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Phenotypic and genotypic characterisation of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*.

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Declaration of Originality

This thesis entitled "Phenotypic and genotypic characterisation of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenza*e and *Haemophilus haemolyticus*", describes original research conducted by the candidate within the School of Health Sciences (formerly School of Human Life Sciences) at the University of Tasmania, and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material has previously been published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Co-Authorship of Jointly Published Work

Manuscript 1:

I was the primary author and lead investigator of manuscript 1, which comprises Chapter 3 of this thesis and was published as:

Witherden EA, Kunde D, Tristram SG (2012). An evaluation of SNP-based PCR methods for the detection of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother*; 18 (4): 451-455.

Manuscript 2:

I was the primary author and lead investigator of manuscript 2, which comprises Chapter 4 of this thesis and was published as:

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Manuscript 5:

I was the primary author and lead investigator with respect to study design, data analysis and manuscript write up of manuscript 5, which comprises Chapter 7 of this thesis. However, I wish to duly acknowledge Annette Sondergaard (Department of Clinical Medicine, Aarhus University, Denmark), for conducting the transformation experiments that generated the raw experimental data that significantly contributes to this chapter.

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Statement of Candidature Contribution to Thesis

The thesis comprises interlinked research investigations where the candidate **Elizabeth Witherden** is the lead investigator, however, the following people and institutions also contributed to the published and non-published work contained within the thesis as follows:

- Elizabeth Witherden (School of Health Sciences, University of Tasmania): Lead investigator responsible for design of each individual research project, laboratory and experimental analysis, data collection, data analysis, data interpretation, and is the lead author on all resultant manuscripts.

- Dr. Stephen Tristram (School of Health Sciences, University of Tasmania): Assisted with research project design, experimental techniques, and manuscript revisions.

- **Dr. Dale Kunde** (School of Health Sciences, University of Tasmania): Technical assistance in experimental work performed within the molecular biology laboratory, and manuscript revisions (Chapters 3 and 5).

- **Dr. Murray Adams** (School of Health Sciences, University of Tasmania): Thesis revisions.

- Dr. Paula Bajanca-Lavado (National Institute of Health, Department of Infectious Diseases, Portugal): Technical expertise and manuscript revision (Chapter 6).

- Dr. Alexandra Nunes (National Institute of Health, Department of Infectious Diseases, Portugal): Technical expertise in bioinformatics and data analysis (Chapter 6) as well as data interpretation and manuscript revision (Chapter 6).

- Annette Sondergaard (Department of Clinical Medicine, Aarhus University, Denmark): Conducted the transformation experiments that generated the raw data for Chapter 7.

- Bowen Zhang (School of Health Sciences, University of Tasmania): Assisted with the 16S rRNA data collection (Chapter 5).

Specific contributions to Published Chapters:

- Chapter 3; Elizabeth Witherden (75%), Stephen Tristram (20%), Dale Kunde (5%).

- Chapter 4; Elizabeth Witherden (80%), Stephen Tristram (15%), Bowen Zhang (5%).

- Chapter 5; Elizabeth Witherden (75%), Stephen Tristram (20%), Dale Kunde (5%).

- Chapter 6; Elizabeth Witherden (70%), Alexandra Nunes (15%), Stephen Tristram (10%), Paula Bajanca-Lavado (5%).

- Chapter 7; Elizabeth Witherden (70%), Stephen Tristram (10%), Annette Sondergaard (20%).

We, the undersigned agree with the above stated, "proportion of work undertaken" for each of the published or non-published chapters, which contribute to this thesis:

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Publications and Presentations at Conferences During PhD Candidature

A. Published Manuscripts:

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Witherden EA, and Tristram SG (2013). Prevalence and mechanisms of β-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (5): 1049-1053. [doi: 10.1093/jac/dks532].

Witherden EA, Kunde D, Tristram SG (2013). PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (10): 2255-2258. [doi: 10.1093/jac/dkt189].

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrobial Chemother*; In Press. [doi: 10.1093/jac/dku022].

B. Conference Presentations:

Witherden EA and Tristram SG (2012). PCR screening for β-lactamase-negative ampicillin-resistant strains of *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p673.

Witherden EA and Tristram SG (2012). *Haemophilus haemolyticus* as a potential reservoir for *ftsI* gene mutations and altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p1301.

Witherden EA and Tristram SG (2012). A comparison of MIC based screening tests for β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p672.

Witherden EA (2013). Molecular characterization of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. Oral presentation proceedings from the Australian Society for Medical Research, Medical Research Week Postgraduate Student Awards, May 30 – June 7th University of Tasmania, Clinical School, Hobart, Tasmania.

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. Proceedings from the Australian Society for Antimicrobials Annual Meeting, 20th-22nd February, Melbourne Exhibition Centre, Victoria, Australia, Poster p22.

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Yours Sincerely,

Elizabeth A. Witherden

General Abstract

Haemophilus influenzae is a significant opportunistic pathogen that causes a range of respiratory infections, including community-acquired pneumonia (CAP), acute exacerbations of chronic obstructive pulmonary disease (COPD) and acute otitis media (AOM). These infections frequently require antibiotic therapy for management, with antibiotics of the β-lactam class such as amoxicillin, cefaclor, and amoxicillin-clavulanate historically used as first line therapies. However, the efficacy of these antibiotics is currently threatened by the increasing prevalence of β-lactam resistance mediated by specific mutations in the *ftsI* gene that produce an N526K substitution in the encoded penicillin-binding protein 3 (PBP3), a protein that is the target of these antibiotics. This type of resistance, termed β-lactamase-negative ampicillin-resistance (BLNAR), is difficult to detect in the diagnostic laboratory, as many BLNAR isolates do not actually show an ampicillin resistant phenotype using standard susceptibility testing methods. As a result genotypic testing methods are being increasingly adopted for BLNAR detection. Furthermore, the recent recognition of *Haemophilus haemolyticus,* a close non-pathogenic relative of *H. influenzae*, in diagnostic specimens from the respiratory tract, has compounded the issue. This is because *H. haemolyticus* isolates are frequently mis-identified as *H. influenzae*, which further complicates the role of the diagnostic laboratory in guiding antibiotic therapy for infections involving *H. influenzae*.

A working strain collection comprising a total of 393 *Haemophilus* isolates was established and used for all the subsequent studies conducted in this thesis. Isolates were taken from; 1) the University of Tasmania (UTAS) culture collection (n=44),

that had been previously collected from cases of respiratory illness, and 2) the Genetics of Recurrent Otitis Media and Immunology in Toddlers (GROMIT) Study conducted in Perth, Western Australia, where 349 XV-dependent *Haemophilus* isolates were cultured from paediatric patients suffering AOM with healthy aged-matched controls.

In Chapter 3, the SNP-based PCR algorithm of Hasegawa et al. (2003) that has been widely used to detect BLNAR mediated resistance was evaluated against the less commonly used PCR algorithm of Nakamura et al. (2009). Although, the primer set (PBP3-S) of Hasegawa et al. (2003), that is designed to amplify susceptible (N526) isolates was 100% sensitive and specific for detecting N526K-positive BLNAR isolates by non-amplification, the primer set (PBP3-BLN) designed to amplify N526K isolates was unreliable (sensitivity 84%; specificity 26%). Similar findings were reported for the sensitivity and specificity of the Nakamura et al. (2009) primers for detecting the N526K substitution in BLNAR isolates by amplification (sensitivity 96%; specificity 26%). The poor performance of these PCR primers was attributed to the fact that the N526K substitution can be encoded by an AAT-AAG codon change at base pair (bp) position 1576-1578 of *ftsI,* as well as the recently described AAT-AAA codon change. As the PCR primers investigated were designed for detection of the N526K substitution encoded by the AAG SNP only, they failed to detect N526K encoded by AAA. A search of *ftsI* gene sequences available on GenBank revealed that the AAT to AAG or AAA codon changes occurred with equal prevalence in N526K-positive BLNAR isolates, suggesting that the prevalence of BLNAR isolates would go under-reported when these PCR algorithms were used for BLNAR detection.

Little was known about the β-lactam resistance profile of *H. haemolyticus*, a respiratory commensal commonly mis-identified as *H. influenzae* in the diagnostic laboratory. As a result, Chapter 4 examined the phenotypic and genotypic characteristics of β-lactam resistance mechanisms in a large collection of *H. haemolyticus* and *H. influenzae* isolates, collected during the GROMIT study using a well defined patient population. The prevalence and mechanisms of β-lactam resistance were identified to be similar for both bacterial species, with 13.1% of *H. haemolyticus*, and 15.7% of *H. influenzae* isolates harbouring the TEM-1 β-lactamase (with the same replicon and promoter types commonly reported in *H. influenzae* identified in *H. haemolyticus*), whilst 31.0% of both *H. haemolyticus* and *H. influenzae* isolates were positive for the N526K BLNAR-defining substitution. Further analysis of the *ftsI* gene encoding PBP3 in these N526K-positive BLNAR isolates revealed that some of the commonly recognized BLNAR-associated substitutions in *H. influenzae* form part of the baseline PBP3 sequence in *H. haemolyticus*. This suggests that respiratory isolates of *H. haemolyticus* might possibly represent a significant reservoir for β-lactam resistance determinants in co-localised *H. influenzae*.

Examination of the *ftsI* gene sequences of the *H. haemolyticus* isolates from Chapter 4 revealed some differences that might interfere with the SNP-based PCR assays of Hasegawa et al. (2003) that were previously evaluated in Chapter 3 using only *H. influenzae* isolates. When evaluated against a panel of susceptible (N526) and resistant (N526K) isolates of *H. influenzae* and *H. haemolyticus* in Chapter 5, the primer set (PBP3-S) designed to amplify N526-positive (BLNAS) isolates performed well for the identification of susceptible *H. influenzae* isolates. However it failed to

amplify any *H. haemolyticus* isolates, irrespective of their N526/N526K status, due to a species-specific sequence variation in the forward primer-binding region. The discovery of this PCR limitation is significant, as these primers are frequently used in respiratory surveillance studies where *H. haemolyticus* is often mis-identified as *H. influenzae*, and will result in the mis-categorisation of susceptible *H. haemolyticus* isolates as low-BLNAR isolates of *H. influenzae.* A new PCR primer set was therefore developed to overcome this limitation and was 100% sensitive and specific for the separation of N526 isolates (by amplification) from N526K-positive isolates (which fail to amplify) of both species. This is an important new tool for the surveillance of the N526K-positive BLNAR genotype in XV-dependent *Haemophilus* species commonly encountered in the diagnostic laboratory.

Chapter 6 explores the main observation made in Chapter 4, that some of the BLNAR-associated substitutions reported in N526K-positive isolates of NTHi, appear to form part of the baseline PBP3 genotype in susceptible isolates of *H. haemolyticus*. The *ftsI* gene sequences from 100 clinical isolates, including susceptible (N526) and resistant (N526K) *H. influenzae* and *H. haemolyticus* isolates, were examined using a range of bioinformatic approaches for evidence of inter-species recombination events. Mosaic *ftsI* gene sequences were identified in 33% of the isolates tested and shown to represent inter-species recombination events. All recombination events occurred in N526K-positive isolates of either species and frequently resulted in the horizontal transfer of only partial *ftsI* gene fragments. There was no evidence to support the horizontal transfer of the entire *ftsI* gene among the clinical isolates *in vivo*.

Chapter 7 extended on the work of Chapter 6 using an *in vitro* approach. Transformation experiments, using reference recipients and fully characterised N526K-positive isolates of *H. influenzae* and *H. haemolyticus* as donors, were performed to investigate potential inter- and intra-species *ftsI* recombination events. Both inter- and intra-species recombination of the *ftsI* gene frequently occurred in *H. influenzae* and *H. haemolyticus* isolates, and resulted in the formation of mosaic *ftsI* genes that carry the N526K-positive resistance genotype.

In summary, the major findings of this thesis are that a widely used SNP-based PCR algorithm is unreliable for N526K-positive BLNAR detection because of a previously unrecognized SNP encoding the N526K substitution, and because of *ftsI* sequence divergence with *H. haemolyticus* that might mis-identify as *H. influenzae*. As a result, a new SNP-based PCR algorithm was developed and shown to be 100% sensitive and specific for detection of the N526K substitution. Additionally, this thesis presents for the first time the phenotypic and genotypic β-lactam susceptibility profiles of *H. haemolyticus* isolates, and highlights the potential role *H. haemolyticus* plays in the emergence and dissemination of β-lactam resistance determinants in *H. influenzae*. Finally, this thesis has characterised homologous (inter- and intra-species) recombination events involving the *ftsI* gene in both *in vivo* and *in vitro* models. Such *ftsI* recombination events were shown to occur frequently between *H. influenzae* and *H. haemolyticus* isolates, and frequently resulted in the formation of mosaic *ftsI* genes that contribute to the dissemination and diversification of the N526K-positive resistance mechanism in *Haemophilus* species.

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List of Abbreviations

aa	amino acid
AECB	Acute exacerbations of chronic bronchitis
aLRTIs	Acute lower respiratory tract infections
AMC	Amoxicillin-clavulanate
AMP	Ampicillin
AMOX	Amoxicillin
AOM	Acute otitis media
AST	Antimicrobial susceptibility testing
BLNAS	β-lactamase-negative ampicillin-susceptible
BLPAR	β-lactamase-positive ampicillin-resistant
BLNAR	β-lactamase-negative ampicillin-resistant
BLPACR	β-lactamase-positive amoxicillin-clavulanate-resistant
BSAC	British Society for Antimicrobial Chemotherapy
bp	base pair
CEC	Cefaclor
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic obstructive pulmonary disease
CTX	Cefotaxime
DD	Disc diffusion
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GROMIT	Genetics of Recurrent Otitis Media and Immunology in Toddlers
HGT	Horizontal gene transfer
Hib	*Haemophilus influenzae* type b
HTM	Haemophilus test medium
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
NTHi	Non-typeable *Haemophilus influenzae*
ORF	Open reading frame
PBP	Penicillin-binding protein
PBP3	Penicillin-binding protein 3
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PSG	Phosphate-buffered saline glucose
RTIs	Respiratory tract infections
sBHI	Supplemented brain heart infusion
SNP	Single nucleotide polymorphism
STs	Sequence types
URTI	Upper respiratory tract infection
U.S	United States
USSs	Uptake signal sequences