The bacteriology of bronchiectasis and impact of azithromycin on upper and lower airway bacteria and resistance in Australian Indigenous children

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Thesis submitted for the degree of
Doctor of Philosophy

March 2014

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DECLARATION

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to the ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

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ABSTRACT

Bronchiectasis unrelated to cystic fibrosis (CF) is prevalent in Australian Indigenous children. Early detection, knowledge of the bacteriology, and appropriate treatment are needed to prevent disease progression and early death in adulthood.

This thesis presents the first research on the upper and lower airway bacteriology of non-CF bronchiectasis in Australian Indigenous children, and the impact of a macrolide antibiotic (azithromycin). Gaps in methodology were addressed. Principal findings were:

1. The predominant bacterial pathogens causing lower airway infection were nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, as in other paediatric populations with non-CF bronchiectasis.

2. Nasopharyngeal (NP) swabs were more sensitive than oropharyngeal swabs for detecting upper airway carriage of these three pathogens.

3. Strain concordance of bacteria isolated from NP and lower airway specimens from children with lower airway infection was very high. This strongly supports the hypothesis that aspiration of NP secretions contributes to the pathology of non-CF bronchiectasis.

4. There was a differential effect of recent azithromycin use on NP carriage (reduced) and lower airway infection (not reduced, with the probable exception...
of *M. catarrhalis*). Both NP carriage of and lower airway infection by macrolide-resistant *S. pneumoniae* were significantly increased.

5. Long-term azithromycin use was associated with reduced NP carriage of the three main bacteria, but increased carriage of macrolide-resistant *S. pneumoniae* and *Staphylococcus aureus*, in a ‘cumulative dose-response’ relationship.

6. In a randomised controlled trial, similar long-term impacts on NP carriage were seen, and respiratory exacerbation frequency was significantly reduced, in the azithromycin compared to the placebo group. This demonstrated azithromycin’s effectiveness despite increased macrolide resistance.

These findings will inform management of children with non-CF bronchiectasis. Research on the impact of increased macrolide resistance in the community is recommended until a better understanding of azithromycin’s mode of action in reducing exacerbation frequency is reached.
There are many people who deserve acknowledgement for the work presented here. 
First and foremost, there would have been no Bronchoscopy Study, and I would not have embarked on PhD studies, without Professor Anne Chang. She convinced me that I could do this work, and provided the necessary support and encouragement, not to mention a great deal of time and energy, along the way. Her dedication to the health of children, particularly Indigenous children, is inspirational, and they are fortunate indeed to have someone of Anne’s calibre to promote their cause.

Sincere thanks to my supervisor Heidi Smith-Vaughan, who was the first port of call for all my queries and provided valuable guidance and enthusiasm over many years. I’m sorry I don’t share Heidi’s passion for the molecular side of things, but am grateful that she also likes to see the bugs on the bench. Thank you Heidi for the many hours you have put into reading drafts, and your prompt and helpful responses. We are fortunate indeed to have you leading the Child Health laboratory team.

Thank you to Amanda Leach who, together with Peter Morris, has headed the Ear Health research program at Menzies for over 20 years. Amanda’s knowledge of respiratory bacteria, antibiotics and vaccines has been invaluable also for the Respiratory Health program, and her insightful comments and support have been much appreciated. As a co-investigator for the Bronchiectasis studies, Peter has contributed valuable advice on statistics and as a co-author, and I am grateful for his support and assistance over many years. Allen Cheng has also been a wonderful resource for statistical questions, including modelling, and a great help in using Stata. Thank you, Allen, for your valued support and input as a co-author and supervisor.
Although not a formal supervisor, Keith Grimwood has been enormously helpful, putting in a huge amount of effort on the papers he co-authored. As a clinician and a microbiologist, his perspective and contributions are invaluable. Keith explained to me the U-shaped density curve separating contamination from infection, and that the oropharynx was the probable source of bacteria in the lower airways that I had not previously encountered in nasopharyngeal cultures.

Thank you to my fellow PhD students (and co-authors) Michael Binks and Susan Pizzutto for your support and being there to unload on as required. Michael, I greatly appreciate our discussions on the joys and pains of working with Stata, and the work we sweated on over weekends in our respective houses and gardens. Susan, thank you for also refusing to work most nights and weekends on your thesis, I feel much less guilty as a result.

I wish to thank two other co-authors for being especially supportive and positive. Paul Torzillo, another champion for Indigenous health, shared with me the ‘hitting a tennis ball at a target on a wall’ analogy for nasopharyngeal bacteria reaching the lung. Malcolm McDonald suggested we investigate *Staphylococcus aureus* as well as the three main respiratory bacteria, and amazed me with his pithy replies from far-flung reaches of the globe such as Cape York and Tanzania.

Thank you to the other members of the Child Health laboratory team who have helped me over many years. Jemima Beissbarth and I both started with the Ear Team in 2000, and it is wonderful to see Jemima back in the lab with her trademark humour and peerless organisational skills. Thank you to Vanya Hampton, my first
(and only) Research Assistant, who freed me from the bench so that I could work on publications. Vanya is a star, and I greatly appreciate her 4 years with us. Thank you also to Estelle Carter, Peter Christensen, Jana Lai, Joanna Bugg and Donna Woltring (who now fills Vanya’s shoes); and to our gap year students, firstly Nerida Liddle, and then Chris Wevill and Yuki Ruzsicska, for their hard work at the bench, youthful enthusiasm and smiley faces. Finally many thanks to Robyn Marsh who, together with Heidi, Peter and Michael, helped with the generation and interpretation of the molecular data. Robyn has also been a great help (and example to follow) with scholarship and fellowship applications, and thesis formatting.

Many other Menzies staff have assisted with our work, including Jo Bex our intrepid Laboratory Manager, and Sue Hutton who ably filled that role for many years. Thank you to Maria Scarlett for assistance with ethics applications, Robyn Liddle for her helpfulness and wonderful database skills, and members of the Research Administration team (Kalotina Halkitis, Kate McKay and Belinda Snell) for assistance with my grant, scholarship and fellowship applications respectively. Special thanks to Catherine Richardson and Caroline Walsh for their unfailing and cheerful support in the Academic Administration department.

I would especially like to thank the incomparable Gabrielle McCallum, project manager extraordinaire, and her team (Kobi Schutz, Clare McKay [née Wilson] and Lesley Versteegh, plus Emma Tilley and Stacey Svenson who worked in Central Australia) for all their assistance with enrolment of the children and the clinical and specimen collection aspects of the Bronchiectasis and Bronchoscopy studies. I envy Gabby her ability to remain smiling and helpful through thick and thin. Thank you to
Dr Paul Bauert and Anne Chang who performed the bronchoscopies at Royal Darwin Hospital, and the hospital staff who assisted. And thank you to many other members of the Menzies community for their support and friendship over the years.

To the children and their carers who participated in these studies, many sincere thanks. I know I didn’t meet them, but without them there would be no research. I hope they feel that they have benefited from taking part. I believe they would indeed have benefited from the extra attention and care provided.

Many thanks to my partner Caroline for her support, forbearance and helpful insights into the problems that invariably arose. I would also like to thank my parents for their care and support, and special thanks to my mother who taught me the value of ‘stickability’. Likewise thank you to my dear brother Winton and sister Merryn, and their families, who I am so glad to know and so lucky to have.

I would especially like to acknowledge the National Health and Medical Research Council of Australia for their financial support of this research through a Project Grant (2009-2011) and the Gustav Nossal (Dora Lush Biomedical) Postgraduate Scholarship (2012-2013). Thank you also to the Australian Academy of Science for the Douglas and Lola Douglas Scholarship in Medical Science (2012-2013).

Finally, many thanks to my three thesis examiners for their very helpful comments and corrections. I believe my PhD thesis has been improved as a result of their input.


3. Hare KM, Smith-Vaughan HC, Leach AJ. Viability of respiratory pathogens cultured from nasopharyngeal swabs stored for up to 12 years at -70°C in skim milk tryptone glucose glycerol broth. J Microbiol Methods 2011; 86(3):364-7 (Section 2.8).


STATEMENT OF CONTRIBUTIONS TO JOINTLY AUTHORED WORKS

This section lists author contributions to publications included in this PhD thesis.


Ms Hare was responsible for the concept, literature review and drafting of the manuscript. Drs Smith-Vaughan and Leach revised the manuscript and made helpful suggestions.


Drs Leach and Morris contributed to the study concept and design. Ms Hare, Ms Stubbs and Ms Beissbarth collected the data. Ms Hare and Drs Morris and Leach contributed to the analysis and interpretation of the data. Ms Hare and Dr Leach drafted the article and all authors contributed to its revision.

Ms Hare and Dr Leach contributed to the study concept and design and drafted the article. All authors contributed to data collection, analysis and interpretation of data, and to revision of the article.


Drs Leach and Morris were responsible for the study concept and design. Ms Hare collected the data and drafted the article. All authors contributed to the analysis and interpretation of the data and revision of the article.


Ms Hare was responsible for the study concept, performed the pneumococcal serotyping, and drafted the article. Dr Smith-Vaughan and Mr Binks contributed to the PCR testing using methods from Drs Park and Nahm. All authors contributed to the analysis and interpretation of the data and revision of the article.

Drs Grimwood, Chang, Torzillo and Leach were responsible for the study concept and design. Ms Hare collected the data and drafted the article. Drs Grimwood and Chang and Ms Hare contributed to analysis of the data. All authors contributed to interpretation of the data and revision of the article.


Ms Hare contributed to the study concept and design. Dr Smith-Vaughan and Mr Binks contributed to the methods development and interpretation of the PCR data. Ms Hare collected the culture data and Mr Binks collected the PCR data. Ms Hare and Drs Chang, Grimwood and Leach contributed to the analysis and interpretation of the data. Ms Hare drafted the article and all authors contributed to its revision.


Ms Hare and Dr Chang contributed to the study concept and design. Ms Hare collected the culture data, Dr Marsh collected the qPCR data and Ms Pizzutto collected the airway cellularity data. Dr Smith-Vaughan, Dr Marsh and Mr Binks contributed to the methods development and interpretation of the qPCR data. Ms
Hare and Drs Chang and Grimwood contributed to the analysis and interpretation of the data. Ms Hare drafted the article and all authors contributed to its revision.


Drs Grimwood, Chang, Bauert and Leach were responsible for the study concept and design. Ms Hare collected the data and drafted the article. Drs Grimwood, Cheng, Morris and Chang and Ms Hare contributed to analysis of the data. All authors contributed to interpretation of the data and revision of the article.


Drs Singleton, Grimwood, Morris and Chang were responsible for the study concept and design. Ms McCallum, Ms Reasonover, Ms Chikoyak and Ms Hare collected the data. Ms Hare and Drs Singleton, Grimwood, Chang, Valery and Leach drafted the article. Drs Grimwood, Cheng, Morris and Chang, Mr Chatwood and Ms Hare contributed to analysis of the data. All authors contributed to interpretation of the data and revision of the manuscript.
PRESENTATIONS

This section lists conference presentations made relevant to my PhD thesis.


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### Abbreviations

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<tr>
<td>AmpR</td>
<td>Ampicillin resistant</td>
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<tr>
<td>ATP</td>
<td>According-to-protocol</td>
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<tr>
<td>Azi(I)R</td>
<td>Azithromycin (intermediate) resistant</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BIS</td>
<td>Bronchiectasis Intervention Study</td>
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<tr>
<td>BL</td>
<td>Beta-lactamase</td>
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<tr>
<td>BLNAR</td>
<td>Beta-lactamase negative ampicillin-resistant</td>
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<td>Bnpos</td>
<td>Beta-lactamase positive</td>
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<tr>
<td>BOS</td>
<td>Bronchiectasis Observational Study</td>
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<tr>
<td>CDS</td>
<td>Calibrated disc sensitivity</td>
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<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
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<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive lung disease</td>
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<tr>
<td>CSLD</td>
<td>Chronic suppurative lung disease</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>erm</td>
<td>erythromycin ribosomal methylation</td>
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<tr>
<td>EryR</td>
<td>Erythromycin resistant</td>
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<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<tr>
<td>Hi(b)</td>
<td><em>Haemophilus influenzae</em> (type b)</td>
</tr>
<tr>
<td>(c)HRCT</td>
<td>(chest) High resolution computerised tomography</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research Ethics Committee</td>
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<tr>
<td>HRM</td>
<td>High resolution melt</td>
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PenNS  Penicillin nonsusceptible
PBB  Protracted bacterial bronchitis
PBP  Penicillin-binding protein
(q)PCR  (quantitative) Polymerase chain reaction
PCV(7,13)  Pneumococcal conjugate vaccine (7-valent, 13-valent)
PFGE  Pulsed-field gel electrophoresis
PHiD-CV  Pneumococcal *Haemophilus influenzae* protein D conjugate vaccine
PK/PD  Pharmacokinetic / pharmacodynamic
PNG  Papua New Guinea
PPV  Positive predictive value
PPV23  23-valent pneumococcal polysaccharide vaccine
rs  Spearman's rank correlation coefficient
RCH  Royal Children’s Hospital
RCT  Randomised controlled trial
RDH  Royal Darwin Hospital
RD  Risk difference
Sens  Susceptible to penicillin and azithromycin
Spn  *Streptococcus pneumoniae*
SSI  Statens Serum Institute (Denmark)
STGGB  Skim milk tryptone glucose glycerol broth
TCC  Total cell count
URT  Upper respiratory tract
WHO  World Health Organization
CHAPTER 1

Introduction and Literature Review
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Chapter overview

This review outlines the condition bronchiectasis, its burden of disease in Australian Indigenous children, bacterial aetiology and the possible role of microaspiration in disease initiation and progression. It refers to a more comprehensive review of the bacteriology of lower respiratory infections included in the Appendix (Hare, Smith-Vaughan & Leach 2010). This review then provides a brief summary of immune responses and the role of vaccines. It describes antibiotic treatment, particularly with the macrolide antibiotic azithromycin, and the problem of antimicrobial resistance. The review concludes with a summary of the research hypotheses and aims addressed by this thesis.

1.2 Overview of bronchiectasis

Bronchiectasis is an irreversible abnormal dilatation of one or more of the bronchial airways (Chang et al. 2008; Cole 1986). Extra mucus tends to form and pool in the affected areas, rendering them prone to infection and chronic inflammation (Cole 1986). Diagnosis of bronchiectasis requires a chest high resolution computerised tomography (HRCT) scan. The term chronic suppurative lung disease (CSLD) is used to describe a diagnosis where there are clinical symptoms of bronchiectasis without HRCT evidence (Chang, Redding & Everard 2008). Untreated CSLD may progress to bronchiectasis as a result of cumulative airway injury from recurrent or persistent bacterial infection (Chang et al. 2008). In its severest form bronchiectasis can progress to end stage pulmonary failure in adult life (Stafler & Carr 2010).
1.3 Burden of disease in Indigenous children

Indigenous children in the Northern Territory (NT) of Australia have a much higher burden of disease than non-Indigenous children (Dempsey & Condon 1999). This burden includes respiratory diseases such as CSLD or bronchiectasis. In Central Australia, radiologically-confirmed bronchiectasis is present in almost 1.5% of Indigenous children aged <15 years (147 per 10 000), one of the highest recorded rates in the world (Chang et al. 2003). This is 40-fold greater than the rate for cystic fibrosis (CF, 35 per 100 000) in Australian non-Indigenous children (Massie et al. 2000). CF is considered the most important cause of clinically significant bronchiectasis in developed countries, since non-CF bronchiectasis has declined due to improved hygiene and nutrition, better vaccine coverage and early institution of antibiotic therapy (Kapur & Karadag 2011). However, with estimated prevalences of 1 in 6000 in New Zealand (Edwards, Asher & Byrnes 2003) and 1 in 5800 in Britain (Eastham et al. 2004), paediatric non-CF bronchiectasis is only slightly less common than CF bronchiectasis, even in developed countries.

The management of CF bronchiectasis aims to prevent disease progression by treating infective respiratory episodes, optimising growth and development, normalising lung function and, if possible, halting disease progression. Until recently, there were no parallel concerted programs or dedicated resources to manage children with non-CF bronchiectasis (mostly Indigenous children in Australia) (Chang et al. 2002). These children have significant morbidity and some succumb to premature death in adulthood (Loebinger et al. 2009). Diagnosis may be delayed for years or even decades, and many likely remain undiagnosed (Chang et al. 2010). Non-CF bronchiectasis is now relatively uncommon in developed countries, but
persists in developing countries and other disadvantaged populations (Karadag et al. 2005). These differences are partly attributed to overcrowding, poor nutrition and living standards, and poor access to health services (Chang et al. 2002).

1.4 The microbiology of bronchiectasis

The high burden of respiratory disease in Australian Indigenous children is associated with infections caused by bacterial pathogens. Of these, three bacteria dominate: *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis*. While these bacteria are commonly carried in the nasopharynx of all children, Indigenous children are colonised concurrently with multiple species from a few weeks of age (Leach et al. 1994), have higher carriage rates throughout childhood (Stubbs et al. 2005), and frequently carry multiple species and strains (Hare et al. 2008; Smith-Vaughan et al. 1996) at a higher bacterial load (Smith-Vaughan et al. 2006). In a longitudinal birth cohort study, early nasopharyngeal (NP) colonisation with respiratory bacterial pathogens predicted onset of otitis media (OM); those infants with NP colonisation progressed to OM at a rate of 16% per day compared to 2% per day in non-colonised infants (Leach et al. 1994). Other studies have also found that NP colonisation is a likely precursor to upper respiratory tract infections such as OM (Gray, Converse & Dillon 1980; Soininen et al. 2002) and is associated with lower respiratory tract infections such as pneumonia (Wolf & Daley 2007).

Prior to commencement of this thesis, no microbiological studies of NP colonisation had been carried out in children with non-CF bronchiectasis, although some authors (Kapur, Masters & Chang 2009) have postulated that NP bacteria are also likely
important in these children. One of the main aims of this thesis was therefore to
determine the upper and lower airway bacteriology in children with non-CF bronchiectasis (Chapter 3).

The significance of *H. influenzae* in bronchiectasis of children was noted more than
50 years ago (Allibone, Allison & Zinnemann 1956). These authors concluded that
NTHi “is responsible for keeping the chronic inflammatory process smouldering in
bronchiectatic individuals”. The main bacteria associated with non-CF bronchiectasis
in six more recent studies in children (Eastham et al. 2004; Edwards, Asher &
Byrnes 2003; Hare, Grimwood et al. 2010; Kapur, Masters & Chang 2009; Karadag
et al. 2005; Li et al. 2005), including our study reported in Chapter 3, were reviewed
and are listed in Table 1.1 (Hare, Smith-Vaughan & Leach 2010). All studies prior to
commencement of my thesis were however retrospective, with no data on concurrent
use of antibiotics when specimens were collected. Nevertheless, *H. influenzae* was
the most common pathogen identified in all six studies (specified as NTHi in two),
followed by *S. pneumoniae* and (in varying order of frequency) *M. catarrhalis,*
*Pseudomonas* species and *Staphylococcus aureus* (Hare, Smith-Vaughan & Leach
2010). Co-infection with more than one pathogen was reported in three studies.
Figure 1.1 shows the perceived relationship (based on the evidence reviewed)
between acute and chronic respiratory diseases and the bacteria associated with them
(Hare, Smith-Vaughan & Leach 2010).

*Pseudomonas aeruginosa* is one of the three main pathogens (together with *S. aureus*
and *H. influenzae*) in paediatric and adult CF patients with bronchiectasis (Govan &
Nelson 1992). It is also common in adults with non-CF bronchiectasis (King et al.
2007; Steinfort et al. 2008). However *P. aeruginosa* and *S. aureus* are relatively uncommon in paediatric non-CF bronchiectasis, especially in young children (Grimwood 2011; Hare, Smith-Vaughan & Leach 2010; Kapur et al. 2011). Several other bacteria have been implicated in the pathobiology of non-CF bronchiectasis in small numbers, namely *Haemophilus parainfluenzae* (Eastham et al. 2004), *Streptococcus pyogenes* (Edwards, Asher & Byrnes 2003) and *Klebsiella pneumoniae* (Karadag et al. 2005). Non-tuberculous mycobacteria (NTM) are commonly found in adult CF and non-CF bronchiectasis (Masekela & Green 2012; Mirsaedi et al. 2013) and paediatric CF patients (Radhakrishnan et al. 2009), but rarely found in paediatric non-CF bronchiectasis (Kapur et al. 2011).

![Figure 1.1. Relationship between acute and chronic lower respiratory diseases and associated pathogens: *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.](image)

From Hare, Smith-Vaughan & Leach (2010)

COPD = chronic obstructive pulmonary disease; CSLD = chronic suppurative lung disease
Table 1.1. Studies reporting lower airway bacteriology in children with non-cystic fibrosis bronchiectasis.

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Population</th>
<th>Age (years)</th>
<th>No.</th>
<th>Specimen</th>
<th>Haemophilus influenzae</th>
<th>Streptococcus pneumoniae</th>
<th>Moraxella catarrhalis</th>
<th>Pseudomonas sp.</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards</td>
<td>2003</td>
<td>New Zealand*</td>
<td>1 to 17 (median 10)</td>
<td>60</td>
<td>Sputum</td>
<td>68% (NTHi)</td>
<td>12%</td>
<td>6%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>Karadag</td>
<td>2005</td>
<td>Turkey</td>
<td>1 to 17.5 (mean 7.4)</td>
<td>111</td>
<td>Sputum</td>
<td>23% (type not specified)</td>
<td>14%</td>
<td>4%</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>Li</td>
<td>2005</td>
<td>England</td>
<td>3.1 to 18.1 (median 12.1)</td>
<td>136</td>
<td>Cough swab, sputum or BAL</td>
<td>39% (type not specified)</td>
<td>17%</td>
<td>2%</td>
<td>11%</td>
<td>4%</td>
</tr>
<tr>
<td>Eastham</td>
<td>2007</td>
<td>England</td>
<td>1.6 to 18.8 (median 7.2)</td>
<td>93</td>
<td>Cough swab, sputum or BAL</td>
<td>48% (type not specified)</td>
<td>22%</td>
<td>17%</td>
<td>6%</td>
<td>8%</td>
</tr>
<tr>
<td>Kapur</td>
<td>2009</td>
<td>Australia</td>
<td>3 to 17 (median 5.5)</td>
<td>30</td>
<td>Sputum and BAL (85 specimens)</td>
<td>32% (type not specified)</td>
<td>15%</td>
<td>8%</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>Hare</td>
<td>2010</td>
<td>Australia (Indigenous children)</td>
<td>0.7 to 10.1 (median 2.3)</td>
<td>45</td>
<td>BAL</td>
<td>78% any growth (NTHi)</td>
<td>33% any growth</td>
<td>27% any growth</td>
<td>4% any growth</td>
<td>9% any growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>47% &gt;10⁴ CFU/ml</td>
<td>18% &gt;10⁴ CFU/ml</td>
<td>20% &gt;10⁴ CFU/ml</td>
<td>0% &gt;10⁴ CFU/ml</td>
<td></td>
</tr>
</tbody>
</table>

*80% Pacific Islander or Maori descent
CF = cystic fibrosis; NTHi = nontypeable *Haemophilus influenzae*; BAL = bronchoalveolar lavage fluid; CFU = colony-forming units
All percentages are based on number of specimens

From Hare, Smith-Vaughan & Leach (2010)
The role of viral infections in bronchiectasis is not well defined (Grimwood 2011; King 2009). Adenoviruses may be a major cause of post-infectious bronchiectasis in childhood (Becroft 1971) and are the most commonly reported (Alharbi et al. 2012; Diaz et al. 1999; Edwards, Asher & Byrnes 2003; Hogg et al. 1989; Kapur et al. 2011). Increased nasopharyngeal NTHi density has been shown in the presence of any one of several respiratory viruses in Indigenous children with acute OM (Binks et al. 2011). Bronchiectasis is associated with human T-cell lymphotropic virus type 1 infection in Jamaican children (La Grenade 1996) and Indigenous Australian adults (Einsiedel et al. 2012). Respiratory syncytial virus (Diaz et al. 1999; Edwards, Asher & Byrnes 2003), human immunodeficiency virus (Berman et al. 2007; Jeena et al. 1998; Masekela et al. 2012), influenza (Kapur et al. 2011; Laraya-Cuasay et al. 1977) and parainfluenza (Eastham et al. 2004; Kapur et al. 2011) have also been detected in children with bronchiectasis. Respiratory viral infections often lead to bacterial superinfection by mechanisms including the promotion of bacterial adhesion to respiratory epithelial cells (Peltola & McCullers 2004). It is therefore feasible that viral infections may contribute to the initiation of bronchiectasis, and possibly subsequent exacerbations, in susceptible hosts.

Fungal pathogens such as *Aspergillus fumigatus* (Edwards, Asher & Byrnes 2003) and *Candida albicans* (Chipps et al. 1979; Li et al. 2005) are occasionally associated with paediatric non-CF bronchiectasis, particularly in patients with immuno-deficiency.

Bacterial biofilms have long been linked to persistent infections (Costerton, Stewart & Greenberg 1999). Biofilm formation has been demonstrated for *H. influenzae*
(Cardines et al. 2012; Starner et al. 2006), *S. aureus* (Molina et al. 2008) and *P. aeruginosa* (Lutz et al. 2012; Murray Egan & Kazmierczak 2007) from CF patients; for *H. influenzae* in the sputum of patients with chronic obstructive pulmonary disease (COPD) (Murphy & Kirkham 2002); and for *S. pneumoniae* (Allegrucci et al. 2006), NTHi and *M. catarrhalis* (Pearson et al. 2006) in chronic and recurrent OM (Murphy, Bakaletz & Smeesters 2009). While many studies were performed *in vitro* or using animal models, biofilm formation has been demonstrated on the middle ear mucosa of children with chronic OM (Hall-Stoodley et al. 2006), from patients with chronic suppurative OM (smears from children and biopsies from adults) (Homoe et al. 2009), and in middle ear effusion from children with recurrent acute OM (Thornton et al. 2013). It is likely that biofilms also form in the lower airways of children with non-CF bronchiectasis. Bacteria in biofilm formations are protected from phagocytosis and are more resistant to antimicrobials (Starner et al. 2006), therefore biofilm infections are harder to treat. This could explain the persistence of NTHi in COPD (Murphy et al. 2004), chronic OM, CF bronchiectasis and (theoretically) non-CF bronchiectasis.

### 1.5 Microaspiration of upper airway secretions

The classic definition of bronchiectasis is of permanent bronchial dilatation; however, at the milder end of the spectrum it appears that radiographic changes may be reversible (Stafler & Carr 2010). Management of bronchiectasis should therefore aim for early detection and aggressive treatment (Chang et al. 2002). Known risk factors for bronchiectasis in Indigenous children include severe and recurrent pneumonia (Valery et al. 2004), and previous pneumonia has also been reported as the most common association in non-Indigenous children (Eastham et al. 2004).
However, the initial pathogenetic mechanisms leading to bronchiectasis are unknown. One possibility is recurrent microaspiration into the lower airways of pathogenic bacteria originating from the nasopharynx (Ben-David et al. 2005; Bogaert, de Groot & Hermans 2004; Thach 2008). It is therefore of relevance (as outlined in section 1.4) that Indigenous infants have high and early rates of NP colonisation by potentially pathogenic respiratory bacteria, have higher bacterial loads and carry a greater abundance and diversity of species and strains than found in other children. It is possible that during acute respiratory infections, repeated aspiration of relatively large inocula of bacteria-laden NP secretions may overwhelm local pulmonary defences, helping to initiate endobronchial infection, inflammation and airway injury. This tenet is central to Cole’s ‘vicious circle’ hypothesis for the origins of bronchiectasis (Cole 1986). Figure 1.2, adapted from an OM model (Wiertsema & Leach 2009) which was in turn adapted from Cole’s original model (Cole 1986), shows an ‘extended vicious circle’ hypothesis to explain high rates of respiratory infection among Australian Indigenous children (Hare, Smith-Vaughan & Leach 2010).

It has long been hypothesised that bacterial pneumonias result from entry into the lung of bacteria from the upper respiratory tract (URT), possibly by aspiration of fluid from the pharynx, and possibly also associated with impairment of pulmonary clearance mechanisms (Johanson, Pierce & Sanford 1969). Although few studies have compared bacterial cultures from upper and lower airway specimens from pneumonia patients, especially children, numerous studies have been conducted in CF patients. However, no studies have compared upper and lower airway
bacteriology in patients with non-CF bronchiectasis to determine if aspiration is a possible aetiological mechanism for this condition.

Figure 1.2. Extended vicious circle hypothesis explaining high rates of respiratory infection in Australian Indigenous children.

From Hare, Smith-Vaughan & Leach (2010), adapted from Wiertsema & Leach (2009)

1.6 Comparison of upper and lower airway bacteria

1.6.1 Controlling for contamination

Contamination with upper airway microflora is a potential problem when lower airway specimens are obtained for bacterial culture (Loens et al. 2009). Sputum is the lower airway specimen most often collected in adults. Since sputum may be contaminated with saliva, most studies employ a washing step. In a study of 19 adult CF patients, sputum and mouthwash samples were compared using a molecular approach to include species (e.g. anaerobic bacteria) not detected using traditional
culture methods (Rogers et al. 2006). After washing to remove adherent saliva, sputum samples were not found to be subject to profound contamination by oral cavity bacteria; rather, highly diverse but different bacterial communities were identified from the oral and lung environments (Rogers et al. 2006). However, about one species in 10 from the CF lung matched bacteria in the oral cavity, which may act as a “stepping stone” for lung colonisation and infection (Rogers et al. 2006).

Although sputum is most often collected in adults, it is difficult to collect sputum from young children as they are generally unable to expectorate. Therefore several studies have employed flexible bronchoscopy in anaesthetised children to provide bronchoalveolar lavage (BAL) for culture to detect bacteria colonising the lower airways. Despite careful technique, contamination may occur as the tube passes through the nasopharynx (when bronchoscopy is performed transnasally) and oropharynx. Bacteria present in low numbers in BAL fluid due to upper airway contamination can be excluded by quantitative culture. In a study of 67 adult patients with a clinical diagnosis of lower respiratory tract infection, and 8 healthy controls, quantitative bacterial culture was found to be very specific (100%) using a threshold value for positive culture of $10^4$ colony forming units (CFU)/mL, but was positive in only 36% of patients (Rasmussen et al. 2001).

### 1.6.2 Species concordance

Only one study was found comparing upper and lower airway bacteria from children with pneumonia. In a prospective study of 25 consecutive paediatric patients with pneumonia, bacterial culture results from nasal lavage (NL) were compared with BAL fluid (Wang, Clement & Lauwers 1994). Potential bacterial pathogens were
isolated from 96% of NLs and 56% of BAL fluids, with the same pathogens found in both specimens from 13 (52%) children. The authors concluded that nasal bacteriological findings were poor predictors of BAL bacteriology, but acknowledged the accepted logic that bacterial pathogens from the URT spread to the lower airways (Wang, Clement & Lauwers 1994). In this study sensitivity of NL cultures for lower airway bacteriology, and concordance of pathogens, were high; however the positive predictive value (PPV) was low due to the high NL positivity rate. This study did not use quantitative culture to exclude possible contamination from upper airway bacteria in BAL cultures.

A more recent study of BAL microbiology in 123 children with recurrent community-acquired pneumonia used a significance cutoff point of ≥10⁴ CFU/mL (De Schutter et al. 2011). Although this study did not use upper airway cultures for comparison, it is the first study to report a predominance of NTHi in children with pneumonia since the 1980s (Shann et al. 1984). The authors chose a cutoff value of ≥10⁴ CFU/mL, despite a value of ≥10³ CFU/mL being used in some adult studies (Loens et al. 2009), because colonisation of the URT is more common in children (De Schutter et al. 2011).

Most studies of URT specimens from CF patients collect oropharyngeal (OP) rather than NP specimens. One study compared OP swabs and NP suction in 47 young CF patients and found no significant difference in the rate of positive cultures for the main CF pathogens (S. aureus, P. aeruginosa or H. influenzae) (Taylor et al. 2006). The authors concluded that NP sampling is thus not routinely warranted for CF; however, NP specimens yielded more positive cultures of S. pneumoniae and M.
catarrhalis (Taylor et al. 2006), which are important pathogens in non-CF bronchiectasis.

The accuracy of OP cultures in detecting lower respiratory pathogens in infants and young children with CF has been controversial, with different studies reaching somewhat different conclusions (Rosenfeld et al. 1999). Ramsey et al. (1991) found that positive OP cultures were highly predictive for positive bronchial cultures (83% for P. aeruginosa and 91% for S. aureus) in 43 CF patients, while predictive values of negative OP cultures were lower. However in this study, quantification of lower airway bacteria was not performed to adjust for upper airway contamination. Armstrong et al. (1996) measured bacterial counts and inflammatory cell (neutrophil) and IL-8 concentrations in BAL fluid to determine a diagnostic threshold for lower airway infection in young CF patients of $\geq 10^5$ CFU/mL. In 75 children with OP and BAL specimens collected, sensitivities of OP cultures for lower respiratory tract infections with S. aureus, H. influenzae and P. aeruginosa were 86%, 92% and 71% respectively, and specificities were 61%, 92% and 93% respectively. While negative predictive values (NPVs) were high ($\geq 95$%), PPVs were low (33%, 50% and 57% respectively) and the authors concluded that OP cultures do not reliably predict the presence of bacterial pathogens in the lower airways of young CF patients (Armstrong et al. 1996).

By combining data from three independent, prospective studies, Rosenfeld et al. (1999) concluded that the specificity and NPV of OP cultures are high, but sensitivity and PPV are poor. These authors also noted, however, that predictive values are affected by prevalence, and age affects the prevalence of both upper
airway carriage and lower airway infection (Rosenfeld et al. 1999). Another factor to consider is antibiotic use, since oral antibiotics reduced OP carriage of \( S. \text{aureus} \) and \( H. \text{influenzae} \) but did not appear to alter BAL culture results (Armstrong et al. 1996). Conclusions based on predictive values should therefore be limited to populations with similar prevalences of organisms, and should also take into account antibiotic prescribing.

Two more recent studies in adults and children emphasised the importance of upper airway cultures for CF patients. A study of 157 children with CF found 44% of upper airway (nasal endoscopy) and 80% of lower airway (sputum) specimens positive for bacteria, mostly \( P. \text{aeruginosa}, S. \text{aureus} \) and \( H. \text{influenzae} \) (Bonestroo et al. 2010). However, in 65% of patients with positive upper airway cultures and negative lower airway cultures for \( P. \text{aeruginosa} \), the lower airway culture became \( P. \text{aeruginosa} \) positive within the next 3 months (Bonestroo et al. 2010). In 104 adult patients with CF, \( P. \text{aeruginosa} \) was by far the most frequently isolated bacteria from upper airway cultures (48% positive) and 37% of lower airway cultures were concurrently positive; however, \( P. \text{aeruginosa} \) was cultured from the upper airways of three patients after successful eradication from the lower airways (Berkhout et al. 2013). These studies further support the possible role of the upper airways as a reservoir for pathogens that may colonise or infect the lower airways.

1.6.3 Strain concordance

If aspiration of upper airway secretions is important in establishing or maintaining lower airway infection, the bacterial flora of the upper and lower airways should not only share the same species, but also the same strains. Jung et al. (2002) used pulsed-
field gel electrophoresis (PFGE) to type *P. aeruginosa* isolates from OP, sputum and BAL specimens obtained from 38 stable CF patients ≥5 years of age. While strain concordance with BAL isolates was good for both OP specimens (8/9) and sputum (10/10) when *P. aeruginosa* was detected, the sensitivity of OP specimens was only 35% (9 positive of 26 matching positive BALs) compared to 92% (11 of 12 positive) for sputum. The authors concluded that sputum and BAL specimens were of equal value in detecting *P. aeruginosa*, while OP specimens were not suitable (Jung et al. 2002). Armstrong et al. (1996) also used PFGE to type 10 randomly selected colonies each of *S. aureus*, *H. influenzae* and *P. aeruginosa* from paired OP and BAL cultures from 9 children, and found strain concordance in 6/9, 6/9 and 8/9 respectively.

Another study by Mainz et al. (2009) used genotyping to compare NL and sputum strains from 187 CF patients (median age 17 years). Good concordance was found for both *S. aureus* (31 of 36 patients had identical *spa* types) and *P. aeruginosa* (23 of 24 patients had identical SNP-genotypes) when both anatomical sites were positive. While the sensitivity of NL for detection of lower airway pathogens was only 43% (28 positive of 65 positive sputums) for *P. aeruginosa* and 64% (41 positive of 64 positive sputums) for *S. aureus*, the genotype concordance suggests that the upper airways act as a reservoir for *P. aeruginosa* and *S. aureus* in CF (Mainz et al. 2009), supporting the conclusion of previous authors.

**1.6.4 Summary of section 1.6**

Published studies summarised above have used various URT specimens to predict lower airway pathogens in pneumonia and CF patients. Although sensitivity and PPV
tend to be low, specificity and NPV, as well as strain concordance, are generally high. It is highly likely but unknown if the data above are relevant to children with non-CF bronchiectasis. This is a clinical gap that is addressed in my thesis.

### 1.7 Gaps in methodology

In our Bronchoscopy Study we compared the sensitivity of NP and OP swabs for the detection of upper airway carriage of *S. pneumoniae, H. influenzae, M. catarrhalis* and *S. aureus* (*Pseudomonas* was rarely detected) in Indigenous children with non-CF bronchiectasis (Chapter 2). As children in our study were too young to expectorate sputum, BAL fluid obtained during bronchoscopy (scheduled by treating paediatricians) was used for the detection of lower airway pathogens. To exclude URT contamination, quantitative culture of BAL fluid was performed and a threshold of >$10^4$ CFU/mL was used to define lower airway infection.

NP swabs were found to be preferable to OP swabs for the detection of upper airway carriage of the main non-CF bronchiectasis pathogens (Chapter 2). There were too few Indigenous children in the Bronchoscopy Study who did not have bronchiectasis to enable a meaningful comparison, therefore we compared the bacterial flora in NP swabs and BAL fluid from Indigenous children with bronchiectasis to a group of non-Indigenous children without bronchiectasis to determine the species and strain concordance between upper and lower airway bacteria in both groups (Chapter 3).

Other gaps in methodology concern specimen collection in field studies (such as our Multicentre Bronchiectasis Studies described in Chapter 4), transport of specimens to the laboratory, and recovery of bacterial pathogens from specimens following long-
term ultra-freeze storage. Studies addressing these gaps are described in Chapter 2. Furthermore, many studies select and type multiple colonies to detect multiple strains. This could be for the purpose of detecting multiple carriage (e.g. serotype studies following pneumococcal vaccination) or to determine strain concordance between upper and lower airway cultures (e.g. a study summarised in section 1.6.3). However, there was previously no standardised method for colony selection. New molecular methods are also being developed to identify an increasing number of pneumococcal serotypes. These methods are discussed by Satzke et al. (2013); however, the Quellung reaction used in our laboratory remains the gold standard. Studies addressing these issues are also described in Chapter 2.

1.8 Immune responses

A comprehensive review of the immunology of non-CF bronchiectasis is beyond the scope of this literature review as immune responses are not the subject of this thesis. Briefly, host pulmonary defences mounted in response to microbial challenges include physical mechanisms (cough and mucociliary clearance) and barriers (epithelial cells, mucus and cilia), and innate and adaptive immune responses (Grimwood 2011). The interaction between microbes and the innate immune system results in opsonisation of the microbes and immediate release of multiple pro-inflammatory cytokines and chemokines, and neutrophils are recruited into the airways (Grimwood 2011). The highly regulated adaptive immune system is slower to respond and involves both T- and B-lymphocytes; B-cells generate antibodies while T-cells determine the nature of the immunological response, either Th1 responses that promote killing of intracellular pathogens, or Th2 responses which destroy extracellular pathogens (Grimwood 2011).
Disorders associated with bronchiectasis have in common an impaired clearance of airway secretions including pulmonary pathogens. There are many causes of bronchiectasis including primary immunodeficiency disorders, therefore laboratory investigations for these in children with bronchiectasis are considered essential (Li et al. 2005). Regulation of the inflammatory response within bronchiectatic airways is poorly understood, but inflammation appears to be excessive and persistent, contributing to progressive and irreversible bronchial wall damage (Grimwood 2011). King et al. (2003) hypothesised that recurrent airway infection with NTHi may be associated with nonclearing adaptive immunity. They found that bronchiectasis patients with chronic NTHi infection mount an ineffective Th2 antibody response against this pathogen, which can reside within macrophages and airway epithelial cells (King et al. 2003). Thus persistent infection and ongoing inflammation are likely important in the pathogenesis and eventual irreversibility of this disease.

While this thesis does not include immunology, studies of immune responses in non-CF bronchiectasis are being undertaken within our research group. A comparison between quantitative PCR and semi-quantitative culture for the definition of *H. influenzae* lower airway infection, and correlation of both measures with total and differential cell counts as measures of the airway inflammatory response, was made (described in Chapter 3) as there were previously no such data in the literature.
1.9 Prevention of respiratory infections by vaccination

1.9.1 *Streptococcus pneumoniae*

Development of vaccines to prevent or reduce the severity of respiratory infections initially targeted adults. Attempts to prevent pneumococcal pneumonia by the use of whole cell vaccines began in the South African mines in 1911 (Douglas & Riley 1979). Subsequent vaccines were developed using capsular polysaccharides which are related to the invasiveness of any particular serotype. *S. pneumoniae* has more than 90 different serotypes, many of which cause invasive pneumococcal disease (IPD) and pneumonia, but only a limited number are included in current vaccines. The 23-valent pneumococcal polysaccharide vaccine (PPV23, Pneumovax®, Merck, USA) was introduced in 1983 (Austrian 1999). Polysaccharide vaccines substantially increase host resistance to serious pneumococcal infection in adults and older children and reduced the incidence of pneumonia in high risk populations; however the antibody response is less satisfactory between 6 months and 2 years of age, and poor in infants under 6 months (Douglas & Riley 1979).

To stimulate an effective antibody response in young children, pneumococcal polysaccharides were conjugated to a carrier protein to produce conjugate vaccines which are immunogenic in all age groups (Wuorimaa & Kayhty 2002). Conjugate vaccines have had a major impact on targeted diseases in countries where the vaccines have been introduced. The 7-valent pneumococcal conjugate vaccine (PCV7, Prevenar®, Pfizer, USA) which includes serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, the so-called “paediatric types”, was licensed in the United States in 2000 (Black et al. 2000). Incidences of IPD and pneumonia caused by PCV7 serotypes have been substantially reduced (Whitney et al. 2003). PCV7 was introduced in
Australia in July 2001 for Indigenous and other high risk infants, and IPD caused by vaccine serotypes decreased between 2002 and 2006 by 78% in children aged under two years (Roche et al. 2008). Paediatric hospitalisations for pneumonia in Australia have also decreased substantially (by 22%) in the 5 years following national PCV7 introduction in January 2005; however, there has been a small (absolute) increase in empyema (Strachan, Snelling & Jaffe 2013).

Many pneumococcal serotypes causing a large burden of disease in some parts of the world, particularly developing countries, are not included in PCV7, e.g. serotype 1 (Antonio et al. 2008). Additionally, in countries where universal PCV7-vaccination has been introduced, there has been replacement with non-vaccine serotypes (NVTs) such as 19A which became a major cause of IPD (Pai et al. 2005). New conjugate vaccines on the market provide additional protection. The 10-valent pneumococcal nontypeable *H. influenzae* (NTHi) protein D conjugate vaccine, PHiD-CV (Synflorix®, GlaxoSmithKline, Belgium), was licensed in the European Union in 2009 (Palmu et al. 2013). PHiD-CV provides protection against three additional serotypes (1, 5 and 7F) and its effectiveness against IPD caused by vaccine serotypes has been demonstrated in a clinical trial (Palmu et al. 2013).

A 13-valent conjugate vaccine (PCV13, Prevenar13®, Pfizer, USA) has the same 10 serotypes as PHiD-CV plus an additional three (3, 6A and 19A), but does not have the NTHi component. Compared to PCV7, PCV13 resulted in lower acquisition and prevalence of NP colonisation for PCV13 serotypes 1, 6A, 7F and 19A, as well as serotypes 6C and 19F, and was comparable for all other common serotypes (Dagan et al. 2013). From these findings, the authors predicted vaccine effectiveness through
both direct and indirect protection. PCV13 was introduced in 2010 in the United States, and early results show that invasive pneumococcal infections in children, including culture-confirmed pneumonia, have further decreased compared with cases from 2007 to 2009; this included >50% reductions in IPD caused by serotypes 19A, 7F and 3 (Kaplan et al. 2013).

While PCVs are part of the routine infant immunisation schedule in many countries, current recommendations for additional pneumococcal vaccination with PPV23 include children and adults with CSLD (Chang et al. 2009). Current but limited evidence supports the use of PPV23 as routine management in adults with bronchiectasis, while circumstantial evidence also supports the use of routine PPV23 vaccination in children with bronchiectasis (Chang et al. 2009).

A reduction in pneumococcal disease resulting from pneumococcal vaccination would be useful for children with non-CF bronchiectasis. In the absence of data relating vaccination status to pneumococcal serotypes identified in the airways, studies are ongoing (the Bronchoscopy Study) to address this clinical research gap.

1.9.2 Nontypeable *Haemophilus influenzae* (NTHi)

An oral vaccine containing killed NTHi (Broncostat®, Auspharm, Australia) significantly reduced the incidence of acute bronchitis in adult patients with COPD (Clancy et al. 1985) or chronic bronchitis (Lehmann et al. 1991). A review of six studies found that the vaccine reduced the number and severity of exacerbations for up to 6 months after vaccination, and concluded that a large clinical trial was needed (Foxwell, Cripps & Dear 2006). NTHi was the most commonly isolated bacterium
during an exacerbation, and the oral vaccine reduced carriage of NTHi in the upper respiratory tract (Foxwell, Cripps & Dear 2006). Recently a phase 2 clinical trial demonstrated that a similar NTHi oral immunotherapeutic (HI-164OV, Bioxyne, Australia) reduced the number and severity of acute exacerbations of COPD (Tandon et al. 2010). Results are consistent with the hypothesis that oral NTHi enhances mucosal protection (Clancy & Dunkley 2010). It is possible that such a vaccine may reduce the incidence and/or severity of other respiratory infections caused by NTHi, such as non-CF bronchiectasis.

An 11-valent precursor to PHiD-CV (with all pneumococcal serotypes conjugated to NTHi-derived protein D) has been shown to protect against OM due to NTHi (Prymula et al. 2006). Although a reduction in *H. influenzae* carriage was seen following primary and booster vaccination with the 11-valent vaccine (Prymula et al. 2009), no substantial effect of PHiD-CV (which has 8 of 10 serotypes conjugated to protein D) was observed on NP carriage of NTHi (Prymula et al. 2011). A recent study similarly found no difference in NP carriage (NTHi prevalence or *H. influenzae* density) in Dutch children vaccinated with PHiD-CV or PCV7 (van den Bergh et al. 2013). Whether or not PHiD-CV will afford protection to children with NTHi lower airway infection is yet to be determined.

1.9.3 Viruses including influenza

Viral infections also likely contribute to the pathogenesis and exacerbations of non-CF bronchiectasis. However, other than the influenza vaccines, there are no licensed vaccines that provide active immunisation. Influenza vaccination is recommended for adults aged 65 years and over, and other at-risk groups including all adults and
children with chronic pulmonary diseases such as bronchiectasis (Chang, Morris & Chang 2007). A study of 105 Japanese patients with chronic respiratory disease found that the additive inoculation of influenza vaccine and PPV23 significantly reduced the number of respiratory infections and hospitalisations in the 2 years after PPV23 compared to the 2 years prior (Sumitani et al. 2008). While no randomised controlled trials (RCTs) have been conducted, it is plausible that vaccination against respiratory viruses may help prevent lower respiratory infections, since viral infections often precede bacterial superinfection.

1.10 Antibiotic therapy and its impact on airway bacteriology

Antibiotic therapy forms the cornerstone of bronchiectasis treatment (Masekela & Green 2012). Although many antibiotic classes are used to treat respiratory infections, beta-lactam (penicillins) and macrolide antibiotics are included here as these are the most widely used in the setting in which my PhD studies have been undertaken. Beta-lactam antibiotics are the most commonly used antimicrobials in clinical practice, especially in treating upper and lower respiratory tract infections (Jacobs 2001). However, the macrolide antibiotic azithromycin is increasingly used for a variety of clinical problems in the NT and elsewhere including OM (Arguedas, Loaiza & Soley 2004; Arguedas et al. 2011; Morris et al. 2010), trachoma (Batt et al. 2003; Coles et al. 2013; Haug et al. 2010; Mak 2006), sexually transmitted infections (Bowden & Fethers 2008) and bronchiectasis (Chang et al. 2012). A brief overview of these antibiotics is necessary to place into context our studies examining the impact of antibiotics on airway bacteriology and antibiotic resistance (Chapter 4).
At the time of commencement of this thesis, the widely-used Central Australian Rural Practitioners Association Manual (*CARPA Standard Treatment Manual* 2003) recommended amoxicillin 3 times daily as the first choice for treatment of respiratory exacerbations. Amoxicillin remains the drug of choice for many respiratory infections such as OM due to its long history of clinical success, acceptability, limited side effects and relatively low cost (Klein 1998). High doses of amoxicillin can be prescribed for penicillin-resistant pneumococci, but clinical failure may indicate another infecting pathogen that is a beta-lactamase producer (Blumer 1998). Many *H. influenzae* and *M. catarrhalis* strains produce beta-lactamase, an enzyme that inactivates beta-lactam antibiotics. This mode of resistance can be overcome by antibiotics containing beta-lactamase inhibitors such as clavulanic acid (Klein 2003). A study by Peric, Browne et al. (2003) applied breakpoints derived from pharmacokinetic and pharmacodynamic (PK/PD) data to test *in vitro* nine beta-lactam and macrolide (including azithromycin) antimicrobials, and found that amoxicillin/clavulanate had the best overall activity against beta-lactam sensitive and resistant strains of *S. pneumoniae, H. influenzae, M. catarrhalis* and *S. aureus*.

Nevertheless, many clinicians in the NT routinely prescribe azithromycin (frequently long-term) for children with bronchiectasis. Azithromycin does not require refrigeration and is taken orally once a week, and therefore can be more easily managed by many families and health care staff, especially in remote communities. Azithromycin has been used in paediatric patients since 1991, and has been found to be safe and well-tolerated in the new single dose regimen (30mg/kg) as well as the conventional 3-day and 5-day regimens (Ruuskanen 2004). The rationale for use of a
single high dose is based on the drug’s long elimination half-life (>50 hours) and the fact that it is concentrated within phagocytic cells and achieves targeted delivery by these cells to sites of infection (Gordon & Blumer 2004).

Azithromycin has a spectrum of activity particularly suited to respiratory disease. When introduced in the late 1980s, it was claimed to have equivalent activity against *S. pneumoniae* and superior activity against *H. influenzae* and *M. catarrhalis* compared with other macrolides (Neu 1991). However, the complex pharmacology of macrolides produced interpretive problems, and more clinical efficacy studies were essential (Williams & Sefton 1993). The application of PK/PD principles has been ably reviewed by Calbo and Garau (2005). Briefly, sufficient concentrations of antimicrobial at the site of infection must be maintained for an adequate period of time to achieve bacteriologic and clinical success. Bacteriologic failures are due to infection by resistant pathogens or suboptimal therapy. On the basis of studies in patients and animal models, the susceptibility breakpoint for macrolides using approved oral dosing regimens for infections other than meningitis has been determined as \( \leq 0.5 \text{ mg/L} \) (Calbo & Garau 2005). Application of this minimum inhibitory concentration (MIC) breakpoint results in 20-35% of *S. pneumoniae* strains and 95% of *H. influenzae* strains being classified as macrolide resistant (Jacobs et al. 1999).

Macrolides are not only antibiotics. For the last two decades, macrolides have been known to have immunomodulatory properties as well as antibacterial effects (Masekela & Green 2012). Macrolides decrease inflammation, inhibit bronchial hyper-responsiveness and improve mucus clearance (Hoban & Zhanel 2006). Anti-
inflammatory drugs can play a pivotal role in the management of bronchiectasis by breaking the cycle of inflammation. However, there are few studies looking at the role of macrolides in non-CF bronchiectasis. Patient numbers and length of treatment were limited in eight studies described in two recent reviews (Figueiredo & Ibiapina 2011; Masekela & Green 2012). Only two of these trials were conducted in children with non-CF bronchiectasis (Koh et al. 1997; Yalcin et al. 2006), using roxithromycin and clarithromycin respectively. All studies showed a reduction in the frequency of exacerbations and/or sputum volume, often together with other improvements in pulmonary function. Three more recent studies similarly found fewer respiratory exacerbations in adult non-CF bronchiectasis patients receiving azithromycin or erythromycin for 6 to 12 months (Altenburg et al. 2013; Serisier et al. 2013; Wong et al. 2012). Clearly, more studies are needed in children with non-CF bronchiectasis, and during my PhD candidature a RCT in children was conducted by our research group (Valery et al. 2013).

Whilst long-term azithromycin therapy appears safe to individuals, its influence on individual and community-wide bacterial antibiotic susceptibility needs to be considered. In a remote Australian Indigenous community, single-dose azithromycin was given to children with trachoma and their rates of pneumococcal carriage and resistance were monitored (Leach et al. 1997). Immediately before treatment and 2-3 weeks, 2 months and 6 months after treatment carriage rates were 68%, 29%, 78% and 87% respectively, while the proportion of carriage-positive children with azithromycin-resistant S. pneumoniae strains was 2% before treatment and then 55%, 35% and 6% at follow-up visits (Leach et al. 1997).
Other studies have observed a similar increase in macrolide resistance following mass treatment for trachoma (Coles et al. 2013). A subsequent decrease in resistance after antibiotic pressure was removed has also been observed in Ethiopian communities after 6 biannual mass distributions of oral azithromycin for trachoma, where resistance declined from 77% to 31% 12 months later and 21% after 24 months (Haug et al. 2010). However, in communities where macrolide resistance is rare, azithromycin distribution for trachoma control is unlikely to increase the prevalence of resistant organisms (Batt et al. 2003). Although it is quite possible that the fitness cost of macrolide resistance is sufficient to ensure its eventual elimination in the absence of antibiotic selection, this process takes time. Using data from the Ethiopian trachoma elimination study, investigators used modelling to predict a 95% chance of elimination of macrolide resistance in *S. pneumoniae* by intra-species competition within 5 years of the last antibiotic treatment (Maher et al. 2012).

The effect of maintenance azithromycin therapy on airway microbiology, including resistance, is an important clinically relevant endpoint in evaluating the risks and benefits of this intervention. The final main aim of this thesis was therefore to assess the impact of azithromycin on upper and lower airway bacteriology, including carriage of and infection by macrolide-resistant respiratory pathogens, in Indigenous children with non-CF bronchiectasis. Additionally, understanding how antimicrobial resistance of respiratory pathogens translates into clinical outcomes is crucial to optimise the management of respiratory infections and ensure the most appropriate use of antibiotic therapies (Klugman 2007).
1.11 Antibiotic resistance in pathogens common in children with bronchiectasis

The biggest concern with the use of antibiotics is resistance. The two major classes of antimicrobials used to treat non-CF bronchiectasis, beta-lactams (penicillins) and macrolides, are considered here. Both beta-lactam and macrolide resistance can be a problem in *S. pneumoniae*, *H. influenzae* and *S. aureus*, while beta-lactam resistance is prevalent in *M. catarrhalis*. Macrolide resistance is also a concern in NTM (Masekela & Green 2012); however, NTM is rarely found in paediatric non-CF bronchiectasis (Kapur et al. 2011). Macrolides have no bactericidal effects against *Pseudomonas aeruginosa* but do inhibit biofilm formation (Masekela & Green 2012).

1.11.1 *Streptococcus pneumoniae*

Penicillin resistance in *S. pneumoniae* was first described in 1967 in Australia (Hansman & Bullen 1967). However, it was not until the 1990s that there was a major increase in its prevalence; from 1998 to 2000 the worldwide prevalence of pneumococcal respiratory tract isolates with high level penicillin resistance was estimated at 18%, but with conspicuous differences between countries (Jacobs 2003). Pneumococcal resistance to beta-lactams is primarily mediated through alterations in penicillin-binding proteins (PBPs), with highly penicillin-resistant strains (MIC ≥2 mg/L) having more PBP alterations than strains exhibiting intermediate resistance (MIC 0.12-1 mg/L). Beta-lactam antibiotics can maintain efficacy against PBP-mediated resistance if adequate drug concentrations are achieved and maintained at the site of infection for an adequate period of time (Jacobs 2003).
MIC breakpoints used by the United States' Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to define penicillin resistance are similar. Susceptible isolates have MIC $\leq 0.06$ mg/L, while resistant strains are defined as MIC $\geq 2$ mg/L (CLSI 2012) or $>2$ mg/L (EUCAST 2013). In a 2003 to 2005 survey of mostly healthy NT Indigenous children, 15% of *S. pneumoniae* strains were penicillin nonsusceptible (MIC $\geq 0.12$ mg/L) (Leach et al. 2009). Penicillin resistance (MIC $\geq 2$ mg/L) was only detected in serotype 9V; all other serotypes were penicillin intermediate resistant (MIC 0.12-1 mg/L), in descending order of prevalence 19A, 19F, 6B, 16F, 11A, 23B, and four additional serotypes (Leach et al. 2009).

Azithromycin is reported to be effective against susceptible strains of *S. pneumoniae*; however the relevant MIC breakpoint is controversial and depends on the site of infection. Treatment failure has mostly been reported for community-acquired pneumonia (Rzeszutek et al. 2004) and generally with MIC $\geq 8$ mg/L (Nuermberger & Bishai 2004). Treatment failure has been reported for OM with MIC $\geq 2$ mg/L (Dagan et al. 2001). The CLSI defines erythromycin and azithromycin resistance in *S. pneumoniae* as MIC $\geq 1$ mg/L and MIC $\geq 2$ mg/L respectively (CLSI 2012) whilst the EUCAST defines resistance to both as MIC $>0.5$ mg/L (EUCAST 2013), as per the recommendation of Calbo and Garau (2005).

Macrolide resistance in *S. pneumoniae* paediatric respiratory tract isolates worldwide was 25% overall (erythromycin MIC $\geq 1$ mg/L) from 1998 to 2000, exceeding penicillin resistance in 19 of 26 countries surveyed (Jacobs 2003). In a United States study from 2005 to 2007 azithromycin resistance (MIC $\geq 2$ mg/L) was 43% (Harrison...
et al. 2009). In contrast, 16% of clinically significant S. pneumoniae strains from Australian hospitals in 1997 were resistant to erythromycin (Turnidge, Bell & Collignon 1999), and only 5% of NP isolates from mostly healthy NT Indigenous children were found to be azithromycin resistant (MIC ≥2 mg/L) in a 2003 to 2005 survey (Leach et al. 2009). Azithromycin-resistant strains in the latter study were serotypes (in descending order of prevalence) 23B, 17F, 9N, 6B, 6A, 11A, 23F and 10 additional serotypes including 6C (Leach et al. 2009).

Two genetic acquisitions are responsible for most pneumococcal macrolide resistance (Jacobs 2003). Acquisition and expression of an erythromycin ribosomal methylation (ermB) gene results in complete resistance to macrolides, lincosamides and streptogramin B (MLS resistance), while acquisition of a macrolide efflux system (mefE) gene also affects macrolide susceptibilities (M resistance) (Jacobs & Johnson 2003). Resistance phenotypes can be determined using the erythromycin-clindamycin-rokitamycin triple-disk test; MLS types have the ermB gene and high level resistance (erythromycin MIC ≥128 mg/L), while the M phenotype with the mefE gene generally has low level resistance (MIC 2-16 mg/L) (Montanari et al. 2003; Montanari et al. 2001). Most macrolide-resistant isolates tested (n=94) in our NT study had the M phenotype (83%) and MICs 2 – 48 mg/L (unpublished data); eight isolates, serotypes 6B (6) and 16F (2), had MIC ≥256 mg/L (Leach et al. 2009).

1.1.11.2 Haemophilus influenzae

In H. influenzae, resistance to ampicillin and other beta-lactam antibiotics is generally limited to the production of beta-lactamase or, in the case of beta-lactamase negative ampicillin-resistant (BLNAR) strains, the presence of altered PBPs
A very small proportion of strains possess both mechanisms and are beta-lactamase positive and amoxicillin-clavulanate resistant (Tristram, Jacobs & Appelbaum 2007). In the United States in 1989, up to 40% of NTHi isolates from middle ear fluid were beta-lactamase producers and thus resistant to amoxicillin (Bluestone, Stephenson & Martin 1992). However, since 1994-5 there has been a gradual decline in *H. influenzae* beta-lactamase production to 28% in 2000-1 (Jacobs 2003). In NT Indigenous children, *H. influenzae* beta-lactamase production is even lower at 5-10% (Morris et al. 2010). The overall prevalence of beta-lactamase positive strains was 16.6% in a large international surveillance study from 1999 to 2000, ranging from 3% in Germany to 65% in South Korea; only 0.07% of strains were BLNAR (Hoban & Felmington 2002). An Australian study of 200 clinical isolates found no BLNAR strains using CLSI criteria (ampicillin MIC $\geq 4$ mg/L), but 5 (2.5%) strains with low BLNAR (MIC $\geq 2$ mg/L) (Witherden et al. 2011). EUCAST defines ampicillin resistance as MIC $>1$ mg/L.

In a large international study, most clinical *H. influenzae* strains (not specified as typeable or nontypeable) were found to have an intrinsic macrolide efflux mechanism and azithromycin MICs between 0.25 and 4mg/L; only 1.3% had high-level macrolide resistance (MIC $>4$ mg/L) due to ribosomal alterations, while 1.8% were defined as hypersusceptible (MIC $<0.25$ mg/L) (Peric, Bozdogan et al. 2003). Current CLSI breakpoints categorise the majority of isolates as susceptible (MIC $\leq 4$ mg/L), but many authors have suggested these breakpoints need revision and recommended lowering the cut-off to include isolates with the intrinsic macrolide efflux mechanism (Calbo & Garau 2005; Dagan et al. 2000; Jacobs & Johnson 2003; Peric, Bozdogan et al. 2003). EUCAST guidelines recognise *H. influenzae*...
intermediate azithromycin resistance (MIC $>0.12 - 4$ mg/L), with resistance defined as MIC $>4$ mg/L. Ampicillin and azithromycin susceptibility in *H. influenzae* have not previously been reported in NT Indigenous children.

### 1.11.3 *Moraxella catarrhalis*

Most *M. catarrhalis* strains are beta-lactamase producers. In the United States in 1989, more than 90% of isolates from middle ear fluid were beta-lactamase positive (Bluestone, Stephenson & Martin 1992). In a study of respiratory tract pathogens in the Western Pacific (including Australia) and South Africa, 98% of *M. catarrhalis* isolates were beta-lactamase positive (Bell et al. 2002). *M. catarrhalis* beta-lactamase production has not previously been reported in NT Indigenous children.

*M. catarrhalis* is almost universally susceptible to azithromycin (Jacobs & Johnson 2003; Peric, Bozdogan et al. 2003; Peters, Friedel & McTavish 1992; Soley & Arguedas 2005) and is usually not tested. Only one macrolide resistant isolate of *M. catarrhalis* was found in a study of long-term azithromycin treatment for CF patients (Hansen et al. 2009), while a recent Western Australian OM study (which included Indigenous children) reported that all 261 *M. catarrhalis* strains tested were susceptible to azithromycin (Pingault et al. 2010). *M. catarrhalis* azithromycin resistance has not previously been reported in NT Indigenous children, and was not tested as part of our bronchiectasis studies.

### 1.11.4 *Staphylococcus aureus*

Although *P. aeruginosa* and *S. aureus* are relatively uncommon in paediatric non-CF bronchiectasis, *S. aureus* is included here for two main reasons. Firstly, NP carriage
of *S. aureus* is common in older children. In the Netherlands, *S. aureus* was isolated from 36% of healthy children aged 1-19 years, with peak carriage >50% at age 11 years (Bogaert et al. 2004). In the United States, colonisation prevalence was 32% in persons ≥1 year old, with the highest prevalence in participants 6-11 years (Kuehnert et al. 2006). Secondly, there has been increasing interest in *S. aureus* as a respiratory pathogen as the prevalence of infection caused by methicillin-resistant *S. aureus* (MRSA) has increased (Lo et al. 2013). MRSA rates (proportion of *S. aureus* infections) have been increasing rapidly over the past few decades and MRSA is currently endemic in many hospitals throughout the world. The highest rates (>50%) are in North and South America, Asia and Malta; intermediate rates (25-50%) in China, Australia, Africa and some European countries such as Portugal and Greece; and low rates (<25%) in other European countries such as The Netherlands and Scandinavia (Stefani et al. 2012).

There is very little information on macrolide resistance in *S. aureus* other than in the context of CF patients (next section) or MRSA. A recent surveillance study reported macrolide resistance rates of 0-57% for methicillin-susceptible *S. aureus* respiratory tract isolates in Asia (Wang et al. 2011). In a European study, *S. aureus* resistance to azithromycin ranged from 1.6% in Sweden to 16.9% in France (den Heijer et al. 2013). Macrolide resistance in *S. aureus* has not previously been reported in NT Indigenous children.

1.12 Macrolide resistance in bronchiectasis patients

Only two trials of macrolide treatment have been reported in children with non-CF bronchiectasis (Koh et al. 1997; Yalcin et al. 2006); neither reported microbiologic
outcomes. Increased macrolide resistance in sputum bacteria from adult patients receiving macrolide antibiotics was reported in two RCTs (Altenburg et al. 2013; Serisier et al. 2013). In the former study, 88% (53/60) of pathogens tested from patients in the azithromycin group were macrolide resistant, compared with 26% (29/112) in the placebo group (Altenburg et al. 2013). Most resistance was found in *H. influenzae*, with only small numbers (<10 each in total) of macrolide resistant *S. pneumoniae, S. aureus, M. catarrhalis* and *H. parainfluenzae* (Altenburg et al. 2013). In the latter study, erythromycin increased the proportion of macrolide-resistant oropharyngeal streptococci (Serisier et al. 2013).

Studies of long-term macrolide treatment in patients with CF bronchiectasis have found high rates of macrolide resistance in *S. aureus* (up to 100%) (Hansen et al. 2009; Phaff et al. 2006; Prunier et al. 2003; Tramper-Stranders et al. 2007) and *Haemophilus* spp. (Phaff et al. 2006). In the latter study, erythromycin resistance in *S. aureus* increased from 7% to 54% and clarithromycin resistance (MIC ≥32 mg/L) in *Haemophilus* spp. increased from 4% to 38% over a 4-year period.

Macrolide antibiotics have an established role in the management of airway inflammatory diseases such as CF and, although the exact mechanisms of action remain uncertain, a non-antibiotic effect seems likely (Serisier 2013). Increased macrolide resistance resulting from long-term use of macrolide antibiotics is already a major concern, and will only increase as more non-CF bronchiectasis patients are treated with macrolides. The development of novel macrolides that have no antimicrobial activity but only anti-inflammatory and immunomodulatory properties may be needed (Masekela & Green 2012; Serisier 2013; Tarran et al. 2013).
1.13 Hypotheses and aims

Many gaps in the literature were found for a condition that has substantial morbidity in Indigenous children. To address these gaps the overall aims of the studies within my PhD were to determine the bacteriology of non-CF bronchiectasis, and the impact of antibiotics (particularly azithromycin) on respiratory bacteria and antibiotic resistance, in these high risk children.

The main hypotheses were:

1. That the bacteriology of non-CF bronchiectasis in NT Indigenous children is the same as in other paediatric populations.

2. That bacterial pathogens in the lower airways of NT Indigenous children with non-CF bronchiectasis derive from the upper airways, specifically the nasopharynx.

3. That azithromycin therapy is not associated with any significant differences in the proportions of NT Indigenous children with non-CF bronchiectasis carrying or infected by common respiratory pathogens with azithromycin resistance compared to children not receiving azithromycin. (While increased azithromycin resistance might be expected, this may be offset by reduced carriage and/or reduced lower airway infection.)

The specific aims addressed by the studies included in this thesis are to:

1. Develop improved methods for the study of respiratory bacterial pathogens (Chapter 2).
2. Describe the bacteriology of the upper and lower airways in NT Indigenous children with bronchiectasis (Chapter 3).

3. Compare the bacteriology of the upper and lower airways in NT Indigenous children with bronchiectasis to the bacteriology in children without bronchiectasis (Chapter 3).

4. Describe the impact of recent antibiotic treatment on upper airway carriage, lower airway infection, and resistance of bacterial respiratory pathogens in NT Indigenous children with bronchiectasis (Chapter 4).

5. Describe the impact of long-term azithromycin treatment on upper airway carriage and resistance of bacterial respiratory pathogens in NT Indigenous children with bronchiectasis (Chapter 4).

1.14 Thesis design

Studies were performed to address the specific thesis aims and in so doing address some of the knowledge gaps previously outlined.

Methods used are described in Chapter 2. These consist of ethics clearances; specimen collection, transport, storage and culture; pathogen identification and typing; and antibiotic susceptibility testing. Our studies have contributed to the methodological literature in the following ways:

- A comparison of NP and OP sampling for the detection of upper airway colonisation with respiratory bacteria;
- A method of swab transport that would enable routine swab collection by clinic staff in remote communities for surveillance purposes;
• A study of the viability of respiratory pathogens in swab specimens kept long-term in ultra-freeze storage;
• A method for the detection of multiple pneumococcal serotypes in NP swabs;
• Support for the continued use of the gold standard Quellung reaction for pneumococcal serotyping.

Chapter 3 addresses the first two hypotheses using data from our Bronchoscopy Study. Firstly, the bacteria found in upper and lower airway specimens collected during bronchoscopy from NT Indigenous children with bronchiectasis were determined, and compared with those from other paediatric populations with non-CF bronchiectasis. The bacteriology was then compared with that from non-Indigenous children without bronchiectasis, as too few Indigenous children without bronchiectasis were seen. Secondly, concordance between upper and lower airway bacterial strains was assessed. An issue of identification was also addressed by using PCR to differentiate NTHi from nonhaemolytic strains of the closely-related primarily commensal *Haemophilus haemolyticus*. Isolates from NP, OP and BAL specimens were firstly differentiated, and then quantitative PCR (qPCR) was used to quantify *H. influenzae* in BAL specimens and confirm NTHi lower airway infection.

The third main hypothesis of this thesis is addressed in Chapter 4 using data from our Bronchoscopy and Multicentre Bronchiectasis Studies. Firstly, the impact of recent antibiotic use on upper and lower airway bacteriology, including antibiotic resistance, is described using NP and BAL specimens from the Bronchoscopy Study. Secondly, the effects of long-term azithromycin use on upper airway carriage and macrolide resistance in Indigenous children with bronchiectasis are described using
NP specimens from our Bronchiectasis Observational Study (BOS). The same long-term effects were examined in children who received azithromycin in the Bronchiectasis Intervention Study (BIS), a randomised, placebo-controlled trial to reduce pulmonary exacerbations. The BIS bacteriology results will be written up in detail for publication at a later date.

Chapter 5 summarises and discusses the thesis findings and limitations, and the work still needed in this area.

1.15 Summary of introduction

Non-CF bronchiectasis is a serious and prevalent condition in NT Indigenous children resulting in substantial morbidity and early death in adulthood. Early and intensive treatment impacts on the long term clinical outcomes of children with bronchiectasis. One key to this treatment is the use of antibiotics for respiratory exacerbations and, in some, use of long-term or maintenance antibiotics, specifically macrolides.

Research into the bacterial pathogens commonly found in the upper and lower airways of Australian Indigenous children with non-CF bronchiectasis, and the impact of antibiotic treatment on the bacteriology including antimicrobial resistance rates, is lacking. This thesis aims to fill these gaps and thus contribute to better treatment and management of this condition, in the hope that health outcomes for these disadvantaged children might be improved.
CHAPTER 2

Methods
CHAPTER 2: METHODS

2.1 Chapter overview

Many of the methods used in this study were developed in Australia by Amanda Leach (Leach et al. 1994; Leach et al. 1997) and Mike Gratten (Gratten et al. 1994). This work contributed to standardised methods published for *Streptococcus pneumoniae* nasopharyngeal (NP) carriage studies in the era of pneumococcal conjugate vaccine introduction (O'Brien & Nohynek 2003). Work for this thesis has contributed to updated methods (Satzke et al. 2013) which also include data on oropharyngeal (OP) sampling and Supplementary Material on *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus* and respiratory viruses. The relevant sections are summarised here, together with contributory findings from our studies.

Ethical approvals and specimens collected are listed in sections 2.2 and 2.3. As outlined in Chapter 1, recurrent microaspiration of NP secretions is believed to be important in establishing and maintaining lower airway infection in bronchiectasis. Aspiration of OP secretions, which may result in aspiration pneumonia or other lung infections, is also possible. Therefore culture of specimens from both the nasopharynx and oropharynx, and comparison of species and strains with those isolated from lower airway culture, are essential to determine the source of lower airway infection. The sensitivity of NP and OP sampling for the detection of upper airway carriage of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* is compared in section 2.4. Section 2.5 describes sampling from nasal and tissue swabs when children will not tolerate NP sampling.
The transport and storage medium, skim milk tryptone glucose glycerol broth (STGGB), used for all our studies since 1992, is described in Section 2.6. Section 2.7 comprises a publication describing multiple pathogen recovery using a combination of Amies gel swabs transported by the routine pathology service, and storage within 48 hours in STGGB, compared with recovery from standard swabs transported frozen in STGGB (Hare, Stubbs et al. 2010). This evaluation demonstrated that surveillance using this system is possible. We also demonstrated that recovery of respiratory bacterial pathogens from NP swabs stored at −70°C in STGGB for up to 12 years was equivalent to recovery from the original cultures (Hare, Smith-Vaughan & Leach 2011) in a publication comprising section 2.8. This information is useful when samples from original specimens are used for additional or follow-up studies, e.g. further quantification of specific bacteria or determination of resistance mechanisms.

Standard methods for culture, identification and typing of respiratory bacterial pathogens are briefly described in sections 2.9 and 2.10. Multiple colonies are selected for the purpose of detecting carriage of multiple strains (defined by phenotype and antibiotype) of each species. Research into the dynamics of carriage, e.g. pneumococcal serotype replacement following vaccine introduction or co-colonisation of susceptible and resistant strains, is dependent upon the sensitivity of the method used for detecting multiple strains. Section 2.11 comprises a publication describing a random colony selection method combined with selection of morphologically distinct colonies for the detection of multiple pneumococcal
Section 2.12 illustrates the continued importance of the Quellung reaction for pneumococcal serotyping (Hare et al. 2009).

Antibiotic susceptibility testing and molecular and immunological methods are briefly covered in sections 2.13 and 2.14, followed by a chapter summary.

### 2.2 Ethical approvals

This study used specimens obtained from three studies conducted by the Menzies School of Health Research (Menzies), all approved by the Human Research Ethics Committee (HREC) of the Northern Territory (NT) Department of Health and Menzies, which includes an Aboriginal subcommittee. These studies were the Bronchoscopy Study (HREC 07/63) conducted at Royal Darwin Hospital (RDH), the Bronchiectasis Observational Study (BOS, HREC 04/46) and the Bronchiectasis Intervention Study (BIS, HREC 07/77). Specimens were also obtained from a study at the Royal Children’s Hospital (RCH) in Brisbane which was approved by the RCH HREC, Protocol No. 2003/017.

### 2.3 Specimens collected

Ethical approval was obtained to collect NP swabs from children in all studies. In addition, approval was obtained to collect bronchoalveolar lavage (BAL) fluid, blood (for immunology studies) and OP swabs from children undergoing high resolution computerised tomography (HRCT) scans and bronchoscopy. The procedures for specimen collection at RDH are described in publications from the Bronchoscopy Study (Hare, Grimwood et al. 2010; Hare, Marsh et al. 2013) included in Chapter 3.
While the original World Health Organization (WHO) guidelines (O'Brien & Nohynek 2003) described sampling from the nasopharynx only for the detection of upper respiratory tract carriage of the pneumococcus, the revised guidelines compared NP and OP sampling. Nine studies (including unpublished findings from our Bronchoscopy Study) were identified for children, and five studies for adults, comparing the sensitivity of sampling the nasopharynx and oropharynx for the detection of pneumococcal carriage (Satzke et al. 2013). The nasopharynx was confirmed as the preferred sampling site for *S. pneumoniae* in children, while sampling both routes was recommended for adults (Satzke et al. 2013).

In contrast, current data suggest that both NP and OP swabs are required for optimal recovery of *H. influenzae* from children and adults (Satzke et al. 2013). However, it was acknowledged that this finding is confounded by the recent recognition that a substantial proportion of apparent nontypeable *H. influenzae* (NTHi) isolates from the nasopharynx (12 - 27%) and oropharynx (65%) of children are the closely-related *H. haemolyticus* (Hare, Binks et al. 2012; Kirkham et al. 2010; Murphy et al. 2007). More studies are needed to clarify the respective prevalences of *H. influenzae* and *H. haemolyticus* in NP and OP specimens. The optimal sampling site for *M. catarrhalis* is also unclear, with few existing studies and variable carriage rates across age groups (Satzke et al. 2013). The anterior nares were considered to be the primary colonization site of *S. aureus*. However, more recent studies in adults and children have found OP swabs more sensitive while other studies have found nasal swabs more sensitive. Together, this evidence suggests swabbing both nasal and OP sites is needed for optimal detection of *S. aureus* carriage (Satzke et al. 2013).
Detection of all four bacterial species in NP and OP swabs from 120 Australian Indigenous children with HRCT-confirmed bronchiectasis who underwent bronchoscopy from June 2008 to June 2013 is shown in Table 2.1. Findings were similar to other studies in children (Satzke et al. 2013), with increased NP detection of *S. pneumoniae* in particular compared to OP sampling. In specimens from a subset of these children, *H. influenzae* was differentiated from presumptive *H. haemolyticus* (Hare, Binks et al. 2012) with results reported in Chapter 3.

### 2.5 Type of sample collected

While NP swabs are preferred for pneumococcal carriage studies, these are not always well tolerated by children and some studies use nasal swabbing. In Australian Aboriginal children living in remote communities and mostly non-Aboriginal children attending urban child care centres, high carriage of *S. pneumoniae* (90% and 43% respectively) and *H. influenzae* (80% and 41% respectively) was detected using nasal swabs (Stubbs et al. 2005). Three studies were identified that directly compared NP and nasal sampling methods for detecting pneumococci in children (Carville et al. 2007; Rapola et al. 1997; van den Bergh et al. 2012). While all three studies found no difference in isolation rates, in two of these studies (Rapola et al. 1997; van den Bergh et al. 2012) children had respiratory symptoms which are known to enhance pneumococcal carriage and possibly affect the sensitivity of detection of nasal specimens. There is therefore insufficient evidence to conclude that nasal swabbing is as effective as NP swabbing for the detection of pneumococcal carriage in healthy children (Satzke et al. 2013).
Sometimes even nasal swabs are not well tolerated. Leach et al. (2008) found that nose blowing into a paper tissue, followed by swabbing and culture of the material on the tissue, was an effective alternative to nasal swabbing when nasal secretions were present. Compared with nasal swabs, nose blowing samples had high sensitivity (>90%) for the detection of *S. pneumoniae* and *H. influenzae* in Aboriginal children aged 3-7 years and children aged <4 years who were attending urban child care centres (Leach et al. 2008; Satzke et al. 2013). For children without visible secretions, direct NP or nasal sampling was required (Leach et al. 2008). More recently, sampling of secretions from a tissue was found to be comparable to NP and nasal swabs for detection of *H. influenzae*, *M. catarrhalis* and *S. aureus* in children aged 0 - 4 years with rhinorrhoea (van den Bergh et al. 2012).

NP swabs were always collected during bronchoscopy, as children were anaesthetised at the time. While specimens collected at other times from children in the BOS and BIS studies sometimes included nasal and tissue swabs, the evidence presented here suggests that recovery of respiratory pathogens would be similar to that from NP swabs. The type and quality of specimens collected were recorded on specimen collection forms.
Table 2.1. Sensitivity of nasopharyngeal (NP) and oropharyngeal (OP) swabs for the detection of upper airway carriage of respiratory bacteria in 120 Australian Indigenous children with bronchiectasis.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Number positive</th>
<th>Sensitivity (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP swabs</td>
<td>OP swabs</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>43 (36%)</td>
<td>7 (6%)</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em>¹</td>
<td>56 (47%)</td>
<td>54 (45%)</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>29 (24%)</td>
<td>5 (4%)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>16 (13%)</td>
<td>11 (9%)</td>
</tr>
</tbody>
</table>

¹Nontypeable *H. influenzae* was differentiated from presumptive *Haemophilus haemolyticus* in a subset of these children (Chapter 3).
2.6 Transport and storage medium

STGGB was originally developed for frozen storage of bacterial isolates (Gibson & Khoury 1986). However specimens such as NP swabs can also be collected and placed immediately in tubes containing 1 mL STGGB for transport and storage at −70°C (Leach et al. 1997; O'Brien et al. 2001). STGGB was previously recommended (O'Brien & Nohynek 2003) because it is non-proprietary, is easily made with commonly available ingredients, is inexpensive and has been successfully used by many groups investigating carriage of pneumococci and other upper respiratory tract bacterial organisms (Satzke et al. 2013). Although no systematic comparisons have been conducted, the consensus is that STGGB remains the medium of choice for transport and storage of NP swabs (Satzke et al. 2013). Concentrated STGGB (same ingredients mixed in 40mL instead of 100mL distilled water) has been used for liquid specimens such as NP aspirates (Moore et al. 2010) and BAL, mixing equal volumes of specimen and STGGB (Hare, Grimwood et al. 2010). The ability to store original specimens allows additional studies without the need for further expensive field collection.

Specimens are not cultured immediately on receipt at the Menzies laboratory, even those received ‘fresh’ from the adjacent RDH. Rather, specimens are stored at −70°C (−80°C since the mid 2000s) and then batch processed according to standard protocols.
2.7 Journal article: Swab transport in Amies gel followed by frozen storage in skim milk tryptone glucose glycerol broth (STGGB) for studies of respiratory bacterial pathogens

(following page)

Table 1 in this journal article is numbered Table 2.2 in the List of Tables.
2.8 Journal article: Viability of respiratory pathogens cultured from nasopharyngeal swabs stored for up to 12 years at –70°C in skim milk tryptone glucose glycerol broth

(following page)

Tables 1, 2, 3 and 4 in this journal article are Tables 2.3, 2.4, 2.5 and 2.6 in the List of Tables. Figure 1 is numbered Figure 2.1 in the List of Figures.
2.9 Standard culture methods

Specimens were cultured on selective media and pathogens isolated as previously described (Leach et al. 1994). Briefly, batches of specimens stored at −70°C to −80°C in STGGB were thawed on ice, mixed, and 10 µL aliquots inoculated onto chocolate agar plates (non-selective) for isolation of *M. catarrhalis*, plates containing 5% horse blood agar and colisten-nalidixic acid (Oxoid, Australia) for isolation of streptococci and staphylococci, and bacitracin vancomycin clindamycin chocolate agar for isolation of *Haemophilus* species. Plates were incubated overnight at 37°C in a humid environment containing 5% CO₂ and read the next day.

Semi-quantitative colony counts were recorded from primary plates as previously described (Smith-Vaughan et al. 2006) using the following density scores: 0: no growth, 1: <20 colonies, 2: 20–49 colonies, 3: 50–100 colonies, 4: colonies greater than 100 but not present beyond the primary inoculum, 5: colonies present in first streak zone beyond primary inoculum, 6: colonies present in 2nd streak zone, 7: colonies present in 3rd streak zone. For BAL specimens, density scores were correlated with colony forming units (CFU)/mL in BAL fluid determined by serial dilution and quantitative colony counts of organisms (Hare, Grimwood et al. 2010).

2.10 Pathogen identification and typing

Bacterial species were identified according to standard methods (Engelkirk & Duben-Engelkirk 2007; Murray et al. 2003). Identification of *S. pneumoniae* was confirmed by colony morphology, susceptibility to optochin and a positive Quellung reaction with pneumococcal antisera (Statens Serum Institute [SSI], Denmark). *H. influenzae* was confirmed by colony morphology and requirement for X and V
growth factors. *M. catarrhalis* was confirmed by colony morphology, oxidase positivity and Gram stain. In our bronchiectasis studies, the addition of a tributyrin test enabled differentiation of *M. catarrhalis* from commensal *Neisseria* species. *S. aureus* was confirmed by colony morphology and a positive coagulase test.

Pneumococcal serotyping was carried out on all *S. pneumoniae* isolates using antisera from SSI. While *H. influenzae* and *Haemophilus parainfluenzae* can be differentiated by standard culture methods, molecular methods are needed to differentiate NTHi from the primarily commensal non-haemolytic *Haemophilus haemolyticus* (Binks et al. 2012; Murphy et al. 2007). Molecular methods are becoming increasingly important for pathogen identification and quantification of bacterial load (Smith-Vaughan et al. 2006). DNA extraction can be incorporated into the standard processing schedule or carried out at a later date. PCR-ribotyping (Smith-Vaughan et al. 1995) was performed on a subset of NTHi isolates to determine concordance of NP, OP and BAL strains. High resolution melt (HRM) typing was developed in our laboratory to allow faster and more cost-effective routine typing of NTHi (Pickering et al. 2014).
2.11 Journal article: *Random colony selection versus colony morphology for detection of multiple pneumococcal serotypes in nasopharyngeal swabs*

(following page)

*Table 1 in this journal article is Table 2.7 in the List of Tables.*
2.12 Journal article: "Dodgy 6As": Differentiating pneumococcal serotype 6C from 6A by use of the Quellung reaction

(following page)

Figure 1 in this journal article is Figure 2.2 in the List of Figures.
The Quellung reaction has long been the standard method for pneumococcal serotyping (7); however, the new serotype 6C is reportedly indistinguishable from serotype 6A using this method (8). We report an anomalous Quellung reaction associated with serotype 6C, which may aid in diagnostic interpretation.

The 7-valent pneumococcal conjugate vaccine was introduced in Australia for indigenous and other high-risk infants in 2001. In 2002, a serotyping anomaly was noted in a subset of carriage isolates. These isolates appeared negative for all nine pool antisera A to I (covering 90 pneumococcal serotypes) but reacted positively with Omni serum. Following advice from the Pneumococcal Reference Laboratory in Queensland, Australia, we found that these isolates reacted strongly with group 6 antiserum but that factor reactions were weak. Group 6 is included in the B pool. A selection of isolates was sent to Queensland and serotyped as 6A. In 2003, the Statens Serum Institute (SSI) confirmed 10 such isolates as 6A; pool B reactions were mostly weak (+), while pool Q reactions were strongly positive (+++). (Pool Q, which includes group 6, is part of an alternative set of antisera used to identify types present in the 23-valent pneumococcal polysaccharide vaccine.) To distinguish “normal” 6As with strong pool B reactions, we referred to isolates with weak or apparent negative pool B reactions as “dodgy” 6As.

In 2007, when the new serotype 6C was announced (8), we queried whether our dodgy 6As were actually 6Cs. Using a wciN PCR (9), we revealed that 1 of 20 prevaccine isolates recorded as 6A was 6C, while 17 of 18 postvaccine isolates recorded as 6A were 6C, suggesting serotype replacement. All 17 postvaccine serotype 6C isolates had been recorded as dodgy 6As, while the one postvaccine serotype 6A isolate was recorded as a normal 6A. Atypical reactions had not been recorded prevaccine.

To confirm our results, fresh serum broths were prepared and serotyped blinded. All 20 isolates typed as 6A by PCR were normal 6As by the Quellung reaction, while all 18 isolates typed as 6C by PCR were dodgy 6As by the Quellung reaction. Subsequently, another 144 6A isolates from a 2003 to 2005 surveillance study were tested using wciN PCR. All 61 normal 6As were confirmed as 6A, while all 83 dodgy 6As were 6Cs. We have therefore matched PCR results to pool B reactions for 182 isolates previously recorded as 6A (81 [100%] normal, 101 [100%] dodgy 6A isolates were determined to be 6C by PCR).

We have used five different batches of pool B antiserum since 2002; thus, batch variation is unlikely to have influenced our findings. Furthermore, in a competitive inhibition enzyme-linked immunosorbent assay, 6C polysaccharide antigen did not significantly attenuate the binding of pool B to 6A and 6B polysaccharide antigens. This suggests that pool B may react weakly with serotype 6C. Interestingly, 6C antigen considerably inhibited the binding of pool Q to these antigens, suggesting a strong pool Q reaction with 6C (as reported by SSI).

The Quellung reaction is produced using the Neufeld test, described by Lund (6) and Austrian (1), and the SSI pneumococcal antisera insert (August 2001 revision). Briefly, a drop of specimen is mixed with a drop of typing serum, a coverslip is placed over the mixture, and the preparation is examined using a ×100 magnification oil immersion lens. A positive reaction occurs when type-specific antibody binds to the pneumococcal capsule, causing a change in its refractive index so that it appears “swollen” and more visible. A World Health Organization working group acknowledged the Quellung reaction as the standard method for serotyping pneumococcal isolates but did not detail a recommended method (7).
Recently, 11 reference laboratories in Europe participated in the validation of pneumococcal serotyping (5). A high degree of consensus was found between the Neufeld test and other serotyping methods (agglutination or gel diffusion). Six laboratories used the Neufeld test; however, the magnification used was not specified, nor was the use of oil immersion (5). Australian laboratories routinely use ×40 magnification (4), which is quicker and simpler than ×100. This protocol is accurate and reliable and has been used by experts for more than 30 years (2). According to Henrichsen (3), the Quellung reaction continues to be carried out essentially as described by Austrian, and we find no reference to any modified Neufeld method used outside Australia.

To our eyes, the differing strengths of the pool B reaction with 6A and 6C were clearly visible at both magnifications (Fig. 1). Furthermore, this phenomenon is not confined to pool B antiserum. We have observed that a strong positive pool G reaction indicates serotype 29 or 35B, while a weak positive pool G reaction invariably indicates serotype 34.

We have demonstrated that pneumococcal serotype 6C is associated with a weak pool B reaction which differentiates it from 6A. This information should prove useful for laboratories that use the Quellung reaction, particularly those without the capability for molecular typing.

We thank Denise Murphy (Pneumococcal Reference Laboratory, Queensland Health Scientific Services, Australia) and Mette Barendorf Kerrn (Statens Serum Institute, Denmark) for serotyping advice and Peter Christensen (Menzies School of Health Research, Australia) for laboratory assistance.

This study was supported by an NHMRC project grant and NIH grant AI-31473 (M.H.N.). M.H.N. and I.H.P. are employed by the University of Alabama at Birmingham, which has applied for a patent covering the discovery of the new pneumococcal serotype, 6C.

REFERENCES

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Published ahead of print on 8 April 2009.
2.13 Antibiotic susceptibility testing

Isolates of *S. pneumoniae*, *H. influenzae* and *S. aureus* were tested using the calibrated dichotomous susceptibility (CDS) method (Bell 1975; Bell, Pham & Fisher 2013) to detect beta-lactam and macrolide resistance. *M. catarrhalis* and NTHi isolates were tested for beta-lactamase activity using a nitrocephin-based test. Etest® strips (formerly AB Biodisk, Sweden, now AB bioMérieux, France) were used to determine minimum inhibitory concentrations (MICs) for *S. pneumoniae* and NTHi isolates nonsusceptible to penicillin (ampicillin) and erythromycin (azithromycin) by the CDS method. The MICs for susceptible isolates using the CDS method, and the breakpoints employed by the Clinical and Laboratory Standards Institute (CLSI 2012) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2013), are shown in Table 2.8. *S. aureus* isolates were tested using cefoxitin (to detect methicillin resistance) and erythromycin discs.

As discussed in Chapter 1, breakpoints for *S. pneumoniae* resistance are similar according to CLSI and EUCAST, with differences of no more than one doubling dilution. Differences are more apparent for *H. influenzae* resistance. In the past we have used CLSI breakpoints for interpretation of MIC values. This proved adequate until we embarked on closer examination of NTHi as an important pathogen in non-CF bronchiectasis. Investigation of azithromycin and its impact on carriage of and infection by NTHi led to an appreciation of the probable importance of intermediate macrolide resistance, and we therefore wished to use EUCAST interpretive criteria. To avoid confusion, we have used EUCAST breakpoints to define resistance of *S. pneumoniae* and *H. influenzae* to both beta-lactam and macrolide antibiotics in our bronchiectasis publications.
As can be seen in Table 2.8, the CDS method has not been calibrated to test macrolide resistance in *H. influenzae*. However, standardised disc susceptibility and MIC breakpoints have been published by the British Society for Antimicrobial Chemotherapy, using 15 µg azithromycin discs and the same interpretation of zone diameters (as we did), for both *S. pneumoniae* and *H. influenzae* (Andrews et al. 2011). Moreover, in our bronchiectasis studies we used Etest® strips to determine the MIC for at least one NTHi isolate from each positive specimen. The isolate with the smallest azithromycin disc annulus was selected to ensure no intermediate resistance was missed and so provide an accurate assessment of resistance on a per specimen basis. However, this meant there was a bias against detection of fully susceptible strains, since isolates susceptible by disc diffusion may have been intermediate resistant (using EUCAST criteria) or susceptible.

### 2.14 Molecular and immunological methods

As mentioned in sections 2.4 and 2.10, a PCR for *H. influenzae* protein D (*hpd#3*) was used on isolates to differentiate NTHi from presumptive *H. haemolyticus* (Hare, Binks et al. 2012). Subsequently, we used *hpd#3* PCR quantitatively (qPCR) on BAL fluid as a measure of *H. influenzae* lower airway infection (Hare, Marsh et al. 2013). We compared our semi-quantitative NTHi culture-based definition of *H. influenzae* lower airway infection with *hpd#3* qPCR estimation, and then compared both measures of bacterial density with total and differential cell counts to gauge the airway inflammatory response to infection (Hare, Marsh et al. 2013). The molecular and immunological methods employed are described in these papers which are included in Chapter 3.
### Table 2.8. MIC breakpoints for *S. pneumoniae* and *H. influenzae*

<table>
<thead>
<tr>
<th>Organism and antibiotic</th>
<th>CDS breakpoint</th>
<th>CLSI breakpoints</th>
<th>EUCAST breakpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(≥6mm disc annulus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-lactam (benzylpenicillin)</td>
<td>S(\leq 0.125) mg/L</td>
<td>S(\leq 0.06) mg/L</td>
<td>S(\leq 0.06) mg/L</td>
</tr>
<tr>
<td></td>
<td>I =0.12–1 mg/L</td>
<td>I =0.12–2 mg/L</td>
<td>S&lt;4 mg/L</td>
</tr>
<tr>
<td></td>
<td>R≥2 mg/L</td>
<td>R&gt;2 mg/L</td>
<td>S≤0.5 mg/L</td>
</tr>
<tr>
<td>Macrolide (erythromycin)</td>
<td>S(\leq 0.5) mg/L</td>
<td>S(\leq 0.25) mg/L</td>
<td>S(\leq 0.25) mg/L</td>
</tr>
<tr>
<td></td>
<td>R≥1 mg/L</td>
<td>R&gt;0.5 mg/L</td>
<td>S≤0.5 mg/L</td>
</tr>
<tr>
<td>Not calibrated (azithromycin)</td>
<td>S(\leq 0.5) mg/L</td>
<td>S(\leq 0.25) mg/L</td>
<td>S≤0.25 mg/L</td>
</tr>
<tr>
<td></td>
<td>R≥2 mg/L</td>
<td>R&gt;0.5 mg/L</td>
<td>R&gt;0.5 mg/L</td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-lactam (ampicillin)</td>
<td>S(\leq 1) mg/L</td>
<td>S(\leq 4) mg/L</td>
<td>S(\leq 1) mg/L</td>
</tr>
<tr>
<td></td>
<td>R≥4 mg/L</td>
<td>R&gt;1 mg/L</td>
<td>S≤4 mg/L</td>
</tr>
<tr>
<td>Macrolide (azithromycin)</td>
<td>Not calibrated</td>
<td>S(\leq 4) mg/L</td>
<td>S≤0.12 mg/L</td>
</tr>
<tr>
<td></td>
<td>R&gt;4 mg/L</td>
<td>I &gt;0.12–4 mg/L</td>
<td>R&gt;4 mg/L</td>
</tr>
</tbody>
</table>

CDS, calibrated dichotomous susceptibility; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MIC, minimum inhibitory concentration; S, susceptible; I, intermediate; R, resistant.
2.15 Summary of methods

Standardised methods are essential to enable valid comparison of results between studies. This thesis has relied heavily on methods described by a WHO working group for the detection of pneumococcal carriage using NP swabs (O’Brien & Nohynek 2003). Our work has contributed to updated methods which also include data on OP sampling, as well as NP and OP detection of the bacterial pathogens *H. influenzae*, *M. catarrhalis*, *S. aureus* and respiratory viruses (Satzke et al. 2013). Our work has added to the literature on swab transport (Hare, Stubbs et al. 2010) and long-term storage (Hare, Smith-Vaughan & Leach 2011). Standard methods have also been used for pathogen identification and typing, and antibiotic susceptibility testing. We have contributed to methods of colony selection to detect multiple types (Hare et al. 2008), and supported the continued utility of the Quellung reaction for pneumococcal serotyping (Hare et al. 2009).

Our studies have included processing of BAL fluid collected during bronchoscopy. The same methods used for detection of upper airway carriage are applicable, with few exceptions. The use of STGGB requires a slight modification, using a concentrated preparation mixed in equal part with BAL fluid. In addition, semi-quantitative growth scores were correlated with CFU/mL in BAL fluid determined by serial dilution and quantitative colony counts of organisms, since quantitative measures are needed to define lower airway infection by excluding possible contamination from upper airway bacteria. Current evidence supports a threshold of $10^4$ CFU/mL BAL fluid which has been used in the next chapter.
CHAPTER 3

The Bacteriology of Bronchiectasis

In Australian Indigenous Children
CHAPTER 3: THE BACTERIOLOGY OF BRONCHIECTASIS IN AUSTRALIAN INDIGENOUS CHILDREN

3.1 Chapter overview

This chapter is concerned with the bacteriology of bronchiectasis unrelated to cystic fibrosis (CF) in Australian Indigenous children. The overall aims are to describe various aspects of lower airway bacteriology in these children, and to compare the bacterial species and strains from the lower airways with those detected in the nasopharynx. Data from three published studies (sections 3.2, 3.3 and 3.4) included in this chapter were collected from a cohort of children enrolled prospectively in our Bronchoscopy Study when they came to hospital for high resolution computed tomography (HRCT) and bronchoscopy. This cohort of children is described in the paper presented here as section 3.2. Pathogens identified from bronchoalveolar lavage (BAL) fluid provide the best representation of lower airway infections in conditions such as bronchiectasis. Collection of matching upper airway specimens, such as nasopharyngeal (NP) and oropharyngeal (OP) swabs, enables comparison of bacterial species and strains to determine the origin of pathogens associated with lower airway infections.

Section 3.2 consists of a published paper (Hare, Grimwood et al. 2010) from the Bronchoscopy Study, with preliminary results from 45 Indigenous children with HRCT-confirmed bronchiectasis. As so few Indigenous children undergoing bronchoscopy at Royal Darwin Hospital did not have bronchiectasis, and there were few non-Indigenous children undergoing this procedure, a comparison group of 30 non-Indigenous children without bronchiectasis from the Royal Children’s Hospital.
in Brisbane was included. This paper describes the bacteria isolated from NP and BAL specimens from these two groups. Multiple strains of each bacterial species were detected by selecting multiple colonies (Hare et al. 2008), and strain concordance between the upper and lower airways was assessed in both groups.

Culture of BAL fluid from Indigenous children with bronchiectasis revealed a multiplicity of pathogen species and strains far more diverse than matching NP cultures. Some of these organisms may originate from the oropharynx. Moreover, it became clear that nontypeable *Haemophilus influenzae* (NTHi), the most prevalent bacteria associated with bronchiectasis, needed to be differentiated from nonhaemolytic strains of the closely related (primarily commensal) *Haemophilus haemolyticus* (Murphy et al. 2007). We therefore used a PCR for *H. influenzae* protein D (*hpd*#3) to differentiate NTHi from presumptive *H. haemolyticus*. Apparent NTHi isolates from NP, OP and BAL specimens were tested and the results published (Hare, Binks et al. 2012), appearing here as Section 3.3.

Although this PCR study answered some questions, it raised others. Of 84 BAL specimens tested, 30 were culture-positive for NTHi infection (defined by semi-quantitative growth scores). However, both *H. influenzae* and presumptive *H. haemolyticus* were detected in 27% (8/30) of these specimens (at least one phenotypic NTHi isolate tested positive and one negative by *hpd*#3 PCR). Semi-quantitative counts are made on primary plates where *H. influenzae* and *H. haemolyticus* colonies cannot be differentiated, potentially leading to overestimation of *H. influenzae* density. We thus expanded on our previous work by using the *hpd*#3 PCR quantitatively (*hpd*#3 qPCR) to further define the presence of NTHi in
paediatric non-CF bronchiectasis. For 81 of the original 84 BAL specimens we compared: (i) the semi-quantitative NTHi culture-based definition with \( hpd\#3 \) qPCR estimation of \( H. \text{influenzae} \) infection; and (ii) both measures of bacterial density with total and differential cell counts to gauge the airway inflammatory response to infection. This work has been published (Hare, Marsh et al. 2013) and appears here as Section 3.4.

As more children have been recruited since the first paper was published (Hare, Grimwood et al. 2010), Section 3.5 presents updated results from the Bronchoscopy Study. NP and BAL culture results from 145 Indigenous children with bronchiectasis and 83 non-Indigenous children without bronchiectatic symptoms are presented. Strain concordance (pneumococcal serotypes and antibiotypes) between NP and BAL cultures from 24 Indigenous children with \( S. \text{pneumoniae} \) lower airway infection is presented. Sensitivities, specificities and predictive values of NP swabs for the detection of lower airway infection by respiratory bacteria in the 145 Indigenous children with non-CF bronchiectasis are also included.

Section 3.6 summarises the main findings from this work, which support the first two hypotheses of this thesis. That is, the bacteria associated with lower airway infection in Australian Indigenous children with non-CF bronchiectasis are the same as in other paediatric populations, and the evidence that these bacteria derive from the nasopharynx is compelling.
3.2 Journal article: *Respiratory bacterial pathogens in the nasopharynx and lower airways of Australian Indigenous children with bronchiectasis*

(following page)

*Tables I and II in this journal article are Tables 3.1 and 3.2 in the List of Tables.*
3.3 Journal article: *Culture and PCR detection of Haemophilus influenzae and Haemophilus haemolyticus in Australian Indigenous children with bronchiectasis*

(following page)

*Table 1 in this journal article is Table 3.3 in the List of Tables.*
Culture and PCR Detection of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Australian Indigenous Children with Bronchiectasis

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Child Health Division, Menzies School of Health Research, Charles Darwin University, Darwin, Northern Territory; Queensland Children's Medical Research Institute, The University of Queensland, Royal Children's Hospital, Brisbane, Queensland; Department of Infectious Diseases, Royal Children's Hospital, Brisbane, Queensland; Royal Darwin Hospital, Darwin, Northern Territory; and Department of Respiratory Medicine, Royal Children's Hospital, Brisbane, Queensland, Australia

A PCR for protein D (hpd#3) was used to differentiate nontypeable *Haemophilus influenzae* (NTHI) from *Haemophilus haemolyticus*. While 90% of nasopharyngeal specimens and 100% of lower-airway specimens from 84 Indigenous Australian children with bronchiectasis had phenotypic NTHI isolates confirmed as *H. influenzae*, only 39% of oropharyngeal specimens with nontypic NTHI had *H. influenzae*. The nasopharynx is therefore the preferred site for NTHI colonization studies, and NTHI is confirmed as an important lower-airway pathogen.

Nontypeable *Haemophilus influenzae* (NTHI) colonizes the upper airways, where it is an important cause of otitis media (10). It is also isolated frequently from the lower airways of adults with chronic obstructive pulmonary disease (COPD) (8) and children and adults with bronchiectasis (3, 4).

Accurate identification of NTHI is important, since nonhemolytic strains of the closely related (primarily commensal) *Haemophilus haemolyticus* may be misidentified as NTHI by phenotypic methods used in most clinical microbiology laboratories (9). Molecular detection techniques have revealed that 12 to 27% of nasopharyngeal isolates from healthy and otitis-prone children, which were initially identified by classical phenotypic methods as NTHI isolates (here referred to as phenotypic NTHI isolates), were actually *H. haemolyticus* (5, 9), while *H. influenzae* was not identified in middle ear fluid samples from children with acute otitis media (5). In contrast, *H. haemolyticus* comprised 40% of phenotypic NTHI sputum isolates from adults with COPD (9).

These findings raise important questions about NTHI as a true lower respiratory pathogen. We therefore investigated the proportion of *H. haemolyticus* isolates among phenotypic NTHI isolates in the upper and lower airways of Indigenous Australian children with bronchiectasis, a population with high rates of NTHI colonization and associated respiratory disease (2, 3, 12).

The Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research approved the study. Following written, informed parent/guardian consent, paired nasopharyngeal swabs and bronchoalveolar lavage (BAL) fluid specimens were collected from a convenience sample of 84 (62% male) Indigenous children aged 5 to 155 (median, 27) months who were undergoing routine diagnostic evaluation following radiographic confirmation of bronchiectasis at the Royal Darwin Hospital between July 2007 and December 2010. Oropharyngeal swabs were also collected from 56 of these children. Specimens were stored and cultured using standard microbiologic methods (3). Lower-airway infection was defined by a semiquantitative growth score of ≥4, which correlated with >10^4 CFU/ml BAL fluid, as determined by serial dilution and quantitative counts (3).

Phenotypic NTHI isolates were identified by morphology, requirement for X and V growth factors, and failure to react with Phadebact (Bactus AB, Sweden) antisera specific for *H. influenzae* type b or types a and c through f. Up to 4 colonies (including any with differing morphology) were isolated from each culture-positive specimen and tested using PCR. Confirmation of phenotypic NTHI isolates was performed using defined DNA extraction methods (12) and a TaqMan-based, real-time PCR assay targeting protein D (hpd#3), which discriminates between *H. influenzae* and *H. haemolyticus* isolates (1, 13). Phenotypic NTHI isolates returning negative PCR results were considered to be *H. haemolyticus*, since the only other X- and V-factor-dependent *Haemophilus* species (*H. aegyptius*, an important cause of conjunctivitis) has a different appearance, requires additional growth factors, and is unlikely to be cultured from these sites (6).

Table 1 shows the numbers, proportions, and distribution of phenotypic NTHI, hpd#3 PCR-confirmed *H. influenzae*, presumptive *H. haemolyticus*, and concurrent *H. influenzae* and *H. haemolyticus* in the 224 specimens collected from the upper and lower airways. A total of 214 isolates from 108 phenotypic NTHI-positive specimens (an average of 1.8 colonies isolated per nasopharyngeal and oropharyngeal swab and 2.4 colonies per BAL specimen) were tested. Most nasopharyngeal (87%) and BAL fluid (88%) isolates were confirmed as *H. influenzae*, but almost two-thirds (65%) of oropharyngeal isolates were presumptive *H. haemolyticus*. *H. influenzae* and *H. haemolyticus* were isolated concurrently from 10% (4/42) of nasopharyngeal swabs, 17% (6/36) of oropharyngeal swabs, and 27% (8/30) of BAL fluid cultures from children with phenotypic NTHI carriage or lower-airway infection.

This study shows that, similar to healthy and otitis-prone Western
Australian children (5), most phenotypic NTHI nasopharyngeal isolates from Indigenous children with bronchiectasis were confirmed as *H. influenzae*. In contrast, many apparent NTHI isolates from oropharyngeal swabs were *H. haemolyticus* in our study population. Previous oropharyngeal culture-based studies of *H. influenzae* may have therefore overestimated NTHI carriage, and our data instead support the nasopharynx as the preferred site for *H. influenzae* carriage studies in children. Such studies are important to monitor antimicrobial resistance and detect changes in pharyngeal biota that may result from antibiotic administration or vaccination.

Quantifying pathogens in BAL fluid helps adjust for upper-airway contamination during bronchoscopy. Our finding that 100% of BAL specimens with phenotypic NTHI lower-airway infection (>10⁴ CFU/ml BAL fluid) were PCR positive for *H. influenzae* confirms NTHI as a lower-airway pathogen. However, the role of *H. haemolyticus* is unclear. Prior studies report that *H. haemolyticus* is rarely found in sterile sites, including middle ear fluid, or associated with clinically defined infections (5, 7, 9, 11). While our findings suggest that *H. haemolyticus* has a propensity for the oropharynx, without specific molecular detection and quantification, we cannot determine whether its presence in lower-airway cultures represents upper-airway contamination or a pathogenic role.

In conclusion, we have used *H. influenzae*-specific PCR to reaffirm the importance of NTHI as a lower-airway pathogen in Australian Indigenous children with bronchiectasis. In addition, we have shown that the nasopharynx, rather than the oropharynx, is the preferred site for NTHI carriage studies in this population.

**ACKNOWLEDGMENTS**

We thank the children and their caregivers for participating in the study, Menzies staff (Gabrielle MacCallum, Kobi Schutz, and Susan Pizzutto) and Royal Darwin Hospital staff for assistance with specimen collection, Menzies laboratory staff (Vanya Hampton, Joanna Bugg, Estelle Carter, and Peter Christensen) for assistance with laboratory processing, and Robyn Marsh for helpful comments.

This study was supported by NHMRC project grant 545223.

**REFERENCES**


**TABLE 1** Culture and PCR results for phenotypic NTHI isolates from nasopharyngeal and oropharyngeal swabs and bronchoalveolar lavage fluid specimens from 84 Australian Indigenous children with bronchiectasis

<table>
<thead>
<tr>
<th>Isolate source</th>
<th>No. of specimens</th>
<th>Phenotypic NTHI confirmed by PCR as <em>H. influenzae</em></th>
<th>Presumptive <em>H. haemolyticus</em></th>
<th>Concurrent <em>H. influenzae</em> and presumptive <em>H. haemolyticus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>NP swab</td>
<td>84</td>
<td>42 (50)</td>
<td>38 (45)</td>
<td>8 (10)</td>
</tr>
<tr>
<td>OP swab</td>
<td>56</td>
<td>36 (64)</td>
<td>14 (25)</td>
<td>28 (50)</td>
</tr>
<tr>
<td>BAL fluid</td>
<td>84</td>
<td>303 (36)</td>
<td>30 (36)</td>
<td>8 (10)</td>
</tr>
<tr>
<td>Total</td>
<td>224</td>
<td>108 (48)</td>
<td>82 (37)</td>
<td>44 (20)</td>
</tr>
</tbody>
</table>

* NP, nasopharyngeal; OP, oropharyngeal; BAL, bronchoalveolar lavage; NTHI, nontypeable *Haemophilus influenzae*.

Specimens with phenotypic NTHI lower-airway infection (>10⁴ CFU/ml BAL fluid).

TABLE 1 Culture and PCR results for phenotypic NTHI isolates from nasopharyngeal and oropharyngeal swabs and bronchoalveolar lavage fluid specimens from 84 Australian Indigenous children with bronchiectasis

<table>
<thead>
<tr>
<th>Isolate source</th>
<th>No. (%) of specimens with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phenotypic NTHI</td>
</tr>
<tr>
<td></td>
<td>confirmed by PCR as</td>
</tr>
<tr>
<td></td>
<td><em>H. influenzae</em></td>
</tr>
<tr>
<td></td>
<td>Presumptive <em>H. haemolyticus</em></td>
</tr>
<tr>
<td></td>
<td>Concurrent <em>H. influenzae</em> and presumptive <em>H. haemolyticus</em></td>
</tr>
</tbody>
</table>

* NP, nasopharyngeal; OP, oropharyngeal; BAL, bronchoalveolar lavage; NTHI, nontypeable *Haemophilus influenzae*.

Specimens with phenotypic NTHI lower-airway infection (>10⁴ CFU/ml BAL fluid).
3.4 Journal article: *Quantitative PCR confirms culture as the gold standard for detection of lower airway infection by nontypeable *Haemophilus influenzae* in Australian Indigenous children with bronchiectasis*

(following page)

*Tables 1 and 2 in this journal article are Tables 3.4 and 3.5 in the List of Tables, and Figure 1 is Figure 3.1 in the List of Figures.*
3.5 Updated results

The most recent results from the Bronchoscopy study are presented in Table 3.6, with data from 145 Indigenous children with HRCT-confirmed bronchiectasis (enrolled up to June 2013) compared with 83 non-Indigenous children without bronchiectasis. The Indigenous children were all enrolled at RDH; only nine (results not included here) did not have HRCT-confirmed bronchiectasis. Of 12 non-Indigenous children enrolled at RDH, six had HRCT-confirmed bronchiectasis and six did not; the latter are included in Table 3.6. At RCH, bacteriology results are available for 77 children without bronchiectasis; 10 children with bronchiectasis are not included in Table 3.6.

Comparison with Table 3.1 (section 3.2) reveals a decline in NP carriage and lower airway infection for all three of the main pathogens in Indigenous children with bronchiectasis since the preliminary analysis published in 2010. It is possible that new pneumococcal vaccines introduced in 2009 and 2011 may have affected carriage of *S. pneumoniae* and *H. influenzae*; this is a topic for future analysis. However, there has been a particular decline in carriage of *M. catarrhalis* (not in any vaccine) documented in the NT since 2005, for reasons that remain unclear.

In children without bronchiectasis, NP carriage is similar to that previously reported (Hare, Grimwood et al. 2010) for *S. pneumoniae* and *M. catarrhalis*, but lower airway infection is lower. In contrast, NP carriage and lower airway infection are higher (both 16%) than previously reported for NTHi (7% and 3%, respectively). These rates remain significantly lower than those for children with bronchiectasis (47% and 29%, respectively; P<0.05 for both comparisons).
Table 3.6. Major respiratory bacterial pathogens isolated from nasopharyngeal (NP) and bronchoalveolar lavage (BAL) fluid cultures from Indigenous children with bronchiectasis and non-Indigenous children without bronchiectasis.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Indigenous children with bronchiectasis (n=145)</th>
<th>Non-Indigenous children without bronchiectasis (n=83)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP swab, n (%)</td>
<td>BAL fluid, any growth, n (%)</td>
</tr>
<tr>
<td>NTHi</td>
<td>68 (47)</td>
<td>94 (65)</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>52 (36)</td>
<td>49 (34)</td>
</tr>
<tr>
<td>M. catarrhalis</td>
<td>38 (26)</td>
<td>32 (22)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>23 (16)</td>
<td>11 (8)</td>
</tr>
</tbody>
</table>

CFU, colony forming units; NTHi, nontypeable *H. influenzae*. 
Many (62%) of the children enrolled at RCH had protracted bacterial bronchitis (PBB), thought to be a precursor for bronchiectasis (Chang, Redding & Everard 2008), so it is unsurprising that many had lower airway bacterial infections. NP carriage and lower airway infection were higher in the PBB children than other non-Indigenous children without bronchiectasis, and \textit{S. pneumoniae} was the dominant pathogen (Table 3.7).

\begin{table}[h]
\centering
\begin{tabular}{llll}
\hline
 & Non-Indigenous children & Non-Indigenous children \\
 & with PBB (n=47) & without PBB (n=36) \\
\hline
NP swab & & & \\
>10^4 CFU/mL & & & \\
\textit{S. pneumoniae} & 30\% & 23\% & 11\% & 11\% \\
\textit{NTHi} & 21\% & 21\% & 8\% & 8\% \\
\textit{M. catarrhalis} & 21\% & 11\% & 8\% & 0\% \\
\textit{S. aureus} & 28\% & 2\% & 22\% & 3\% \\
\hline
\end{tabular}
\caption{Major respiratory bacterial pathogens isolated from nasopharyngeal (NP) and bronchoalveolar lavage (BAL) fluid cultures from non-Indigenous children with and without protracted bacterial bronchitis (PBB).}
\end{table}

Concordance results have also been updated for \textit{S. pneumoniae} and \textit{M. catarrhalis} from Indigenous children with bronchiectasis. Updated results will be available for \textit{H. influenzae} once HRM-typing, which is faster and cheaper than PCR-ribotyping (Pickering et al. 2014), becomes routine in our laboratory. The same strains (same serotype and antibiotype) were found in 100\% of cases with \textit{S. pneumoniae} lower airway infection and \textit{S. pneumoniae} also isolated from NP swabs (Table 3.8).
Table 3.8. Serotypes isolated from 24 paired nasopharyngeal (NP) swab and bronchoalveolar lavage (BAL) fluid cultures associated with *Streptococcus pneumoniae* lower airway infection.

<table>
<thead>
<tr>
<th>Pair #</th>
<th>Antibiotics received in 2 weeks prior to bronchoscopy</th>
<th>Serotypes in NP swab</th>
<th>Serotypes in BAL fluid (&gt;10⁴ CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Azithromycin</td>
<td>21 (sens)</td>
<td>21 (sens), 10A (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>2</td>
<td>Azithromycin</td>
<td></td>
<td>31 (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>5</td>
<td>Azithromycin</td>
<td></td>
<td>7F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>9</td>
<td>Azithromycin</td>
<td></td>
<td>9N (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>10</td>
<td>Azithromycin</td>
<td>22F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>22F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>14</td>
<td>Azithromycin</td>
<td>11A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>11A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>15</td>
<td>Azithromycin</td>
<td></td>
<td>22F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>16</td>
<td>Azithromycin</td>
<td>17F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>17F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>18</td>
<td>Azithromycin</td>
<td>15A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>15A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>21</td>
<td>Azithromycin</td>
<td>15A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>15A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>23</td>
<td>Azithromycin</td>
<td>19A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>19A (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>24</td>
<td>Azithromycin</td>
<td>15C (Azi&lt;sup&gt;R&lt;/sup&gt;), 17F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>15B (Azi&lt;sup&gt;R&lt;/sup&gt;), 15C (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>4</td>
<td>Azithromycin, amoxicillin</td>
<td>6A (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>6A (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>11</td>
<td>Amoxicillin</td>
<td>23F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>23F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>13</td>
<td>Cefotaxime, benzylpenicillin</td>
<td></td>
<td>6C (Pen&lt;sup&gt;NS&lt;/sup&gt;), 19F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>6C (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>6C (Azi&lt;sup&gt;R&lt;/sup&gt;), 19F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>6</td>
<td>None</td>
<td>16F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>16F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>7</td>
<td>None</td>
<td>16F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>16F (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>8</td>
<td>None</td>
<td>9N (sens)</td>
<td>9N (sens)</td>
</tr>
<tr>
<td>12</td>
<td>None</td>
<td>19A (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>19A (Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>17</td>
<td>None</td>
<td>15A (sens)</td>
<td>15A (sens)</td>
</tr>
<tr>
<td>19</td>
<td>None</td>
<td>7B (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
<td>7B (Azi&lt;sup&gt;R&lt;/sup&gt;Pen&lt;sup&gt;NS&lt;/sup&gt;)</td>
</tr>
<tr>
<td>20</td>
<td>None</td>
<td>23F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>23F (Azi&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td>22</td>
<td>None</td>
<td>23A (sens)</td>
<td>23A (sens)</td>
</tr>
</tbody>
</table>

Azi<sup>R</sup>, azithromycin resistant (MIC >0.5 mg/L); Pen<sup>NS</sup>, penicillin nonsusceptible (MIC >0.06 mg/L); sens, susceptible to penicillin and azithromycin.
Similarly in 12 Indigenous children with *M. catarrhalis* lower airway infection and NP carriage, all 12 had beta-lactamase positive strains isolated from both sites. Combining this data with the original 15/16 concordant NTHi strains, the evidence for lower airway pathogens originating in the nasopharynx is compelling.

As described in section 3.2.4, a high proportion of BAL specimens from Indigenous children contained more than one bacterial strain: 67% for NTHi lower airway infection, 25% for *S. pneumoniae*, and 22% for *M. catarrhalis*. Many of these strains were not detected in the corresponding upper airway specimens. This suggests recurrent aspiration of NP specimens, with strains previously aspirated persisting in the lower airways but apparently cleared from the upper airways. Furthermore, in some cases lower airway infection occurred in the absence of detection of the same species in the upper airways. This may occur if NP carriage is eliminated (e.g. by antibiotic treatment) while lower airway infection persists, as appeared to be the case (for OP not NP carriage) in some CF children (Armstrong et al. 1996).

The sensitivity and specificity of NP swabs for lower airway infection in our Bronchoscopy Study were moderate to high (78-100% and 66-87% respectively), while positive predictive values (PPVs) were low and negative predictive values (NPVs) were high (Table 3.9). It has been noted that predictive values are affected by prevalence (Rosenfeld et al. 1999). In Table 3.8 it can be seen that PPVs decrease and NPVs increase as prevalence (of carriage and lower airway infection) decreases from highest (NTHi) to lowest (*S. aureus*).
Table 3.9. Sensitivity, specificity and positive and negative predictive values (PPV and NPV) of nasopharyngeal swabs for the detection of lower airway infection in 145 Indigenous children with bronchiectasis.

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTHi</td>
<td>79%</td>
<td>66%</td>
<td>49%</td>
<td>88%</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>78%</td>
<td>72%</td>
<td>35%</td>
<td>95%</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>92%</td>
<td>80%</td>
<td>32%</td>
<td>99%</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>100%</td>
<td>87%</td>
<td>17%</td>
<td>100%</td>
</tr>
</tbody>
</table>

NTHi, nontypeable *H. influenzae*.

3.6 Summary of bacteriology

The study described in this chapter is the first to compare upper and lower airway bacteriology in Indigenous children with non-CF bronchiectasis. The main lower airway pathogens were the same as those found in other paediatric populations, predominantly NTHi followed by *S. pneumoniae* and *M. catarrhalis*, supporting our first hypothesis. *S. aureus* infection was uncommon, while *Pseudomonas*, which is mostly found in older children and adults with non-CF bronchiectasis (King et al. 2007; Steinfort et al. 2008), was rarely seen. We also confirmed the importance of NTHi in NP carriage and lower airway infection using *hpd#3* PCR to differentiate NTHi from presumptive *H. haemolyticus*.

By comparing NP, OP and BAL cultures we found that the nasopharynx, rather than the oropharynx, is the preferred sampling site for carriage studies of the three main bacterial pathogens in Indigenous children with non-CF bronchiectasis. Furthermore, in cases of lower airway infection where the same bacterial species was found in NP
swabs, it was almost invariably the same strain. This strongly supports our second hypothesis that aspiration of NP secretions contributes to lower airway infection in children with non-CF bronchiectasis. Although NP swab culture results may not be good predictors of lower airway infection in the individual child, bacteria isolated from NP swabs in this paediatric population give a good indication of strains (including antimicrobial resistance profiles) likely to cause lower airway infection.

The effects of antibiotics on NP carriage and lower airway infection have been briefly mentioned in this chapter, and will now be explored in Chapter 4.
CHAPTER 4

Antibiotic Therapy and its Impact
On Airway Bacteriology
CHAPTER 4: ANTIBIOTIC THERAPY AND ITS IMPACT ON AIRWAY BACTERIOLOGY

4.1 Chapter overview

The overall aim of this chapter is to describe the impact of antibiotics on airway bacteriology in Australian Indigenous children with bronchiectasis unrelated to cystic fibrosis (CF). As described in Chapter 1, antibiotics are frequently used to treat respiratory exacerbations. In some children, antibiotics are used for long-term prophylaxis in patients with chronic lung diseases such as bronchiectasis. However, bacterial resistance is a major concern. Although many studies only report clinical outcomes, treatment failure may be due to the presence of pathogens resistant to the antibiotic used. Microbiological investigations are needed to determine the pathogens involved in infection and their antibiotic resistance profiles. Data from two published papers (sections 4.2 and 4.3) and one unpublished study (section 4.4) are included in this chapter to determine whether or not macrolide therapy is associated with increased macrolide resistance.

Section 4.2 describes the impact of recent antibiotics (administered <2 weeks before specimen collection) on nasopharyngeal (NP) carriage of and lower airway infection by respiratory bacterial pathogens (Hare, Leach et al. 2012). Of 117 children enrolled in the cross-sectional prospective Bronchoscopy Study from July 2007 to September 2011, 104 were Indigenous and had bronchiectasis confirmed by high resolution computed tomography (HRCT). These 104 children were included in this analysis; 39 children had received macrolide antibiotics, 26 children had received beta-lactam antibiotics, and 39 children had received no antibiotics in the 2 weeks prior to their
bronchoscopy procedure. NP carriage and lower airway infection in the two groups of children who had received antibiotics were compared to that in the children who had received no antibiotics. Antimicrobial resistance in the upper and lower airways of children who had received antibiotics (macrolide or beta-lactam) was compared to that of children who had not received that class of antibiotics. Pneumococcal strains (defined by serotype and antibiotype) from NP and bronchoalveolar lavage (BAL) specimens from children with *Streptococcus pneumoniae* lower airway infection were also compared.

The paper in section 4.3 examines the impact of long-term azithromycin treatment on NP carriage and antibiotic resistance of respiratory bacterial pathogens in the longitudinal Bronchiectasis Observational Study (BOS) (Hare, Singleton et al. 2013). This study included multiple swabs per child from 41 Alaska Native as well as 79 Australian Indigenous children, and there were interesting similarities and differences in carriage and resistance between the two groups. While many Australian children received long-term azithromycin therapy, Alaskan children did not. Australian children were grouped by frequency of azithromycin use during the study period; those who received no azithromycin in the 2 weeks preceding any study visit with swab collection (Azi-None), those who received azithromycin preceding 1-50% of study visits (Azi-Infequent), and those who received azithromycin at 51-100% of study visits (Azi-Frequent). NP carriage and antibiotic (macrolide and beta-lactam) resistance in the three groups were compared.

A third study, the Bronchiectasis Intervention Study (BIS), was a randomised controlled trial (RCT) of azithromycin versus placebo to reduce pulmonary
exacerbations. This trial included 3-6 monthly swab collection from 47 Maori and Pacific Islander children from New Zealand as well as 42 Australian Indigenous children. The study found a significant reduction in exacerbations in the azithromycin compared to the placebo group (Valery et al. 2013). Primary microbiological outcomes were reported (carriage and resistance of respiratory pathogens isolated from swabs collected at enrolment and at the end of the study), and children in the azithromycin group developed significantly higher carriage of azithromycin-resistant bacteria than those receiving placebo (Valery et al. 2013). A preliminary comparison of microbiological outcomes in the Australian BIS children (azithromycin and placebo groups), using intention-to-treat (ITT) and according-to-protocol (ATP) analyses, is included in section 4.4. Future work will include detailed analysis and publication of these results.

The main findings from this work on the impact of recent and long-term antibiotics are summarised in section 4.5.
4.2 Journal article: *Impact of recent antibiotics on nasopharyngeal carriage and lower airway infection in Indigenous Australian children with non-cystic fibrosis bronchiectasis*

(next page)

*Tables 1, 2 and 3 in this journal article are Tables 4.1, 4.2 and 4.3 in the List of Tables.*
4.3 Journal article: *Longitudinal nasopharyngeal carriage and antibiotic resistance of respiratory bacteria in Indigenous Australian and Alaska Native children with bronchiectasis*

(next page)

*Tables 1, 2, 3 and 4 in this journal article are Tables 4.4, 4.5, 4.6 and 4.7 in the List of Tables. Figures 1, 2 and 3 are Figures 4.1, 4.2 and 4.3 in the List of Figures.*
Longitudinal Nasopharyngeal Carriage and Antibiotic Resistance of Respiratory Bacteria in Indigenous Australian and Alaska Native Children with Bronchiectasis

Kim M. Hare1,4, Rosalyn J. Singleton2,3, Keith Grimwood4,5, Patricia C. Valery1, Allen C. Cheng6,7, Peter S. Morris1,8, Amanda J. Leach1, Heidi C. Smith-Vaughan1, Mark Chatfield1, Greg Redding9,10, Alisa L. Reasonover3, Gabrielle B. McCallum1, Lori Chikoyak11, Malcolm I. McDonald12, Ngiare Brown13,14, Paul J. Torzillo15,16,17, Anne B. Chang1,18

Abstract

Background: Indigenous children in Australia and Alaska have very high rates of chronic suppurative lung disease (CSDL)/bronchiectasis. Antibiotics, including frequent or long-term azithromycin in Australia and short-term beta-lactam therapy in both countries, are often prescribed to treat these patients. In the Bronchiectasis Observational Study we examined over several years the nasopharyngeal carriage and antibiotic resistance of respiratory bacteria in these two PCV7-vaccinated populations.

Methods: Indigenous children aged 0.5–8.9 years with CSLD/bronchiectasis from remote Australia (n = 79) and Alaska (n = 41) were enrolled in a prospective cohort study during 2004–8. At scheduled study visits until 2010 antibiotic use in the preceding 2-weeks was recorded and nasopharyngeal swabs collected for culture and antimicrobial susceptibility testing. Analysis of respiratory bacterial carriage and antibiotic resistance was by baseline and final swabs, and total swabs by year.

Results: Streptococcus pneumoniae carriage changed little over time. In contrast, carriage of Haemophilus influenzae declined and Staphylococcus aureus increased from 0% in 2005–6 to 23% in 2010 in Alaskan children; these changes were associated with increasing age. Moraxella catarrhalis carriage declined significantly in Australian, but not Alaskan, children (from 64% in 2004–6 to 11% in 2010). While beta-lactam antibiotic use was similar in the two cohorts, Australian children received more azithromycin. Macrolide resistance was significantly higher in Australian compared to Alaskan children, while H. influenzae beta-lactam resistance was higher in Alaskan children. Azithromycin use coincided significantly with reduced carriage of S. pneumoniae, H. influenzae and M. catarrhalis, but increased carriage of S. aureus and macrolide-resistant strains of S. pneumoniae and S. aureus (proportion of carriers and all swabs), in a 'cumulative dose-response' relationship.

Conclusions: Over time, similar (possibly age-related) changes in nasopharyngeal bacterial carriage were observed in Australian and Alaskan children with CSLD/bronchiectasis. However, there were also significant frequency-dependent differences in carriage and antibiotic resistance that coincided with azithromycin use.
Introduction

Indigenous children in Australia, Alaska and New Zealand have amongst the world’s highest recorded rates of chronic suppurative lung disease (CSLD), including bronchiectasis unrelated to cystic fibrosis (CF) [1]. Antibiotics play a key role in managing persistent respiratory symptoms and acute exacerbations associated with bronchiectasis [2]. However, few longitudinal data exist on the impact of antibiotics upon the airway bacteriology of these patients. This is an important knowledge gap as Indigenous children have high rates of nasopharyngeal bacterial carriage [3] and micro-aspiration of upper airway bacteria may contribute to both the pathogenesis and on-going morbidity of bronchiectasis [4].

Based upon the clinical impression that patients receiving azithromycin have fewer acute respiratory exacerbations, there is anecdotal evidence that many clinicians in Australia routinely prescribe azithromycin (frequently long-term) for children with bronchiectasis, while in Alaska such practice is uncommon. In contrast, both sites utilize short-term intermittent therapy with beta-lactam antibiotics (e.g. amoxicillin or amoxicillin-clavulanate) for acute pulmonary exacerbations associated with bronchiectasis. The Bronchiectasis Observational Study was established in Australia and Alaska to study prospectively the clinical course of CSLD/bronchiectasis in Indigenous children [1]. Using the opportunity thus provided, we examined the nasopharyngeal carriage of respiratory bacteria and their antibiotic resistance patterns in children. We hypothesised that the two populations would differ in their nasopharyngeal carriage of potential respiratory bacterial pathogens and antibiotic resistance in these organisms as a result of differences in prescribing practices between the two settings.

Methods

Subjects

Children aged 0.5–8.9 years of age with a diagnosis of non-CF bronchiectasis (confirmed on high-resolution computerized tomography) or chronic (>3-months) daily wet (or productive) cough were enrolled in a prospective cohort study during 2004–8 [1]. Children were recruited opportunistically while attending an outpatient clinic or in hospital or undergoing bronchoscopy for suspected CSLD/bronchiectasis or for respiratory exacerbations. Subsequently, enrolled children were examined and nasopharyngeal swabs collected by research staff at scheduled study visits until 2010. These visits were scheduled quarterly in Australia (to ensure at least one annual review in highly mobile enrolled children) and annually in Alaska. Such visits were planned independently of the children’s health status. Only visits where swabs were collected were included in this analysis. Children’s vaccination status at baseline was recorded from immunisation registers. Antibiotic use in the 2-weeks preceding swab collection (to capture recent antibiotic events and allow comparison with other studies in this population [5] and studies using parent recall) was recorded from clinic notes.

Ethics statement

The study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research in Australia (HREC 04/46) and the Alaska Area Institutional Review Board (which acts under the Indian Health Service Institutional Review Board FWA number 0008994). At enrolment written informed consent was obtained from the carer of each child and when appropriate assent was also provided by the older children in the cohort. The process of obtaining consent, and the information sheets and consent forms used were approved by the respective ethics bodies.

Specimen collection and laboratory testing

Nasopharyngeal swabs collected at study visits were transported and processed according to published methods [3,6], unless otherwise noted. Swabs stored in skim-milk tryptone glucose glycerol broth at −80°C were thawed and 10 µL aliquots plated on selective media and incubated overnight at 37°C and 5% CO2. In Australia, four colonies each (including any with differing morphologies) of presumptive Streptococcus pneumoniae and Haemophilus influenzae, two of Moraxella catarrhalis and one of Staphylococcus aureus were isolated and identified using standard methods [5]. In Alaska, multiple colonies were picked when differing morphologies were observed. When multiple isolates underwent antibiotic susceptibility testing, the child was reported as carrying a resistant strain if one or more of the isolates proved resistant. S. pneumoniae serotypes were determined by the Quellung reaction using antiserum from Statens Serum Institute (Denmark).

Antimicrobial sensitivities for S. pneumoniae, H. influenzae and S. aureus isolates were determined by disk diffusion (Australia [7]; Alaska [8]). Minimum inhibitory concentrations (MICs) were determined for resistant S. pneumoniae and H. influenzae isolates using Etest strips (AB bioMérieux, France) in Australia. In Alaska, erythromycin and penicillin MICs for S. pneumoniae were determined by microbroth dilution and amoxicillin MICs for H. influenzae were determined using Etest strips. MIC breakpoints from the European Committee on Antimicrobial Susceptibility

PLOS ONE | www.plosone.org August 2013 | Volume 8 | Issue 8 | e70478


Received March 25, 2013; Accepted June 20, 2013; Published August 5, 2013

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Funding: This work was supported by the National Health and Medical Research Council of Australia (http://www.nhmrc.gov.au/), National Health and Medical Research Council (NHMRC) project grants 389837 (clinical component) and 545223 (microbiology component); and Centre for Research Excellence in Lung Health of Aboriginal and Torres Strait Islanders (grant 1040830). The Alaskan study site received funding from the National Institutes of Health (http://www.nih.gov/) and the National Heart Lung and Blood Institute (http://www.nhlbi.nih.gov/). KMH is supported by the NHMRC Gustav Nossal Postgraduate Scholarship 1038072 and the Australian Academy of Science’s (http://science.org.au/) Douglas and Lola Douglas Scholarship; PCV is supported by an Australian Research Council (http://www.arc.gov.au/) Future Fellowship No.100100511; ACC and HSV are supported by NHMRC Career Development Fellowships 1009945 and 1024175; AJL is supported by the NHMRC Elizabeth Blackburn Research Fellowship 1020561; and ABC is supported by a NHMRC Practitioner Fellowship 545216.

The findings and conclusions in this article are those of the authors and do not necessarily represent the official position of the funding agencies. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Testing (EUCAST, http://www.eucast.org) were used to define resistance for *S. pneumoniae* (penicillin resistance MIC > 2 mg/L, intermediate resistance MIC > 0.06–2 mg/L; macrolide [azithromycin or erythromycin] resistance MIC > 0.5 mg/L) and *H. influenzae* (ampicillin resistance MIC > 1 mg/L; macrolide resistance MIC > 4 mg/L; intermediate resistance MIC > 0.12–4 mg/L). A nitrocephin-based test detected beta-lactamase activity in *H. influenzae* and *M. catarrhalis* isolates.

**Data analysis**

Carriage data are reported as the proportion of children at baseline (first swab) and as they left the study (last swab), and also presented graphically as the proportion of total swabs collected per study year. As storage of 29 swabs from Central Australia during 2004-6 was at ~20°C, which is suboptimal for recovery of *H. influenzae* [9], these samples were excluded from analyses involving this pathogen and the first subsequent swab for each child was included for *H. influenzae* baseline data. Also, as only 20 swabs were collected from both cohorts in 2004-5, their data were combined with those from 2006.

Antibiotic resistance data are reported as the proportion of carriers as children entered and left the study, proportion of total isolates for each bacterial species, and graphically as the proportion of all positive swabs by study year. Multiple strains detected in the same swab (differing serotype or antibiotype for *S. pneumoniae* and *H. influenzae*, differing beta-lactamase status for *M. catarrhalis*) were counted as additional isolates where results for total isolates are presented.

Confidence intervals (CIs) were calculated using the exact binomial method for first and last swabs from each child and where numbers were small (<5), and otherwise adjusted for repeated sampling using the clustered sandwich estimator for estimating the variance-covariance matrix, implemented as vce(cluster id) in Stata. We used Stata’s logistic command for bivariate and multivariate analyses, and nptrend to test for trend across ordered groups. Statistical tests were performed using Stata 12 (College Station, Texas).

To investigate the cumulative effect of repeated and sustained long-term azithromycin exposure on bacterial carriage and resistance, Australian children were divided into three groups based on frequency of azithromycin use during the study period: Azi-None = no azithromycin in the 2-weeks preceding swab collection at any of the study visits; Azi-Infrequent = azithromycin preceding 1–30% of study visits; Azi-Frequent = azithromycin preceding >50% of study visits. Tables and figures are presented as Supporting Information.

**Results**

**Subjects**

A total of 120 children were enrolled and 597 swabs collected (Table 1). At enrolment, 24% of Alaskan and 30% of Australian children had respiratory exacerbations. At subsequent visits the numbers were 8% and 17% respectively. The mean number of swabs collected per child per year enrolled (person-years of follow-up) was 2.5 in Australia and 1.3 in Alaska. Most (88%) children had received ≥3 doses of the 7-valent pneumococcal conjugate vaccine (PCV7). In addition, two Australian children received ≥1 dose of the pneumococcal *Haemophilus influenzae* protein D conjugate vaccine introduced in 2009. No Alaskan children received PCV13, introduced in 2010. Australian children were more likely to receive azithromycin in the 2-weeks preceding swab collection, while beta-lactam antibiotic use was similar in the two cohorts (Table 1). There were no clear temporal trends in antibiotic use; peaks were observed in beta-lactam antibiotic and azithromycin use in 2007 and 2008 (preceding 18% and 55% of swabs collected respectively) in Australian children, and in beta-lactam antibiotic use in Alaskan children in 2008 (preceding 25% of swabs collected).

**Nasopharyngeal carriage over time**

Nasopharyngeal bacterial carriage as children entered and left the study is shown in Table 2. Australian children were at significantly lower risk than Alaskan children of *M. catarrhalis* carriage at the study’s end, and this finding persisted after adjusting for carriage at baseline. When all data were analysed as a proportion of swabs by year (Figure 1), *S. pneumoniae* carriage remained relatively stable, *H. influenzae* carriage declined in both Australian (nptrend P = 0.007) and Alaskan (P = 0.033) children, *M. catarrhalis* carriage in Australian children showed a marked decline from 64% (95% CI 47–81) in 2004-6 to 11% (95% CI 2–21) in 2010 (P < 0.001), and *S. aureus* carriage in Alaskan children increased from 0% (95% CI 0–9) in 2005-6 to 23% (95% CI 6–40) in 2010 (P = 0.010).

**Association between frequency of azithromycin use and carriage**

The number of children and time in the study were comparable when Australian children were grouped by azithromycin exposure

---

| Table 1. Enrollment, swab collection, vaccination and antibiotic use in Australian Indigenous and Alaska Native children. |
|---|---|---|---|
| **Children enrolled** | **Australia** | **Alaska** | **Total** |
| 79 | 41 | 120 |
| **Median age at enrolment in years (range)** | 2.7 (0.8–8.9) | 2.8 (0.5–7.9) |
| **Person-years of follow-up** | 179 | 121 | 300 |
| **Median time in study in years (range)** | 2.2 (0-5.8) | 3.4 (0–4.8) |
| **Number of children who had received ≥3 doses of 7-valent PCV* at enrolment** | 71 (90%) | 35 (85%) | 106 (88%) |
| **Swabs collected** | 443 | 154 | 597 |
| **Median number of swabs collected (range)** | 5.1 (1–15) | 4.1 (1–11) |

| **Number of swabs where children received antibiotics in the 2-weeks preceding collection:** |
| **Azithromycin** | 192 (43%) | 1 (<1%) | 193 (32%) |
| **Beta-lactam antibiotics** | 44 (10%) | 14 (9%) | 58 (10%) |

*Pneumococcal conjugate vaccine.

DOI: 10.1371/journal.pone.0070478.t001
Figure 1. Pathogen carriage (proportion of swabs) by study year in Australian and Alaskan children.
doi:10.1371/journal.pone.0070478.g001

Table 2. Nasopharyngeal carriage of respiratory bacteria from Australian and Alaskan children at baseline and end of study.

<table>
<thead>
<tr>
<th></th>
<th>First swab for each child</th>
<th>Last swab for each child¹</th>
<th>OR (95% CI)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Australia</td>
<td>Alaska</td>
<td>Australia</td>
</tr>
<tr>
<td>Children enrolled</td>
<td>79 (39%)</td>
<td>41 (54%)</td>
<td>76 (37%)</td>
</tr>
<tr>
<td>Median age at study visit (years)</td>
<td>2.7 (0.8–8.9)</td>
<td>2.8 (0.5–7.9)</td>
<td>5.4 (1.7–13.0)</td>
</tr>
<tr>
<td>Number of male children</td>
<td>45 (57%)</td>
<td>22 (34%)</td>
<td>42 (55%)</td>
</tr>
</tbody>
</table>

Nasopharyngeal carriage; n (%, 95% CI)

<table>
<thead>
<tr>
<th></th>
<th>First swab</th>
<th>Last swab</th>
<th>OR (95% CI)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pneumoniae</td>
<td>47 (59, 48–70)</td>
<td>28 (68, 52–82)</td>
<td>1.45 (0.66–3.21)</td>
</tr>
<tr>
<td>Haemophilus influenza</td>
<td>47 (59, 48–70)</td>
<td>31 (76, 60–88)</td>
<td>0.81 (0.36–1.81)</td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>38 (48, 37–60)</td>
<td>25 (61, 45–76)</td>
<td>0.25 (0.11–0.58)*</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>6 (8, 3–16)</td>
<td>3 (7, 2–20)</td>
<td>0.46 (0.17–1.20)</td>
</tr>
</tbody>
</table>

Antibiotics received <2 weeks before swab collection; n (%, 95% CI)

<table>
<thead>
<tr>
<th></th>
<th>Australia</th>
<th>Alaska</th>
<th>Australia</th>
<th>Alaska</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrolide</td>
<td>31 (39)</td>
<td>0 (0)</td>
<td>19 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Beta-lactam</td>
<td>15 (19)</td>
<td>3 (7)</td>
<td>12 (16)</td>
<td>1 (3)</td>
</tr>
</tbody>
</table>

*P = 0.001; CI, confidence interval.
¹Swabs from 7 children who only ever had one swab collected (included in baseline) were excluded.
²Multiple logistic regression compared carriage at the end of the study in Australian versus Alaskan children, adjusting for carriage at baseline.
doi:10.1371/journal.pone.0070478.t002
However, there was a significant difference in the frequency of visits by group: Azi-None: median 3.5 (range 1–10); Azi-Infrequent: median 5 (range 2–14); Azi-Frequent: median 7 (range 1–15); nptrend P = 0.014. The median proportion of visits at which children had received azithromycin was 33% (range 12–50) in the Azi-Infrequent group and 75% (range 57–100) in the Azi-Frequent group. Differences in bacterial carriage among these groups were apparent at baseline. These differences were accentuated by the end of the study and were significant for all four bacteria (Table S1). A ‘cumulative dose-response’ relationship was observed: increasing azithromycin use coincided with decreasing carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, and increasing carriage of *S. aureus*. The same trends were apparent when data from all swabs were included (Figure S1, nptrend P<0.001 for all four bacteria).

Since azithromycin use coincided with carriage differences in the Australian children, carriage in the Alaskan children was compared with carriage in the Australian Azi-None group. Carriage of *S. pneumoniae* was higher in the Australian children, while *S. aureus* carriage was higher in Alaskan children. These differences were significant when all data were included; *S. pneumoniae* and *S. aureus* carriage were 79% (95% CI 71–87) and 2% (95% CI 0–6) respectively in the Australian Azi-None group compared to 60% (95% CI 52–67) and 15% (95% CI 9–21) respectively in Alaskan children.

**Antibiotic resistance**

Resistance of bacterial pathogens as children entered and left the study is shown in Table 3 and as a proportion of positive swabs by year in Figure 2. No penicillin resistant *S. pneumoniae* isolates (MIC>2 mg/L) were detected in either cohort. Macrolide resistance in *S. pneumoniae* and *S. aureus* carriers was significantly higher in Australian compared to Alaskan children. However, beta-lactam antibiotic resistance was higher in Alaskan *H. influenzae* carriers. Macrolide-resistant strains of *S. pneumoniae* and *S. aureus* were also more prevalent in Australian children when all swabs were included in analysis, isolated from 30% (95% CI 24–36) and 14% (95% CI 9–20) of swabs respectively, compared to 4.5% (95% CI 1–8) and 4% (95% CI 0–8) respectively in Alaskan children. *H. influenzae* and *M. catarrhalis* beta-lactam antibiotic resistance were both higher in Alaskan children as a proportion of all swabs: 19% (95% CI 13–26) had ampicillin-resistant *H. influenzae* and 65% (95% CI 56–74) had beta-lactamase positive *M. catarrhalis* strains compared to 4% (95% CI 2–6) and 29% (95% CI 23–35) respectively in Australian children.

Resistance as a proportion of all isolates is reported in Table 4. All *H. influenzae* isolates from Alaskan children and 80% from Australian children were macrolide-susceptible by disk diffusion. Since most *H. influenzae* possess an intrinsic macrolide efflux mechanism [10] which confers intermediate resistance by EUCAST criteria, MICs were determined for all Australian isolates.
Table 3. Antibiotic resistance (proportion of carriers) in respiratory bacteria from Australian and Alaskan children at baseline and end of study.

<table>
<thead>
<tr>
<th></th>
<th>First swab for each child</th>
<th>Last swab for each child</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Australia</td>
<td>Alaska</td>
</tr>
<tr>
<td>MacR(^1) S. pneumoniae</td>
<td>17 (36, 23–51)</td>
<td>2 (7, 1–24)**</td>
</tr>
<tr>
<td>Azir(^2) M. influenzae</td>
<td>40 (85, 72–94)</td>
<td>not determined(^7)</td>
</tr>
<tr>
<td>Azir(^9) H. influenzae</td>
<td>7 (15, 6–28)</td>
<td>0 (0, 0–11) (^1)</td>
</tr>
<tr>
<td>EryR(^5) S. aureus</td>
<td>5 (83, 36–100)</td>
<td>2 (67, 9–99)</td>
</tr>
<tr>
<td>Beta-lactam antibiotic resistance; n (%), 95% CI</td>
<td>22 (47, 32–62)</td>
<td>6 (21, 8–41)*</td>
</tr>
<tr>
<td>PenR(^6) S. pneumoniae</td>
<td>7 (15, 6–28)</td>
<td>9 (29, 14–48)</td>
</tr>
<tr>
<td>AmpR(^6) M. catarrhalis</td>
<td>6 (13, 5–26)</td>
<td>9 (29, 14–48)</td>
</tr>
<tr>
<td>Beta-lactam antibiotic resistance; n (%), 95% CI</td>
<td>35 (92, 79–98)</td>
<td>25 (100, 86–100)</td>
</tr>
<tr>
<td>MethR(^6) S. aureus</td>
<td>3 (50, 12–88)</td>
<td>0 (0, 0–70)</td>
</tr>
</tbody>
</table>

\(^1\)P<0.05, ** P<0.01 for difference in resistance between Australian and Alaskan carriers; CI, confidence interval.
\(^2\)MacR, macrolide-resistant: azithromycin (Australia) and erythromycin (Alaska) minimum inhibitory concentration (MIC) >0.5 mg/L.
\(^3\)Azir, azithromycin intermediate resistant: MIC:0.12–4 mg/L, Azir, azithromycin resistant: MIC: >4 mg/L for Australian children.
\(^4\)All isolates from Alaskan children were susceptible on disk diffusion; MICs were not determined.
\(^5\)isolates from one swab did not grow on sensitivity plates.
\(^6\)EryR, erythromycin resistant on disk diffusion.
\(^7\)PenR, penicillin intermediate resistant: MIC >0.06–2 mg/L, no resistant (MIC>2 mg/L) isolates were detected in either cohort.
\(^8\)AmpR, ampicillin MIC >1 mg/L.
\(^9\)Beta-lactamase positive.
\(^1\)MethR, methicillin resistant on disk diffusion.

doi:10.1371/journal.pone.0070478.t003

Association between frequency of azithromycin use and resistance
Differences in bacterial resistance in the first and last swabs from Australian children grouped by azithromycin exposure are shown in Table S2. Macrolide resistance tended to be highest in the Azi-Frequent group for S. pneumoniae, H. influenzae and S. aureus carriers, but increased in S. pneumoniae in all three groups from the beginning to the end of the study. When all swabs were included, macrolide-resistant S. pneumoniae and S. aureus strains were found in 21% (95% CI 14–28) and 1% (95% CI 0–5) respectively from the Azi-None group, 26% (95% CI 17–38) and 14% (95% CI 6–22) from the Azi-Infrequent group, and 38% (95% CI 26–49) and 23% (95% CI 14–33) from the Azi-Frequent group (nptrend \(P=0.002\) and \(P<0.001\) respectively). There were no trends for beta-lactam antibiotic resistance in any of the four pathogens. Trends by study year are shown in Figure S2.

S. pneumoniae and H. influenzae serotypes and resistance patterns
Of 285 S. pneumoniae isolates serotyped from the Australian children, PCV7 serotypes 19F and 23F were among the top 10 (Figure 3). The most prevalent serotype (8% of isolates) was 23B, followed by 6A, 19A and 6C (each 7%), and 16F, 17F, 19F and 23F (each 6.5%). Of 96 isolates serotyped from the Alaskan children the most prevalent serotype was 19A (15% of isolates) followed by 23B (9%) and 3, 15A and 34 (each 6.5%). The serotype hierarchy in Australian children appeared to coincide with azithromycin use; serotypes that were prevalent in the Azi-Frequent group also had the highest prevalence of azithromycin resistance. Commonly carried macrolide-resistant serotypes in the Australian group were 23B, 6A, 6C, 17F, 23F, 22F, 9N, 15A and 33D. In Alaskan children, azithromycin resistance was found in only 7 isolates (3 different serotypes). Intermediate penicillin resistance (MIC>0.06–2 mg/L) was most prevalent in serotypes 19A (100%), 19F, 23B (70%) were both macrolide and intermediate penicillin resistant) and 16F in Australian children, and serotypes 19A (67%) and 33B in Alaskan children. Multiple strains were detected in 30 (12%) positive specimens (two had three different serotypes).
serotypes) from Australian children and four (4%) from Alaskan children; this difference was likely due to more colonies being selected in Australia. Overall, PCV7 serotypes comprised 13% of Australian and 3% of Alaskan isolates.

Most *H. influenzae* isolates from both cohorts were nontypeable (NTHi) (Australia 89% of 191 isolates, Alaska 83% of 100 isolates). Capsular type b (Hib) was isolated from four children in both cohorts (2% and 4% of isolates respectively); three (75%) Hib isolates from Alaskan children were ampicillin resistant, but none from Australian children. Using EUCAST criteria, three isolates from Australian and eight from Alaskan children (three and seven NTHi respectively, no Hib) were beta-lactamase negative, but ampicillin resistant (BLNAR). However, only one BLNAR NTHi isolate from an Alaskan child had ampicillin MIC $4 \text{ mg/L}$.

Multiple strains (different serotype and/or antibiotype) were detected in 14% of Australian and 11% of Alaskan children.

Associations between age and other bacteria and carriage

In bivariate analyses adjusted for repeated sampling of children, age was significantly associated with carriage. Older children were less likely to carry *H. influenzae* (Odds Ratio (OR) 0.91 per additional year of age, 95% CI 0.83–0.99), but more likely to carry *S. aureus* (OR 1.13 per additional year of age, 95% CI 1.01–1.25). These associations largely remained when study year was included in the analysis: OR 0.94 (95% CI 0.86–1.02) for *H. influenzae* and OR 1.11 (95% CI 0.99–1.25) for *S. aureus*.

In multivariate analyses adjusted for the presence of other respiratory bacteria and for repeated sampling of children, similar associations were seen in both cohorts for *S. pneumoniae* colonization, which was positively associated with *H. influenzae* (OR 3.18, 95% CI 2.07–4.88) and *M. catarrhalis* (OR 3.86, 95% CI 2.41–6.19) and negatively associated with *S. aureus* (OR 0.38, 95% CI 0.21–0.70). Similar associations were also seen for *H. influenzae* which was positively associated with the presence of *M. catarrhalis* (OR 3.27, 95% CI 2.21–4.81), and for *M. catarrhalis* which was negatively associated with *S. aureus* (OR 0.35, 95% CI 0.11–0.99).

Discussion

This is the first longitudinal study reporting nasopharyngeal carriage in children with CSLD/bronchiectasis. While the two patient populations described are distinct and geographically dispersed, they share surprisingly similar environmental risk factors [1]. Carriage of respiratory pathogens in these two populations receiving different antibiotic treatment strategies showed similarities, but also important differences. *S. pneumoniae* carriage was similar overall and relatively stable in both Alaskan and Australian children during the study period. Carriage of *H. influenzae* declined in both cohorts over time, while *S. aureus* carriage increased. Carriage of *M. catarrhalis* in Alaskan children remained high throughout the study period, while carriage in Australian children started at the same level, but declined dramatically. Despite broadly similar carriage of three of the four main bacteria in the two populations, azithromycin use in the Australian cohort coincided with significant differences in the carriage of all four organisms, with an apparent ‘cumulative dose-response’ effect.

In the Australian Azit-None group, carriage of *S. pneumoniae* (79% of all swabs) was equivalent to that previously reported in this population [11]. Carriage of *S. pneumoniae* has been reported in several populations to peak at 2–3 years of age and decline.
thereafter [12,13]. However, in Indigenous children and children from developing countries, carriage is higher than in developed countries and remains common up to 10 years of age [14,15]. In the Azi-None group, carriage of H. influenzae was lower (60%) than the 83% reported in a younger cohort of Indigenous children [3], but similar to that reported for Aboriginal children 3–8 years of age [14]. The observed decline in carriage in both cohorts may therefore be age-related (as we found in bivariate analysis) as children grew older during the study. M. catarrhalis carriage (63%) in the Australian Azi-None group was lower than previously reported, even in older Indigenous children [14]. Nevertheless, M. catarrhalis carriage in this group and the Alaskan children was higher than generally reported in healthy children or children with upper respiratory tract infections [16].

S. aureus carriage has not been reported previously in this Australian population, and Alaskan reports are from a community with high rates of methicillin-resistant S. aureus infection [17]. In other populations, S. aureus carriage is high in infants <3-months of age, but declines rapidly as carriage of the other three bacteria increases, reaching a low point at 1–2 years [18,19]. Carriage then increases again to reach its highest prevalence in children 6–11 years of age [12,20]. If these trends apply to Australian and Alaskan children, then S. aureus carriage could be expected to be near its lowest point at the age when most of the children in this study were enrolled and highest at the age when their last swabs were collected. This would explain the observed increase in S. aureus carriage over the study period in both cohorts.

Consistent with other studies [3,5], we found that azithromycin use coincided with significant reductions in carriage of S. pneumoniae, H. influenzae and M. catarrhalis in the Australian children. Our study also found an apparent ‘cumulative dose response’ effect on carriage and resistance. However, a potential confounding factor was attendance at clinic, as children who did not take azithromycin had fewer clinic attendances (fewer swabs collected). While we cannot exclude the possibility that they only attended clinic when they were sick and therefore more likely to be carrying bacteria, most swabs were collected at scheduled visits that were arranged independently of the child’s health status. Factors other than azithromycin may also be involved in the decline of M. catarrhalis carriage in Australian children, since this decline was also seen in the Azi-None group. Conversely, while the increase in S. aureus carriage in Alaskan children is most likely age-related, the increase in Australian children may also be partly attributable to azithromycin since no such increase was seen in the Azi-None group (where S. pneumoniae carriage remained high).

Levels of macrolide resistance in bacteria colonizing the Australian children were not only higher than in the Alaskan children, but also higher than previously reported for most S. pneumoniae and H. influenzae paediatric respiratory tract isolates worldwide [10,21,22], and similar to the high levels of macrolide resistance in S. aureus often found in CF patients treated with azithromycin [23,24]. Levels of beta-lactam antibiotic resistance in Alaskan children were similar to previous reports from North America [21,22], and higher than H. influenzae and M. catarrhalis (but not S. pneumoniae) beta-lactam antibiotic resistance in Australian children. Beta-lactamase produced by one organism may protect co-colonizing bacteria from beta-lactam antibiotics [25,26]. However, we found conflicting evidence using simple multivariate analyses (data not shown). The higher rates of intermediate resistance to penicillin in Australian S. pneumoniae isolates may be due to other factors, e.g. co-occurrence of macrolide and intermediate penicillin resistance in prevalent serotypes such as 23B.

As with other observational studies, it is possible that the associations observed may be confounded by factors not measured. Patients who took azithromycin consistently may differ from those who did not. Antibiotics taken before enrolment likely affected baseline carriage. Additionally, biases may have been introduced if the carriage status of children for whom we achieved good follow-up was systematically different to those for whom our follow-up was less successful. The different swabbing schedules (quarterly in Australia and annually in Alaska) may also have led to bias, e.g. if carriage varied seasonally and children were mostly seen at a particular time of year, or if resistant strains were persistently carried by the same child. However, children from both cohorts were seen in all months of the year, and a recent Kenyan study found the mean duration of S. pneumoniae carriage to be just over 30-days [27]. Thus swabbing every 3-months is unlikely to detect the same S. pneumoniae carriage episode, and this was confirmed by inspection of our serotype data by child visit. However, without further strain discrimination we cannot comment on carriage duration in other species. Finally, while changes in H. influenzae and S. aureus carriage over time appeared to be age-related, differences could also be explained by other temporal changes.

In conclusion, this study adds important new information on long-term carriage and antibiotic resistance trends in Indigenous children with CF/bronchiectasis from two environmentally diverse regions of the world. While similar, possibly age-related, changes in carriage were observed over time in both patient populations, azithromycin use was associated with a ‘cumulative dose-dependent’ pattern of carriage and resistance in the Australian children. Moreover, while two recent randomised controlled trials showed that adults with non-CF bronchiectasis had fewer respiratory exacerbations after receiving azithromycin continuously for 6–12 month periods [28,29], this was accompanied by increased macrolide resistance amongst respiratory pathogens [29]. Such studies need to be repeated in children and, in light of these findings in adults and our own studies in children, special attention should be paid to monitoring antibiotic resistance as this may ultimately offset any clinical benefit derived from this important class of antibiotics.

Supporting Information

Figure S1 Pathogen carriage (proportion of swabs) by study year in Australian and Alaskan children. 1Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection at: Azi-None 0 no study visits; Azi-Infrequent 1 1–50% of study visits; Azi-Frequent 1 51–100% of study visits. (TIF)

Figure S2 Pathogen resistance (proportion of carriers) by study year in Australian and Alaskan children. 1Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection at: Azi-None 0 no study visits; Azi-Infrequent 1 1–50% of study visits; Azi-Frequent 1 51–100% of study visits. (TIF)

Table S1 Nasopharyngeal carriage of respiratory bacteria from Australian children, grouped by azithromycin exposure, at baseline and end of study. 1Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection at: Azi-None 0 no study visits; Azi-Infrequent 1 1–50% of study visits; Azi-Frequent 1 51–100% of study visits. (DOC)
Table S2 Antibiotic resistance (proportion of carriers) in respiratory bacteria from Australian children, grouped by azithromycin exposure*, at baseline and end of study. *Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection at: Azithromycin None = no study visits; Azithromycin Frequent = 1–50% of study visits; Azithromycin Frequent = 51–100% of study visits.

Acknowledgments
We wish to thank the children and their families for participating in the study. We also thank the paid research personnel including Valerie Logan and Abbey Diaz for their help with data management and cleaning.

References
8. CLSI (2012) Performance Standards for Antimicrobial Disk Susceptibility Testing, Twentieth-Supplemental Informational M02-S22; Table 2: Zone Diameter and MIC Interpretive Standards for Staphylococcus spp. (2E), Haemophilus influenzae and Haemophilus parainfluenzae (2E), and Staphylococcus pneumoniae (2E).
4.4 Nasopharyngeal carriage and antibiotic resistance of respiratory bacteria in Australian Indigenous children with bronchiectasis from an azithromycin versus placebo randomised controlled trial (unpublished)

4.4.1 Background

The BIS trial in Indigenous Australian and New Zealand children up to 9 years of age with non-CF bronchiectasis or chronic suppurative lung disease was recently completed. Compared to placebo, weekly azithromycin significantly reduced frequency of respiratory exacerbations; however, carriage of azithromycin-resistant bacteria was higher in the azithromycin group (Valery et al. 2013). Here, we compare NP carriage and antibiotic resistance in a nested cohort of Australian participants using ITT and ATP analyses.

4.4.2 Methods

Methods have been described in the primary paper (Valery et al. 2013). Briefly, the study was a multicentre, double-blind, randomised, parallel-group, placebo-controlled trial. Children were randomised to receive either azithromycin (30mg/kg) or placebo once a week for up to 24 months. The primary outcome was exacerbation (respiratory episodes treated with antibiotics) rate, and analysis of the primary endpoint was by ITT. Deep nasal or NP swabs were collected at scheduled 3-6 monthly visits for 1-2 years for analysis of antibiotic-resistant bacteria.
Standardised methods for swab collection, transport and processing were used for the Australian children, as described for the observational study (Hare, Singleton et al. 2013) and in Chapter 2. Four colonies each (including any with differing morphologies) of presumptive *S. pneumoniae* and *Haemophilus influenzae*, two of *Moraxella catarrhalis*, and one of *Staphylococcus aureus* were isolated and identified using standard methods (Hare, Grimwood et al. 2010). Antimicrobial sensitivities for *S. pneumoniae* and *S. aureus* isolates were determined by disk diffusion (Bell, Pham & Fisher 2013). Minimum inhibitory concentrations (MICs) were determined for resistant *S. pneumoniae* isolates using Etest strips (AB bioMérieux, France), and European Committee on Antimicrobial Susceptibility (EUCAST) breakpoints were used to define resistance (penicillin resistance MIC>2 mg/L, intermediate resistance MIC>0.06-2 mg/L; azithromycin resistance MIC>0.5 mg/L).

All swabs collected from the Australian children were included in the ITT analysis, with confidence intervals corrected for repeat measures in the same children. For the ATP analysis, swabs were only included if children were recorded as having taken their study medication (azithromycin or placebo) within the 2 preceding weeks.

### 4.4.3 Results

The following results were reported in the primary paper which included Australian and New Zealand children (Valery et al. 2013); 45 children were assigned to azithromycin and 44 to placebo. The mean treatment duration was 20.7 (standard deviation 5.7) months; azithromycin=902 child-months, placebo=875 child-months. Compared to the placebo group, children receiving azithromycin had significantly
lower exacerbation rates; incidence rate ratio 0.50 (95% CI 0.35, 0.71; \( p<0.0001 \)).

However, when their last swabs were collected, children in the azithromycin group had developed significantly higher carriage of azithromycin-resistant bacteria (19 of 47, 40%) than those receiving placebo (4 of 37, 11%; \( p=0.001 \)).

In the nested cohort of Australian participants there were 21 children in each group (Table 4.8). The median enrolment age (range) in months was 51 (20-103) for the placebo group and 50 (17-107) for the azithromycin group. By ITT, exacerbations were lower in the azithromycin group, but this was not statistically significant. However, compared to placebo, children in the azithromycin group had statistically significant reductions (Risk Difference, RD 19-24%) in NP carriage of \textit{H. influenzae}, \textit{M. catarrhalis} and penicillin-nonsusceptible \textit{S. pneumoniae}, but increased carriage of \textit{S. aureus} (RD 23%) and macrolide-resistant \textit{S. pneumoniae} (RD 14%).

Medication was documented in the 2-weeks preceding 44% and 38% of swabs from the placebo and azithromycin groups, respectively (Table 4.9). Fifteen children in the placebo group and 14 in the azithromycin group had received their study medication in the 2-weeks preceding at least one swab, and were therefore included in the ATP analysis together with their qualifying swabs. Differences in the azithromycin group compared to the placebo group were more pronounced in this analysis, with reductions of 30-41% in NP carriage of \textit{H. influenzae}, \textit{M. catarrhalis} and penicillin-nonsusceptible \textit{S. pneumoniae}, and greater increases in carriage of \textit{S. aureus} (RD 37%) and macrolide-resistant \textit{S. pneumoniae} (RD 19%). There was a greater reduction in exacerbations in the azithromycin group in the ATP compared to the ITT analysis, but this did not reach statistical significance (Table 4.9).

<table>
<thead>
<tr>
<th>Pathogen carriage</th>
<th>Placebo group</th>
<th>Azithromycin group</th>
<th>Total</th>
<th>Risk Difference&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Streptococcus pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycin resistant&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16 (7, 25)</td>
<td>30 (20, 40)</td>
<td>14 (3, 25)</td>
<td></td>
</tr>
<tr>
<td>Penicillin nonsusceptible&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30 (15, 45)</td>
<td>11 (5, 17)</td>
<td>-19 (-29, -8)</td>
<td></td>
</tr>
<tr>
<td><strong>Haemophilus influenzae</strong></td>
<td>44 (29, 59)</td>
<td>20 (11, 29)</td>
<td>-24 (-36, -12)</td>
<td></td>
</tr>
<tr>
<td><strong>Moraxella catarrhalis</strong></td>
<td>27 (15, 39)</td>
<td>7 (2, 12)</td>
<td>-20 (-30, -11)</td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>10 (3, 17)</td>
<td>33 (20, 46)</td>
<td>23 (13, 33)</td>
<td></td>
</tr>
<tr>
<td>Erythromycin resistant&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9 (2, 16)</td>
<td>32 (19, 44)</td>
<td>23 (13, 32)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fisher’s exact test with 95% confidence interval (CI).

<sup>b</sup> Confidence intervals corrected for repeat measures in the same children.

<sup>c</sup> MIC >0.5 mg/L.

<sup>d</sup> MIC>0.06-2 mg/L (no isolates had MIC>2 mg/L).

<sup>e</sup> Resistant by disk diffusion.
Table 4.9. Respiratory bacterial carriage and antibiotic resistance in Australian Indigenous children with bronchiectasis: according-to-protocol analysis.

<table>
<thead>
<tr>
<th></th>
<th>Placebo group</th>
<th>Azithromycin group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number children</td>
<td>15</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td>Number swabs</td>
<td>44</td>
<td>50</td>
<td>94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exacerbations (%) (95% CI)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo group</td>
<td>20 (9, 32)</td>
<td>10 (2, 19)</td>
<td></td>
</tr>
<tr>
<td>Azithromycin group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk Difference^a (95% CI)</td>
<td>-10 (-25, 4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pathogen carriage</th>
<th>% swabs (95% CI)</th>
<th>% swabs (95% CI)</th>
<th>Risk Difference^a (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycin resistant</td>
<td>9 (0, 19)</td>
<td>28 (12, 44)</td>
<td>19 (4, 34)</td>
</tr>
<tr>
<td>Penicillin nonsusceptible</td>
<td>34 (11, 57)</td>
<td>4 (0, 11)</td>
<td>-30 (-45, -15)</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55 (31, 78)</td>
<td>14 (1, 27)</td>
<td>-41 (-58, -23)</td>
<td></td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34 (17, 51)</td>
<td>0 (na)</td>
<td>-34 (-48, -20)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin resistant</td>
<td>11 (0, 23)</td>
<td>48 (27, 69)</td>
<td>37 (20, 53)</td>
</tr>
</tbody>
</table>

^a Fisher’s exact test with 95% confidence interval (CI).

^b Confidence intervals corrected for repeat measures in the same children.

^c MIC >0.5 mg/L.

^d MIC>0.06-2 mg/L (no isolates had MIC>2 mg/L).

^e Resistant by disk diffusion.
4.4.4 Conclusions

The BIS trial is, to our knowledge, the first RCT of azithromycin in children with non-CF bronchiectasis and the first in children and adults exceeding 12-months’ duration (Valery et al. 2013). Once-weekly azithromycin administered to Indigenous children with non-CF bronchiectasis for 12 to 24 months significantly reduced pulmonary exacerbations. In addition, children given azithromycin had significantly fewer non-pulmonary illnesses (mostly otitis media and skin infections) treated acutely with non-macrolide antibiotics (Valery et al. 2013). These positive findings were accompanied by an increase in NP carriage of macrolide-resistant pathogens in the azithromycin group.

Adherence to study medication was poorer in the Australian children compared to the New Zealand study participants (data not shown). Therefore data from the Australian participants were further analysed to elucidate the impact of adherence on exacerbation frequency and antibiotic resistance. As expected, both the reduction in exacerbations and the significant changes in NP carriage seen in children randomized to azithromycin were greater in those who were adherent to therapy. Subgroup analyses of clinical outcomes according to NP carriage including carriage of resistant strains will be conducted.

4.5 Summary of antibiotic impact

As far as we are aware, these studies of Australian Indigenous children are the first to report microbiological outcomes of macrolide treatment in children with non-CF bronchiectasis. Moreover, bacteriology results from all three studies (the cross-sectional prospective Bronchoscopy Study, the longitudinal BOS, and the BIS RCT)
were similar, and refute our third main hypothesis: that azithromycin therapy is not associated with increased carriage of or infection by azithromycin-resistant bacterial pathogens. It was thought that any increase in macrolide-resistant pathogens would be offset by reductions in carriage, leaving a similar proportion of children (swabs) carrying resistant organisms; however, this was not the case.

In the Bronchoscopy Study, significant impacts of macrolide antibiotics on NP carriage were observed, namely, a reduction in carriage of the three main respiratory pathogens, in particular *S. pneumoniae* and *M. catarrhalis*. However, the same effects were not seen for lower airway infection, with the probable exception of reduced *M. catarrhalis* infection. Significant increases in macrolide-resistant *S. pneumoniae* were observed in both NP carriage and lower airway infection in children who had received macrolide antibiotics.

The impacts of long-term azithromycin use on NP carriage (in BOS and BIS) were similar to those seen for recent azithromycin use in the Bronchoscopy Study: reduced carriage of all three main respiratory bacteria, but increased carriage of macrolide-resistant *S. pneumoniae*. In addition, azithromycin use was associated with increased NP carriage of *S. aureus* (mostly macrolide-resistant). Moreover, a ‘cumulative dose-response’ relationship was seen in the observational study: increasing azithromycin use (from none to infrequent to frequent over the course of the study) coincided with decreasing carriage of *S. pneumoniae, H. influenzae* and *M. catarrhalis*, but increasing carriage of *S. aureus* and macrolide-resistant *S. pneumoniae* and *S. aureus* (Appendix 2: Supplementary Figures and Tables).
These results were paralleled in the BIS RCT, with reductions in carriage of the three main pathogens (and penicillin-nonsusceptible *S. pneumoniae*), and increases in carriage of *S. aureus* and macrolide-resistant *S. pneumoniae* and *S. aureus*, in the azithromycin group compared to the placebo group. Moreover, these differences were more pronounced in the ATP analysis (where swabs were only included if children had received their study medication) compared to the ITT analysis.

The results of the BIS clinical trial indicate that azithromycin improves clinical outcomes despite increased carriage of macrolide-resistant pathogens. The antibiotic effects of azithromycin may be instrumental in reducing otitis media (possibly through reduced NP carriage) and skin infections. Although we found in our Bronchoscopy Study that lower airway infection was not reduced, reduced NP carriage likely means reduced aspiration of respiratory bacteria which may trigger exacerbations. Thus exacerbations may also be reduced as a direct result of azithromycin's antibiotic activity (Amanda Leach, personal communication). Alternatively (or additionally), the beneficial effect of azithromycin for pulmonary infections may reside in its anti-inflammatory and immunomodulatory properties. Several authors have suggested that a macrolide is needed that has these latter properties, but no antimicrobial activity (Masekela & Green 2012; Serisier 2013). Although this may be suitable for adults, or children with low NP carriage, it may not be appropriate for children in high risk populations with high NP carriage. Further studies in children are required, as well as research to determine the possible clinical consequences of increased macrolide resistance in the community and transmission to untreated individuals.
CHAPTER 5

Final Discussion
CHAPTER 5: FINAL DISCUSSION

5.1 Chapter overview

Bronchiectasis is a serious condition that is prevalent in Indigenous children. When suboptimally treated, it can reduce life expectancy and lead to poor quality of life. Although much research effort has been directed towards bronchiectasis related to cystic fibrosis (CF), limited data were available on the bacteriology and impact of antibiotic treatment in children with non-CF bronchiectasis. The studies described in this thesis were directed towards filling these knowledge gaps with respect to Indigenous children in Australia’s Northern Territory (NT).

Three main hypotheses were proposed:

1. That the bacterial aetiology of non-CF bronchiectasis in NT Indigenous children is the same as in other paediatric populations.
2. That aspiration of nasopharyngeal (NP) secretions contributes to the initiation and progression of non-CF bronchiectasis.
3. That azithromycin therapy is not associated with any significant differences in the proportions of children with macrolide-resistant respiratory pathogens. (While increased resistance is expected, it was predicted that this would be offset by reductions in carriage and infection in children receiving azithromycin.)

The first two hypotheses were addressed using data from our Bronchoscopy Study (Section 3.2). The bacterial pathogens isolated from bronchoalveolar lavage (BAL) fluid from Indigenous children with non-CF bronchiectasis were compared with
those reported from published studies in other populations of children with non-CF bronchiectasis. In addition, results were compared with a group of non-Indigenous children without bronchiectatic symptoms. Strain concordance between NP and BAL cultures was assessed when the same bacterial species was isolated from both sites in children with lower airway infection.

The third hypothesis was addressed in several ways. The impacts of recent azithromycin intake (<2 weeks before specimen collection) on the bacteria identified in NP carriage and lower airway infection were assessed using data from the cross-sectional Bronchoscopy Study (Section 4.2). The impact of long-term azithromycin use was assessed using NP carriage data from the longitudinal Bronchiectasis Observational Study (BOS) by grouping children according to the frequency of their azithromycin exposure during the study (Section 4.3). The impact of long-term azithromycin use was also assessed using data from the Bronchiectasis Intervention Study (BIS), a randomised controlled trial (RCT) of azithromycin versus placebo for the prevention of respiratory exacerbations. NP carriage and bacterial resistance in the azithromycin and placebo groups were compared using intention-to-treat (ITT) and according-to-protocol (ATP) analyses (Section 4.4).

This final chapter presents the main study findings relating to the bacteriology of non-CF bronchiectasis in Australian Indigenous children, and the impact of azithromycin on upper and lower airway bacteria. Limitations are addressed and future research outlined. The chapter ends with the final conclusions from this thesis.
5.2 Main study findings

5.2.1 Bacteriology of bronchiectasis

The bacterial pathogens associated with non-CF bronchiectasis in Australian Indigenous children are similar to those found in other paediatric populations with bronchiectasis, supporting the first hypothesis. Nontypeable *Haemophilus influenzae* (NTHi) was the predominant bacterium, followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Hare, Grimwood et al. 2010). Lower airway infection with *Staphylococcus aureus* was uncommon, and no infection with *Pseudomonas* species was detected. By way of comparison, non-Indigenous children without bronchiectasis (many of whom had protracted bacterial bronchitis) had significantly fewer infections with NTHi (Hare, Grimwood et al. 2010).

A comparison of bacteria isolated from NP and oropharyngeal (OP) swabs established that the three main bacterial pathogens above were more commonly found in the nasopharynx than the oropharynx in Australian Indigenous children with non-CF bronchiectasis. Using standard methods for pathogen identification, NP sampling was clearly superior in this population for the detection of *S. pneumoniae* and *M. catarrhalis*; however, detection of *H. influenzae* (mostly NTHi) was similar from NP and OP swabs (Satzke et al. 2013). Although not part of the original thesis proposal, it became clear that apparent NTHi needed to be differentiated from the closely-related primarily commensal *Haemophilus haemolyticus* (Murphy et al. 2007). NTHi isolates from NP swabs, OP swabs and BAL fluid were differentiated from isolates of presumptive *H. haemolyticus* using *hpd*#3 PCR. *H. influenzae* was confirmed in 90% of NP swabs and 100% of BAL specimens, but only 39% of OP swabs, with phenotypic NTHi (Hare, Binks, et al. 2012). These findings indicate that,
as for *S. pneumoniae* and *M. catarrhalis*, the nasopharynx is the preferred site to examine for NTHi colonisation studies in this population.

NP carriage of all three main respiratory pathogens was significantly higher in the Indigenous children with bronchiectasis compared to the non-Indigenous children without bronchiectasis. However, when the same bacterial species was isolated from NP and BAL specimens from children with lower airway infection, strain concordance was high in both groups (Hare, Grimwood et al. 2010). This supports the hypothesis that aspiration of NP secretions contributes to lower airway infection. Moreover, there was greater strain diversity in the lower airways of Indigenous children with bronchiectasis compared to non-Indigenous children without bronchiectasis and compared to the upper airways (Hare, Grimwood et al. 2010). This suggests recurrent aspiration of NP secretions and persistence of strains in the lower airways of these children, while strain replacement or clearance likely occurs in the upper airways. Interventions that reduce NP carriage prevalence and density may therefore help to reduce the risk of lower airway infection.

Strain diversity in BAL specimens from Indigenous children with phenotypic NTHi infection included a high proportion of co-detection of *H. influenzae* and presumptive *H. haemolyticus*. Semi-quantitative counts are made on primary plates where NTHi and *H. haemolyticus* colonies cannot be differentiated, potentially leading to overestimation of *H. influenzae* density. The *hpd#3* PCR previously used to identify NTHi isolates was therefore used quantitatively on BAL fluid to estimate *H. influenzae* density and thus confirm lower airway infection (Hare, Marsh, et al. 2013). Semi-quantitative growth scores for phenotypic NTHi were correlated with
quantitative \textit{hp}d\#3 PCR detection of \textit{H. influenzae} in BAL fluid, and both measures of bacterial density were correlated with total and differential cell counts as measures of the airway inflammatory response to infection. The results confirmed NTHi as the most important pathogen associated with non-CF bronchiectasis in Australian Indigenous children, and support the continued use of quantitative culture as the gold standard for defining \textit{H. influenzae} lower airway infection (Hare, Marsh, et al. 2013).

\textbf{5.2.2 Impact of azithromycin therapy}

The impact of azithromycin use on NP carriage of respiratory bacteria was assessed in three studies. In the Bronchoscopy Study, recent azithromycin use (in the 2 weeks preceding bronchoscopy) was associated with significantly reduced carriage of the three main pathogens, particularly \textit{S. pneumoniae} (Hare, Leach, et al. 2012). In the longitudinal observational study (BOS), azithromycin use was also significantly associated with reduced carriage of \textit{S. pneumoniae}, \textit{H. influenzae} and \textit{M. catarrhalis}, but increased carriage of \textit{S. aureus}, in a ‘cumulative dose-response’ relationship (Hare, Singleton, et al. 2013). Carriage was highest (lowest for \textit{S. aureus}) in children who had not received azithromycin preceding any study visit when swabs were collected, intermediate in children who received azithromycin infrequently (preceding 1-50\% of study visits), and lowest (highest for \textit{S. aureus}) in children who received the most azithromycin (preceding 51-100\% of study visits). Similar impacts of long-term azithromycin therapy were seen in the BIS RCT. NP carriage of the three main bacterial pathogens was significantly lower in the azithromycin compared to the placebo group, while \textit{S. aureus} carriage was significantly higher (Section 4.4). These differences in children randomised to azithromycin were greater in those who were adherent to therapy.
The impact of azithromycin therapy on antibiotic resistance of NP respiratory bacteria was assessed in the same three studies. Children who received azithromycin in the 2 weeks preceding their bronchoscopy procedure were significantly more likely to carry azithromycin-resistant *S. pneumoniae*, despite reduced carriage overall (Hare, Leach, et al. 2012). This finding refutes our third hypothesis that azithromycin use is not associated with significant differences in the proportions of children with macrolide-resistant respiratory pathogens. Although carriage of azithromycin-resistant *H. influenzae* was also higher in children who received azithromycin, numbers were too small to reach statistical significance. Long-term azithromycin use in BOS children also coincided significantly with increased NP carriage of macrolide-resistant *S. pneumoniae* and *S. aureus*, in a similar ‘cumulative dose-response’ relationship seen for carriage, despite reduced *S. pneumoniae* carriage overall (Hare, Singleton, et al. 2013). Similar impacts of long-term azithromycin therapy on NP bacterial resistance were seen in the BIS RCT, with significantly higher carriage of macrolide-resistant *S. pneumoniae* and *S. aureus* in children randomized to azithromycin (Valery et al. 2013). Again (unsurprisingly), these increases were greater in those children who were adherent to therapy (Section 4.4).

The impact of azithromycin use on lower airway infection by respiratory bacteria could only be assessed in the Bronchoscopy Study, using BAL fluid as children rarely expectorate sputum. With respect to antimicrobial resistance, the same effect was seen as in the upper airways. Compared to children who had not received azithromycin, children who received azithromycin in the 2 weeks preceding bronchoscopy were significantly more likely to be infected by azithromycin-resistant
*S. pneumoniae* (Hare, Leach, et al. 2012). Infection with azithromycin-resistant *H. influenzae* was also higher in children who received azithromycin, but low numbers precluded significance. However, recent azithromycin use was not associated with significantly reduced lower airway infection with any of the three main pathogens (Hare, Leach, et al. 2012). Infection with *M. catarrhalis* was substantially lower in children who received macrolide antibiotics (likely due to the almost universal susceptibility of *M. catarrhalis* strains to azithromycin); however this was not statistically significant, probably due to low numbers. There was thus a differential effect of antibiotics on upper and lower airway bacteria, and this was especially apparent in the case of *S. pneumoniae* (Hare, Leach, et al. 2012). This may be due to the persistence of resistant strains causing infection in the lower airways, while upper airway *S. pneumoniae* strains are hampered by the fitness cost of macrolide resistance and ongoing competition in the nasopharynx from susceptible strains (Maher et al. 2012). In the case of NTHi, recurrent or persistent airway infection with NTHi may be associated with nonclearing adaptive immunity (King et al. 2003).

It is likely that the increased prevalence of macrolide-resistant *S. pneumoniae* observed in NP carriage and lower airway infections in children who received azithromycin therapy is due to elimination of susceptible strains, consistent with earlier reports (Leach et al. 1997; Morris et al. 2010), rather than *in vivo* development of resistance. In communities where macrolide resistance is rare, azithromycin use (e.g. for trachoma) is unlikely to increase the prevalence of resistant organisms (Batt et al. 2003). However, macrolide resistance in 5% of *S. pneumoniae* NP isolates has previously been reported in healthy NT Indigenous children (Leach et al. 2009). As we have shown, the prevalence of macrolide-resistant organisms increases with
increasing exposure to azithromycin, and many different serotypes with macrolide resistance have been detected (Hare, Singleton, et al. 2013). This suggests selection of resistant strains already circulating in the population rather than a mutation resulting in \textit{de novo} resistance. Monitoring of antibiotic resistance is clearly required.

\subsection*{5.2.3 Implications for treatment}

In the BIS RCT, the frequency of respiratory exacerbations was significantly lower in the azithromycin group (Valery et al. 2013). This indicates that azithromycin is clinically effective in children with non-CF bronchiectasis, despite increased macrolide resistance in NP carriage and lower airway bacteria. These findings complement those from RCTs in adults with non-CF bronchiectasis, in which long-term macrolide treatment was also found to be effective in reducing exacerbation frequency (Altenburg et al. 2013; Serisier et al. 2013). While the mode of action is not clear, macrolides are known to have anti-inflammatory and immunomodulatory properties as well as antibacterial effects (Masekela & Green 2012). As described in Chapter 1, macrolides decrease inflammation, inhibit bronchial hyper-responsiveness and improve airway clearance (Hoban & Zhanel 2006). These properties are clearly biologically advantageous in the treatment of inflammatory conditions such as bronchiectasis.

Inhaled corticosteroids have no obvious role in the management of bronchiectasis in children when asthma is not concurrently present, and the role of non-steroidal anti-inflammatory drugs remains undefined (Chang et al. 2012). Although newer anti-inflammatory drugs are available or under development, RCTs in patients with bronchiectasis are required (Chang et al. 2012). At this point in time it would appear
that macrolides represent the best proven treatment option available for patients with non-CF bronchiectasis. However, due to the risks of population antimicrobial resistance associated with chronic macrolide use, it has been suggested that macrolide antibiotics be reserved for patients at greatest risk of morbidity and deterioration until such time as non-antibiotic macrolides become available for the long-term treatment of patients with airway inflammatory disease (Serisier 2013).

Other infections such as otitis media (OM) and skin infections were also reduced in the BIS RCT (Valery et al. 2013). Such reductions are presumably due to the antibiotic effect of azithromycin (Arguedas et al. 2011; Shelby-James et al. 2002), and represent additional advantages to the use of azithromycin in Australian Indigenous children who have high rates of OM (Morris et al. 2005) and skin sores (Clucas et al. 2008). Pyoderma in NT Indigenous children is often caused by group A streptococci, and sequelae such as acute post-streptococcal glomerulonephritis (Marshall et al. 2011) and acute rheumatic fever (Lawrence et al. 2013) are also prevalent in the NT and may lead to chronic kidney and heart disease (Andrews et al. 2009). Reductions in skin infections following azithromycin use may assist in reducing the prevalence of these serious and life-threatening conditions. Long-term azithromycin therapy may also have a protective effect against bronchiectasis (and OM) by reducing NP carriage. Whilst antibiotics failed to reduce lower airway bacterial density and infection, episodes of exacerbation may be prevented by the reduced bacterial load in the nasopharynx, thereby reducing aspiration into the lung.

For all these reasons it is arguably justifiable that long-term azithromycin therapy be recommended for Australian Indigenous children with non-CF bronchiectasis, who represent a high risk group requiring the best available treatment.
5.3 Limitations

Although valuable BAL specimens have been obtained from the Bronchoscopy Study, a major limitation of the study design is its cross-sectional nature. Repeat BAL specimens from the same children would be invaluable to monitor disease progression or improvement. However, the bronchoscopy procedure requires general anaesthesia and is not without risk, and in most cases it is employed once only to confirm a diagnosis of suspected bronchiectasis. Ethical concerns would need to be addressed before proposing a study where repeat bronchoscopies are required.

Another limitation is the relatively small sample size of Indigenous children undergoing bronchoscopy, especially as children are of various ages, have received different pneumococcal conjugate vaccines in infancy, and been subjected to differing treatment regimens. Nevertheless, significant results have been obtained with respect to the impact of antibiotics on upper and lower airway bacteriology and antibiotic resistance. Future studies will examine the impact of vaccines. Statistical modelling is needed to define the relative effects of patient age, azithromycin use and vaccination status.

While the main limitation of BOS was its observational nature, BIS was a RCT which provided higher level evidence of the impact of azithromycin on NP carriage and bacterial resistance. The fact that similar results were found in both of these longitudinal studies supports the validity of these findings. Again, statistical modelling is needed to define the relative effects of patient age, azithromycin use and vaccination status in these studies, plus the additional element of time.
Detection of respiratory bacteria was limited to culture-dependent techniques, and no anaerobic studies were conducted. The roles of viruses and bacterial biofilms, fungi and co-infections (i.e. the microbiomes) in the pathophysiology of non-CF bronchiectasis are yet to be elucidated (Chang et al. 2012). Other team members from our laboratory are conducting culture-independent studies of bacteria and viruses, and biofilm and microbiome studies, using these lower airway specimens.

Our antibiotic susceptibility testing protocol could be criticised for mixing methods and interpretive criteria, particularly with respect to \textit{H. influenzae} and azithromycin resistance. We used the calibrated dichotomous susceptibility (CDS) method for screening NTHi susceptibility to azithromycin; however, the CDS method has not been calibrated for this combination of antibiotic and organism (Bell, Pham & Fisher 2013). This oversight is unlikely to have affected our results, since we used Etest® strips to determine minimum inhibitory concentrations for at least one NTHi isolate per positive specimen. This was done to detect intermediate azithromycin resistance, which is defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2013) but not the Clinical and Laboratory Standards Institute (CLSI 2012). In fact, CDS susceptibility criteria for \textit{S. pneumoniae} and erythromycin, and \textit{H. influenzae} and ampicillin, correspond more closely with EUCAST compared to CLSI breakpoints (Table 2.8). We will continue to use the CDS method for consistency with data collected over the past 20 years. However, until azithromycin is calibrated for \textit{H. influenzae} using the CDS method, we will consider using the EUCAST disc screening method for this combination in future studies requiring these data.
The bacteriology of non-CF bronchiectasis in this population has been considered largely in isolation, with no host-response studies except for the preliminary study reported in Section 3.3. A larger and more detailed study comparing the bacteriology and host immune responses in Indigenous and non-Indigenous children, with and without bronchiectasis, is needed to further define a diagnostic threshold for lower airway infection in children with non-CF bronchiectasis.

5.4 Future research

To address some of the many remaining questions on the bacteriology of the airways of children with non-CF bronchiectasis, further studies are planned.

5.4.1 Diagnosis of lower airway infection

Is there a difference in detection of respiratory bacteria between the first and second bronchoalveolar lavage during a single bronchoscopy procedure? BAL fluid collected during bronchoscopy is typically sequential, with two lavages. Traditionally, the first lavage is used for bacterial culture and the second lavage is used for cellularity, fluid phase and other studies such as immunology-based work. Both specimens will be cultured prospectively and results analysed to determine if there are any qualitative or quantitative differences between the two samples in the detection of respiratory bacteria. It is feasible that the first lavage may break up biofilm in the lower airways (theoretically present in non-CF bronchiectasis patients), allowing better recovery of bacteria in the second lavage. If differences are found, this may have implications for hospital microbiology procedures.
5.4.2 Definition of lower airway infection

*Determining the best bacterial density cut-off for defining lower airway infection in children with non-CF bronchiectasis: What is the relationship between bacterial density and measures of airway inflammation?* We have published a small study (Hare, Marsh, et al. 2013) on the relationship between the bacterial density of *H. influenzae* in BAL fluid, and total and differential cell counts, in Indigenous children with bronchiectasis (Section 3.4). A larger and more detailed study will be carried out whereby bacterial counts (measured by semi-quantitative culture and quantitative PCR) and inflammatory cell and cytokine concentrations in BAL fluid, from Indigenous and non-Indigenous children with and without bronchiectasis, will be measured. Results will further define a diagnostic threshold for lower airway infection in children with non-CF bronchiectasis, along the lines of a study in CF children (Armstrong et al. 1996). Such a study would look at lower airway infection with *S. pneumoniae* and *M. catarrhalis* in addition to *H. influenzae*.

5.4.3 Analysis of vaccine impact on nasopharyngeal carriage and lower airway infection

*Have the different pneumococcal conjugate vaccines impacted NP carriage and/or lower airway infection in Indigenous children with non-CF bronchiectasis?* As described in Chapter 1, two pneumococcal conjugate vaccines have been introduced to the market since the 7-valent pneumococcal conjugate vaccine (PCV7) was licensed in 2000: the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine (PHiD-CV) and the 13-valent pneumococcal conjugate vaccine (PCV13). PCV7 was introduced in the NT in 2001 for Indigenous and other high-risk infants, and was the vaccine in use when the Bronchoscopy Study commenced in July 2007.
PHiD-CV replaced PCV7 on the NT immunization schedule in October 2009, and PCV13 replaced PHiD-CV in October 2011 (Strachan, Snelling & Jaffe 2013).

Few data are available to date regarding the impact of these newer vaccine formulations on NP carriage or disease. Both PHiD-CV and PCV13 have been shown to protect against invasive pneumococcal disease caused by vaccine serotypes (Kaplan et al. 2013; Palmu et al. 2013). An 11-valent precursor to PHiD-CV was shown to protect against otitis media due to NTHi (Prymula et al. 2006), and a reduction in *H. influenzae* carriage was seen (Prymula et al. 2009). However, no substantial effect of PHiD-CV was observed on NP carriage of NTHi (Prymula et al. 2011). A study in Dutch children (a RCT of PCV7 versus PHiD-CV) similarly found no NP carriage differences between groups in either NTHi prevalence or *H. influenzae* density (van den Bergh et al. 2013).

Vaccine policy-makers are faced with a difficult choice in regions with high rates of NTHi and pneumococcal infection such as Australia’s Northern Territory. Changes in the NT immunization schedule have provided a unique opportunity to evaluate a possible impact of conjugate vaccines on respiratory diseases, such as bronchiectasis, which are associated with NTHi and *S. pneumoniae* lower airway infections. To date, numbers of children undergoing bronchoscopy who have been vaccinated with the newer formulations are too few for meaningful analysis. Once enrolment numbers are sufficient children will be grouped by vaccination status, with those who received ≥2 doses of a particular vaccine assigned to that vaccine group. (A small number of children who received ≥2 doses of two different vaccines during the changeover...
periods, e.g. 2 doses of PCV7 followed by 2 doses of PHiD-CV, will be in two groups.) NP carriage and lower airway infection will be analysed by vaccine group.

Temporal changes also need to be considered. As reported in Chapter 3, there has been a decline in NP carriage and lower airway infection for all three of the main bacterial respiratory pathogens in Indigenous children with bronchiectasis since the initial results were published in 2010. Although carriage of *S. pneumoniae* and/or *H. influenzae* may have been affected by the different vaccines, there has been a particular decline in carriage of *M. catarrhalis* (not in any vaccine) documented in the NT since 2005, for reasons that remain unclear. Children who received ≥2 doses of PCV7 (the largest group) will be split into two groups: those who underwent bronchoscopy before and after October 2009. NP carriage and lower airway infection will then be compared between the latter PCV7 group and the PHiD-CV and PCV13 groups to control for the observed declines over time. Age differences between groups will be accounted for using regression modelling.

Results for NT Indigenous and non-Indigenous children with and without non-CF bronchiectasis will also be compared with results from non-Indigenous children with and without bronchiectasis recruited at the Royal Children’s Hospital in Brisbane. Most of these latter children will have been vaccinated with PCV7, introduced in Australia for all children less than 2 years of age in January 2005. PHiD-CV was only ever used in Australia, in the NT, for a period of 2 years.
5.4.4 Modelling of nasopharyngeal carriage and resistance by duration of azithromycin use

What effect does long-term azithromycin have on NP carriage and resistance, independent of year and child age? In the BOS, changes in carriage of bacterial respiratory pathogens over time were observed in Indigenous Australian and Alaskan Native children (Hare, Singleton, et al. 2013). Although some of these changes appeared to be age-related, an apparent ‘dose-response’ relationship between frequency of azithromycin use, and carriage and resistance, was also seen in the Australian children. Statistical modelling will be used to further define the relative effects of time, age of patient and azithromycin use.

To estimate the effect of azithromycin use (in the 2 weeks prior to swab collection) on carriage of respiratory pathogens in Australian children, a mixed effects logistic model will be used with date of swab (in years), age of child (in years) and azithromycin use (as a time varying covariate) as independent variables. This model implicitly corrects for repeated swabs in the same patient. The coefficient for time will represent the estimated odds ratio for pathogen carriage in all study children per year, the coefficient for age will represent the estimated odds ratio for pathogen carriage associated with child age, and the coefficient for azithromycin will represent the estimated odds ratio for pathogen carriage associated with azithromycin use.

Results from this analysis will allow us to estimate the effect of azithromycin use on carriage and resistance, independent of time and age of patient.
5.4.5 Analysis of carriage and resistance in placebo and azithromycin groups

What are the relationships between NP carriage and resistance of bacterial pathogens, azithromycin, and respiratory exacerbations in Indigenous children with non-CF bronchiectasis? The recently completed BIS RCT in Indigenous Australian and New Zealand children up to 9 years of age with non-CF bronchiectasis showed that, compared to placebo, weekly azithromycin significantly reduced frequency of respiratory exacerbations (Valery et al. 2013). In section 4.4, NP bacterial carriage and antibiotic resistance in a nested cohort of Australian participants were compared using ITT and ATP analyses. Subgroup analyses of clinical outcomes according to NP carriage, including carriage of resistant strains, will be conducted.

5.6 Final conclusions

The studies described in this thesis have contributed to the knowledge of culturable aerobic bacteria in the upper and lower airways of children with non-CF bronchiectasis. Some methodological issues were first addressed, and our research has contributed to the literature in several ways. Firstly, we compared sampling using NP and OP swabs for the detection of upper airway colonisation with respiratory bacteria. We published a study on swab transport from remote communities and another on the viability of respiratory bacteria kept long-term in ultra-freeze storage. We also described a method for the detection of multiple pneumococcal serotypes in NP swabs, and provided supporting evidence for the continued use of the gold standard Quellung reaction for pneumococcal serotyping.
The clinical studies that followed demonstrated that the predominant bacterial pathogens associated with lower airway infection in Australian Indigenous children with non-CF bronchiectasis are the same as in other paediatric populations with this condition. When the same species was found in NP and BAL specimens from children with lower airway infection, strain concordance was very high. Ours is the first study to report this finding, which strongly supports the hypothesis that aspiration of NP secretions contributes to the initiation and progression of non-CF bronchiectasis.

Azithromycin use (recent and long-term) was associated with significantly reduced NP carriage of the three main bacterial pathogens, but an increased proportion of children carrying macrolide-resistant bacteria, contrary to our third main hypothesis. Recent azithromycin use was also associated with increased macrolide resistance in bacteria causing lower airway infection, but was not associated with reduced lower airway infection. Possible reasons for this differential effect of azithromycin on upper and lower airway bacteria were discussed. Nevertheless, the reduction in exacerbation frequency in Indigenous children with non-CF bronchiectasis who were randomised to long-term azithromycin treatment is encouraging. Whether this effect is due to azithromycin’s antibiotic action, its anti-inflammatory and immunomodulatory properties, or both, remains to be determined.

Many questions remain and future studies have been proposed, some of which I hope to undertake following my PhD work.
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The bacteriology of lower respiratory infections in Papua New Guinean and Australian Indigenous children

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SUMMARY

Indigenous children in Australia and children in Papua New Guinea (PNG) share a high burden of respiratory disease. In PNG the focus has been on pneumonia as a major cause of mortality. While pneumonia incidence remains high in Australian Indigenous children, improved access to better health care has resulted in reduced mortality. However, severe and recurrent pneumonia are risk factors for chronic suppurative lung disease or bronchiectasis in Australian Indigenous children. Bronchiectasis is associated with significant morbidity, and early death in adulthood. This paper includes an outline of the disease manifestations of acute and chronic lower respiratory infections. The main bacterial pathogens involved in pneumonia, bronchiolitis, bronchitis and bronchiectasis have been determined. Capsular organisms such as Streptococcus pneumoniae and Haemophilus influenzae type b are more often implicated in acute infections, while chronic infections are frequently associated with nontypeable (noncapsular) H. influenzae. Moraxella catarrhalis is more often isolated from very young children. Possible reasons for the high burden of respiratory disease in Papua New Guinean children and Australian Indigenous (primarily Aboriginal) children include early and dense colonization with multiple species and strains of respiratory pathogens. There is a role for vaccines in preventing lower respiratory infection.

Introduction

Children in Papua New Guinea (PNG) and Indigenous (mainly Aboriginal) children in Australia share a high burden of respiratory disease. In PNG the focus has been on pneumonia as a major cause of mortality (1,2). In Australia, while the incidence of pneumonia and other lower respiratory infections remains high in Indigenous children (3), mortality has been reduced (4) as a result of improved access to better health care. However, pneumonia (particularly severe and recurrent pneumonia) has been shown to be a risk factor for chronic suppurative lung disease (CSLD) or bronchiectasis in Australian Indigenous children (5). Bronchiectasis is associated with significant morbidity, and early death in adulthood (6).

Figure 1 shows the relationship between acute and chronic respiratory diseases and the bacteria associated with them. A brief description of the main acute and chronic infections is presented, followed by evidence of their bacterial aetiology. The microbiological factors contributing to the burden of disease and the role of vaccines in preventing disease are then discussed.

Acute lower respiratory infections (ALRIs)

The two most common ALRIs resulting in hospitalization are bronchiolitis and pneumonia (7). Their importance is reflected not only in direct morbidity and mortality but also in long-term consequences. Recurrent hospitalization for ALRIs and severity of illness are risk factors for bronchiectasis in Australian Indigenous children (5). Data from The Gambia also suggest a link between severe early childhood pneumonia and later chronic lung disease (8).
Bronchiolitis is the most common ALRI in young children (usually ≤12 months of age) and is characterized by extensive inflammation of the airways and increased mucus production (7). It is a clinical diagnosis, characterized by rapid breathing and wheeze or crepitations following an upper respiratory illness, and is primarily caused by viral infection of the respiratory epithelial cells (7). There are no published data on bronchiolitis in PNG children. Indigenous infants in the Northern Territory (NT) of Australia have a higher incidence of bronchiolitis than non-Indigenous children and higher rates of coexistent clinical pneumonia (9). Bronchiolitis accounted for most of the disease burden in Indigenous infants hospitalized in the NT in the first year of life, with a rate of 227 per 1000 child-years for the years 1999-2004 (3).

Pneumonia is characterized by inflammation of the lung parenchyma (alveoli, excluding the bronchi) and congestion/abnormal alveolar filling with fluid (consolidation and exudation). It is caused by viruses and/or bacteria. As a major cause of mortality, pneumonia has a long history of research in PNG. In 1991 it was estimated that pneumonia accounted for approximately 30% of all deaths in children <5 years of age (10). From verbal autopsy reports in the Tari Basin between 1971 and 1995, pneumonia accounted for 50% and 33% of infant and toddler deaths respectively (1).

Despite a significant reduction in infant mortality in Aboriginal Australians since the 1960s, ‘respiratory disease’ (predominantly pneumonia) still caused 18% of all Aboriginal infant deaths in the NT between 1979 and 1983 (4,11). Morbidity rates remain high, with an ALRI incidence of 427 episodes (227 attributed to bronchiolitis) per 1000 child-years reported for Indigenous infants admitted to hospital in the NT during the years 1999-2004 (3). Compared to non-Indigenous children, Indigenous children have increased rates of pneumonia, a higher frequency of repeated hospitalizations for pneumonia and a greater propensity to develop CSLD (12).

Another lower respiratory infection, bronchitis, is defined as inflammation of the mucous membranes of the bronchi. Acute bronchitis often occurs during the course of an acute viral illness such as the common cold...
or influenza. Viruses cause about 90% of cases while bacteria account for less than 10% (13). There is very little literature relating to acute bronchitis in children, presumably because it is a self-limiting disorder for which antibiotics are not usually justified (13). There are no data available specific to acute bronchitis in Papua New Guinean or Australian Indigenous children.

**Chronic lower respiratory infections**

Chronic wet cough may be caused by several interrelated endobronchial infections: persistent or protracted bacterial bronchitis (PBB), CSLD and bronchiectasis (14). Neutrophilic airway inflammation features in all three conditions and impaired mucociliary clearance seems to be the common risk factor, allowing bacteria to colonize the lower airway (14). Clinically these conditions overlap; whether they are different conditions or reflect severity as part of a spectrum is yet to be determined (14). Misdiagnosis of asthma is common, complicated by the fact that coexistence of asthma is not uncommon (14).

PBB is defined as the presence of chronic (>4 weeks) wet/moist cough, resolution of cough with antibiotic treatment and absence of pointers suggestive of an alternative specific cause (14). This condition has only recently been adequately characterized in children, both clinically and by bronchoscopic examination (15). As determined by bronchial lavage bacterial infection of the airways is the most common cause of chronic cough in Australian children, with few respiratory viruses detected (15). Airway neutrophilia is present in these children, and treatment is based on eradicating the bacteria with antibiotics. No data specific to Papua New Guinean or Australian Indigenous children have been found. PBB is increasingly recognized as an important diagnosis, the treatment of which may prevent progression to bronchiectasis (16).

Untreated PBB is a likely precursor to CSLD and bronchiectasis (Figure 1) (14). The term CSLD is used to describe a diagnosis where there are clinical symptoms of bronchiectasis without evidence from a chest high-resolution computed tomography (HRCT) scan (14). Bronchiectasis is defined as irreversible dilatation of peripheral airways (bronchi) which has been HRCT confirmed. At the milder end of the spectrum it appears that radiographic changes may be reversible (17). Extra mucus tends to form and pool in the parts of the airways that are widened, rendering them prone to infection (18). Antibiotics are the mainstay of treatment: with mild bronchiectasis, a course of antibiotics is needed occasionally to clear chest infections as and when they occur; however, with more severe bronchiectasis, chest infections may return quickly following cessation of antibiotics and prophylactic antibiotics may need to be taken regularly (18).

No reference could be found for bronchiectasis in children in PNG. However, it is likely that high rates exist as with Australian Indigenous children. In Central Australia, radiologically confirmed bronchiectasis is present in 1.5% of Indigenous children aged <15 years, which is one of the highest rates recorded in the world (12). While there are management programs for cystic fibrosis (CF) (the commonest cause of bronchiectasis in non-Indigenous children) which aim to prevent disease progression, there are no parallel concerted programs or dedicated resources to manage children with non-CF bronchiectasis (who are mostly Aboriginal children in Australia). These children have significant morbidity and some succumb to premature death in adulthood (19). Non-CF bronchiectasis is now uncommon in developed countries, but persists in developing countries and other disadvantaged populations (20). These differences are partly attributed to overcrowding and poor living conditions.

While chronic obstructive pulmonary disease (COPD) is primarily a disease of adults, it is included here as a potential consequence of lower respiratory infections in childhood (Figure 1). COPD refers to chronic bronchitis and emphysema, two commonly coexisting diseases of the lungs in which the airways become narrowed. Chronic bronchitis is characterized by the presence of a productive cough and usually develops due to recurrent injury to the airways caused by inhaled irritants. While cigarette smoking is the most common cause, COPD is not simply a ‘smoker’s cough’ but an underdiagnosed, life-threatening lung disease (21).

Acute exacerbations of chronic bronchitis (AECB) are a major contributor to morbidity and mortality in patients with COPD (22).
AECB can be caused by allergens, toxins, or acute viral or bacterial infections. However, bacterial agents are the predominant cause and the acquisition of new strains has been linked with episodes of AECB (23).

Chronic lung disease (primarily COPD) is a major problem in PNG. A survey of 510 adults in the Asaro Valley found a high prevalence of loose cough (36%) and chronic bronchitis (25%) (24). Demographic surveillance in the Tari Basin found that respiratory disease (particularly chronic lung disease in adults) accounted for 39% of all deaths (1). The role of smoking is unclear, and complicated by differences between traditional tobacco and western-style cigarettes. Grimley found that smoking was significantly related to loose cough and chronic bronchitis (24). However, Anderson and colleagues found that, unlike chronic obstructive lung disease in European populations, tobacco smoking is not an important aetiological factor in PNG (25). Although there is no direct evidence, the most likely alternative aetiologies are domestic wood smoke and acute respiratory infections in childhood (24,26).

In Australia, COPD is a serious disease that is increasing in prevalence, yet is underdiagnosed and under-recognized (27). Data on CSLD and bronchiectasis in Australian adults are lacking (28). However, bronchiectasis remains a significant cause of morbidity and mortality in Indigenous adults in Central Australia (6).

**Aetiology of acute lower respiratory infections**

Whilst the focus here is bacterial, recent animal and epidemiological studies indicate that respiratory viral infections increase susceptibility to bacterial superinfection, and interactions between bacteria and viruses result in greater severity of disease (29,30). Attempts to establish the aetiology of pneumonia and other ALRIs have been frustrated by the difficulty of detecting causative organisms (31). Lung aspiration is rarely done and sputum examination is difficult in young children (7). Blood cultures can only be successful when the infection has become bacteraemic, and even then their sensitivity is low, especially when antibiotics have already been administered (7). A study of the aetiology of 322 ALRI cases in 280 Central Australian Aboriginal children found only 20 blood cultures positive for bacteria (32). Interestingly, antimicrobial substances were present in 6 of 10 blood-culture-positive cases examined, and the proportion of patients with a positive pneumolysin antibody test was not significantly different in those with or without antimicrobial substances (32). However, antibiotic resistance was not reported.

**Aetiology of pneumonia**

Despite problems of detection, the importance of Streptococcus pneumoniae and Haemophilus influenzae type b (Hib) as major causes of pneumonia in children has been established by blood culture, by lung aspiration studies and, more recently, by vaccine probe studies (33). S. pneumoniae is the main cause of pneumonia in almost all studies around the world (34). Recent vaccine trial data indicate that in Africa it may be responsible for over 50% of severe pneumonia cases, and probably a higher proportion of fatal cases (35). This proportion may vary in different parts of the world. H. influenzae is also a major cause of pneumonia, with most disease caused by Hib (34). Vaccine studies from Bangladesh, Chile and The Gambia suggest that Hib causes around 20% of severe pneumonia cases (36,37). With the introduction of Hib conjugate vaccines, the incidence of pneumonia (and meningitis) caused by Hib has been greatly reduced; however, not all countries have these vaccines.

Other important pathogens causing pneumonia in children (32,34,36-41) include viruses such as respiratory syncytial virus (RSV) and influenza; other bacteria such as Staphylococcus aureus; Klebsiella pneumoniae, Moraxella catarrhalis, Mycoplasma pneumoniae, Neisseria meningitidis, Escherichia coli and other enterobacteriaceae; Chlamydia species; and the fungus Pneumocystis, which is particularly important in young children with AIDS. Detailed coverage of these pathogens is beyond the scope of this review, which is primarily concerned with the three main respiratory bacteria, namely S. pneumoniae, H. influenzae and M. catarrhalis.

Nontypeable H. influenzae (NTHi) may be an important pneumonia pathogen in children in some populations but evidence is limited (7). NTHi is rarely cultured from blood and therefore lung aspiration studies are especially valuable (42,43). The seminal
Aetiology of pneumonia

While NTHi pneumonia may also be important in Australian Indigenous children, it was not found in the Central Australian study by Torzillo and colleagues (32). Only 20 blood cultures were positive for bacteria: 11 were positive for *H. influenzae* (all typeable, 10 Hb) and 6 positive for *S. pneumoniae*. However, pneumolysin results (92 of 322 positive) suggest that at least one-third of ALRI in this population is caused by the pneumococcus. Of the 322 ALRI cases an aetiological agent was identified in 219 (68%) and in 20% of these co-infection with chlamydia and/or viral and/or other bacterial infection was found (32).

The Gambia has 20 years of experience of lung aspiration, with no deaths and few complications (46). An early lung aspiration study of 64 patients with pneumonia (51 children <10 years of age) found *S. pneumoniae* in 51% and *H. influenzae* in 24% of children: 4 of 13 typed isolates (31%) were NTHI (47); 5 (12%) of the 43 patients with a bacterial aetiology had multiple pathogens identified. Detection has improved with new molecular methods. In a more recent Gambian study, detection of *S. pneumoniae* in 50 lung aspirates from children with severe pneumonia was improved from 24% on culture to 92% using PCR, while detection of *H. influenzae* was improved from 0% on culture to 20% using PCR (48). All *H. influenzae* were found in co-infection with pneumococcus, and 5/10 were NTHi (48). Therefore NTHi would appear to be an important pneumonia pathogen in Gambian children, though not to the same extent as in PNG (found in 8-10% versus >20% of affected children).

NTHi is a common cause of pneumonia in adults (49). In a prospective study of 170 patients with acute pneumonia in PNG, *H. influenzae* was found (in cultures of blood or lung aspirate or both) in 15 cases (9%): 7 were Hb and 3 NTHi (50). In all 15 cases *H. influenzae* was the sole organism isolated and, interestingly, chronic lung disease was significantly more common in patients with *H. influenzae* pneumonia than in patients with pneumonia due to other organisms. An earlier study in the US suggested that *H. influenzae*, both typeable and nontypeable, is a more frequent cause of pneumonia in adults than previously appreciated (51). Following widespread vaccination against Hib, NTHi was found to cause 79% of *H. influenzae* pneumonia cases in Spain (52). In a review of pneumonia studies from around the world (mainly in adults), the most common pathogens (in order of frequency) were *S. pneumoniae*, *H. influenzae*, *H. parainfluenzae*, *S. aureus*, *M. catarrhalis* and *K. pneumoniae* (53).

Aetiology of bronchiolitis

RSV bronchiolitis is the leading cause of paediatric admissions in the first year of life (54). RSV is identified in about 70% of hospitalized infants with bronchiolitis (55); other viruses implicated include adenovirus, rhinovirus, and influenza and parainfluenza viruses (7,56,57). Single viral infections are most common, but co-infection with two or more viruses is found in about a third of cases (56,58). Bacterial co-infection is generally uncommon, except in severe cases. In five studies of bronchiolitis or wheezing (presumably bronchiolitis) in infants and young children with bacterial co-infection, the bacteria detected most frequently were *H. influenzae*, *M. catarrhalis*, *S. pneumoniae* and *S. aureus* (56,57,59,60 and KMH and colleagues, unpublished) (Table 1). Only our Australian study reported whether *H. influenzae* strains were typeable or nontypeable (unpublished data).

In studies listed in Table 1 all children were hospitalized, except in the Danish study, in which children with no wheeze were compared to children with wheezy episodes or clinical pneumonia; wheezy episodes were
<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Population</th>
<th>Median Age</th>
<th>Number and Condition</th>
<th>Specimen</th>
<th>Haemophilus influenza</th>
<th>Streptococcus pneumonia</th>
<th>Moraxella catarrhalis</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thorburn (59)</td>
<td>2006</td>
<td>United Kingdom</td>
<td>1.6 months</td>
<td>165 severe RSV bronchitis</td>
<td>Lower airway secretions</td>
<td>17% any growth</td>
<td>7% any growth</td>
<td>11% any growth</td>
<td>13% any growth</td>
</tr>
<tr>
<td>(59)</td>
<td></td>
<td></td>
<td>(interquartile range 0.5 to 4.6 months)</td>
<td></td>
<td></td>
<td>(10% &gt;10^5 CFU/ml)</td>
<td>(4% &gt;10^5 CFU/ml)</td>
<td>(5% &gt;10^5 CFU/ml)</td>
<td>(6% &gt;10^5 CFU/ml)</td>
</tr>
<tr>
<td>Franz (56)</td>
<td>2010</td>
<td>Germany</td>
<td>0.8 years</td>
<td>244 (60%) wheezing</td>
<td>Nasopharyngeal aspirates</td>
<td>21% any growth</td>
<td>11% any growth</td>
<td>17% any growth</td>
<td>11% any growth</td>
</tr>
<tr>
<td>(56)</td>
<td></td>
<td></td>
<td>(range 0 to 16 years)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bisgard (57)</td>
<td>2010</td>
<td>Denmark</td>
<td>12 months</td>
<td>279 No wheeze</td>
<td>Hypopharyngeal aspirates</td>
<td>26%</td>
<td>38%</td>
<td>29%</td>
<td>20%</td>
</tr>
<tr>
<td>(57)</td>
<td></td>
<td></td>
<td>14 months</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>15 months</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duttweller (60)</td>
<td>2004</td>
<td>Switzerland</td>
<td>1.7 months</td>
<td>56 severe RSV bronchitis</td>
<td>Tracheal aspirates</td>
<td>30%</td>
<td>16%</td>
<td>21%</td>
<td>14%</td>
</tr>
<tr>
<td>(60)</td>
<td></td>
<td></td>
<td>(range 0 to 5.8 years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hare and colleagues, unpublished data</td>
<td>2010</td>
<td>Australia (35 Indigenous, 13 non-Indigenous)</td>
<td>4.9 months</td>
<td>48 bronchitis</td>
<td>Nasopharyngeal swabs</td>
<td>44% (all NTHi)</td>
<td>23%</td>
<td>44%</td>
<td>10%</td>
</tr>
<tr>
<td>(unpublished data)</td>
<td></td>
<td></td>
<td>(range 0.5 to 11.2 years)</td>
<td></td>
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</tbody>
</table>

RSV = respiratory syncytial virus; CFU = colony-forming units; NTHi = non-typeable Haemophilus influenzae
independently associated with both bacterial and viral infection (57). In the German study of children hospitalized for acute virus-induced wheezing (60%) or pneumonia (40%), the median age was 0.8 years, with pneumonia more often found in older children (56). A positive bacterial culture was found in 198 (63%) of the nasopharyngeal aspirates collected on admission; 97 (31%) had “abundant pathogenic bacteria” and were included in viral co-infection analyses, where *H. influenzae* dominated in infections due to RSV identified as the sole virus and *M. catarrhalis* in infections due to RSV identified simultaneously with other viruses (56). The UK study found that up to 40% of children with severe RSV bronchiolitis had lower airway bacterial infection and were at increased risk of developing bacterial pneumonia (59).

Interestingly, *M. catarrhalis* was isolated more often than *S. pneumoniae* and almost as often as *H. influenzae* in all studies listed in Table 1. This probably reflects the young age of bronchiolitis patients, since *M. catarrhalis* mainly colonizes the very young or the very old (61). Data suggest that *M. catarrhalis* carriage is highest in children <18 months of age. While *M. catarrhalis* has long been known as an important pathogen in adults with COPD (62), it is also now considered an important pathogen in lower respiratory tract infections in children (63). However, even though *M. catarrhalis* was isolated from 11% of children in Shann and colleagues’ pneumonia study (41), *M. catarrhalis* carriage is uncommon in Papua New Guinean children (D. Lehmann, personal communication); the reasons for this are unclear.

**Aetiology of chronic lower respiratory infections**

Only two studies were found reporting bacteriological findings in children with persistent bacterial bronchitis. In a prospective study of 108 (mainly non-Indigenous) Australian children with chronic wet cough (median age 2.6 years), 40% were diagnosed with PBB, with a positive bacterial culture ≥10⁵ colony-forming units (cfu)/ml bronchoalveolar lavage (BAL) fluid (15). Pathogens included *H. influenzae* (type unspecified, 47% of children), *S. pneumoniae* (35%) and *M. catarrhalis* (26%). More than one organism grew in significant numbers in an unspecified number of patients. A second study of 81 UK children with PBB (median age 3.75 years) similarly found that the most commonly isolated organisms were *H. influenzae* (type unspecified) and *S. pneumoniae* (16). Pathogens were grown in over half of 51 cough swabs, with *H. influenzae* cultured in 81%, *S. pneumoniae* in 37% and both in 30% of positive swabs. Other organisms detected were *Moraxella* (unspecified) and other streptococci. Bronchoscopy culture results from 19 patients were reported to be similar to cough swab results but details were not given (16).

CSDL and bronchiectasis are associated with persistent infection with the same organisms in the airways as those found in PBB. The significance of *H. influenzae* in bronchiectasis of children was noted more than 50 years ago (64). After *H. influenzae* was found in 63% of 100 bronchoscopic aspirations from young adults, the authors detected *H. influenzae* in 84% of 32 children aged 4-15 years with purulent bronchiectasis (64). In both studies, the majority of strains were non-encapsulated (NTHi). These authors quoted from a previous study that stated “non-encapsulated Hemophilus (influenzae) is a pathogen” and “the etiology of bronchitis and that of bronchiectasis cannot possibly be understood if the part played by Hemophilus infection is overlooked.” After observing a total of 204 relapses during 306 patient-months, mostly associated with reappearance of *H. influenzae*, the authors concluded that NTHi “is responsible for keeping the chronic inflammatory process smouldering in bronchiectatic individuals” (64).

The main bacteria associated with non-CF bronchiectasis in six more recent studies in children are listed in Table 2 (20,65-69). *H. influenzae* was the most common pathogen identified in all six studies (specified as NTHi in two studies), followed by *S. pneumoniae* and (in varying order of frequency) *M. catarrhalis, Pseudomonas* sp. and *S. aureus*. It is noteworthy that *M. catarrhalis* was isolated more frequently from the Australian Indigenous children, who were also younger than children in the other studies (Table 2). *H. parainfluenzae*, *Streptococcus pyogenes* and *K. pneumoniae* were listed as pathogens in one study each (20,65,67). Co-infection was reported in three studies: two or more organisms were isolated in 5% of New Zealand children (65), 15% of UK patients (66) and 18% of Australian Indigenous children with
bronchiectasis (69); if the denominator included only those with lower airway infection (defined as $>10^4$ cfu/ml BAL fluid), 33% had co-infection in the Australian study (69). The Australian study also reported a high proportion of multiple strains within pathogen species isolated from the lower airways: 67% for NTHi, 25% for *S. pneumoniae* and 22% for *M. catarrhalis* (69). The highest rates of NTHi isolation were among Australian Indigenous children and children in the New Zealand study, 80% of whom were of Pacific Islander or Maori descent (65,69). While *H. influenzae* also features in CF bronchiectasis, two other main pathogens are *P. aeruginosa* and *S. aureus* (39). In a study of non-CF bronchiectasis in 61 Central Australian adults (97% Indigenous), *H. influenzae* (type unspecified) was isolated from 38 (81%) of 47 positive sputum cultures, *P. aeruginosa* from 12 (26%) and *S. pneumoniae* from 9 (19%), and 21 (45%) had multiple pathogens isolated (6).

Common bacterial pathogens identified during acute exacerbations of COPD include *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* (70). Exacerbations may also be associated with infection with *S. aureus* and *P. aeruginosa* (71). Viruses associated with exacerbations include rhinovirus, influenza virus, parainfluenza virus, coronavirus, adenovirus and RSV (71). An early study found that, while NTHi may have pathogenic potential in patients with COPD, *H. parainfluenzae* should be considered as normal flora (72). More recent studies suggest that *H. parainfluenzae* may have a pathogenic role in COPD (73), and it has been listed as a pathogen commonly associated with AECB (74).

**Microbiological factors contributing to the burden of disease**

It is likely that recurrent aspiration into the lower airways of pathogenic bacteria originating in the nasopharynx is an important contributor to lower respiratory infections (69,75-77). It is therefore of relevance that Papua New Guinean and Australian Aboriginal infants have high rates of nasopharyngeal colonization by potentially pathogenic respiratory bacteria from early infancy (78,79). During acute respiratory infections, repeated aspiration of bacteria-laden nasopharyngeal (NP) secretions may overwhelm local pulmonary defences, helping to initiate endobronchial infection, inflammation and airway injury, the central tenet of Cole’s ‘vicious circle’ hypothesis for the origins of bronchiectasis (80). High concordance between NP and lower airway (BAL) strains of respiratory bacteria in Australian Indigenous children with bronchiectasis suggests recent aspiration of NP secretions (69). Compared to the NP secretions, a higher proportion of BAL fluids harbour multiple strains, many of which are not found in the NP (69). It is plausible that recurrent aspiration over time leads to accumulation and persistence of strains in the lower airway, where immune clearance is poor. Recurrent acute respiratory infections therefore contribute to the development of chronic lung disease.

Australian Indigenous children (and most likely Papua New Guinean children) have higher bacterial loads than non-Indigenous children (81). Children from these populations also carry a greater diversity of species and strains. While carriage of multiple pneumococcal serotypes is reportedly rare (<3%) in other populations (82), 20%-30% of carriage-positive children in PNG and The Gambia as well as Australian Aboriginal children carry multiple serotypes (83-85). Carriage-positive Australian Aboriginal and Papua New Guinean children also harbour multiple *H. influenzae* strains: 44% have multiple ribotypes and 53% have multiple biotypes (83,86).

It is possible that the child’s immune response to repeated acquisition of new strains may be overwhelmed, since antibody responses to pneumococci and *H. influenzae* are strain-specific (87,88). Nevertheless, while NTHi is a major cause of respiratory disease, it rarely causes systemic infection because it does elicit effective humoral immunity (89). However, chronic infection with NTHi in bronchiectasis is associated with non-clearing adaptive immunity that may be important in the pathogenesis of bronchial infection (90).

In addition, antibiotic regimens developed in low-risk populations may be inadequate for eradication of infections where there is a high bacterial load. Antimicrobial efficacy is based on pharmacodynamic and pharmacokinetic parameters including the requirement to maintain a sufficient concentration for an adequate period of time at the site of infection (91). Minimum inhibitory concentrations determined in first-world settings may not be appropriate for the
<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Population</th>
<th>Age (years)</th>
<th>Number</th>
<th>Specimen</th>
<th>Haemophilus influenzae</th>
<th>Streptococcus pneumoniae</th>
<th>Moraxella catarrhalis</th>
<th>Pseudomonas sp.</th>
<th>Staphylococcus aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards (65)</td>
<td>2003</td>
<td>New Zealand*</td>
<td>1 to 17 (median 10)</td>
<td>60</td>
<td>Sputum</td>
<td>55% (NTHi)</td>
<td>8%</td>
<td>5%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>Karadag (20)</td>
<td>2005</td>
<td>Turkey</td>
<td>1 to 17.5 (mean 7.4)</td>
<td>111</td>
<td>Sputum</td>
<td>23% (type not specified)</td>
<td>14%</td>
<td>4%</td>
<td>6%</td>
<td>10%</td>
</tr>
<tr>
<td>Li (66)</td>
<td>2005</td>
<td>England</td>
<td>3.1 to 18.1 (median 12.1)</td>
<td>136</td>
<td>Cough swab, sputum or BAL</td>
<td>39% (type not specified)</td>
<td>17%</td>
<td>2%</td>
<td>11%</td>
<td>4%</td>
</tr>
<tr>
<td>Eastham (67)</td>
<td>2004</td>
<td>England</td>
<td>1.6 to 18.8 (median 7.2)</td>
<td>93</td>
<td>Cough swab, sputum or BAL</td>
<td>48% (type not specified)</td>
<td>22%</td>
<td>17%</td>
<td>6%</td>
<td>8%</td>
</tr>
<tr>
<td>Kapur (68)</td>
<td>2009</td>
<td>Australia</td>
<td>3 to 17 (median 5.5)</td>
<td>30</td>
<td>Sputum and BAL (85 specimens)</td>
<td>32% (type not specified)</td>
<td>15%</td>
<td>8%</td>
<td>8%</td>
<td>5%</td>
</tr>
<tr>
<td>Hare (69)</td>
<td>2010</td>
<td>Australia (Indigenous children)</td>
<td>0.7 to 10.1 (median 2.3)</td>
<td>45</td>
<td>BAL</td>
<td>78% any growth (NTHi)</td>
<td>33% any growth</td>
<td>27% any growth</td>
<td>4% any growth</td>
<td>9% any growth</td>
</tr>
</tbody>
</table>

*80% Pacific Islander or Maori descent
CF = cystic fibrosis; NTHi = nontypeable Haemophilus influenzae; BAL = bronchoalveolar lavage fluid; CFU = colony-forming units
All percentages are based on number of specimens
significantly higher bacterial loads found in disadvantaged populations (81). For example, azithromycin was highly effective (clinical success rate >80%) in treating acute otitis media (OM) in non-Indigenous children (92), whereas the same regimen achieved a success rate of only 50% in Australian Aboriginal children (93).

It has been suggested that early and dense colonization and carriage of multiple strains of potentially pathogenic respiratory bacteria may help to explain the chronicity of carriage and persistence of OM in Australian Aboriginal infants (79,86). These factors may also explain the recurrence and persistence of lower respiratory infections and the development of chronic lung disease in Australian Indigenous and Papua New Guinean children. The importance of household transmission and crowding as risk factors for carriage of respiratory pathogens has been demonstrated (94). A study in the Asaro Valley of PNG found that cohabitation with an adult complaining of chronic cough was a risk factor for ALRI in children (Rohan Grimley, ‘Cohabitation with an adult complaining of chronic cough as a risk factor for acute respiratory infections (ARI) in children’, unpublished report, 1989). While respiratory infections are primarily spread by airborne droplets, indirect spread by hands is also believed to play a role. Hand contamination has been demonstrated in PNG (95), and shown to be higher in Indigenous than in non-Indigenous children in the NT (96). Poor hygiene, overcrowding and hand contamination are likely to facilitate frequent transmission of respiratory bacteria, contributing to high rates of infection. Figure 2, adapted from an OM model (97) which was in turn adapted from Cole’s original model (80), shows an ‘extended vicious circle’ hypothesis to explain high rates of respiratory infection among Papua New Guinean and Australian Indigenous children.

Vaccines

New vaccines are needed to help reduce this heavy burden of disease. Conjugate vaccines have had a major impact on targeted diseases (including pneumonia) in countries where the vaccines have been introduced. Hib disease is now almost unknown in developed countries, and incidence of invasive disease and pneumonia caused by serotypes included in the 7-valent

![Figure 2. Extended vicious circle hypothesis explaining high rates of respiratory infection in Papua New Guinean and Australian Indigenous children. Adapted from Wiertsema and Leach (97)](image-url)
pneumococcal conjugate vaccine Prevenar® (which includes serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) have been substantially reduced (98,99). Hib vaccine was introduced to Australia in 1993 and resulted in a substantial decline in incidence of Hib disease, while Prevenar was introduced in 2001 for Indigenous and other high-risk infants (100,101). PNG only recently (in 2008) introduced a Hib vaccine, and has not yet introduced a pneumococcal conjugate vaccine into their infant vaccination schedule.

New conjugate vaccines on the market provide additional protection. The pneumococcal-nontypeable H. influenzae protein D conjugate vaccine, PHiD-CV (Synflorix®), provides protection against three additional serotypes (1, 5 and 7F) compared to Prevenar, and has been shown to protect against OM due to NTHi (102). At time of writing it is unknown whether Synflorix affords protection against pneumonia or other lower respiratory infections. Synflorix replaced Prevenar on the NT immunization schedule in October 2009. Prevenar13® has the same 10 serotypes as Synflorix plus an additional three (3, 6A and 19A), but does not have an NTHi component. This presents a difficult choice for vaccine policy-makers in regions with high rates of NTHi and pneumococcal infection. A trial in the NT aims to evaluate a combination schedule with both Synflorix and Prevenar13, while a study in PNG aims to assess safety and immunogenicity of each of these two vaccines in the routine accelerated 1-2-3-month schedule, followed by a booster at age 9 months with pneumococcal polysaccharide vaccine or no booster.

An oral vaccine containing killed NTHi (Broncostat®) significantly reduced the incidence of acute bronchitis in patients with chronic bronchitis in the Asaro Valley of PNG (103). This vaccine had earlier been found to afford protection in an Australian trial (104). A review of the PNG study and five Australian trials found that the vaccine reduced the number and severity of exacerbations for up to 6 months after vaccination, and the authors concluded that a large clinical trial was needed (105). NTHi was the most commonly isolated bacterium during an exacerbation, and the oral vaccine reduced carriage of NTHi in the upper respiratory tract (105). Recently a phase 2 clinical trial demonstrated that an NTHi oral immunotherapeutic vaccine (HI-1640V) reduced the number and severity of acute exacerbations of COPD (106). Results are consistent with the idea that oral NTHi enhances mucosal protection (107). It is possible that such a vaccine may reduce the incidence and/or severity of other respiratory infections caused by H. influenzae, namely bronchiolitis, pneumonia and bronchiectasis.

In studies of carriage, aetiology and vaccine impact, there is a need to differentiate between NTHi and non-haemolytic H. haemolyticus, a respiratory tract commensal (108). In a study of otitis-prone and healthy children in Western Australia, researchers found that 12% of NP isolates previously identified as NTHi were H. haemolyticus (109). Studies are underway to differentiate NTHi from H. haemolyticus in NP swabs from children who took part in a pneumococcal conjugate vaccine trial in PNG, and from NP and lower airway specimens from Indigenous Australian children with bronchiectasis.

Discussion

There are similarities and differences in the types of bacteria causing bronchiolitis, bronchitis, pneumonia and bronchiectasis in children. S. pneumoniae and H. influenzae feature in all respiratory bacterial infections, while M. catarrhalis is more likely to be associated with infections in infants with bronchiolitis or young children with pneumonia or bronchiectasis. The capsular organisms S. pneumoniae and Hib appear to be more important in acute respiratory infections and invasive disease, while NTHi features more in chronic lung disease. Acute infections are usually caused by single pathogens, although this is less true for Papua New Guinean and Australian Indigenous children, who frequently carry multiple species and strains of respiratory bacteria and suffer a high burden of respiratory disease. Chronic infections are more often associated with multiple pathogen species and strains. It also appears that NTHi may be more important in PNG children and Indigenous children in Australia and New Zealand than in more advantaged populations, though evidence is limited since in many studies H. influenzae serotype is not reported.

Ideally, children with pneumonia (especially severe and/or recurrent pneumonia) or persistent symptoms should be evaluated for an underlying condition such as bronchiectasis, since they are at risk of
developing chronic lung disease. There is also little doubt that improved living conditions (better nutrition and hygiene, less crowding and reduced indoor air pollution) would help reduce the high bacterial load and transmission of potential pathogens, and thus have a positive impact on disease outcomes. In the short to medium term, the rollout of new vaccines may provide more practical protection.

ACKNOWLEDGEMENTS

We thank Deborah Lehmann and Michael Alpers for helpful comments.

REFERENCES

27 Frith PA, Cafarella PA, Duffy JM. Chronic obstructive pulmonary disease (COPD) is a major personal and public health burden in Australia. Aust NZ J Public Health 2008;32:139-141.


Appendix 2

Supplementary Figures and Tables

Bronchiectasis Observational Study (section 4.3, pp. 110-1)
Figure S1. Pathogen carriage (proportion of swabs) by study year in Australian children, grouped by azithromycin exposure.¹

¹Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection at: Azi-None = no study visits; Azi-Infreq(uent) = 1-50% of study visits; Azi-Freq(uent) = 51-100% of study visits.
Table S1. Nasopharyngeal carriage of respiratory bacteria from Australian children, grouped by azithromycin exposure\(^1\), at baseline and end of study.

<table>
<thead>
<tr>
<th></th>
<th>First swab for each child</th>
<th>Last swab for each child</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azi-None</td>
<td>Azi-Infrequent</td>
</tr>
<tr>
<td>Children enrolled</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Median age at study visit in years (range)</td>
<td>3.3 (0.8-8.9)</td>
<td>2.6 (1.0-8.5)</td>
</tr>
<tr>
<td>Male sex; n (%)</td>
<td>15 (58%)</td>
<td>12 (46%)</td>
</tr>
<tr>
<td>Median time in study in years (range) at last swab</td>
<td>2.5 (0.5-5.8)</td>
<td>2.1 (0.5-5.8)</td>
</tr>
<tr>
<td><strong>Nasopharyngeal carriage; n (%, 95% CI)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>21 (81, 61-93)</td>
<td>12 (46, 27-67)</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>17 (65, 44-83)</td>
<td>18 (69, 48-86)</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>17 (65, 44-83)</td>
<td>15 (58, 37-77)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>0 (0, 0-13)</td>
<td>3 (12, 2-30)</td>
</tr>
<tr>
<td><strong>Antibiotics received &lt;2 weeks before swab collection; n (%, 95% CI)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrolide</td>
<td>0 (0, 0-13)</td>
<td>9 (35, 17-56)</td>
</tr>
<tr>
<td>Beta-lactam</td>
<td>7 (27, 12-48)</td>
<td>7 (27, 12-48)</td>
</tr>
</tbody>
</table>

* P<0.05, ** P<0.01, *** P≤0.001 for trend over 3 groups; CI, confidence interval.

\(^1\)Azithromycin preceding: no study visits (Azi-None); 1-50% of study visits (Azi-Infrequent); 51-100% of study visits (Azi-Frequent).
Figure S2. Pathogen resistance (proportion of carriers) by study year in Australian children, grouped by azithromycin exposure\(^1\).

\(^1\)Azithromycin preceding: no study visits (Azi-None); 1-50% of study visits (Azi-Infreq); 51-100% of study visits (Azi-Freq).
Table S2. Antibiotic resistance (proportion of carriers) in respiratory bacteria from Australian children, grouped by azithromycin exposure, at baseline and end of study.

<table>
<thead>
<tr>
<th></th>
<th>First swab for each child</th>
<th>Last swab for each child</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azi-None</td>
<td>Azi-Infrequent</td>
</tr>
<tr>
<td><strong>Macrolide resistance; n (%, 95% CI)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MacR² S. pneumoniae</td>
<td>4 (19, 5-42)</td>
<td>2 (17, 2-48)</td>
</tr>
<tr>
<td>AzIR³ H. influenzae</td>
<td>15 (88, 64-99)</td>
<td>16 (89, 65-99)</td>
</tr>
<tr>
<td>AzIR³ H. influenzae</td>
<td>2 (12, 1-36)</td>
<td>1 (6, 0-27)</td>
</tr>
<tr>
<td>EryR⁶ S. aureus</td>
<td>none carried</td>
<td>3 (100, 29-100)</td>
</tr>
<tr>
<td><strong>Beta-lactam antibiotic resistance; n (%, 95% CI)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PenIR⁷ S. pneumoniae</td>
<td>10 (48, 26-70)</td>
<td>7 (58, 28-85)</td>
</tr>
<tr>
<td>AmpR⁸ H. influenzae</td>
<td>1 (6, 0-29)</td>
<td>3 (17, 4-41)</td>
</tr>
<tr>
<td>BLpos⁹ H. influenzae</td>
<td>0 (0, 0-20)</td>
<td>3 (17, 4-41)</td>
</tr>
<tr>
<td>BLpos⁹ M. catarrhalis</td>
<td>14 (82, 57-96)</td>
<td>15 (100, 78-100)</td>
</tr>
<tr>
<td>MethR¹⁰ S. aureus</td>
<td>none carried</td>
<td>2 (67, 9-99)</td>
</tr>
</tbody>
</table>

* P<0.05 for trend over 3 groups; # statistical tests not performed due to small numbers; CI, confidence interval.

¹ Australian children were grouped by proportion of study visits with azithromycin use <2-weeks before swab collection: Azi-None=no azithromycin at any study visit; Azi-Infrequent=azithromycin at 1-50% of study visits; Azi-Frequent=azithromycin at 51-100% of study visits.
MacR, macrolide-resistant: azithromycin (Australia) and erythromycin (Alaska) minimum inhibitory concentration (MIC) >0.5 mg/L.

AzIR, azithromycin intermediate resistant: MIC >0.12-4 mg/L; AziR, azithromycin resistant: MIC >4 mg/L.

Isolates from one swab did not grow on sensitivity plates.

One swab had AzIR and AziR strains of H. influenzae.

EryR, erythromycin resistant on disk diffusion.

PenIR, penicillin intermediate resistant: MIC >0.06-2 mg/L; no resistant (MIC >2 mg/L) isolates were detected in any group.

AmpR, ampicillin MIC >1 mg/L.

BLpos, beta-lactamase positive.

MethR, methicillin resistant on disk diffusion.