and six cases in Scotland [1,2]. This is the second report of macrolide-resistant *M. pneumoniae* strains detected in England and Wales, with one case previously documented for a single patient specimen from 2008 [1]. Macrolide resistance in *M. pneumoniae* has been reported in Scotland at 19% (6/32) [2], considerably higher than the 9.3% documented here. This may reflect low sample numbers or sampling differences and it is important to note that the specimens examined for macrolide resistance in Scotland were from patients in whom macrolide resistance was considered most likely based on their clinical presentation or history, being one of the following: repeated specimen positive, remaining symptomatic following antibiotic treatment, admitted to critical care or having an underlying condition.

In this study a high number of samples were from a single city in England and a local epidemic cannot be excluded. There is no requirement for referral of *M. pneumoniae*-positive specimens to the reference laboratory in England and Wales. Systematic testing and referral of positive specimens does not occur. Therefore regional comparison was not possible. Nonetheless, the focus of this article was to highlight macrolide resistance rather than a specific regional cluster analysis.

Macrolides are currently recommended as the first-line treatment for *M. pneumoniae* infection in the UK [17]. The 2011 British Thoracic Society guidelines for the management of community acquired pneumonia in children and adults suggest empirical macrolide treatment at any age if there is no response to first-line beta-lactam antibiotics or in the case of very severe disease [17,18]. Tetracyclines (minocycline and doxycycline) and fluoroquinolones (levofloxacin and moxifloxacin) can be used to treat *M. pneumoniae* infections as an alternative to macrolides when clinically relevant [19], however, their use in children is limited due to effects on bone toxicity and cartilage development, respectively [20,21].

We did not isolate *M. pneumoniae* by culture from those specimens wherein *M. pneumoniae* was detected by PCR and therefore we were not able to confirm phenotypic macrolide resistance. However, point mutations within the 23SrRNA gene in clinical specimens and isolates, including the A2058G mutation, have previously been shown to confer resistance [16]. Acquisition of resistance has been documented in patients receiving macrolides and resistance may develop as a consequence of antibiotic selective pressure [22]. This is supported by the highest macrolide resistance rates being reported in countries with extensive macrolide use [15]. Increased vigilance pertaining to macrolide-resistant *M. pneumoniae* in the UK is recommended.

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Conflict of interest

None declared.

Authors' contributions

RJB wrote the manuscript, LMS undertook PCR and local study conception, SP performed macrolide resistance analysis, VJC designed, oversaw the study and wrote the manuscript.

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Mycoplasma pneumoniae Epidemiology in England and Wales: A National Perspective

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Investigations of patients with suspected *Mycoplasma pneumoniae* infection have been undertaken in England since the early 1970s. *M. pneumoniae* is a respiratory pathogen that is a common cause of pneumonia and may cause serious sequelae such as encephalitis and has been documented in children with persistent cough. The pathogen is found in all age groups, with higher prevalence in children aged 5–14 years. In England, recurrent epidemic periods have occurred at ~4-yearly intervals. In addition, low-level sporadic infection occurs with seasonal peaks from December to February. Voluntarily reports from regional laboratories and hospitals in England from 1975 to 2015 were collated by Public Health England for epidemiological analysis. Further data pertaining cases of note and specimens submitted to Public Health England from 2005 to 2015 for confirmation, molecular typing is included.

Keywords: ***Mycoplasma pneumoniae*, epidemiology, England, wales, microbiology**

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INTRODUCTION

Mycoplasma pneumoniae is a respiratory bacterial pathogen causing upper and lower respiratory disease in humans of all ages. It is a major cause of community-acquired pneumonia (CAP) and is considered to be responsible for 15–20% of CAP cases in adults and up to 40% of cases in children, especially in children of school age (Foy, 1993; Korppi et al., 2004; Dumke et al., 2012). Up to 25% of *M. pneumoniae* infections may manifest as extra-pulmonary sequelae after the onset of or in some cases in the absence of respiratory illness (Cassell and Cole, 1981; Narita, 2010). Encephalitis is one of the most severe complications (Narita, 2009; Meyer Sauteur et al., 2014b) estimated in 5–10% of pediatric encephalitis patients (Bitnun et al., 2001; Christie et al., 2007) of which up to 60% of have additional neurologic sequelae (Bitnun et al., 2001, 2003). *M. pneumoniae* infection can result in dermatological manifestations including Stevens-Johnson syndrome (Olson et al., 2015). Hemolytic anemia is a rare but serious complication of *M. pneumoniae* infection and is more frequent children than in adults (Gu et al., 2014). *M. pneumoniae* infections occur both endemically and epidemically worldwide, with epidemic peaks every 4–7 years (Chalker et al., 2011a, 2012a; Jacobs, 2012). Typical outbreaks of *M. pneumoniae* infection occur in areas of close personal contact for example, schools and military barracks. Both symptomatic and asymptomatic individuals with *M. pneumoniae* carry the organism in the respiratory tract and it can be transmitted from person to person via aerosols and cough (Clyde, 1979; Waites and Talkington, 2004; Meyer Sauteur et al., 2014c). Long-term morbidity due to *M. pneumoniae* infection is uncommon however; the acute illness is often disruptive and can consume significant resources (Waites and Talkington, 2004).

In England and Wales (EW), seasonal peaks of infection are detected from December to February each year with epidemics at ~4-yearly intervals (Chalker et al., 2011b, 2015). Cyclical patterns, as observed in EW, are also seen in Denmark, Sweden, Norway, Finland, Korea, and Japan (Ito et al., 2001; Rasmussen et al., 2010; Blystad et al., 2012; Linde et al., 2012; Polkowska et al., 2012; Kim et al., 2015). It has recently been suggested that minor variations in the duration of immunity may be essential to the cyclic epidemic peaks (Omori et al., 2015). A con-current increase in reported *M. pneumoniae* cases was documented in several European countries in 2011 (Lenglet et al., 2012) and in EW the most recent increase has been noted in 2015 (this study).

The recent global increase in macrolide resistance observed in cases of *M. pneumoniae* infection is of increasing concern and importance to the international community (Bébéar, 2012). In China resistance has been documents in over 90% of clinical isolates of *M. pneumoniae* studied (Zhao et al., 2013) however resistance is lower in European counties including France, Germany, Switzerland, and Sweden (Peuchant et al., 2009; Meyer Sauteur et al., 2014a; Nilsson et al., 2014; Dumke et al., 2015). Macrolides are currently recommended as the first-line treatment for *M. pneumoniae* infection in the UK (Harris et al., 2011). The 2011 British Thoracic Society guidelines for the management of CAP in children and adults suggest empirical macrolide treatment at any age if there is no response to first-line β-lactam antibiotics (which are ineffective against cell wall-less bacteria such as *M. pneumoniae*) or in the case of very severe disease (Lim et al., 2009; Harris et al., 2011). Macrolide-resistance in EW has recently been documented at 9.3% and is therefore not included in this article (Brown et al., 2015a). This is considerably lower than in Scotland (19%) (Ferguson et al., 2013).

This study aims to provide up to date overview of the number of laboratory reports and incidence of *M. pneumoniae* infection in EW, molecular typing data, and briefly highlight cases of note in recent years.

MATERIALS AND METHODS

A total of 16,878 serological, culture, genomic, and unspecified laboratory diagnostic methodology *M. pneumoniae* positive cases reported to Public Health England, via the Communicable Disease Report Network comprising ~250 laboratories from January 1989 to June 2015 were aggregated into 3 weekly periods. These report the organisms identified from specimens (e.g., throat swabs, serum, or sputum) submitted by general practitioners and hospitals with the patient's age and sex, the reporting laboratory, and date of the first sample; the system has changed little over time. Duplicate specimens were removed and reports plotted to examine the general pattern (3-weekly moving average). National reporting categories include antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*M. pneumoniae* antibody level. Rising titres are not demonstrated for all patients as it is not always possible to obtain a second specimen. It is possible that a fraction of cases reported as antibody detection only include some cases of rising titre that have not been appropriately coded. A distinction between IgA, IgG and IgM cannot be made when collating figures, however the number of cases with rising titre demonstrating active infection mirror the overall total case pattern of epidemic periodicity. A total of 39,758 laboratory reports of *M. pneumoniae* infections in England and Wales from January 1975 to June 2009 previously examined indicated that cyclic epidemics occurred every 4 years, were synchronous across all regions in the country, and occurred during the winter (Nguipdop-Djomo et al., 2013). Epidemic periods were defined as a clear increase in cases resulting in more than 20 cases in a 3 weekly average rolling period (**Figure 1**). From this dataset, we computed average age specific incidence for epidemic and non-epidemic periods using the England and Wales (EW) population censuses of 1981, and 2001 for the denominator for the periods 1975–1988 and 1998–2009 respectively. Data from 1989 to 1997 were excluded from age-specific analyses because age was missing in ~90% records during that period. Age distribution incidence rates from 2010 to June 2015 were calculated using the Office for National Statistics (ONS) mid-year population estimates for EW.

Diagnostic methodology of choice has altered with time in EW, with the decline in culture and use of the complement fixation test being superseded by enzyme immunosorbent assays and the introduction of molecular testing. To ascertain the proportion of reports now obtained using molecular methods, differing methodologies in use with time was examined from 1989 to 2015 (**Figure 2**). Molecular typing of *M. pneumoniae* positive clinical specimens and isolates was undertaken using MLST (50) (Brown et al., 2015b), MLVA (156) (Chalker et al., 2015), and P1 type (84) determinations (Dumke et al., 2006) from 1977 to 2011 (**Figures 3–5**). Referred cases to the Bacteriology Reference Department, Public Health England from 2005 to 2015 were examined for cases with unusual or severe presentation.

RESULTS

From January 1989 to June 2015 seven epidemics of *M. pneumoniae* were noted of declining amplitude with recent peak in 2015 (**Figure 1**). For some epidemic periods clear annual fluctuations can also be seen apparent as a double peak over two winter seasons. The clarity of epidemic periods have in recent years declined with less reported cases overall. From 1975 to 2009 incidence was found to be similar by gender, both during epidemic and inter-epidemic periods. The annual notification rate in 2010–2015 was consistently highest in those aged 15–44 years, detailed in **Table 1**. The use of culture has declined in recent years and despite serology being the most commonly used methodology the implementation and increased use of molecular methods has resulted in a proportional increase in reports based on molecular tests from 0.32% (3/936 95%CI 0.06–0.98) in 2010 to 28.5% (95/333 95%CI 24.0–33.6) in the first 6 months of 2015 (**Figure 2**). Molecular typing data were grouped into 4-yearly

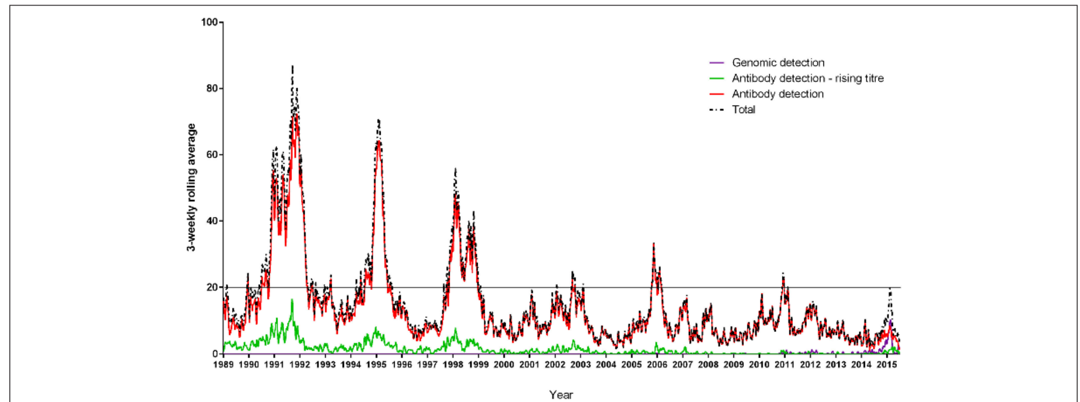

FIGURE 1 | Laboratory reports of *Mycoplasma pneumoniae* infection detection by genomic and serological methods in England and Wales from January 1989 to June 2015. The line at 20 cases per 3 weekly average rolling period defines seven epidemic periods of declining magnitude and clarity, lasting up to 2 years (1991–1992, 1994–1995, 1998–1999, 2001–2003, 2005–2006, 2011, 2015). National reporting categories include antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*Mycoplasma pneumoniae* antibody level.

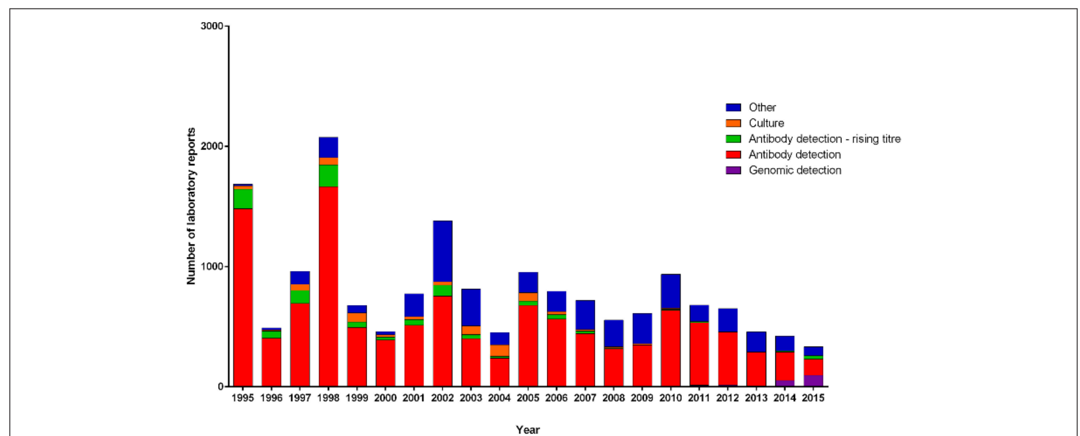

FIGURE 2 | Number of laboratory reports per year from January 1995 to June 2015 separated by detection methodology. National reporting categories included are: antibody detection and antibody-detection rising titre. A rising titre is defined as a four-fold increase in detectable anti-*Mycoplasma pneumoniae* antibody level (methods not specified). Other indicates specimens for which *M. pneumoniae* infection was determined using antigen detection (method not specified), microscopy and unknown categories. Culture indicates cases from which specimens yielded isolates of *M. pneumoniae* and genomic detection those for which DNA of *M. pneumoniae* was detected by PCR.

intervals, representing the epidemic cycles observed in the UK. Multiple MLST, MLVA, and P1 types were observed in each 4-yearly interval (3–5) however a predominance of P1 type 1 can be seen for all intervals except 1981–1984 where equal numbers of P1 type 1 and type 2 strains were observed. This data is limited by low sample number in 4-yearly intervals, therefore the variation in P1 types observed is likely to be an underestimate of the actual *M. pneumoniae* population present.

Cases of Note: 2005–2015

From January 2005 to June 2015 eleven cases were referred to the Bacterial Reference Department, Public Health England that were identified as positive for *M. pneumoniae* that were of particular note. The majority of cases were patients with lower respiratory tract infection. Stevens-Johnson syndrome is an immune-mediated hypersensitivity complex typically involving the skin and mucous membranes. Two cases of Stevens-Johnsons

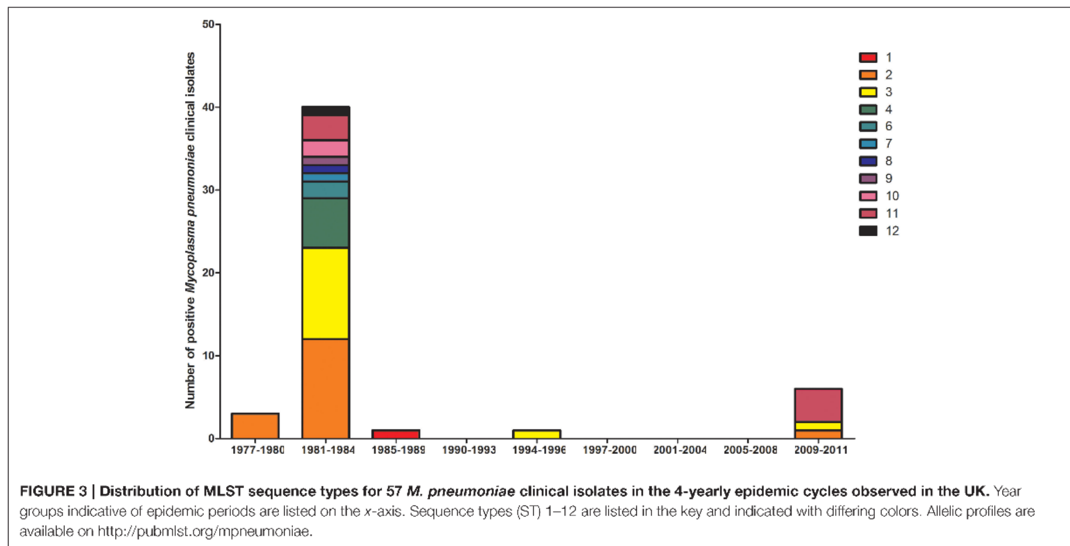

FIGURE 3 | Distribution of MLST sequence types for 57 *M. pneumoniae* clinical isolates in the 4-yearly epidemic cycles observed in the UK. Year groups indicative of epidemic periods are listed on the *x*-axis. Sequence types (ST) 1–12 are listed in the key and indicated with differing colors. Allelic profiles are available on http://pubmlst.org/mpneumoniae.

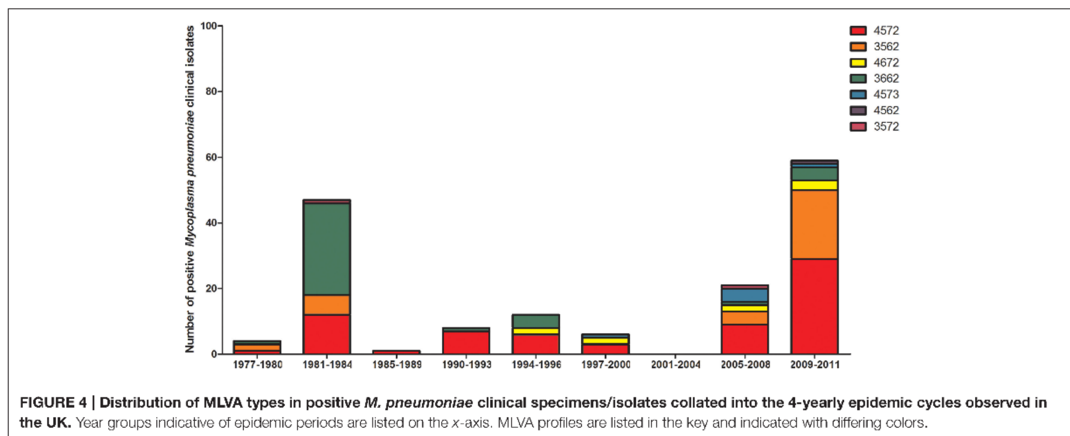

FIGURE 4 | Distribution of MLVA types in positive *M. pneumoniae* clinical specimens/isolates collated into the 4-yearly epidemic cycles observed in the UK. Year groups indicative of epidemic periods are listed on the *x*-axis. MLVA profiles are listed in the key and indicated with differing colors.

syndrome were noted in 2009 and 2010 in male children aged 8 and 6 respectively. Two cases were noted in respiratory specimens in immunocompromised patients following extra-pulmonary organ transplantation (2013 and 2015). Infection in donor transplant patient respiratory secretions was also noted in 2015. *M. pneumoniae* was detected by qPCR in the nasopharyngeal aspirate but not the cerebral spinal fluid (CSF) of a patient with pneumonia and reactive transverse myelitis in a child in 2005, and in the bronchoalveolar lavage of a child with encephalitis and seizures in 2011. In 2011 a young adult patient presented post respiratory tract infection with encephalitis and transverse myelitis that progressed to tetraplegia with ventilator dependency. *M. pneumoniae* was confirmed by qPCR on throat swab specimens taken 19 and 21 days post onset but was not detected in concurrent CSF specimens (Chalker et al., 2012b). Detection of *M. pneumoniae* in CSF is unusual and it is postulated that neurological manifestation of *M. pneumoniae* infection is antibody mediated rather than by direct presence of the bacteria itself (Waites and Talkington, 2004). Of 68 CSF specimens referred only 1 positive case was detected in 2010, in a child with a ventriculoperitoneal shunt, in which contamination of the CSF during sampling could not be excluded. In 2012 *M. pneumoniae* was detected by qPCR in the lung tissue of *two* co-habiting adults that both suffered sudden fatal collapse. This was presumed a secondary infection as one of the two patients also had confirmed *Staphylococcus aureus* infection.

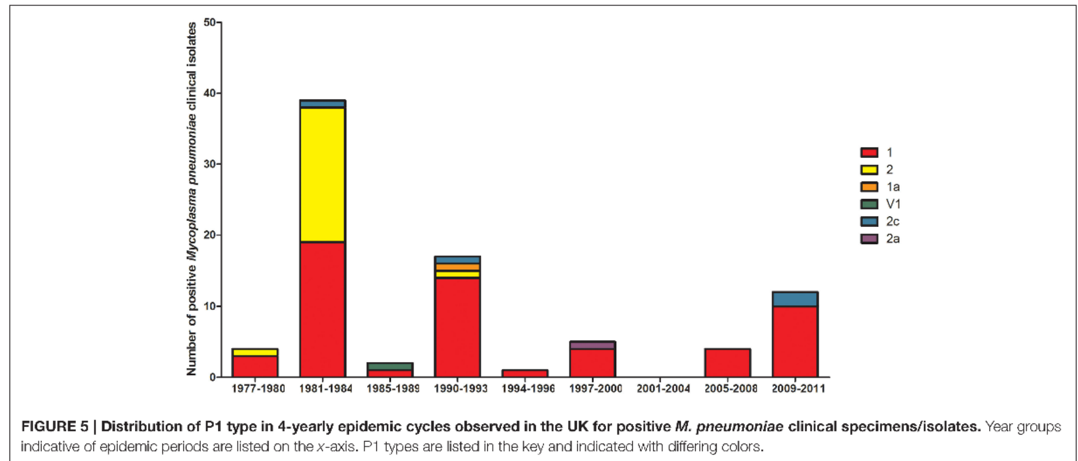

FIGURE 5 | Distribution of P1 type in 4-yearly epidemic cycles observed in the UK for positive *M. pneumoniae* clinical specimens/isolates. Year groups indicative of epidemic periods are listed on the *x*-axis. P1 types are listed in the key and indicated with differing colors.

TABLE 1 | Incidence of *M. pneumoniae* positive laboratory reports per million persons.

	Year	Incidence (per million persons in overall population)								
		0–4 years	5–9 years	10–14 years	15–24 years	25–44 years	45–64 years	65+ years	Unknown	Total
Serology + PCR	2010	1.17	1.35	0.99	1.40	2.09	1.44	0.61	0.00	9.05
	2011	1.14	1.83	1.16	1.51	2.40	1.28	0.87	0.02	10.21
	2012	1.45	1.47	1.15	1.45	2.84	2.05	1.22	0.00	11.63
	2013	0.61	0.95	0.74	0.93	1.77	1.82	1.42	0.00	8.25
	2014	0.82	0.78	0.47	0.89	2.09	1.10	1.31	0.02	7.47
	2015*	1.78	1.22	0.70	1.15	3.07	1.88	1.32	0.00	11.11
	Average	1.16	1.27	0.87	1.22	2.38	1.60	1.13	0.01	9.62

Data for 2015 from January to June and rates adjusted for half-year data were collected.

DISCUSSION

Laboratory reports show that cyclic epidemics of *M. pneumoniae* infections in EW recur every 4 years on average, concurrent with annual seasonal fluctuations with incidence peaking and dipping in the winter and summer respectively. The reduction in clarity and magnitude of epidemic periods in recent years could be indicative of a genuine reduction in cases, increasing population pulmonary health, or reflect the changing nature of testing strategies moving away from techniques such as complement fixation. Overall incidence, although declining over the period 1989–2015, has remained static since 2010 and age-specific differences in epidemic period incidence were noted for the limited periods studied. Annual notification rate in 2010–2015 was highest in 15–44 year olds perhaps reflecting reliance on serological confirmation or infection. Globally, epidemics of *M. pneumoniae* are considered to occur every 3–7 years, however recent epidemiological studies have documented varying trends in epidemic patterns. Serological studies performed in Denmark showed a pattern of *M. pneumoniae* infections over a 50 year period from 1946 through 1995 with endemic disease transmission punctuated with cyclic epidemics every 3–5 years (Lind et al., 1997). In Jerusalem, historically, epidemics were observed every 3–5 years with seasonal peaks in October and early spring; however, since autumn 2014 a constant rate of infection has been observed, diverging from the historical pattern (Nir-Paz et al., 2012). Indeed, similar to the data for EW; 3-yearly cyclic epidemic periods with declining magnitude have been documented in Japan from 1979 to 1999 (Ito et al., 2001).

Speculations regarding the mechanisms driving fluctuations in population incidence of *M. pneumoniae* infections have included decline in immunity or increase of the immunologically naive population level (Chalker et al., 2011a) or shifts in the proportion of individual strains with specific P1 type or concurrent increased incidence of several strains. Additionally, it is believed that the genotype of *M. pneumoniae* may be changing, generating diverse genetic material in each epidemic with a study reporting the detection of polyclonal strains in a single epidemic (Pereyre et al., 2012). Recent modeling of epidemic

peaks has suggested that fluctuations may be attributable to minor variations in the duration of immunity at the population level (Omori et al., 2015). Speculation that a shift in P1 adhesin type may be the cause of epidemics has been disputed with evidence indicating the presence of multiple P1 adhesin types in observed increases of infection (Sasaki et al., 1996; Dégrange et al., 2009; Pereyre et al., 2012). It was hypothesized that a decline in immunity or an increase of the immunologically naïve population may result in the 4-year cycle of epidemic periods (Chalker et al., 2011a). In other geographical locations, it has also been observed that multiple P1 types can be detected during outbreaks, and it has been suggested that although immunological pressure may favor shifts of P1 type, a co-circulation of P1 types appears to be common (Nilsson et al., 2010; Dumke et al., 2015). This is further supported by the presence of multiple MLST types within specimens in EW, reflecting the concurrent presence of strains of varied genetic lineage. As expected an increase in molecular detection of infection is noted, with declining use of culture. Macrolide resistance has recently been documented at 9.3% found in adult patients only (Brown et al., 2015a) and is also of concern in children in other countries (Meyer Sauteur et al., 2014a). However, this was derived from results of specimens submitted to the reference laboratory which may be biased toward those developing resistance during treatment and one patient was documented to have received macrolides prior to sampling therefore this level may be an over-representation to the actual level in the community.

Extrapulmonary complications of *M. pneumoniae* infection can arise involving the skin and the nervous, cardiovascular, renal, gastrointestinal, musculoskeletal, and hematologic systems. The presence of *M. pneumoniae* in these extrapulmonary sites has been confirmed by PCR as well as culture (Koletsky and Weinstein, 1980; Kasahara et al., 1985; Narita et al., 1996; Saïd et al., 1999; Bar Meir et al., 2000). Complications that occur within the central nervous system (CNS) are recognized as the most common extrapulmonary manifestations of *M. pneumoniae* infections. A recent study of 1988 children with encephalitis showed *M. pneumoniae* as the most common causative agent (Bebear and Robertson, 1996). It is thought that the host immune response that develops after *M. pneumoniae* infection contributes to these complications as well as contributing to autoimmunity (Waites and Talkington, 2004). The mechanisms that result in these neurological manifestations of *M. pneumoniae* infection are not completely understood however, immune-mediated mechanism are suspected due to the development of cross-reactive antibodies to the brain and other neurological structures (Waites et al., 2008). PCR testing of 68 CSF specimens in EW resulted in detection of *M. pneumoniae* DNA in a single specimen in which contamination during sampling could not be excluded. Consideration to testing of CSF specimens in tandem with paired respiratory specimens should be given as in practice detection of *M. pneumoniae* in CSF is extremely rare and positive patients may be detected using pulmonary specimens.

In summary, epidemics of *M pneumoniae* infections recur every 4 years on average in EW affecting all age groups, predominantly children and adults <44 years of age. Macrolide resistance has recently been documented at 9.3% and extrapulmonary complications can be severe.

AUTHOR CONTRIBUTIONS

RB worte the manuscript, PN, HZ, and ES undertook epidemiological data, OS and VC oversaw the study and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Conference abstracts

European Mycoplasma Meeting: "Mycoplasmas – a practical approach". June 2013 Dubrovnik, Croatia

The Bactericidal Activity of Normal Human Serum against 21 Clinical Isolates of *Mycoplasma pneumoniae*.

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Poster presentation

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections and may be responsible for up to 15-20% of community-acquired pneumonias. However, as a consequence of *Mycoplasma* pneumoniae infection, extrapulmonary complications may occur (e.g. encephalitis), related to immunopsuppression or direct spread of infection. Understanding of the host immune response to *Mycoplasma pneumoniae* infection has advanced over the recent years although little is known about the role of serum killing and serum resistance to *Mycoplasma pneumoniae* infection. The ability of normal human serum to kill bacteria, via the complement system, plays an important role in the host immune defence against infection. The complement system is activated via three pathways and is directly able to kill many microorganisms.

We have screened for complement killing of 21 *Mycoplasma pneumoniae* clinical isolates by normal human serum. Seropositive and seronegative normal human serums for *Mycoplasma pneumoniae* were identified and the presence of specific antibodies was confirmed by Western blot and Lineblot analysis. The serum killing assay was performed by incubating clinical isolates in 50% NHS (or heat-inactivated control serum) at 37°C for one hour. Complement specific killing was measured as the fold decrease in survival relative to the matched heat-inactivated control serum.

Our initial findings indicate varying susceptibility of the *Mycoplasma pneumoniae* clinical isolates to seropositive serum killing. Five isolates were particularly susceptible, with less than 0.1% survival, including one with survival of approximately 0.001%. One particularly resistant strain has been identified with greater than 10% survival. Further investigation is on-going to determine the pathway of complement activation that is responsible for the highly susceptible isolates. A comparison of the inherent killing ability of seropositive and seronegative normal human serum against these *Mycoplasma pneumoniae* clinical isolates is being made.

The initial results and future plans for the research will be presented.

MiTREG South West and South Wales Annual Meeting. October 2014 Cardiff, UK

Examination of the genetic diversity of *Mycoplasma hominis* reveals high levels of variation in comparison to *Mycoplasma pneumoniae*.

Rebecca J. Brown, Victoria J. Chalker and O. Brad Spiller

Oral presentation

Mycoplasma hominis is an opportunistic, urogenital pathogen that can cause serious diseases such as meningitis and septic arthritis. In comparison, *Mycoplasma pneumoniae* is a respiratory pathogen that is a common cause of pneumonia. A multi-locus sequence typing (MLST) scheme for *M. hominis*, based on the sequence of eight housekeeping genes, revealed high levels of genetic diversity in this pathogen. A discriminatory ability of 0.9 STs per isolate was resolved and phylogenetic analysis confirmed a high level of intra-species diversity. In comparison, a MLST scheme for *M. pneumoniae* resulted in a discriminatory ability of 0.26 STs per isolate. The stability of the *M. hominis* STs were investigated by re-sequencing eight strains after short-term passage; only two of the isolates had conserved STs. The high genetic variability of *M. hominis* observed indicates that *M. hominis* is well adapted to evolve, potentially aiding immune system evasion and rapid development of antibiotic resistance-mediating mutations.

Public Health England PhD Student's Day and Annual UKRO visit. March 2015

Chilton, UK

Genetic diversity of *Mycoplasma pneumoniae* and *Mycoplasma hominis*: consequences for epidemiological studies.

Rebecca J. Brown, Victoria J. Chalker and O. Brad Spiller

Oral presentation

Mycoplasma hominis and *Mycoplasma pneumoniae* are urogenital and respiratory pathogens, respectively. Within a human population it is important to determine pathogen epidemiology and transmission; methodologies have been developed to type organisms into discriminate strains, usually based on genetic diversity. Genetic diversity was examined for *M.hominis* and *M.pneumoniae* by developing two novel multi-locus sequence typing (MLST) schemes. This successfully discriminated between strains of *M.pneumoniae,* comparable to current genetic typing methods. However, for *M. hominis* high levels of genetic diversity and poor genetic fidelity were found, indicating that current conventional genetic typing methodologies for *M.hominis* have little value for epidemiology and transmission studies. However, results indicate that *M.hominis* is well adapted to evolve, potentially aiding immune system evasion and antibiotic resistance.

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Development of a multi-locus typing scheme for *Mycoplasma pneumoniae*

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Poster presentation

Objectives
Mycoplasma pneumoniae is a respiratory pathogen that is a common cause of pneumonia and may cause other serious extra-pulmonary infections such as encephalitis. The pathogen is found in all age groups, with higher prevalence in children aged 5-14 years. The objective of this work was to study the genetic diversity of this pathogen using a multi-locus typing (MLST) scheme in comparison to previously established multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) and P1-typing methodologies.
Methods
An MLST scheme was developed based on the sequence of eight housekeeping genes (*ppa*, *pgm*, *gyrB*, *gmk*, *glyA*, *atpA*, *arcC* and *adk*) and applied to 36 *M. pneumoniae* clinical isolates and the two type strains, M129 and FH. DNA was extracted from isolates and PCR reactions were performed for each individual locus. PCR amplicons were sequenced and allelic types were assigned arbitrary numbers for each locus based on nucleotide differences. Combination of the eight alleles determined an isolate's allelic profile and each unique allelic profile was designated a unique sequence type (ST).
Results
A total of ten STs were resolved for the 38 *M. pneumoniae* isolates tested. The discriminatory ability of this MLST scheme for *M. pneumoniae* is 0.26, with ST2 the predominating ST. Examination of the Hunter-Gaston diversity index indicated moderate diversity between the sequence types (DI: 0.779; 95% CI: 0.692-0.866) with the greatest diversity shown in *pgm* (DI: 0.637; 95% CI: 0.570-0.703) and the lowest diversity in *arcC* (DI: 0.105; 95% CI: 0.000-0.235). Phylogenetic analysis of concatenated sequences of the eight loci for the ten STs using both maximum-likelihood and neighbour-joining methods resulted in two genetically distinct clusters; the first containing ST1, ST3, ST5 and ST9, and the second containing ST2, ST4, ST6, ST7, ST8 and ST10. The grouping of *M. pneumoniae* isolates using this MLST scheme had higher discriminatory power than VNTR-MLVA and was not comparable to P1-type.
Conclusion
MLST is generally considered an intermediate scale typing method and in this case was found to be discriminatory among the *M.* pneumoniae isolates tested. This molecular typing approach provides an alternative method to type *M. pneumoniae* isolates, allowing further characterisation of clinical isolates and epidemiological analysis, in combination with already established typing methods.

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Detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in sequential, neonate clinical samples using a novel multiplex assay.

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Poster presentation

Background and aims

Mycoplasma hominis and *Ureaplasma* species are associated with infections in neonates (pneumonia, bacteremia, meningitis, abscesses and chronic lung disease). This study established a quantitative real-time PCR to simultaneously detect *M. hominis*, *U. urealyticum* and *U. parvum* in neonate clinical specimens to improve current clinical diagnostic services.

Methods

The multiplex assay developed included two gene targets; *yidC* gene of *M. hominis, ureaseB* gene of *Ureaplasma* species with species-specific probes and control. Specificity and sensitivity were determined. Endo-tracheal secretions were tested from 53 intubated, preterm neonates (anonymised; Plymouth) and 6 intubated, preterm neonates treated with clarithromycin (clinical diagnosis samples; Cardiff).

Results

The assay was 100% specific and had positive predictive values of 81.82% and 82.86% and negative predictive values of 98.11% and 92% for *U. urealyticum* and *U. parvum*, respectively, when compared to culture. A total of 238 clinical samples were tested of which 6 were positive for *M. hominis*, 23 were positive for *U. urealyticum* and 52 were positive for *U. parvum*. Four mixed infections were identified. Treatment with clarithromycin cleared *U. parvum* infection by 48 hours post-treatment in 4 out of the 6 neonates however 2 neonates tested PCR positive for *U. parvum* at 48 hours and 72 hours post-treatment, respectively.

Conclusions

In conclusion, we developed a specific, sensitive and reproducible real-time PCR to detect *M. hominis*, *U. ureaplasma* and *U. parvum* in oral clinical samples. This PCR assay was used to successfully diagnose infections in neonatal samples and monitor infection levels over the period of intubation.

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Detection of *Mycoplasma hominis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* in neonate clinical samples: comparison between a novel multiplex assay and existing clinical practices.

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Oral presentation

Background
Mycoplasma hominis and *Ureaplasma* species are associated with infections in neonates including: pneumonia, bacteremia, meningitis, abscesses and chronic lung disease.
Objectives
This study compared three methods (two multiplex assays and culture) for the detection of *M. hominis*, *U. urealyticum* and *U. parvum* in neonate clinical specimens to improve current clinical diagnostic services.
Methods
A panel of neonatal clinical samples (clinical diagnosis samples; 2014; Public Health England) were tested for *Ureaplasma* species and *M. hominis* using the current in-house multiplex assay, a novel multiplex assay and traditional culture methods. The novel multiplex assay developed included two gene targets; *yidC* gene of *M. hominis, ureaseB* gene of *Ureaplasma* species with species-specific probes and control. This assay was able to quantify bacterial load using a positive control plasmid containing one copy of each gene target for *M. hominis*, *U. urealyticum* and *U. parvum*.
Results
M. hominis, *U. urealyticum* and *U. parvum* were detected by all three diagnostic methods. The novel multiplex assay was more sensitive and detected more positive results than the current in-house multiplex assay. Using this novel assay, additional *M. hominis*, and mixed infections with *U. urealyticum* and *U. parvum* positive clinical samples were identified.
Conclusions
The novel multiplex assay is more specific and sensitive for the detection of *M. hominis* and *Ureaplasma* species in oral clinical samples than the current in-house assay. The use of a molecular assay has advantages over culture as it is able to distinguish between *Ureaplasma* species and is able to quantify bacterial load, allowing for more informed treatment of infections by clinicians.