Culture-independent analysis of the bacteriology associated with acute otitis media in Indigenous Australian children.

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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.

Robyn Marsh

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Abstract

Otitis media is endemic in many remote Indigenous Australian communities. Children in remote communities develop otitis media in the first weeks of life and are at high-risk of progression to chronic suppurative otitis media (CSOM). Current therapeutic and preventive interventions are of limited benefit.

This thesis presents a culture-independent analysis of the bacteriology associated with acute otitis media in Indigenous children from the Northern Territory. The objective of the study was to use culture-independent methods to better understand the bacteriology underlying acute otitis media in this population. Principle findings from the study are as follows:

1. Nasopharyngeal total or pathogenic bacterial loads are unsuitable as prognostic indicators of clinical antibiotic treatment outcomes in Indigenous children with acute otitis media.

2. *Alloiococcus otitidis* is present in ear discharge from Indigenous children with acute otitis media, potentially as a secondary middle ear pathogen. Further studies to test for *A. otitidis* in CSOM are warranted.

3. T-RFLP can provide broad characterisation of bacterial communities in upper respiratory specimens; however, it is limited by methodological biases which result in underestimation of bacterial richness.

4. Despite the methodological limitations, T-RFLP analysis demonstrated significant differences between nasopharyngeal and ear discharge bacterial communities in Indigenous children with acute otitis media with perforation, suggesting divergence of the middle ear microbiome following secondary infection by canal flora.

5. 16S rRNA gene microarray (PhyloChip™) analysis of ear discharge from Indigenous children with acute otitis media with perforation indicates a high-level of bacterial richness which is hypothesised to contribute to progression to CSOM.
Prospective longitudinal studies are now required to better understand the canal flora before perforation, the microbiology of acute otitis media immediately following perforation, and microbiomic changes associated with prolonged perforation. Such studies will aid understanding of the pathogenesis of acute otitis media with perforation and progression to CSOM, and potentially reveal new therapeutic targets or prevention strategies.
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Publications and presentations

This section describes publications and presentations made during my PhD candidature.

PUBLICATIONS

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Publications in preparation

Marsh RL; Smith-Vaughan, HC; Kaestli, M; Cheng A; Ward, L; Leach, AJ. Pretreatment nasopharyngeal total and pathogenic bacterial loads do not predict the clinical outcome of antibiotic treatment of acute otitis media in Indigenous Australian children.

Marsh RL; Beissbarth, J; Christensen, P; Binks, M; Morris P; Leach AJ; Smith-Vaughan HC. Quantitative PCR of ear discharge from Indigenous Australian children with acute otitis media supports Alloiococcus otitidis as a secondary otopathogen.

Marsh, RL; Kaestli, M; Binks, M; Ward L; Christensen P; Smith-Vaughan, HC. Optimisation and validation of T-RFLP for application to paediatric nasopharyngeal and middle ear specimens.

Marsh, RL; Kaestli, M; Binks, M; Ward L; Christensen P; Morris, P; Leach, AJ; Smith-Vaughan, HC. T-RFLP analysis suggests microbiomic differences between the nasopharynx and middle ear of Indigenous Australian children with acute otitis media with perforation.

Marsh, RL; Kaestli, M; Morris, P; Leach, AJ; Smith-Vaughan, HC. PhyloChip™ analysis of ear discharge swabs from Indigenous Australian children with acute otitis media with perforation: does microbiomic richness contribute to progression to CSOM?
CONFERENCE PRESENTATIONS

Smith-Vaughan, H; **Marsh, R**; Kaestli, M; Hare, K; Morris, P; Leach, A. A. A PhyloChip™ approach to study ear discharge microbiomes in acute and chronic suppurative otitis media in Australian Aboriginal children. Abstract submitted to the 8th International Symposium on Pneumococci and Pneumococcal Diseases, to be held in Brazil, 2012.

**Marsh, R**; Binks, M; Beissbarth, J; Christensen, P; Morris, P; Leach, AJ; Smith-Vaughan, H. Use of bacterial load measures to investigate the role of *Alloiococcus otitidis* in acute otitis media affecting Indigenous Australian children. 10th International Symposium of Recent Advances in Otitis Media, New Orleans, USA, June 2011. (Poster presentation)

**Marsh, R**; Kaestli, M; Christensen, P; Ward, L; Binks, M; Leach AJ; Smith-Vaughan HC. Use of a multi-template control to assess the usefulness of T-RFLP for investigating polymicrobial otitis media. 10th International Symposium of Recent Advances in Otitis Media, New Orleans, USA, June 2011.

Smith-Vaughan, HC; Binks, M; Christensen, P; **Marsh R**; Morris, P; Leach AJ. Dominance of *H. influenzae*, but not *S. pneumoniae* or *M. catarrhalis*, in ear discharge compared to the nasopharynx in paired swabs. 10th International Symposium of Recent Advances in Otitis Media, New Orleans, USA, June 2011.

**Marsh, R**; Kaestli, M; Christensen, P; Ward, L; Binks, M; Leach AJ; Smith-Vaughan HC. Use of a multi-template control to assess T-RFLP for human pediatric respiratory samples. 13th meeting of the International Society for Microbial Ecology, Seattle, USA, August 2010. (Poster presentation).

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## Definitions

<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indigenous</td>
<td>Australian person of Aboriginal or Torres Strait Islander descent who identifies him/herself as an Indigenous Australian.</td>
</tr>
<tr>
<td>Non-Indigenous</td>
<td>Australian person who does not identify him/herself as Indigenous.</td>
</tr>
<tr>
<td>Acute otitis media</td>
<td>Tympanic membrane bulging and a type B tympanogram.</td>
</tr>
<tr>
<td>Acute otitis media with perforation</td>
<td>The presence of middle ear discharge and tympanic membrane perforation for less than six weeks covering less than 2% of the pars tensa of the tympanic membrane.</td>
</tr>
<tr>
<td>Otitis media with effusion</td>
<td>Presence of fluid behind the ear drum without any signs or symptoms of acute otitis media.</td>
</tr>
<tr>
<td>Chronic suppurative otitis media</td>
<td>Perforation of the tympanic membrane with discharging pus for at least six weeks despite appropriate treatment for acute otitis media with perforation.</td>
</tr>
<tr>
<td>Bacterial richness</td>
<td>The number of taxa present in a specimen.</td>
</tr>
<tr>
<td>Bacterial abundance</td>
<td>A relative measure of the abundance of each taxon in a specimen.</td>
</tr>
<tr>
<td>Bacterial diversity</td>
<td>A bacterial community structure measure incorporating bacterial richness and abundance.</td>
</tr>
<tr>
<td>Taxonomic rank</td>
<td>The levels of classification used to identify Bacteria and Archaea (domain, phylum, class, order, family, genus and species).</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AAP</td>
<td>American Academy of Pediatrics</td>
</tr>
<tr>
<td>AAFP</td>
<td>American Academy of Family Physicians</td>
</tr>
<tr>
<td>ANOSIM</td>
<td>Analysis of similarities</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart-infusion</td>
</tr>
<tr>
<td>BVCCA</td>
<td>Bacitracin-vancomycin-clindamycin-chocolate agar</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>Cq</td>
<td>Quantification cycle</td>
</tr>
<tr>
<td>CSOM</td>
<td>Chronic suppurative otitis media</td>
</tr>
<tr>
<td>dB</td>
<td>Decibels</td>
</tr>
<tr>
<td>FAM</td>
<td>6-Carboxyfluorescein</td>
</tr>
<tr>
<td>HCAN</td>
<td>Hierarchical group-average clustering analysis</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research Ethics Committee</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MEF</td>
<td>Middle ear fluid</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MIQE</td>
<td>Minimum Information for publication of Quantitative real-time PCR Experiments</td>
</tr>
<tr>
<td>NMDS</td>
<td>Non-metric multi-dimensional scaling</td>
</tr>
<tr>
<td>NTHi</td>
<td>Non-typable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>OME</td>
<td>Otitis media with effusion</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RDP</td>
<td>Ribosomal Database Project</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>STGGB</td>
<td>Skim milk tryptone glucose glycerol broth</td>
</tr>
<tr>
<td>TE</td>
<td>1 X Tris-EDTA</td>
</tr>
<tr>
<td>TMP</td>
<td>Tympanic membrane perforation</td>
</tr>
<tr>
<td>T-RFLP</td>
<td>Terminal restriction fragment length polymorphism</td>
</tr>
<tr>
<td>T-RFs</td>
<td>Terminal restriction fragments</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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</tbody>
</table>
CHAPTER 1

Literature Review
Chapter 1: Literature review

1.1 Summary

This review aims to outline why a culture-independent approach is required to better understand the microbiology of acute otitis media affecting Indigenous Australian children. The review provides background literature describing otitis media epidemiology, pathogenesis, risk factors and current intervention strategies. The microbiology of acute otitis media in Indigenous children is described, with emphasis on the need for culture-independent characterisation of polymicrobial infections. The review concludes with a summary of the research questions and hypotheses addressed by this thesis.

1.2 What is otitis media and why is it important?

Otitis media is a broad and often all-encompassing term used to describe manifestations of middle ear inflammation from asymptomatic otitis media with effusion (OME) through to chronic suppurative infections with a range of complications. Otitis media is common to children in all human populations (Gunasekera et al. 2008) but the incidence, severity and persistence of the infection varies (Vergison et al. 2010; World Health Organisation, 2004).

The World Health Organisation (WHO) estimates that 65-330 million individuals have chronic suppurative otitis media (CSOM) and 60% of these will have significant otitis media associated hearing loss of 30-60dB (World Health Organisation, 2004). In the United States, otitis media is the most common reason for paediatric presentation to a clinician (Schappert, 1994), and remains a major reason for prescription of antibiotics to children (Grijalva et al. 2009).

In Australia, a recent national survey identified ear problems as the fourth most common reason for presentation to clinical practitioners (Gunasekera et al. 2007), and surgery for insertion of ventilation tubes to treat otitis media remains one of the most common reasons for paediatric hospital admission (Australian Institute of Health and Welfare, 2011). Indigenous Australian children are estimated to be five times more likely than non-Indigenous children to have severe otitis media
Although rare in the wider Australian population, CSOM prevalence in remote Indigenous communities is at least four times the rate designated by the WHO as indicative of a massive public health problem requiring urgent attention (WHO/CIBA Foundation, 1996; Morris et al. 2005). The economic cost of otitis media in Australia for the period 2008-2012 has been estimated to be between $183-449 million (Access Economics Pty Ltd, 2009).

1.2.1 Otitis media complications

1.2.1.1 Conductive hearing loss
Conductive hearing loss is the most common complication of otitis media (Abdel-Hamid et al. 2007; Homoe et al. 1995; Olusesi, 2008; Pugh et al. 2004), with highest hearing impairment arising from CSOM (O'Connor et al. 2009). Conductive hearing loss has been documented in Indigenous Australian communities since at least the 1960’s (reviewed by Sockalingam et al. 2003) with rates of hearing loss reported for Indigenous children consistently higher than those of non-Indigenous children (Couzos et al. 2001). Mild (25-40dB) to moderate (41-60dB) hearing loss has been reported in 25-50% of Indigenous children attending school in the Northern Territory and Pilbara region of Western Australia (reviewed by Sockalingam et al. 2003). Hearing loss associated with otitis media has also been reported in urban Indigenous populations. A study conducted in three schools in Perth between 1998-2004 identified 19.1% of 94 Indigenous children aged 4-12 years as having mild to moderate hearing loss associated with otitis media (Williams et al. 2009a).

Meta-analyses have identified only small to negligible associations between OME and language development (Casby, 2001; Roberts et al. 2004); however, significant associations have been reported between hearing loss and language development (Roberts et al. 2004). As with other aspects of otitis media, the findings of meta-analyses may be limited by methodological inconsistencies between the included studies (Roberts et al. 2004; Williams et al. 2009b). Published meta-analyses (Roberts et al. 2004) have also been limited to data from “low-risk” populations (CSOM prevalence <4%), and thus may not be generalisable to Indigenous Australian populations where chronic suppurative complications are common.
Australian researchers working with Indigenous children continue to stress an association between otitis media related conductive hearing loss, adverse language development and poor school achievement (O'Connor et al. 2009; Senate Community Affairs Reference Committee Inquiry, 2010; Williams et al. 2009b). This is supported by the high rates of hearing loss and CSOM consistently reported in the Indigenous Australian population (Couzos et al. 2001). While the reasons for poor school achievement by Indigenous children are multi-factorial, disability arising from otitis media-associated hearing loss is likely to be a significant contributing factor (Senate Community Affairs Reference Committee Inquiry, 2010).

1.2.1.2 Rare complications
Although rare, otitis media can also be complicated by mastoiditis and cholesteatoma (O'Connor et al. 2009). In severe cases, meningitis and cerebral abscess can also develop (O'Connor et al. 2009). The WHO has estimated that in 1990 28,000 deaths, globally, arose from complications of CSOM (World Health Organisation, 2004). In Australia, it has been estimated that 3-5 children will die from otitis media complications each year, and 15 children will suffer permanent hearing loss (Access Economics Pty Ltd, 2009).

1.3 Otitis media diagnostic definitions
Standardised otitis media diagnostic definitions are required to achieve clinical consistency in disease management and enable comparisons between studies; however, universally accepted otitis media diagnostic criteria remain elusive (Vergison et al. 2010; World Health Organisation, 2004). This has been highlighted in systematic reviews which identified differences in disease definitions and study methodologies which complicate or prevent comparisons between studies (Gunasekera et al. 2009; World Health Organisation, 2004).

Some of the debate surrounding otitis media definitions originates from differences in the clinical presentation observed between populations. For example, for a diagnosis of acute otitis media, the American Academy of Pediatrics (AAP) and the American Academy of Family Physicians (AAFP) clinical practice guideline requires a history of sudden-onset; evidence of middle ear effusion including bulging or immobility of the tympanic membrane; and signs or symptoms of middle-ear
inflammation such as otalgia or redness of the eardrum (Subcommittee on Management of Acute Otitis Media, 2004).

In contrast, guidelines specific to management of Indigenous Australian populations do not require a sudden onset for a diagnosis of acute otitis media (Morris et al. 2001). This reflects the absence of symptoms (pain and fever) reported for Indigenous children with a bulging tympanic membrane or recent perforation (Morris et al. 2007). For example, a study from the Northern Territory found ear pain was reported for only 5% of 320 Indigenous children with a new diagnosis of acute otitis media (Morris et al. 2010). In this study, acute otitis media without perforation was defined as bulging of the tympanic membrane and a type B tympanogram. Only 10% of children in this study would be considered to have acute otitis media consistent with the AAP and AAFP guideline (Morris et al. 2007). While this implies acute otitis media prevalence in Indigenous communities may be being overestimated, the consistently high rates of CSOM documented in this population suggest this is not the case (Morris et al. 2007). This is supported by recent cross-sectional otitis media data from Indigenous children in the Northern Territory (Morris et al. 2005) which found 15% of 709 children aged 6-30 months had CSOM, suggesting the acute otitis media rate of 26% was not an overestimate.

1.3.1 Otitis media definitions used in this study

Unless stated otherwise, otitis media definitions used in this thesis are as described in the 2001 “Recommendations for clinical care guidelines on the management of otitis media in Aboriginal and Torres Strait Islander populations” (Morris et al. 2001; Table 1). This reflects the thesis’ focus on acute otitis media affecting Indigenous Australian children.
Table 1.1: Otitis media definitions as described in the 2001 “Recommendations for clinical care guidelines on the management of otitis media in Aboriginal and Torres Strait Islander populations” (Morris et al. 2001).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Otitis media</td>
<td>All forms of inflammation and infection of the middle ear</td>
</tr>
<tr>
<td>Otitis media with effusion</td>
<td>Presence of fluid behind the ear drum without any signs or symptoms of acute otitis media</td>
</tr>
<tr>
<td>Acute otitis media</td>
<td>Presence of fluid behind the eardrum plus at least one of the following: bulging eardrum; red eardrum; recent discharge of pus; fever; ear pain; or irritability.</td>
</tr>
<tr>
<td>Recurrent acute otitis media</td>
<td>Three of more episodes of acute otitis media in a six month period.</td>
</tr>
<tr>
<td>Acute otitis media with perforation</td>
<td>Discharge of pus through a perforation within the preceding six weeks</td>
</tr>
<tr>
<td>Chronic suppurative otitis media</td>
<td>Perforation of the tympanic membrane with discharging pus for at least six weeks despite appropriate treatment for acute otitis media with perforation.</td>
</tr>
<tr>
<td>Dry perforation</td>
<td>Tympanic membrane perforation without signs of discharge or fluid behind the eardrum.</td>
</tr>
</tbody>
</table>
1.4 Defining populations at low- and high-risk of otitis media

An objective measure to determine populations at low- and high-risk of otitis media has been proposed based on WHO recommendations for CSOM prevalence. As per the WHO definitions, CSOM prevalence of <1-2% is considered to be low, whereas CSOM prevalence >4% is considered a massive public health problem requiring urgent attention (WHO/CIBA Foundation, 1996). Using this definition, a 2004 WHO review (World Health Organisation, 2004) identified populations in The Gambia, Saudi Arabia, Israel, the United Kingdom, Denmark, Finland, Native Americans and non-Indigenous Australians as having the lowest CSOM prevalence (all <1%). In comparison, CSOM prevalence >4% was reported for populations in Tanzania, India, the Solomon Islands, Guam, Greenland and in the Indigenous Australian population.

Morris et al. (Morris et al. 2001) used the WHO guidelines to define low-risk populations as those with tympanic membrane perforation (TMP) prevalence <4%, and high-risk populations as those with TMP prevalence >4%. This definition provides a constructive tool to allow comparison of populations and allows policy decisions regarding which populations require most urgent interventions. However, it is not clear what constitutes high-risk of otitis media in populations with low rates of TMP. This is an important consideration when critically reviewing the literature from different populations.

1.5 Global prevalence of otitis media

Otitis media has been described as a “rite of passage” in childhood (Rosenfeld, 2005) in that most children will experience at least one episode by three years of age. A 1989 longitudinal birth cohort study from Boston (Teele et al. 1989) found 62% of 877 children had at least one episode of acute otitis media in the first year of life, and 83% of 698 children had at least one episode by age three. Another longitudinal American study from 1997 reported 91% of 2253 infants in Pittsburgh had at least one episode of middle ear effusion between two months and two years of age (Paradise et al. 1997). The global incidence of acute otitis media has been estimated at 0.6-1.7 episodes per child per year (Gunasekera et al. 2008).
In 2006, Gunasekera et al. (Gunasekera et al. 2006a; Gunasekera et al. 2006b) conducted a systematic review of the global burden of otitis media (all forms) in children under 18 years of age. The review reported the prevalence of otitis media to vary from 2-15% in Africa; 3-20% in Asia; 2-49% in Europe; and 15-60% in North America (Gunasekera et al. 2006b), but noted substantial methodological variation between studies. The highest global prevalence identified in the systematic review was 84% in Indigenous Australian children (Gunasekera et al. 2006a). While the review demonstrated the universality of otitis media in human populations, further consideration of differences in study methodologies as well as differences in clinical presentation, disease incidence, severity and persistence is required to better understand the otitis media disease burden between populations.

1.6 Otitis media prevalence in Indigenous Australians

Disparity in otitis media prevalence between Indigenous and non-Indigenous Australian populations has long been recognised with higher rates consistently documented in Indigenous populations since at least the 1960’s (Clements, 1968). For example, a national study from 1979 (Moran et al. 1979) reported the prevalence of otitis media in 60,273 Indigenous and 37,713 non-Indigenous Australians from all regions of the country except south-west Western Australia and Tasmania. The study detected otitis media (all forms) in 16.6% of Indigenous children aged 0-9 years versus 1.3% in non-Indigenous children of the same age. Similarly, higher prevalence was reported for Indigenous adults (5.2%) compared to non-Indigenous adults (0.9%). The study also noted geographic variation in otitis media prevalence in both Indigenous and non-Indigenous populations. For Indigenous people, otitis media prevalence ranged from 7.1% in the Torres Strait Islands to 32.7% in Central Australia. In the non-Indigenous population otitis media prevalence ranged from 0.8-5.7% with highest rates reported for coastal regions of New South Wales. Another study from 1983 detected otitis media in 25.6% of 453 Indigenous children under 10 years of age compared with 3.2% of 126 non-Indigenous children of the same age living in the same region (Torzillo et al. 1983).

Today, otitis media remains a major cause of morbidity in Indigenous children. A 2007 report describing data from a nationwide survey of primary healthcare
consultations identified ear problems as the fourth most common class of clinical problems managed by participating practitioners, with Indigenous children in the study five times more likely to be diagnosed with severe otitis media than non-Indigenous children (Gunasekera et al. 2007). It has been estimated that Indigenous Australians will have 32 weeks of middle ear disease and hearing loss between the age of 2-20 years, compared with only two weeks for non-Indigenous Australians (Coates, 2002).

CSOM continues to cause substantial morbidity in remote Indigenous communities. In the period between 2001-2006, CSOM was the most common reason for Indigenous children aged 5-14 years to present to health clinics in Far North Queensland (Rothstein et al. 2007). In the Northern Territory, the most recent cross-sectional study reported only 8% of 709 Indigenous children aged 6-30 months had bilaterally normal ears, and 24% of children had perforation (Morris et al. 2005).

1.7 Otitis media risk factors

A suite of otitis media risk factors have been identified encompassing host, environmental and microbial factors. Cranio-facial abnormalities associated with cleft palate (Weckwerth et al. 2009) and Down’s syndrome (Bluestone, 2004) predispose infants to otitis media requiring specialist attention. In patients without these abnormalities, systematic reviews have identified the presence of other children in the home; day-care attendance; passive smoking; and use of a pacifier as increasing the risk of acute otitis media, whereas breast-feeding has a protective effect (Lubianca Neto et al. 2006; Uhari et al. 1996). Other risk factors have also been suggested including a family history of otitis media (Albersen et al. 2010; Jensen et al. 2011; Lu et al. 2011; McCormick et al. 2011); prone sleeping position (Gannon et al. 1999); low socio-economic status (Czechowicz et al. 2010; Lasisi et al. 2007; Smith et al. 2010); and exposure to outside fires (Mackenzie et al. 2010).

Despite identification of these specific factors, the underlying risk for developing otitis media is likely to be multi-factorial. Lehmann et al. (Lehmann et al. 2008a) hypothesised a causal network of factors predisposing to otitis media in Indigenous Australian populations (Figure 1.1). This model demonstrates the complexity of environmental and microbial risk factors predisposing to otitis media, and highlights
the difficulties associated with identifying individual modifiable risk factors that could be targeted to reduce the disease burden.

1.7.1 Important risk factors for otitis media in Indigenous children

1.7.1.1 Over-crowded living conditions

Overcrowding has long been recognised as a risk factor for respiratory infections. For example, in 1958 Brimblecombe et al. (Brimblecombe et al. 1958) reported results of a two year survey of 90 families living in London which found a correlation between overcrowding and the incidence of respiratory infections. Today, overcrowding and poor household infrastructure continue to be documented in remote Australian Indigenous communities (Jacoby et al. 2011; Torzillo et al. 2008).

A 2009 report by the Australian Institute of Health and Welfare reviewed Indigenous housing needs (Australian Institute of Health and Welfare, 2009). This report defined overcrowding to be present if households required additional bedrooms to ensure that no more than two people shared a bedroom and people aged 18 years or older had separate bedrooms. Based on 2006 census data, the report described moderate overcrowding (at least one additional bedroom required) to be present in 14% of Indigenous households nationally (Australian Institute of Health and Welfare, 2009). In the Northern Territory, moderate overcrowding was reported for 38.5% of Indigenous households overall, and in 58.3% of Indigenous households in very remote regions (Australian Institute of Health and Welfare, 2009). High-level overcrowding (at least two additional bedrooms required) was reported in 24.5% of Northern Territory Indigenous households compared to 1.3-6.5% in other states. In very remote regions of the Northern Territory, high-level overcrowding was reported for 42% of Indigenous households (Australian Institute of Health and Welfare, 2009). Another study of hygiene and overcrowding in remote Northern Territory communities reported an average of nine people lived in each three bedroom house (range 3-15; McDonald et al. 2010). In the Goldfields Region of Western Australia, a higher risk for otitis media has been reported for Indigenous and non-Indigenous households containing three or more children and when houses have fewer than four rooms (Jacoby et al. 2011).
Figure 1.1: Causal network of otitis media risk factors in Indigenous Australian populations as published by Lehmann et al. (Lehmann et al. 2008a).
Poor household infrastructure is also commonly reported in Indigenous communities. It has been estimated that 31% of dwellings managed by Indigenous Community Housing Organisations require major repair (defined as repairs costing $20,000-$100,000) or replacement (Australian Institute of Health and Welfare, 2009). A study from one Northern Territory Indigenous community reported that 89.3% of 47 houses with children under seven residing had one or more of the essential pieces of infrastructure required for healthy living in need of repair or missing (McDonald et al. 2010). Another study of housing infrastructure in remote Indigenous communities found that it was not possible to bathe a child in 50% of houses in the surveyed communities (Torzillo et al. 2008).

1.7.1.2 Exposure to environmental tobacco smoke

Exposure to environmental tobacco smoke is also an important otitis media risk factor. Tobacco smoke impairs upper respiratory anti-bacterial defences by reducing the activity of respiratory cilia resulting in decreased muco-ciliary clearance of pathogenic bacteria (Pohunek, 2004; Stanley et al. 1986). Smoking has also been shown to alter upper respiratory flora (Charlson et al. 2010); significantly increase the risk of upper respiratory carriage of bacterial pathogens (Lee et al. 2010); increase binding of pathogens to respiratory epithelial cells (El Ahmer et al. 1999; Zhang et al. 2011); and induce chronic respiratory biofilms (Goldstein-Daruech et al. 2011).

High smoking rates have been reported in Indigenous Australian communities (Thomas et al. 2010), and, as with other populations (Lubianca Neto et al. 2006; Uhari et al. 1996), exposure of Indigenous children to environmental tobacco smoke has been associated with increased risk of otitis media (Jacoby et al. 2008). In Inuit populations of Greenland, exposure to cigarette smoke has been identified as the second most important otitis media risk factor after ethnicity (Koch et al. 2011). Efforts to reduce smoking rates in Indigenous communities may also reduce rates of otitis media.
1.7.1.3 Early-age at first onset of otitis media
Peak incidence of otitis media occurs during the first year of life (Rovers et al. 2004). An early-age at first onset of otitis media has been identified as a risk factor for recurrent otitis media in low-risk (Teele et al. 1989) and high-risk populations (Leach et al. 1994). In Indigenous Australian children, persistent otitis media commences in the first weeks of life and is associated with early upper respiratory colonisation by pathogenic species (Leach et al. 1994). Upper respiratory carriage of *Haemophilus influenzae* (*H. influenzae*) and *Streptococcus pneumoniae* (*S. pneumoniae*) is reported in ~60-80% of children under four years of age living in remote Indigenous communities (Mackenzie et al. 2010). Such high carriage rates promote circulation of respiratory pathogens and result in early and frequent upper respiratory infections in young Indigenous infants (Leach et al. 1994; O’Grady et al. 2010; Smith-Vaughan et al. 1996).

The combination of high rates of respiratory pathogen carriage and transmission, overcrowded housing in poor repair, and exposure to environmental tobacco smoke defines a triad of major respiratory risk factors which must be overcome if the burden of otitis media in Indigenous children is to be reduced.

1.7.1.4 Are Indigenous children genetically susceptible to otitis media?
Indigenous children, globally, are at increased risk of developing chronic otitis media (World Health Organisation, 2004). In some populations, indigenous ethnicity is the greatest risk factor for a child developing middle ear pathology (Gunasekera et al. 2007; Koch et al. 2011). This has prompted some researchers to suggest that a genetic susceptibility may account for differences in otitis media prevalence between ethnic groups (Vergison et al. 2010). Investigation of genetic susceptibility to otitis media is a growing field that is identifying some genotypes at increased risk of disease (as reviewed in Ilia et al. 2008; Post, 2011; Rye et al. 2011). However, studies of genetic susceptibility are often performed in low-risk populations where there is a low circulating reservoir of otitis media pathogens. Comparative studies with high-risk populations may not be appropriate as the environmental health and infectious burden between the populations is not equal. Genetic studies in high-risk populations need to be rigorously controlled to ensure that genes associated with increased risk of infection are not surrogates of poor environmental health and the associated high-rates of infectious diseases. Future genetic analyses may reveal
differences in immune and inflammatory responses between individuals and or populations which predict otitis media outcomes; however, studies comparing Indigenous and non-Indigenous populations with similar burdens of infectious disease are required before genetic susceptibility can be used to explain the substantially higher otitis media prevalence in Indigenous populations.

1.8 Pathogenesis and natural history of otitis media

Middle ear infections arise when bacteria from the upper respiratory tract ascend to the middle ear via the Eustachian tube (reviewed by Rovers et al. 2004). The ensuing acute infection may spontaneously resolve with no further signs or symptoms of middle ear abnormality or resolve to non-acute, and potentially asymptomatic, OME with fluid persisting in the middle ear for weeks or months after the acute episode (Kong et al. 2009). Recurrent acute otitis media, where children have at least three episodes in six months (Morris et al. 2001), can occur as a result of repeated infections with complete resolution between episodes, or with recurrent infections associated with an underlying persistent OME (Kong et al. 2009). Acute otitis media can also be complicated by perforation of the tympanic membrane which, if left untreated, can progress to chronic supplicative otitis media (Kong et al. 2009).

The factors which determine the clinical path a child will take remain unclear but are likely to include a combination of host, microbial and environmental risk factors. At the population level, broad differences in the natural history of otitis media have been observed between low-risk (CSOM prevalence <4%) and high-risk (CSOM prevalence >4%) populations. In low-risk populations, chronic supplicative complications are rare (World Health Organisation, 2004) and most children will have spontaneous resolution of acute otitis media within days of onset (Kong et al. 2009; Rovers et al. 2004; Subcommittee on Management of Acute Otitis Media, 2004). In contrast, otitis media in high-risk populations is characterised by a persistent infection which begins early in life, often within weeks of birth, with chronic supplicative manifestations developing in the first year of life (Leach et al. 1994; Lehmann et al. 2008b). These differences must be considered when investigating the underlying aetiology, pathology, optimal prevention and clinical treatment strategies.
1.8.1 Pathogenesis of otitis media in Indigenous Australian children

It has long been recognised that otitis media in Indigenous children commences early in life (Boswell et al. 1995; Leach et al. 1994; Lehmann et al. 2008b; Sunderman et al. 1984); is often asymptomatic without sudden onset or signs of acute inflammation (Gibney et al. 2005; Morris et al. 2010; Sunderman et al. 1984); is persistent (Gibney et al. 2005); and has high-risk of progressing to perforation and CSOM (Morris et al. 2005).

In their seminal 1994 report, Leach et al. (Leach et al. 1994) described the natural history of otitis media in a birth cohort of 41 Indigenous and 12 non-Indigenous children. This study found that otitis media in Indigenous children developed earlier and was more persistent than that observed in non-Indigenous children. 50% of Indigenous children had otitis media by 30 days of age, whereas median age of first onset in non-Indigenous children was 60 days. Overall, 36/41 Indigenous children developed otitis media before 12 weeks of age with 27/36 developing OME; 8/36 developing acute otitis media; and one child presenting with bilateral perforation. Similar evidence of early-onset otitis media was demonstrated by Mackenzie et al. (Mackenzie et al. 2009) who studied 97 Indigenous children vaccinated with 7-valent pneumococcal conjugate vaccine and 51 unvaccinated Indigenous children and found 70% of children in both groups had OME by two months of age and by 12 months of age 90% of children had had acute otitis media; 35% had a documented perforation; and 14% of vaccinated and 23% of non-vaccinated children had CSOM. Gibney et al. (Gibney et al. 2005), studied the clinical course of acute otitis media in 31 Indigenous children under eight years of age and found persistent signs of infection in 77% of 30 children after seven days, and 77% of 26 children after 14 days. Importantly, this study also documented cycles of perforation, healing and re-perforation. In one child the tympanic membrane was observed to perforate, heal and re-perforate eight times over a six week period (Gibney et al. 2005) - a stark contrast to the spontaneous acute otitis media resolution observed for children in low-risk populations (Neumark et al. 2011).
1.8.2 Nasopharyngeal pathogen carriage and early-onset otitis media in Indigenous children

A significant association has been demonstrated between early nasopharyngeal colonisation by respiratory pathogens *S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* (*M. catarrhalis*) and development of otitis media in Indigenous children. Leach et al. (Leach et al. 1994) studied a birth cohort of 41 Indigenous children and reported the median age for first colonisation by any of these species to be 28 days. Nasopharyngeal colonisation by these species correlated significantly with onset of otitis media which was present in 50% of children by 30 days of age (Leach et al. 1994). Polymicrobial colonisation was common with at least two pathogenic species detected in the nasopharynx of 22/36 children at first onset of otitis media. Persistent nasopharyngeal colonisation was common with clearance observed in only 3/134 follow-up examinations. Polymicrobial nasopharyngeal colonisation by pathogenic species was also reported by Gibney et al. (Gibney et al. 2005) who detected simultaneous carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in 67% of 31 Indigenous children under eight years of age with acute otitis media. Multiple carriage of respiratory pathogens is less common in non-Indigenous children (Leach et al. 1994; Watson et al. 2006).

The microbiology of otitis media in Indigenous children is further complicated by the multiplicity of strains colonising the nasopharynx. Smith-Vaughan et al. (Smith-Vaughan et al. 1996) used a ribotyping method to characterise non-typable *H. influenzae* (NTHi) isolates, and identified at least 18 different ribotypes in one small Indigenous community during a nine month period, with co-carriage of multiple strains also occurring. In addition to the high-number of circulating strains, rapid turnover of strains within individual children has also been demonstrated. For example, longitudinal sampling identified 13 different NTHi ribotypes in a single Indigenous child during the first 18 months of age (Smith-Vaughan et al. 2001). Similarly, a high-diversity of pneumococcal serotypes has also been demonstrated in Indigenous children. Mackenzie et al. (Mackenzie et al. 2010) studied pneumococcal carriage in adults and children residing in four remote Indigenous communities and identified a circulating pool of 39 pneumococcal serotypes from 538 individuals.
Density of nasopharyngeal colonisation is also associated with risk of developing otitis media. Smith-Vaughan et al. (Smith-Vaughan et al. 2006) demonstrated that the risk of developing suppurative otitis media increases with higher *S. pneumoniae, H. influenzae, M. catarrhalis* or total bacterial load in the nasopharynx. This study also reported higher geometric mean pathogenic and total bacterial loads in Indigenous children with OME compared to non-Indigenous children (Smith-Vaughan et al. 2006). Another study reporting longitudinal data from 10 randomly selected Indigenous infants between 0-12 weeks of age found a significant increase in the *S. pneumoniae, H. influenzae, M. catarrhalis* and total bacterial load at onset of otitis media (Smith-Vaughan et al. 2008). An increase in the load of pathogenic species as a proportion of total bacterial load was also seen (Smith-Vaughan et al. 2008). High nasopharyngeal bacterial loads have also been reported in Greenlandic children at high-risk of otitis media (Homoe et al. 1996).

Together, these data demonstrate the importance of upper respiratory bacterial colonisation to early-onset of persistent otitis media observed in Indigenous children. The data highlight the need for early interventions to prevent colonisation and reduce bacterial load in the nasopharynx in order to reduce the burden of otitis media in Indigenous communities (Morris et al. 2010).

### 1.8.3 The vicious cycle of otitis media in Indigenous children

An extended “vicious cycle” hypothesis has been proposed by Morris (Morris, 1998) to explain the natural history and microbiology observed in Indigenous children. The hypothesis is based on a bronchiectasis model first proposed by Cole (Cole, 1986) which considers the microbial and inflammatory processes underlying chronic respiratory infections.

In the otitis media model (Figure 1.2; as published by Weitzman et al. 2009), upper respiratory colonisation with a low density of homogenous pathogens results in carriage without inflammation, and will commonly lead to spontaneous clearance. In contrast, upper respiratory colonisation with a high density and multiplicity of bacterial strains results in carriage with neutrophilic inflammation and subsequent mucosal damage.
Figure 1.2: The extended vicious cycle hypothesis as published by Wiertsema et al. (Wiertsema et al. 2009).
Chronic inflammation of the upper respiratory tract then drives a continuing cycle of middle ear infections which ultimately result in CSOM. Importantly, persistent nasal discharge as a result of upper respiratory inflammation provides a vector for bacterial transmission such that a continuing cycle of repeated pathogenic colonisation of the nasopharynx ensues.

The high-dose pathway described by this model is consistent with observations from Indigenous communities and highlights the need to reduce early multiple bacterial transmission events and pathogenic colonisation of the upper respiratory tract in order to break the cycle of chronic respiratory infections.

1.9 Preventing otitis media in Indigenous communities

A range of strategies have been suggested to break the cycle of modifiable otitis media risk factors in Indigenous communities. This includes strategies to address improved nutrition; breast-feeding; feeding position; chewing of xylitol gum; and swimming (Couzos et al. 2001; Lehmann et al. 2003; Stephen et al. 2011). However, the greatest risks for otitis media in high-risk populations have consistently been reported to be overcrowding and exposure to environmental tobacco smoke (Jacoby et al. 2011; Koch et al. 2011). Primary prevention strategies aimed at improved housing and reduced smoking rates are urgently required to reduce the burden of otitis media in high-risk populations (WHO/CIBA Foundation 1996; Carville et al. 2007; Homoe et al. 1996; Morris et al. 2009; Torzillo et al. 2008). Further, hygiene strategies are also likely to reduce the burden of respiratory infections in Indigenous communities (World Health Organisation, 2004; McDonald et al. 2010; Morris et al. 2009; Rothstein et al. 2007). For example, the need for improved hand washing strategies was highlighted in a study by Stubbs et al. (Stubbs et al. 2005) which reported *S. pneumoniae* on the hands of 37% of 89 Indigenous children aged 3-7 years living in remote communities. Hand contamination with respiratory pathogens most likely arises from contact with nasal discharge, and thus hygiene strategies targeting hand-washing and nose-blowing or wiping to reduce exposure to nasal discharge are likely to disrupt the cycle of respiratory pathogen transmission (Figure 1.2).
This, however, is not new knowledge (Torzillo et al. 1983), and consideration of the reasons for persistently poor housing and hygiene in remote Indigenous communities is beyond the scope of this thesis. In the absence of improved environmental health, clinical interventions targeting otitis media pathogens are required.

1.9.1 Vaccination against otitis media pathogens

Vaccination is an attractive anti-microbial tool as it aims to prevent infection occurring. However, vaccines commonly target single pathogens and may be of limited use in polymicrobial infections like otitis media affecting Indigenous children.

In recent years, much attention has been given to vaccine prevention of otitis media following the release of several pneumococcal conjugate vaccines. In Indigenous Australian populations, a 7-valent pneumococcal conjugate vaccine (PCV7) successfully reduced carriage of vaccine serotypes (Mackenzie et al. 2010) and invasive pneumococcal disease (Lehmann et al. 2010); however, persistence of some serotypes and rapid replacement with non-vaccine serotypes has resulted in no overall reduction in pneumococcal carriage or otitis media (Mackenzie et al. 2009). Invasive pneumococcal disease from non-vaccine serotypes has also been reported (Lehmann et al. 2010; Singleton et al. 2007). Another vaccine strategy is maternal immunisation with the pneumococcal polysaccharide vaccine which is currently being trialled as a means of providing infants with immunological protection against 23 pneumococcal serotypes in the first months after birth to prevent early-onset otitis media (Dunbar et al. 2007).

In 2009, a pneumococcal vaccine containing 10 pneumococcal serotypes conjugated to a \textit{H. influenzae} protein D molecule (Synflorix\textsuperscript{®}) replaced PCV7 in the Northern Territory infant vaccination schedule. Initial trials in the Czech Republic suggested the 11-valent prototype of this vaccine may reduce non-typable \textit{H. influenzae} otitis media in addition to targeting the included pneumococcal serotypes (Prymula et al. 2006; Prymula et al. 2009). The Northern Territory infant vaccination schedule was again updated in 2011 and now includes a 13-valent pneumococcal conjugate vaccine (PCV13) which does not include the \textit{H. influenzae} protein D molecule. Studies to assess the efficacy of Synflorix\textsuperscript{®} and PCV13 vaccines in Indigenous
Australian populations are ongoing; however, it is likely that pneumococcal replacement will again occur as serotypes commonly carried by Indigenous children are not included in either vaccine (Marsh et al. 2007). This limitation may be addressed by proposed future vaccine formulations targeting pneumococcal proteins ubiquitous to all serotypes (reviewed by Cripps et al. 2006). Ideally, a polymicrobial vaccine formulation containing antigens from all major otopathogens is needed (Cripps et al. 2006); however, such a vaccine is not currently available and its development will be complicated by the microbial complexity underlying otitis media. Anti-viral vaccination is also being considered as antecedent upper respiratory viral infection may predispose to bacterial otitis media (discussed further below). Overall, continued research is required to achieve an effective otitis media vaccine.

1.10 Clinical management of otitis media

In the absence of effective otitis media prevention strategies, clinical treatment strategies are required. Gunasekera et al. (Gunasekera et al. 2009) summarised evidence from 29 systematic reviews to provide clinical guidance for treating otitis media. For OME, Gunasekera et al. (Gunasekera et al. 2009) recommended children be observed without therapy for 3-6 months, providing no evidence of delayed speech or language development was present. Children remaining symptomatic after this time should be referred for ventilation tube insertion if a bilateral hearing loss of >25dB is present. For children with CSOM, Gunasekera et al. (Gunasekera et al. 2009) recommended ear cleaning and topical antibiotics until the discharge resolved.

Optimal acute otitis media treatment remains controversial. Debate continues as to whether antibiotics should be prescribed when children first present with acute otitis media (Hoberman et al. 2011; Tahtinen et al. 2011), or if a “watch-and-wait” period, with analgesic treatment only, should be applied to prevent over-prescription of antibiotics to patients who would otherwise undergo spontaneous resolution (Del Mar et al. 2011; Gunasekera et al. 2007; Rovers et al. 2006; Subcommittee on Management of Acute Otitis Media, 2004). In 2006, Rovers et al. (Rovers et al. 2006) reported a meta-analysis of six randomised controlled trials of the efficacy of antibiotics for acute otitis media. The six trials included in this meta-analysis were conducted in populations at low-risk of developing CSOM. The meta-analysis found
antibiotic treatment of acute otitis media was beneficial in reducing pain and fever after 3-7 days in children under two years of age; where children had bi-lateral disease; or had acute otitis media with perforation (Rovers et al. 2006).

A 2009 report by Gunasekera et al. (Gunasekera et al. 2009) summarised data from 14 systematic reviews and provided recommendations for clinical management of acute otitis media, including management of disease in Indigenous children. This report recommended a two day observation period with analgesia except in cases where the child has a mid-face abnormality; is less than six months of age; is under two years of age with bilateral infection; has otorrhoea; or is Indigenous. Children fulfilling any of these criteria were recommended to receive immediate treatment with amoxycillin (50mg/kg/day) for seven days to prevent development of suppurative complications.

These recommendations are consistent with current guidelines for treatment of acute otitis media in Indigenous Australian populations (Morris et al. 2001) which recommend at least seven days amoxycillin treatment (50mg/kg/day). Children should be reviewed after 4-7 days with the amoxycillin dose increased to 90mg/kg/day for a further seven days if signs of acute otitis media persist. For children with acute otitis media with perforation, 14 days therapy with 50-90mg/kg/day amoxycillin is recommended, with antibiotic therapy continued until ear drum bulging resolves. Children with recurrent acute otitis media are recommended to receive daily amoxycillin for 2-6 months (25mg/kg/day) with referral for specialist evaluation if the condition persists despite adherence to antibiotic therapy.

1.10.1 Do antibiotics resolve otitis media in Indigenous children?

A randomised controlled trial comparing long-term administration of amoxycillin (50mg/kg/day in two divided doses) versus placebo treatment for OME in Indigenous children found that amoxycillin treatment resulted in more children achieving normal ear health (RD = 9.6%; 95%CI 1.6,17.6); fewer perforations (RD = -16%; 95%CI - 31,-1); and reduced pneumococcal carriage (RD = -12%, 95%CI -31, 7) (Leach et al. 2008b). However, this study considered 24 weeks of treatment requiring bi-daily administration – a regimen for which it may be difficult to achieve good therapeutic
compliance. Ideally, short-term interventions are needed to resolve acute otitis media before perforation and chronic suppurative manifestations develop.

As noted above, amoxicillin is the standard recommended therapy for Indigenous children with acute otitis media; however, other therapies are also being considered. For example, amoxicillin-clavulanic acid has been tested in recent randomised controlled trials from Finland (Tahtinen et al. 2011) and the US (Hoberman et al. 2011). Azithromycin has also been suggested for treatment of otitis media (Kozyrskyj et al. 2010), and is an attractive alternative as can be given as a single dose (overcoming compliance issues) and may also have anti-inflammatory properties (Giamarellos-Bourboulis, 2008).

One randomised controlled trial compared seven days amoxycillin (50mg/kg/day) with azithromycin (30mg/kg as a single dose) for treatment of acute otitis media in Indigenous children (Morris et al. 2010). The study found that azithromycin treatment significantly reduced nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* compared to amoxycillin (27% versus 63%; p <0.001). Despite this, clinical treatment failure occurred for ~50% of children in each group. Clinical treatment failure was more common in children with acute perforations (83% azithromycin group; 92% amoxycillin group) than those with acute otitis media without perforation (40% azithromycin; 46% amoxycillin), highlighting the need for early intervention to prevent perforation. High clinical failure rates have also been reported in Indigenous children following CSOM treatment. A randomised controlled trial of 6-8 weeks topical ciprofloxacin versus topical framycetin-gramicidin-dexamethasone for CSOM in Indigenous children aged 1-16 years reported persistence of ear discharge after therapy in 70% of children in each treatment group, and resolution of perforation in only one of 97 children (Leach et al. 2008a).

In both of the above studies, clinical treatment failure occurred despite good therapeutic compliance and susceptibility of the major pathogens to the antibiotics. This suggests the microbiology underlying otitis media in Indigenous children may be more complex than previously considered. Better understanding of the microbiology underlying acute otitis media in Indigenous children is required to inform design of optimal clinical treatment strategies.
1.11 The microbiology of acute otitis media

Ideally, specimens of middle ear fluid should be tested to determine the microbiology of acute otitis media. Tympanocentesis is required to collect middle ear fluid from children that have acute otitis media without tympanic membrane perforation. Tympanocentesis studies in populations at low-risk of progression to CSOM have identified *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* as important acute otitis media pathogens. For example, Shurin et al. (Shurin et al. 1978) examined middle ear fluid collected by tympanocentesis from 70 infants aged 4-41 days with a diagnosis of otitis media and detected either *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* from 42.9% specimens, with no bacteria cultured from a further 41.4%.

Chonmaitree et al. (Chonmaitree et al. 1990) also reported *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* as the most commonly detected bacteria in middle ear fluid collected by tympanocentesis from 54 children with acute otitis media (detected in 19, 17 and 14 of the 54 children, respectively). More recent tympanocentesis studies from several countries (for example Israel (Leibovitz et al. 2003); Colombia (Sierra et al. 2011), Mexico (Parra et al. 2011); and the United States (Casey et al. 2010)) have also reported *S. pneumoniae* and *H. influenzae* as the most common acute otitis media pathogens.

1.11.1 The microbiology of acute otitis media in Indigenous children

Collection of middle ear fluid by tympanocentesis is an invasive procedure which is not ethically justifiable in Indigenous children at high risk of progression to CSOM. In light of this, the microbiology of acute otitis media in Indigenous children has been determined by analysis of ear discharge sampled following spontaneous tympanic membrane perforation.

*S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are the predominant bacteria detected in ear discharge from Indigenous children with acute otitis media with perforation. A study of 38 ear discharge swabs from 13 Indigenous children under eight years of age with acute otitis media with perforation detected *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in 29%, 32% and 5% of swabs respectively, with at least one of these species detected in 50% of swabs (Gibney et al. 2005). In another study of ear discharge from 70 Indigenous children with acute otitis media with
perforation, *S. pneumoniae* and *H. influenzae* were cultured from 27% and 39% of ear discharge swabs, respectively (Morris et al. 2010). These bacteria are also reported as the predominant species in ear discharge collected following spontaneous perforation of the tympanic membrane in other low-risk (Neumark et al. 2011) and high-risk (Homoe et al. 1996) populations, and in middle ear fluid collected by tympanocentesis from children with acute otitis media without perforation (Grubb et al. 2010; Pichichero et al. 2008; Sierra et al. 2011).

The consistent detection of *S. pneumoniae* and *H. influenzae* in middle ear specimens from low- and high-risk populations suggests the presence of these bacteria alone does not explain the prevalence and severity of otitis media in Indigenous communities, and prompts consideration of other microbial factors which may underly the condition.

1.11.2  **Pathogenic colonisation of the nasopharynx is more common in Indigenous than non-Indigenous children**

Nasopharyngeal microbiology has been analysed in children with acute otitis media without perforation to provide a surrogate of the middle ear microbiology. This is valid as nasopharyngeal colonisation is the critical precursor of acute otitis media and because bacterial strains and species isolated from middle ear specimens are often simultaneously present in the upper respiratory tract (Homoe et al. 1996; Libson et al. 2005).

*S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are commonly detected in nasopharyngeal swabs from Indigenous children with acute otitis media. A study from 316 Indigenous children aged six months to six years from Northern Territory with acute otitis media reported nasopharyngeal colonisation by *S. pneumoniae* or *H. influenzae* to be ~80%, with 95% of children colonised by at least one of these species (Morris et al. 2010). In Western Australia, a birth cohort study following children until two years of age reported nasopharyngeal carriage rates in Indigenous children to be 49%, 50% and 41% for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, respectively, compared to 25%, 25% and 11% respectively in non-Indigenous children. Similarly, birth cohort studies from the Northern Territory have also reported lower carriage rates in non-Indigenous children (Leach et al. 1994).
In addition to higher carriage rates, upper respiratory colonisation of Indigenous children commences early in life with a high density and multiplicity of bacterial strains and species, as discussed above (Leach et al. 1994; Smith-Vaughan et al. 2006; Smith-Vaughan et al. 2008; Watson et al. 2006). This combination of factors likely contributes to the severity and persistence of otitis media observed in Indigenous children.

1.11.3 **Acute otitis media in Indigenous children is polymicrobial**

Nasopharyngeal colonisation by pathogenic species in Indigenous children is further compounded by polymicrobial colonisation, where more than one pathogen is present. Smith-Vaughan et al. (Smith-Vaughan et al. 2006) tested nasopharyngeal swabs from children with acute otitis media and found a significantly higher proportion of swabs from Indigenous (48/51) than non-Indigenous (25/52) children to be positive for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (p=0.0001). Another study of nasopharyngeal swabs detected simultaneous carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in 67% of 31 Indigenous children under eight years of age with acute otitis media (Gibney et al. 2005). Polymicrobial carriage was also reported in a longitudinal birth cohort study from Western Australia that followed children until two years of age (Watson et al. 2006). In this study, 23% of 504 nasal specimens from 100 Indigenous children carried at least two of *S. pneumoniae*, *H. influenzae* or *M. catarrhalis*, compared to 12% of 1045 nasal specimens from 180 non-Indigenous children. A further 23% of nasal specimens from Indigenous children had co-carriage of all three pathogens, compared to only 4% in non-Indigenous children. Polymicrobial infection with *S. pneumoniae*, *H. influenzae* and/or *M. catarrhalis* has also been reported in ear discharge collected from Indigenous children with acute otitis media with perforation (Gibney et al. 2005). Polymicrobial acute otitis media has also been described in other high-risk populations (Homoe et al. 1996).

The polymicrobial nature of acute otitis media in Indigenous children is further exacerbated by the multiplicity of bacterial strains. Upper respiratory colonisation in Indigenous children is characterised by persistent carriage of bacterial pathogens with high turn-over of individual strains, reflecting the high levels of circulating respiratory pathogens. Ribotyping studies have demonstrated the co-carriage and
high-turnover of non-typable *H. influenzae* in Indigenous communities (Smith-Vaughan et al. 1996) and within individuals (Smith-Vaughan et al. 2001), as discussed above. Similarly, a large circulating pool of pneumococcal serotypes has been documented in cross-sectional studies of nasopharyngeal carriage in Indigenous communities (Leach et al. 2009; Mackenzie et al. 2010), with further epidemiological typing demonstrating additional diversity in specific serotypes (Marsh et al. 2010; Marsh et al. 2007). Hare et al. (Hare et al. 2008) reported carriage of multiple, immunologically distinct pneumococcal serotypes in 20% of 98 nasopharyngeal swabs from Indigenous children with acute otitis media. Understanding factors that influence survival of pathogens in the upper respiratory flora may be important to elucidating why otitis media occurs more frequently in certain individuals and populations.

1.11.4 Does antibiotic resistance contribute to clinical treatment failure and persistent acute otitis media in Indigenous children?

Failure to eradicate otitis media pathogens in the nasopharynx or middle ear following an acute episode can lead to recurrent otitis media (Libson et al. 2005). In other populations, bacterial resistance to antibiotics prescribed to treat acute otitis media increased the risk of recurrent infection (Brook et al. 2005). While antibiotic resistance has been documented in *S. pneumoniae* and *H. influenzae* isolates from Indigenous children (Leach et al. 2008b; Leach et al. 2009; Morris et al. 2010), clinical treatment failure as demonstrated by persistent signs of acute otitis media has been reported despite infection with susceptible respiratory pathogens. For example, a randomised controlled trial comparing amoxycillin and azithromycin for treatment of acute otitis media in Indigenous children reported clinical treatment failure of ~50% in both groups, despite only 26% of 164 children treated with azithromycin and 19% of 152 children treated with amoxycillin reported to have baseline nasopharyngeal carriage of antibiotic resistant pneumococci or NTHi (Morris et al. 2010). This suggests that while antibiotic resistance may contribute to recalcitrance in some children, it does not explain the overall high rates of persistent acute otitis media in Indigenous children.
1.11.5 Are viruses important in acute otitis media in Indigenous children?

While bacteria are the most recognised acute otitis media pathogens, viruses may also contribute to the disease pathogenesis (Cripps et al. 2006; Rovers et al. 2004). Upper respiratory viral infection often precedes acute otitis media in low-risk populations (Cripps et al. 2006; Kalu et al. 2011; Winther et al. 2007), consistent with the reported seasonal nature of the condition in some populations (Vesa et al. 2001; Watson et al. 2006). This has prompted consideration of anti-viral vaccines to reduce the incidence of upper respiratory infections to lower the incidence of acute otitis media (Cripps et al. 2006; Hoberman et al. 2003; Jansen et al. 2008). Respiratory viruses have been demonstrated in middle ear fluid collected by tympanocentesis during an acute otitis media episode supporting a direct pathogenic role for viruses (Nokso-Koivisto et al. 2004); however, this remains controversial with other researchers suggesting detection of viruses in MEF reflects the transitory presence of upper respiratory secretions (Winther et al. 2005).

Birth cohort studies have identified rhinovirus, adenovirus, influenza and parainfluenza viruses in nasal specimens from Indigenous children (Jacoby et al. 2007; Leach et al. 1994). Another PCR-based study reported the presence of at least one respiratory virus in 62% of 366 nasopharyngeal swabs from 114 Indigenous children, with rhinovirus, human polyomaviruses and adenovirus most commonly detected (Binks et al. 2011b). The importance of viral pathogens to otitis media pathogenesis, however, is not clear. In contrast to non-Indigenous children, seasonality is not reported for otitis media in Indigenous children (Watson et al. 2006). Additionally, birth cohort studies have shown otitis media commencing prior to upper respiratory viral infection (Leach et al. 1994), suggesting viruses do not contribute to the initial disease pathogenesis. Other studies have considered bacterial-viral interactions which may worsen clinical otitis media outcomes (Rovers et al. 2004), although the exact mechanisms underlying this are unclear.

One hypothesis is that upper respiratory viral infection promotes bacterial overgrowth resulting in higher bacterial load (Wiertsema et al. 2009) with subsequent increased risk of acute otitis media (Smith-Vaughan et al. 2006). This hypothesis is supported by the findings of Binks et al. (Binks et al. 2011b) who found
nasopharyngeal bacterial loads of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were all higher in the presence of respiratory viruses. In this study, significantly higher bacterial loads were demonstrated for *H. influenzae* and the combined bacterial load of all three pathogens in the presence of viruses (Binks et al. 2011b). Jacoby et al. (Jacoby et al. 2007) also reported a positive correlation between nasopharyngeal rhinovirus infection and carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Further research is required to better understand bacterial-viral interactions in the pathogenesis of acute otitis media in Indigenous children.

1.11.6 **Do other bacteria cause acute otitis media?**

*S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are the major acute otitis media pathogens; however, a role for other species must also be considered as the major respiratory pathogens are only detected in a subset of ear discharge specimens (Gibney et al. 2005). For example, a study of ear discharge swabs from Indigenous children with acute otitis media with perforation cultured *S. pneumoniae* from 27% and *H. influenzae* from 39% of swabs, but did not describe the bacteriology of the remaining samples (Morris et al. 2010). Further, elevated nasopharyngeal total bacterial load has been associated onset of otitis media in Indigenous children up to 12 weeks of age (Smith-Vaughan et al. 2006), but this increase is not accounted for by the corresponding increase in *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* bacterial loads alone, suggesting other bacteria may also play a role.

Other bacteria implicated in acute otitis media pathogenesis include *Staphylococcus aureus*, β-haemolytic streptococci, *Pseudomonas aeruginosa* and Enterobacteriaceae which have been cultured from middle ear specimens (Chonmaitree et al. 1990; Leibovitz et al. 2003; Shurin et al. 1978). In Indigenous children, *Pseudomonas aeruginosa* and Enterobacteriaceae have been reported in nasopharyngeal swabs (Watson et al. 2006) and in middle ear fluid collected by tympanocentesis from patients with OME (Stuart et al. 2003). A role for anaerobic species has also been proposed. Anaerobic species have been detected in nasopharyngeal specimens (Kononen et al. 2003) and middle ear effusion specimens collected from children with OME (Brook et al. 2001) when appropriate culture conditions are used or with PCR-based detection methods (Kulekci et al. 2001). A role for anaerobic species in Indigenous Australian children remains to be demonstrated (Stuart et al. 2003).
1.11.7  Does *Alloiococcus otitidis* contribute to acute otitis media pathogenesis in Indigenous children?

*Alloiococcus otitidis* (*A. otitidis*) is a putative otitis media pathogen requiring prolonged incubation for isolation. A study of middle ear fluid collected by tympanocentesis from patients with OME used culture for seven days to isolate *A. otitidis* from 45% of 22 Indigenous children aged 1-10 years (Ashhurst-Smith et al. 2007). *A. otitidis* was the most commonly isolated species, and no significant difference was reported in its recovery from Indigenous versus non-Indigenous children (Ashhurst-Smith et al. 2007). A study to determine if *A. otitidis* is present in nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation is presented in Chapter 4 of this thesis.

1.11.8  Bacterial load

As discussed above, the upper respiratory density of specific pathogens and total bacterial load has been associated with onset (Smith-Vaughan et al. 2008) and severity (Smith-Vaughan et al. 2006) of otitis media in Indigenous children. These findings prompt consideration of mechanisms which may promote bacterial overgrowth, such as viral infection (Binks et al. 2011b; Jacoby et al. 2007; Wiertsema et al. 2009), and suggest pathogenic and total bacterial load may also be important in clinical antibiotic treatment failure. Theoretically, high bacterial loads may decrease the efficacy of standard antibiotic therapies, possibly because the antibiotic concentration is below that required to substantially reduce the bacterial population. Bacterial load is discussed further in Chapter 3 of this thesis which reports a study investigating the pre-treatment nasopharyngeal total and pathogenic bacterial loads in relation to the clinical outcome of antibiotic therapy.

1.11.9  Intra-cellular persistence

Intra-cellular persistence of pathogens may also contribute to persistent otitis media. Transmission electron microscopy has been used to visualise middle ear biopsy specimens and demonstrated bacteria within middle ear epithelial cells of 4/11 children aged 2-9 years of age undergoing tympanostomy tube insertion for persistent OME (Coates et al. 2008). Lower airway studies have also described an
intra-cellular stage of non-typable *H. influenzae* infections and proposed this as a survival mechanism (Morey et al. 2011), which could lead to persistent or recurrent infection (Hotomi et al. 2010). Further research is needed to determine if intra-cellular persistence contributes to chronicity of otitis media in Indigenous children.

### 1.11.10 Biofilm

Biofilm may also contribute to chronic otitis media. Biofilm is a complex form of microbial growth which occurs when bacterial cells aggregate into communities and become embedded in an extra-cellular polymeric substance (Hall-Stoodley et al. 2009). Using confocal scanning laser microscopy and fluorescent *in-situ* hybridisation (FISH), Hall-Stoodley et al. (Hall-Stoodley et al. 2006) demonstrated biofilm in 92% of 52 middle ear mucosal biopsy specimens from children aged six months to 14 years of age with otitis media. Thornton et al. (Thornton et al. 2011) demonstrated biofilm in 15/17 middle ear mucosal biopsies from children aged 0-10 years who had chronic OME or recurrent acute otitis media. Biofilm has also been demonstrated in adenoid biopsies (Nistico et al. 2011) obtained from children with recurrent acute otitis media (Zuliani et al. 2009) and on tympanostomy tubes (Barakate et al. 2007; Thomas et al. 2011). Further *in vitro* studies have demonstrated biofilm production by common otopathogens such as *S. pneumoniae* (Camilli et al. 2011) and *H. influenzae* (Sekhar et al. 2009).

The clinical significance of biofilms arises from the increased antibiotic resistance observed when otherwise susceptible bacteria enter a biofilm mode of growth (Ehrlich et al. 2010). This has led to a range of new anti-biofilm therapies being researched as alternative treatments for chronic mucosal infections (Smith et al. 2011), including otitis media (Antonelli, 2007; Hall-Stoodley et al. 2008). It is unclear if biofilm itself contributes to the pathogenesis of respiratory infections (reviewed by Salcedo et al. 2011), especially as biofilm has been demonstrated in healthy middle ear biopsies (Tonnaer et al. 2009); however, the presence of biofilm is indicative of bacteria progressing to a more complex ecology (Ehrlich et al. 2010) and this should be considered when investigating chronic otitis media.
1.11.11 Polymicrobial interactions

Interactions between bacteria can occur when multiple species and strains co-exist, as is the case in polymicrobial mucosal infections. Several studies have demonstrated interactions between otopathogens; however, the importance of such interactions to otitis media pathogenesis and persistence is poorly understood (Murphy et al. 2009). In Indigenous Australian children, a positive association has been shown between \textit{S. pneumoniae} and \textit{H. influenzae} in nasopharyngeal specimens collected during a longitudinal birth cohort study (Jacoby et al. 2007); whereas a longitudinal study from the United States reported a negative association between these species (Pettigrew et al. 2008). Negative associations have also been demonstrated between upper respiratory pathogenic and commensal species. Brook et al. (Brook et al. 1998) described antagonistic interactions between the pathogens \textit{S. pneumoniae}, \textit{H. influenzae} and \textit{M. catarrhalis}, and nasopharyngeal commensals including Viridans streptococci, \textit{Prevotella} sp. and \textit{Peptostreptococcal} sp.

Polymicrobial interactions may also affect antibiotic susceptibility. For example, β-lactamase production by one species in middle ear aspirates may protect other susceptible species (reviewed by Brook, 2004). One recent \textit{in vitro} study described \textit{M. catarrhalis} β-lactamase in outer membrane vesicles capable of protecting \textit{S. pneumoniae} and \textit{H. influenzae} from amoxycillin-induced killing (Schaar et al. 2011). Virulence can also be modified by bacterial interactions. For example, Lo et al. (Lo et al. 2011) used an \textit{in vitro} assay to demonstrate an interaction between \textit{S. aureus} haemolysin and \textit{Propionibacterium acnes} CAMP factor which enhanced virulence relating to formation of skin lesions.

Better understanding of bacterial communities associated with acute otitis media is required to determine if polymicrobial interactions contribute to the severity of the infection in Indigenous children. As disease promoting interactions may not be limited to pathogenic species, further research is required to better understand bacterial communities (pathogenic and commensal species) in the nasopharynx and middle ear of children with polymicrobial infections. As with other chronic mucosal infections, such research may identify new targets for future therapeutic agents (Brogden et al. 2005).
1.12 Culture-independent characterisation of acute otitis media microbiology

The bacteriology of acute otitis media has been largely determined by culture-based studies. Culture is the foundation of traditional microbiological testing; however, inherent limitations restrict culture to viable cells with growth requirements matching the applied environmental conditions. Culture-based methods used in most otitis media studies have been optimised for recovery of common respiratory pathogens but may fail to detect more fastidious species (Ashhurst-Smith et al. 2007).

A range of culture-independent methods are now available which target nucleic acids to provide more sensitive (Hendolin et al. 1999) and potentially more comprehensive (Cox et al. 2010) description of complex microbial communities than culture alone. Unlike culture, these methods do not require *a priori* knowledge of expected bacterial species, and therefore may detect previously unsuspected and potentially treatable pathogenic species.

### 1.12.1 PCR provides sensitive detection of bacteria in upper respiratory specimens

PCR is widely recognised to provide more sensitive detection of bacteria in middle ear specimens than culture (reviewed by Hendolin et al. 1999). For example, studies of middle ear fluid collected by tympanocentesis from children with OME are commonly culture negative but have bacteria detected by PCR. For example, a study from Finland using standard otitis media culture conditions detected bacteria in 45% of 123 middle ear effusions collected from 123 children with OME, whereas PCR detected bacteria in 89% of these specimens (Leskinen et al. 2002). Similarly, a Japanese study cultured bacteria from 25% of 40 middle ear effusions from children with acute otitis media, whereas PCR detected bacteria in 72.5% (Harimaya et al. 2006). Hendolin et al. (Hendolin et al. 1997) used a multiplex PCR to detect *S. pneumoniae, H. influenzae, M. catarrhalis* and *A. otitidis* in middle ear fluid from 16 children with OME and reported a 225% improvement in bacterial detection compared to culture.
PCR is also being used to provide rapid epidemiological typing of respiratory pathogens. For example, multiplex PCR is being developed for serotyping of pneumococci directly from clinical specimens (Carvalho et al. 2010). Real-time PCR platforms are also available which allow estimation of bacterial load and relative abundance in clinical specimens (Smith-Vaughan et al. 2006; Zemanick et al. 2010) (discussed further in Chapter 3). Quantitative PCRs targeting the ubiquitous bacterial 16S rRNA gene provide better estimates of total bacterial load than culture by detecting all species as opposed to a select group of specifically targeted pathogens (Nadkarni et al. 2002).

In otitis media studies, PCR-based detection of common respiratory pathogens is consistently reported to be more sensitive than culture. Smith-Vaughan et al. (Smith-Vaughan et al. 2006) reported 18%, 23% and 6% of 103 nasopharyngeal swabs to be culture-negative but PCR-positive for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, respectively, although the reasons for this were unclear. PCR-positive but culture-negative findings may reflect the higher sensitivity of PCR (Hendolin et al. 1997); detection of bacteria growing in biofilm which can be difficult to culture (Hall-Stoodley et al. 2006); detection of dormant bacterial cells (Smith-Vaughan et al. 2006); or detection of remnant DNA from non-viable bacteria. Several findings suggest PCR findings do not reflect remnant DNA in middle ear specimens. For example, messenger RNA has been demonstrated in PCR-positive but culture-negative specimens of middle ear fluid (Bakaletz, 2009), indicating the presence of viable bacteria. Other studies have used an animal model to demonstrate DNA from non-viable bacteria is cleared from the middle ear within 24 hrs (Aul et al. 1998; Post et al. 1996). New techniques are also enabling PCR-based differentiation of viable and non-viable cells (Rogers et al. 2010b), but these have not yet been reported in relation to otitis media.

Such results strongly support the use of PCR to characterise middle ear microbiology; however, PCR should be considered complementary to culture-based methods, especially as isolates are still required for determination of antibiotic susceptibility.
1.12.2 **Culture-independent methods provide higher resolution of bacterial richness than culture**

Culture provides limited description of bacterial communities in polymicrobial infections, and requires *a priori* knowledge of a specimen’s likely bacteriology. Seminal work by Woese (Woese, 1987) and Pace (Pace et al. 1985) in the 1980’s demonstrated that culture-independent 16S rRNA gene analyses could provide more comprehensive description of complex bacterial communities than culture. Today, 16S rRNA gene analyses are used to investigate complex bacterial communities, or microbiomes, associated with human mucosal infections (Turnbaugh et al. 2007). Several culture-independent microbiomic methods are available, all of which have consistently demonstrated better bacterial community description than culture (Bogaert et al. 2011; Harris et al. 2007; Lemon et al. 2010; Rogers et al. 2009).

1.12.2.1 **Cloning-and-sequencing of 16S rRNA gene libraries**

The cornerstone of microbiomic analyses for many years was cloning and sequencing of full-length 16S rRNA gene libraries from mixed bacterial populations (Pace et al. 1985). In this method, the 16S rRNA gene is PCR amplified then cloned into *E. coli* or similar vector. Each clone in the resultant library carries one 16S rRNA gene copy which is then sequenced, enabling identification of bacteria within the microbiome (Pace et al. 1985). While this method remains the gold-standard of microbiomic analyses, it is labour intensive and potentially expensive, with sequencing costs often limiting the number of clones analysed (Lemon et al. 2010).

Studies of respiratory specimens have reported sequencing full-length 16S rRNA gene in tens to hundreds of clones (Harris et al. 2007; Hilty et al. 2010; Huang et al. 2011; Lemon et al. 2010; Rogers et al. 2004). The method has been used to describe the lower airway microbiome in patients with cystic fibrosis (Harris et al. 2007) and nasal flora in healthy adults (Lemon et al. 2010). It has also been used to characterise the outer ear microbiome of healthy adults (Frank et al. 2003). Beswick et al. (Beswick et al. 1999) used a cloning and sequencing method to identify bacteria in 12 middle ear effusions collected from 10 children (age 5-10 years) and two adults (age 28 and 59 years) who had had OME for at least six months. 10/12 effusions were culture-negative but the 16S rRNA gene, indicative of bacteria, was amplified from all specimens. Analysis of up to 20 clones from each library identified at least
one bacterial species in each specimen which would not be detected by routine culture techniques. This included *A. otitidis*, *Sphingomonas* sp., and anaerobic species.

### 1.12.2.2 Bacterial community finger-printing

16S rRNA gene profiling methods have been developed to provide “finger-prints” describing microbiomic composition and structure. This includes terminal restriction fragment length polymorphism (T-RFLP) (Li et al. 2007); temperature and denaturing gradient gel electrophoresis (TGGE and DGGE) (Muyzer, 1999); single stranded conformation polymorphism analysis (SSCP) (Kozlowski et al. 2010); amplified ribosomal DNA restriction analysis (ARDRA) (Anderson et al. 2004); and length heterogeneity analysis (reviewed by Stres, 2006). Each method uses species-specific 16S rRNA gene sequence properties to generate a profile of bacterial richness in polymicrobial specimens.

Of these, T-RFLP and length heterogeneity analyses have been used to characterise respiratory samples. Rogers et al. (Rogers et al. 2003) used these methods to characterise the lower airway bacteriology in cystic fibrosis patients and concluded T-RFLP provided better microbiomic resolution than length heterogeneity analysis. T-RFLP is an attractive microbiomic tool as it does not require specialist equipment; can be scaled for high-throughput analyses; and generates digitised data facilitating inter-sample comparisons (Stres, 2006). It has been used to characterise microbiomes in several human mucosal environments (Andoh et al. 2011; Jakobsson et al. 2010; Montagner et al. 2010; Thies et al. 2007), but has not yet been used in otitis media research. T-RFLP is discussed further in Chapters 5 and 6.

### 1.12.2.3 Deep sequencing of the 16S rRNA gene

Microbiomic analyses generated with deep sequencing technologies are providing previously inconceivable measures of bacterial diversity in human mucosal environments. This is exemplified by the Human Microbiome Project which aims to describe the bacterial flora residing in the gut, female genital tract, mouth, skin and nares (Turnbaugh et al. 2007). To date, a limited number of studies have used deep sequencing technologies to describe respiratory microbiomes (Charlson et al. 2010; Erb-Downward et al. 2011; Flanagan et al. 2007; Nakamura et al. 2009; Zhou et al. 2010). Bogaert et al. (Bogaert et al. 2011) used a Roche 454-GS FLX platform to
sequence a partial segment of the 16S rRNA gene (V5-V6) providing microbiomic data for 150 nasopharyngeal swabs from 96 healthy 18-month old children in the Netherlands. The sequencing platform facilitated analysis of ~11,000 16S rRNA gene amplicons per swab and identified 243 phylotypes across the dataset, with 20-87 operational taxonomic units (OTUs; indicative of unique 16S rRNA gene sequences) per child. Overall, Gram-negative bacteria dominated. Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria were the most commonly detected phyla.

One study has reported microbiomic analysis of otitis media specimens using deep sequencing. Liu et al. (Liu et al. 2011) used a Roche 454-GS FLX platform to sequence a segment of the 16S rRNA gene (V3-V4) to describe the microbiome in middle ear effusion, adenoid and tonsil specimens from an eight year old boy with a history of persistent OME. Although relatively few sequences were analysed (a total of 1042 sequences from the three sites), 17 bacterial families were identified, with nine families identified in the middle ear effusion. The middle ear microbiome was dominated by Pseudomonadaceae – a family more commonly associated with CSOM than OME. Although this study was limited to a single child, it demonstrates a more complex bacteriology associated with chronic otitis media than previously considered.

While deep sequencing is revealing much about the human microbiome, technological limitations restrict sequencing to short segments of the 16S rRNA gene, which can limit bacterial identification to the phyla- or family-level. Alternative complementary analyses are required to achieve deeper phylogenetic identification of bacteria in complex microbiomes.

1.12.2.4  Microbiomic profiling with microarray chips

16S rRNA gene microarrays have been developed for microbiomic analyses and several chips optimised for specific environments have been developed. For example, GeoChip (Xie et al. 2011) was developed for characterisation of environmental samples, whereas HitChip (Claesson et al. 2010) has been developed for characterisation of faecal specimens. New chips are also being developed for simultaneous characterisation of bacterial and viral microbiomes (Gardner et al.
Respiratory mucosal microbiomes have been characterised with PhyloChip™, a 16S rRNA gene microarray which can identify up to ~50,000 taxa (Hazen et al. 2010). This microarray has been used to characterise the lower airway microbiome of patients with cystic fibrosis (Cox et al. 2010), chronic obstructive pulmonary disease (Huang et al. 2010), asthma (Huang et al. 2011), and in intubated intensive care patients (Flanagan et al. 2007).

PhyloChip™ has also been used to characterise nasal flora in healthy adults. Lemon et al. (Lemon et al. 2010) used PhyloChip™ to characterise the nostril microbiome in seven healthy adults. This study identified a total of 911 taxa representing 32 phyla in nostril swabs. The study identified Actinobacteria and Firmicutes as the dominant phyla with the families Staphylococcaceae and Lachnospiraceae most commonly detected. An inverse relationship was demonstrated between Actinobacteria and Firmicutes, suggesting a competitive interaction between these phyla. To date, PhyloChip™ analysis of otitis media specimens has not been reported.

Microarray measures of microbiomic richness are limited by the restricted number of probes on each chip. Unlike sequencing technologies, the “closed” nature of microarrays prevents detection of bacteria not included on the chip. Despite this limitation, microarrays provide a good depth of microbiomic resolution and consistently detect more bacterial richness than culture or cloning and sequencing methods. A pilot PhyloChip™ analysis of ear discharge swabs from Indigenous children with acute otitis media with perforation is presented in Chapter 7.

1.13 Culture-independent analysis of acute otitis media bacteriology in Indigenous Australian children: the aims of this study

Traditional culture-based methodologies have revealed much about the major otitis media pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*; however, the limitations of culture restricts more comprehensive description of the polymicrobial bacteriology observed in acute otitis media affecting Indigenous Australian children.
While culture-based studies have provided much information regarding carriage dynamics and species or strain specific virulence, we currently know little regarding the composition and dynamics of the wider nasopharyngeal flora and infective middle ear microbiomes. Many questions remain regarding the importance of bacterial dynamics and richness in persistent middle ear infections in Indigenous children.

This thesis presents results of culture-independent analyses of the bacteriology of nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media. The objective of the study is to use culture-independent methods to explore bacterial load, atypical pathogens and the wider microbiome in nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media.

Five results chapters are presented addressing the following research aims and hypotheses:

**Aim 1:** To determine if baseline nasopharyngeal total or pathogenic bacterial load predicts clinical outcome of antibiotic treatment for Indigenous children with acute otitis media.

**Hypothesis:** That pre-treatment nasopharyngeal total or pathogenic bacterial load predicts clinical antibiotic treatment failure in Indigenous children with acute otitis media.

**Aim 2:** To determine prevalence and bacterial load of the putative otopathogen *Alloiococcus otitidis* in nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media with perforation.

**Hypothesis 1:** That *A. otitidis* is present in nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation.

**Hypothesis 2:** That *A. otitidis* is present at high relative abundance in ear discharge swabs from Indigenous children with acute otitis media with perforation suggesting involvement in middle ear infection.
**Aim 3:** To optimise and validate T-RFLP for application to nasopharyngeal and ear discharge specimens from otitis media patients.

**Hypothesis:** That T-RFLP can be used to characterise polymicrobial bacterial communities in upper respiratory specimens from children with otitis media.

**Aim 4:** To use T-RFLP to describe polymicrobial bacterial communities in ear discharge and nasopharyngeal swabs from Indigenous children with acute otitis media with perforation.

**Hypothesis:** That T-RFLP will demonstrate the presence of polymicrobial bacterial communities in acute otitis media affecting Indigenous children.

**Aim 5:** To pilot PhyloChip™ technology for characterising bacterial communities in ear discharge swabs from Indigenous Australian children with acute otitis media with perforation.

**Hypothesis:** That complex bacterial communities are present in ear discharge swabs from Indigenous children with acute otitis media with perforation.

A final discussion of the results is then presented with consideration of future research directions to progress understanding of acute otitis media microbiology in high-risk populations.
CHAPTER 2

General methods and materials
Chapter 2: General methods and materials

This chapter describes general methods and materials used during this study. Further description of more specific analyses is provided in the relevant chapters. The specific source of reagents, kits and equipment is indicated when they are first described in the text. Methods used to prepare media and solutions are described in Appendix A.

2.1 Ethical approval for the study

This study was approved by the Human Research Ethics Committee (HREC) of the Northern Territory Department of Health and Menzies School of Health Research, which includes an Aboriginal subcommittee (HREC# 07/85; 2007).

2.2 Clinical specimens

2.2.1 Specimens

A retrospective collection of nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media were available for this study. Retrospective analysis of high quality stored specimens is an important strategy in exploratory Indigenous health research. This approach is cost-effective; reduces the research burden on Indigenous communities; and also reduces interference (especially community research fatigue) in other Indigenous health research for which prospective studies are a necessity.

The swabs were originally collected as part of a randomised controlled trial (RCT) of amoxycillin (50mg/kg/day in two divided doses for seven days) versus azithromycin (30mg/kg as a single dose) for treatment of acute otitis media (Morris et al. 2010). The original study was conducted in 14 remote communities and two Aboriginal medical services in northern and central Australia during 2003-2005. Inclusion and exclusion criteria for the original study are described in Table 2.1. At enrolment in the RCT, carers indicated if they required their child’s specimens be destroyed upon study completion or stored at Menzies School of Health Research. Only children whose carers had consented for specimens to be stored at completion of the RCT were considered for this study.
All children in the study had a diagnosis of acute otitis media defined as a bulging tympanic membrane and a type B tympanogram, or acute otitis media with perforation defined as the presence of middle ear discharge and tympanic membrane perforation covering less than 2% of the pars tensa for less than six weeks. The children had not received antibiotics for at least seven days preceding specimen collection. Only pre-treatment specimens were included in analyses reported in this thesis. Further description of specific sample sets is provided in each results chapter.

Table 2.1: Inclusion and exclusion criteria for enrolment in the randomised controlled trial from which the study specimens were sourced (Morris et al. 2010).

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
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<tbody>
<tr>
<td>• Children aged six months to six years</td>
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<tr>
<td>• New diagnosis of acute otitis media with or without perforation</td>
</tr>
<tr>
<td>• Willingness of parents to bring their child for follow-up visit.</td>
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</table>

<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>• Previously allocated to an intervention group in the same study.</td>
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<tr>
<td>• Received antibiotics in the previous seven days.</td>
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<tr>
<td>• Current severe illness requiring intravenous or intramuscular antibiotic treatment to be given within the following seven days.</td>
</tr>
<tr>
<td>• A known allergy to penicillin or azithromycin.</td>
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<tr>
<td>• Perforation covering more than 2% of the pars tensa of the tympanic membrane.</td>
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</table>
2.2.2 Specimen collection and storage

Nasopharyngeal and ear discharge swabs were collected by trained clinical staff as part of the original RCT (Morris et al. 2010). Nasopharyngeal swabs were collected as described by Stubbs et al. (Stubbs et al. 2005). Briefly, cotton-tipped aluminium shafted swabs were inserted into the nose and a quality score applied to indicate the depth and duration of insertion. Swabs were considered to be good quality if inserted 3cm into the nose for 5 s, or if secretions were visible on the swab. An ear discharge swab was also collected from children diagnosed with acute otitis media with perforation. Prior to swabbing, the ear canal was cleaned to remove any visible pus. The ear discharge was then sampled under direct vision (Welch Allyn Lumiview) by positioning the swab as close to the perforation as possible.

Each swab was placed in 1mL skim milk tryptone glucose glycerol broth (STGGB) (Gibson et al. 1986) and immediately frozen before transport to the laboratory in a liquid nitrogen dry shipper (Stubbs et al. 2005). Swabs were then stored at -70ºC, and had been stored for up to five years prior to this study. Specimen integrity is not expected to have been compromised during this time as culture-based studies have demonstrated continued viability of *S. pneumoniae* and *H. influenzae* after up to 12 years storage at -70ºC (Hare et al. 2011) with minimal changes in semi-quantitative bacterial loads (Smith-Vaughan et al. 2006).

2.3 Bacterial isolates

Reference isolates used in this study are described in Table 2.2. *Alloiococcus otitidis*, *Bacteroides fragilis*, *Streptococcus mitis* and *Proteus mirabilis* reference isolates were supplied by Microbiologics. All other reference isolates were sourced from existing Menzies School of Health Research collections. The *Klebsiella pneumoniae*, group A streptococcus, *Staphylococcus aureus* and *Escherichia coli* isolates had previously been identified using phenotypic and molecular methods.

2.4 Culture of clinical specimens

Culture data from the original RCT (Morris et al. 2010) was obtained from the study database. The culture had been done as described by Morris et al. (Morris et al. 2010) and specifically targeted *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Briefly,
the swabs were thawed on ice and 10µL was inoculated onto horse blood agar containing colistin sulphate-nalidixic acid (CNA) agar and bacitracin-vancomycin-clindamycin-chocolate agar (BVCCA) using 10µL sterile plastic loops. Plates were then incubated overnight at 37°C in a humid environment containing 5% CO₂. Capsular pneumococcal isolates were identified based on α-haemolytic colony morphology, optochin susceptibility and the Quellung reaction with pneumococcal Omni-sera (Stubbs et al. 2005). *H. influenzae* isolates were identified based on colony morphology and requirement for factors X and V (Stubbs et al. 2005). Phadebact® Haemophilus Test (Bactus) was used to serotype *H. influenzae* isolates (Smith-Vaughan et al. 1998). *M. catarrhalis* isolates were identified based on colony morphology, Gram stain and oxidase production (Hare et al. 2010). β-lactamase production by *H. influenzae* and *M. catarrhalis* was also determined using nitrocephin discs. Although not specifically sought, the presence of colonies consistent with *Pseudomonas aeruginosa*, β-haemolytic streptococci, *Proteus* species and staphylococcal species were also recorded. Any additional cultured species were recorded as “other”.

**Table 2.2: Bacterial reference isolates used in this study.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alloiococcus otitidis</em></td>
<td>ATCC51267</td>
</tr>
<tr>
<td><em>Bacteroides fragilis</em></td>
<td>ATCC23745</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>MSHR E. coli*</td>
</tr>
<tr>
<td><em>Group A streptococcus</em></td>
<td>MSHR5164*</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>ATCC19418</td>
</tr>
<tr>
<td><em>Haemophilus parainfluenzae</em></td>
<td>ATCC7901</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>MSHR110128*</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
<td>ATCC8176</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>ATCC12453</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>NCTC10662</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>MSHR_SCC1177*</td>
</tr>
<tr>
<td><em>Streptococcus mitis</em></td>
<td>ATCC6249</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>ATCC49619</td>
</tr>
</tbody>
</table>

* Donated by Assoc. Prof. Amanda Leach, Ms Tegan Harris and Ms Rachel Lilliebridge. These isolates were identified using phenotypic and molecular criteria.
2.5 Culture of bacterial isolates

Bacterial isolates were routinely cultured on horse blood agar with incubation for 18 hrs at 37°C in 5% CO₂. Modification of these conditions was performed as follows: *P. aeruginosa* isolates were grown aerobically; *A. otitidis* isolates were grown aerobically for 48-72 hrs; *B. fragilis* isolates were grown anaerobically; *Haemophilus* species were grown on chocolate agar (Oxoid); and *P. mirabilis* isolates were grown on Mackonkey agar (Oxoid).

Anaerobic culture was done in anaerobic jars (Oxoid) with 2.5L Anaerogen sachets (Oxoid). Anaerobic Indicator strips (Oxoid) were used to confirm an anaerobic environment had been achieved. Additionally, *B. fragilis* and *P. aeruginosa* reference isolates were used as positive and negative growth controls, respectively.

2.6 Gram staining

Gram staining was done using standard techniques (Murray et al. 2003). Briefly, a heat-fixed smear was successively stained with crystal violet (Oxoid), Grams iodine (Oxoid), acetone (BDH AnalaR®) and carbol fuchsin (Oxoid). With the exception of acetone, each reagent was applied for 1 min then washed off with tap water. Acetone was applied to the slide and immediately washed off.

2.7 Antibiotic susceptibility testing

The susceptibility of bacterial isolates to penicillin, erythromycin and azithromycin was determined using the Calibrated Dichotomous Susceptibility disk diffusion method (Bell et al. 2009). Etests® (AB Biodisk) were used to determine minimum inhibitory concentrations (Clinical Laboratory Standards Institute, 2009).

2.8 DNA extraction

DNA was extracted using QIAamp DNA extraction kits (QIAGEN) with protocols optimised for different specimen types, as described in the relevant chapters.
2.8.1 DNA extraction from bacterial isolates

Depending on the specific bacterial growth characteristics, between 1 and 100 colonies were harvested from purity plates and suspended in sterile water. Bacterial cells were pelleted by centrifugation at 13000 rpm for 1 min in a benchtop centrifuge (Sigma®). DNA was extracted from pelleted cells using the QIAamp DNA extraction kit as per the manufacturer’s protocol for Gram-positive bacteria (as described in QIAamp Micro Handbook, version 08/2003).

2.8.2 DNA extraction from clinical samples

Swabs were thawed on ice and vortexed for 15 s. Unless otherwise specified, 200µL of the STGGB media was then taken for DNA extraction. Cellular material was pelleted by centrifugation at 13000 rpm for 10 min in a benchtop centrifuge. DNA was extracted from the pellet using the QIAamp DNA extraction kit as per the manufacturer’s tissue protocol (as described in QIAamp Micro Handbook, version 08/2003) with either an enzymatic or a bead-beating pre-treatment, as described below.

2.8.2.1 DNA extraction with enzymatic pre-treatment

Enzymatic pre-treatment was performed as previously described for nasopharyngeal swabs (Binks et al. 2011b). Briefly, pellets were resuspended in 200µL enzymatic lysis buffer (Appendix A) before incubation at 56°C for 45 min. 10µL of 20% w/v sodium dodecyl sulfate (Amresco) was then added, followed by gentle mixing at room temperature for 2 min. If the solution did not become clear, 20µL of 20mg/mL Proteinase K (QIAGEN) was added and samples were incubated at 56°C for 10 min. 4µL of 100µg/mL RNase A (QIAGEN) was then added, followed by incubation at room temperature for 2 min. 200µL of Buffer AL (QIAGEN) was then added and the samples briefly vortexed before incubation at 70°C for 10 min. 200µL of 100% ethanol (Merck) was added and the samples were pulse vortexed for 15 s. DNA was then extracted using QIAamp columns as per the manufacturer’s tissue protocol. DNA was eluted with 200µL of Buffer AE (QIAGEN).
2.8.2.2 DNA extraction with bead-beating pre-treatment

Bead-beating was performed as previously described for lower airway specimens (Cox et al. 2010). Briefly, pellets were resuspended with 600µL of Buffer AL (QIAGEN). The suspension was transferred to a Lysing Matrix B tube (MP Biomedicals). Bead-beating was then done in a FastPrep-24 instrument (MP Biomedicals) for 30 s at 6.0m/s. Samples were then rested for 5-20 min at room temperature before beads were pelleted by centrifugation at 2000 rpm for 1 min in a benchtop centrifuge. The supernatant was taken and 200µL of 100% ethanol was added before the sample was pulse vortexed for 15 s. DNA was then extracted using the QIAamp columns as per the manufacturer’s tissue protocol. DNA was eluted with 50µL of Buffer AE.

2.9 DNA quantification

DNA was quantified using the PicoGreen® reagent (Molecular Probes) as per the manufacturer’s instructions. Fluorescence was measured using a Victor³ 1420 Multilabel counter (Perkin Elmer) with WorkOut software (Perkin Elmer; version 2.5). A preparation of S. pneumoniae genomic DNA was included as a positive control in all PicoGreen® assays.

2.10 Primers and Probes

Primers and probes used in this study are described in the relevant chapters. All primers were supplied by Sigma Genosys. Probes were supplied by Eurogentec. Lyophilised primers and probes were reconstituted to 100µM with sterile molecular grade Tris-EDTA (Sigma®) then aliquoted and stored at -20°C. Aliquots of fluorescently-labelled primers and probes were wrapped in aluminium foil to prevent photo-bleaching.

2.11 PCR

Protocols for specific PCR assays are provided in the relevant chapters. Qualitative PCRs were done in a CG-1 96 thermocycler (Corbett Research). PCR products were then visualised by agarose gel electrophoresis (as described in 2.12). Quantitative PCRs were done in a Rotor-Gene 6000-series real-time thermocycler (Corbett Research). Data were analysed using the Rotor-Gene 6000-series software (Corbett
2.12 Agarose gel electrophoresis

Agarose gel electrophoresis was done using 0.8% gels. The agarose was sourced from Bio-Rad. Gels were prepared in 1 X TAE (Appendix A). SYBR®-safe DNA stain reagent (Invitrogen) was added to a final concentration of 1:10000 immediately prior to pouring each gel. Electrophoresis was done in 1 X TAE at 100V for ~60 min. Gels were visualised in a Geldoc XR instrument (Bio-Rad) using Quantity One software (Bio-Rad; version 4.6.6).

A 1mg/mL 2-Log DNA ladder (New England BioLabs) was included on each gel. The ladder was diluted 1:20 with 1 X Tris-EDTA (TE; Appendix A) and 5µL was loaded. Specimens were mixed with agarose gel loading dye (Appendix A) in a 5:1 ratio before being applied to the gels. Unless otherwise indicated, 5µL of specimen was analysed.

2.13 Purification of PCR products

PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) as per the manufacturer’s instructions (QIAquick Spin Handbook, March 2008). Briefly, 5 volumes of the Buffer PB (QIAGEN) were added to 1 volume of the PCR products. 10µL of 3M sodium acetate (BDH Analar®), pH 5.0, was then added and the solution was vortexed. Purification was then completed in a QIAquick column using either the microcentrifuge or vacuum-manifold protocol. Elution of purified PCR products was done using 30µL of Buffer EB (QIAGEN).

2.14 Purification of restriction digests

Restriction digests were purified using a QIAquick Nucleotide Removal kit (QIAGEN). This kit uses QIAquick columns, but includes a modified loading buffer (Buffer PN; QIAGEN) to ensure all DNA fragments ≥17-bp long are retained. Digests were purified as per the manufacturer’s instructions (QIAquick Spin Handbook, March 2008). Purification was done using either the microcentrifuge or vacuum-manifold protocols. DNA was eluted with 30µL Buffer EB.
2.15 General measures to minimise PCR contamination

PCR mixes were prepared in a dedicated clean-room. All benches were cleaned with DNA-erase (MP Biomedicals) prior to PCR set up. Template was added to the reaction mix in a second dedicated clean room.

Additional measures were taken to reduce contamination of 16S rRNA gene PCRs. A clean laboratory gown was used for each PCR. A dedicated set of pipettes was reserved for 16S rRNA gene applications. When not in use, these pipettes were kept in a sealed box to prevent use in other applications, and to reduce the potential for environmental contamination. Commercially sourced filtered-pipette tips (Mirella Research) were used, with a new box of tips used for each PCR. Reactions were prepared in a Clean View UV Cabinet (Cleaver Scientific) that had been UV-irradiated for 20 min and cleaned with DNA-erase prior to use. All racks and other equipment were also UV-irradiated for 20 min prior to use. All reagents and primers for 16S rRNA gene PCRs were kept in a dedicated “clean” box. All reagents were molecular grade and stored in small aliquots.

2.16 General measures to minimise photobleaching

All fluorescent assays were prepared in light-reduced environments. Fluorescent reagents were wrapped in aluminium foil to prevent light-exposure. Additionally, opaque freezer boxes were used to store specimens where delay was expected between preparation of fluorescent samples and subsequent downstream analyses.
CHAPTER 3

Does nasopharyngeal bacterial load in Indigenous children with acute otitis media predict the clinical outcome of antibiotic treatment?
Chapter 3: Does nasopharyngeal bacterial load in Indigenous children with acute otitis media predict the clinical outcome of antibiotic treatment?

3.1 Summary

**Background:** Traditional microbiological studies consider dichotomous measures of bacterial presence or absence; however, quantitative culture and PCR methods have shown that bacterial load can vary greatly. In the Indigenous paediatric population, increased nasopharyngeal bacterial loads have been positively associated with the onset and severity of otitis media. There is also evidence from other populations that high bacterial loads can adversely affect clinical outcomes in patients with sepsis and pneumonia. No studies to date have investigated bacterial load in relation to otitis media clinical treatment outcomes.

**Hypothesis:** This study tested the hypothesis that baseline nasopharyngeal bacterial load can be used to predict the clinical outcome of acute otitis media antibiotic therapy in Indigenous children.

**Aims:** The aims of the study were i) to describe nasopharyngeal total and pathogenic bacterial load in Indigenous children with acute otitis media; ii) to determine if pre-treatment nasopharyngeal bacterial load is associated with clinical outcome of acute otitis media antibiotic therapy in Indigenous children; and iii) to review the bacterial load methods for consistency with MIQE qPCR guidelines. The overall objective of the study was to determine if pre-treatment nasopharyngeal total or pathogenic bacterial load could be used as a prognostic indicator to guide acute otitis media antibiotic treatment strategies.

**Methods:** Total and pathogenic bacterial loads were determined for 154 pre-treatment nasopharyngeal swabs from Indigenous children with acute otitis media. Fifty-seven of 154 children had clinical treatment failure following amoxycillin or azithromycin therapy.
**Results and Conclusions:** No significant association was observed between baseline nasopharyngeal total or pathogenic bacterial load and clinical treatment outcome. Thus, baseline nasopharyngeal bacterial load is an unsuitable predictor of clinical acute otitis media treatment outcomes in this population. The qPCR methods were largely consistent with MIQE requirements; however, future studies should include assessment of PCR inhibition to achieve optimal compliance.

**Acknowledgements:** Statistical analyses were performed with assistance from Assoc. Prof. Allen Cheng and Dr Mirjam Kaestli. Mr Peter Christensen assisted with processing of qPCR assays under my supervision.

### 3.2 Introduction

Bacterial load refers to the abundance of bacteria in a given specimen. Traditional bacteriological investigations focus on the presence or absence of pathogenic species. While such qualitative measures remain important, quantitative measures of bacterial load can be more informative when applied to understand the pathogenesis, progression and severity of clinical infections. For example, increased pathogenic and/or total bacterial loads have been associated with adverse clinical outcomes in several contexts including intra-uterine infections (Kasper et al. 2010); surgical wounds (Saleh et al. 2011); diabetic ulcers (Xu et al. 2007); endodontic infections (Blome et al. 2008); and sepsis (Carrol et al. 2007; Kirkbright et al. 2011; Rello et al. 2009; Waterer et al. 2011).

#### 3.2.1 Bacterial load in respiratory infections

In the respiratory tract, several studies have demonstrated a positive association between increased bacterial load in lower airway specimens and adverse clinical outcomes (Hill et al. 2000; Munoz-Almagro et al. 2011; Palaci et al. 2007; Perrin et al. 2010). Upper respiratory bacterial load has also been associated with lower airway morbidity. In a case-control study, Vu et al. (Vu et al. 2011) reported the median nasopharyngeal *S. pneumoniae* bacterial load in 274 children with radiologically confirmed pneumonia to be significantly higher than that in 350 healthy control children (7.8x10^6 copies/mL versus 7.9x10^5 copies/mL, respectively; p<0.0001). In another study, Nilsson et al. (Nilsson et al. 2010) reported an
association between elevated upper respiratory load of *Mycoplasma pneumoniae* and more severe pneumonia presentation.

In otitis media, it has been postulated that interactions between the microbial load and host responses contribute to the disease pathogenesis (Rovers et al. 2004). This is supported by upper respiratory studies which have demonstrated a positive association between nasopharyngeal bacterial load and the risk of otitis media. Smith-Vaughan et al. (Smith-Vaughan et al. 2006) analysed nasal swabs from 51 Indigenous and 52 non-Indigenous children (aged 18-36 months) and found a positive association between nasal bacterial load (specific pathogens, combined pathogens, total bacterial load, and proportion of pathogens to total bacterial load) and ear state (no otitis media, OME or suppurative otitis media), leading the authors to hypothesise that “increased bacterial load despite similar clinical conditions may predict persistence of middle ear effusions and progression to suppurative otitis media in the Aboriginal population”. Another study measured nasopharyngeal bacterial load in 10 Indigenous infants with early-onset otitis media during the first 13 weeks of life (Smith-Vaughan et al. 2008). This study reported a significant increase in pathogenic and total bacterial load at the onset of otitis media. The median relative abundance of pathogenic species rose from 0.6% of the total bacterial load in the first three weeks of life to 35% at onset of otitis media between 3-6 weeks of age (Smith-Vaughan et al. 2008). Binks et al. (Binks et al. 2011b) measured nasopharyngeal bacterial load in 366 Indigenous children under two years of age and found nasopharyngeal bacterial load to be significantly higher in children with acute otitis media than in those with no otitis media. This study also tested for the presence of respiratory viruses and reported nasopharyngeal *H. influenzae* load to be significantly higher in children with concurrent viral infection (Binks et al. 2011b). It is not currently known if increased nasopharyngeal bacterial load contributes to persistent middle ear infection following antibiotic treatment of acute otitis media in Indigenous children.

### 3.2.2 How is bacterial load determined?

Several methods have been used to determine bacterial load in clinical specimens. Culture-based measurements can be done semi-quantitatively using a quadrant method (Smith-Vaughan et al. 2006; Tazi et al. 2008) or by serial dilution (Saleh et
In 1991, Hemlin et al. (Hemlin et al. 1991) used a semi-quantitative quadrant method to document a higher density of the respiratory pathogens in nasopharyngeal swabs from children with OME compared to those without. Semi-quantitative methods were also used by Homoe et al. (Homoe et al. 1996) to determine bacterial load in nasopharyngeal swabs from Greenlandic children with acute otitis media. Serial dilution methods have been used to measure bacterial load of respiratory pathogens in upper and lower respiratory specimens (Hill et al. 2000; Smith-Vaughan et al. 2006).

Increasingly, quantitative PCR (qPCR) is being used to measure bacterial load because of its increased sensitivity over culture, and the speed at which results can be generated. Smith-Vaughan et al. (Smith-Vaughan et al. 2006) compared nasopharyngeal bacterial load measures determined by a semi-quantitative quadrant method, serial dilution and qPCR and reported Spearman correlation coefficients of 0.77, 0.66 and 0.83 for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, respectively, but noted that higher sensitivity was consistently observed for qPCR methods.

qPCR targeting the 16S rRNA gene, which is ubiquitous in bacteria, is used to quantify total bacterial load (Horz et al. 2005; Nadkarni et al. 2002). Universal 16S rRNA gene qPCR provides an improved measure of total bacterial load compared to culture-based methods which will only detect a limited number of species. Ideally, the qPCR should amplify evenly from all species in polymicrobial specimens; however, differences in ribosomal operon copy number (1-15 copies) and sequence variation between species prevent even and truly universal amplification of the 16S rRNA gene (Horz et al. 2005; Lee et al. 2009; Nadkarni et al. 2002). Thus, total bacterial load, as determined by universal 16S rRNA gene qPCR, should be considered an estimate of the actual bacterial abundance (Horz et al. 2005). Although a truly universal total bacterial load qPCR remains elusive, a 2005 comparative analysis of published universal 16S rRNA gene qPCRs (Horz et al. 2005) identified the method of Nadkarni et al. (Nadkarni et al. 2002) as providing the best estimate of total bacterial load.

Pathogenic bacterial load can also be determined by qPCR of species-specific genes. Bacterial load assays have been developed for quantification of several respiratory
pathogens (Carvalho et al. 2007; Smith-Vaughan et al. 2006). In otitis media studies, qPCR targeting the autolysin gene (\textit{lytA}) has been used to quantify \textit{S. pneumoniae} bacterial load and an outer membrane protein gene (\textit{copB}) has been used to quantify \textit{M. catarrhalis} (Binks et al. 2011b; Smith-Vaughan et al. 2006). \textit{H. influenzae} qPCRs have targeted the outer membrane protein D (\textit{hpd}). Initial studies used the primers and probe described by Song et al. (Song et al. 1995); however, a more recent study (Binks et al. 2011a) recommended the \textit{hpd} primers and probe described by Wang et al. (Wang et al. 2011) which provide improved differentiation of \textit{H. influenzae} from closely-related \textit{Haemophilus haemolyticus} (Binks et al. 2011a; Kirkham et al. 2010).

### 3.2.3 Standardisation of bacterial load methods

In 2009, a guideline describing the Minimum Information for publication of Quantitative real-time PCR Experiments – the MIQE guideline (Bustin et al. 2009) – was proposed for gene expression studies to ensure “the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency” (Bustin et al. 2009). In 2010, the MIQE précis (Bustin et al. 2010) was published detailing the minimum essential information required for publication of reverse-transcriptase qPCR data (Figure 3.1).

The MIQE guidelines provide a gold standard for qPCR data reporting; however, some essential criteria are not relevant to bacterial load assays which do not include a reverse-transcription step. Although specific criteria for reporting bacterial load qPCR data have not yet been defined, efforts to conform to the MIQE guidelines are likely to provide a standardised framework for comparing bacterial load measures between studies. To date, insufficient information has been provided in published otitis media bacterial load analyses to determine how well the methods conform with the MIQE guidelines.
Figure 3.1: Essential data required for publication of reverse-transcriptase qPCR as described in the 2010 MIQE précis (Bustin et al. 2010).

<table>
<thead>
<tr>
<th>Sample/template</th>
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<tbody>
<tr>
<td>Source</td>
<td>Preservation method</td>
<td>Storage time</td>
</tr>
<tr>
<td>Handling</td>
<td>Extraction method</td>
<td>Concentration</td>
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<tr>
<td>Inhibition free</td>
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<tr>
<th>Assay Optimisation and Validation</th>
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<tr>
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</tr>
<tr>
<td>Primer and probe sequences</td>
</tr>
<tr>
<td>Priming conditions</td>
</tr>
<tr>
<td>Linear dynamic range</td>
</tr>
<tr>
<td>Intra-assay variation</td>
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<th>Reverse-transcriptase/PCR conditions</th>
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<td>Protocols</td>
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<tr>
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<tr>
<th>Data analysis</th>
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<tbody>
<tr>
<td>Specialist software</td>
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</table>
3.2.4 Aims of this study

Elevated nasopharyngeal bacterial loads have been consistently described in Indigenous children with acute otitis media (Binks et al. 2011b; Smith-Vaughan et al. 2006). Clinical resolution of acute otitis media in this population is difficult to achieve, as demonstrated by a randomised controlled trial which reported clinical treatment failure in ~50% of children following standard antibiotic therapies, despite antibiotic susceptibility in the pathogenic species (Morris et al. 2010). These findings prompt consideration of the role of nasopharyngeal bacterial load in the clinical outcomes of acute otitis media antibiotic therapy.

This chapter reports a retrospective study investigating an association between pre-treatment nasopharyngeal bacterial load and clinical outcomes following antibiotic therapy for acute otitis media in Indigenous children. The objective of the study was to determine if pre-treatment nasopharyngeal total or pathogenic bacterial load could be used as a prognostic indicator to guide antibiotic treatment strategies. The specific hypothesis tested was that nasopharyngeal total or pathogenic bacterial load is positively associated with clinical otitis media antibiotic treatment failure.

The study used previously published qPCR methods to determine nasopharyngeal total bacterial load and \textit{S. pneumoniae, H. influenzae} and \textit{M. catarrhalis} bacterial load (Binks et al. 2011b; Smith-Vaughan et al. 2006). The qPCR methods were reviewed to determine their consistency with essential MIQE criteria.

The aims of the study were:

\begin{enumerate}
  \item to describe the nasopharyngeal pathogenic and total bacterial load in Indigenous children with acute otitis media.
  \item to determine if pre-treatment nasopharyngeal bacterial load is associated with clinical outcome of antibiotic therapy for acute otitis media in Indigenous children.
  \item to review the bacterial load qPCR methods to achieve better compliance with MIQE criteria than previously published otitis media studies.
\end{enumerate}
3.3 Methods

3.3.1 Clinical Specimens
The study specimens were derived from a randomised controlled trial (RCT) of amoxycillin versus azithromycin for treatment of acute otitis media in Indigenous children (Morris et al. 2010), as described in Chapter 2.2. At study enrolment, ear examinations were made and nasopharyngeal or nasal swabs collected as described in Chapter 2.2.2. Children were then randomly allocated to receive either azithromycin (30mg/kg as a single dose) or amoxycillin (50mg/kg/day in two divided doses for seven days). Ear examinations were done at baseline (day 0, prior to therapy) and at the end of therapy (between day 6-11). Only children with acute otitis media without perforation were included in this analysis.

3.3.2 Definitions
Acute otitis media was defined as any bulging of the tympanic membrane and a type B tympanogram (Morris et al. 2001). OME was defined as an intact non-bulging tympanic membrane in conjunction with a type B tympanogram (Morris et al. 2010). Clinical treatment failure was defined as persistence of ear pain or a bulging tympanic membrane at the end of therapy.

3.3.3 Bacterial culture
All swabs were cultured as part of the original RCT (Morris et al. 2010), as described in Chapter 2.4. Each swab had been collected into 1mL of STGGB media (as described in Chapter 2.2.2), and a 10µL aliquot of each swab had been cultured. Assuming detection of at least one colony from 10µL, the lower limit of detection was thus 100cfu/swab. Pneumococcal antibiotic susceptibility was determined as part of the original RCT using the Calibrated Dichotomous Sensitivity testing method (Bell et al. 2009). Antibiotic resistant isolates were further characterised by determining the minimum inhibitory concentration (MIC) using Etests®. S. pneumoniae isolates were considered susceptible, of intermediate susceptibility or resistant as follows: penicillin sensitive ≤0.06µg/mL, intermediate susceptibility 0.12-1µg/mL, resistant ≥2µg/mL; azithromycin sensitive ≤0.5µg/mL, intermediate susceptibility 1µg/mL, resistant ≥2µg/mL) consistent with the Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical Laboratory Standards Institute, 2009).
3.3.4 DNA extraction

Whole specimen DNA was extracted from 200µL of each swab using a QIAamp DNA extraction kit with enzymatic pre-treatment as described in Chapter 2.8.2. DNA was eluted with 200µL of elution Buffer AE. DNA extraction from bacterial isolates was done as described in Chapter 2.8.1.

3.3.5 PCR controls

A no template control was included in all assays. This control contained the assay reaction mixture only. Each assay’s standards served as a positive control. A multi-template control containing equivalent concentrations of 13 bacterial species (as described in Chapter 5.3.4) was also tested to ensure inter-assay reproducibility. This multi-template control included genomic DNA from \textit{S. pneumoniae} (ATCC49619), \textit{H. influenzae} (ATCC19418) and \textit{M. catarrhalis} (ATCC8176) reference isolates.

3.3.6 Total bacterial load qPCR

Total bacterial load was estimated as previously described (Binks et al. 2011b; Nadkarni et al. 2002; Smith-Vaughan et al. 2006), with modification. The PCR used universal primers (Table 3.1) which amplify a 466-bp region between positions 331-797 of the 16S rRNA gene, based on \textit{E. coli} numbering (Brosius et al. 1978; Nadkarni et al. 2002). The GenBank accession number for the \textit{E. coli} reference 16S rRNA gene sequence is J01695 (Brosius et al. 1978).

Each 10µL PCR reaction included 1X SensiMix™ SYBR® reagent (Bioline), 300nM of each primer and 1µL of template DNA. The PCR was done in a Corbett Research Rotor-Gene 6000 real-time thermocycler (QIAGEN). The reaction conditions were an initial hold at 50ºC for 2 min followed by incubation at 95ºC for 10 min. This was followed by 35 cycles of 95ºC for 15 s, 58ºC for 15 s and 72ºC for 45 s with data acquisition performed at the end of each cycle. Melt-curve analysis was then done between 80ºC-90ºC with 0.1ºC steps.
Table 3.1: Primers and probes used for bacterial load assays.
TBL = Total bacterial load. Spn = S. pneumoniae. Hi = H. influenzae. Mc = M. catarrhalis. 16S rRNA gene position numbers are based on E. coli numbering (Brosius et al. 1978).

<table>
<thead>
<tr>
<th>Assay</th>
<th>Gene</th>
<th>Primer and Probe sequences</th>
<th>Position</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| TBL   | 16S rRNA | Forward primer 5'- TCCTACGGGAGCCAGCAGT-3'  
Reverse primer 5'- GGACTACCAGGGTATCTAATCCTGT-3' | 331-349  
797-772 | 466 | Nadkarni, 2002 |
| Hi    | hpd  | Forward primer 5'- GGTATAATATGCCGATGGTTGTTG-3'  
Reverse primer 5'- TGCATCTTTACGCACGGTGTA-3'  
Probe 5'Hex- TTGTGTACACTCCGT "T" GG TAAAAGAACTTGCAC –SpC6-3'* | 822-844  
972-953  
896-928 | 151 | Wang, 2011 |
| Spn   | lytA | Forward primer 5'-TCTTACGCAATCTAGCAGATGAAGC-3'  
Reverse primer 5'- GTTGTTTGGTTGGTTATTCGTGC-3'  
Probe 5'- [FAM]-TTTGCCGAAAACGCTTGATACAGGG-[TAMRA]-3' | 306-326  
406-386  
330-354 | 101 | Smith-Vaughan, 2006  
McAvin, 2006 |
| Mc    | copB | Forward primer 5'- GTGAGTGGCGCTTTTACAACC-3'  
Reverse primer 5'- TGTATCGCTGCAAGACAA-3' | 50-70  
121-102 | 72 | Greiner, 2003  
Smith-Vaughan, 2006 |

* Inverted commas in the primer sequence of the hpd probe indicate the position of the Black Hole Quencher.
Genomic DNA from the *S. pneumoniae* ATCC49619 reference isolate was used as the standard for total bacterial load qPCR. Thus, the assay estimated total bacterial load assuming four ribosomal operons per cell (Binks et al. 2011b; Nadkarni et al. 2002). The standard curve was prepared by 1:10 serial dilution (2000ng-200fg) of the genomic DNA with TE (Appendix A). It was critical that standards were prepared in TE as DNA degradation occurs when standards are prepared in water. Based on an *S. pneumoniae* genome size of ~2Mb (GenBank AE005672), the standard curve corresponds to $8.92 \times 10^5 - 8.92 \times 10^1$ cells (Table 3.2). The assay was linear within this range. All standards, controls and samples were tested in duplicate.

The assay was done with SYBR® Green instead of a probe (Nadkarni et al. 2002) to improve the PCR efficiency (Binks, 2008; Binks et al. 2011b). Melt-curve analysis was used to differentiate specific and non-specific amplicons. As multiple amplicons are formed during universal 16S rRNA gene PCR, it was not possible to define a single dissociation temperature which could be used to differentiate specific from non-specific amplicons. To overcome this limitation, the entire melt-curve was considered. Replicate analyses with irreproducible melt-curves were considered indicative of non-specific amplification. Additionally, as a degree of specific amplification was expected in the negative control (due to the presence of remnant bacterial 16S rDNA in assay reagents (Corless et al. 2000)), total bacterial load assays were only accepted if amplification in the negative control was detected at concentrations below that of the lowest standard (D'haene et al. 2010). Assays were repeated if amplification in the negative control occurred at concentrations above the lowest standard. Further, individual specimens were repeated if the melt-curve analysis was irreproducible between replicates.

### 3.3.7 *S. pneumoniae* bacterial load qPCR

*S. pneumoniae* bacterial load was measured as previously described (Binks et al. 2011b; Smith-Vaughan et al. 2006). The PCR primers (Table 3.1) amplify a 101-bp region between positions 306-406 of the *lytA* gene (McAvin et al. 2001; Smith-Vaughan et al. 2006). The hydrolysis probe was located between positions 330-354. The GenBank accession number for the *S. pneumoniae lytA* reference sequence used to design the primers and probe was not reported in earlier publications (McAvin et
al. 2001; Smith-Vaughan et al. 2006). A GenBank search identified accession number AY204888.1 as a suitable \textit{S. pneumoniae} \textit{lytA} reference sequence.

Each 10$\mu$L PCR reaction mix included 1X Taqman$^\circledR$ Universal Master Mix (Applied Biosystems), 100nM of each primer, 200nM of probe, and 1$\mu$L of template DNA. The PCR was done in a Corbett Research Rotor-Gene 6000 real-time thermocycler. The reaction conditions were an initial hold at 50$^\circ$C for 2 min followed by incubation at 95$^\circ$C for 10 min. This was followed by 40 cycles of 95$^\circ$C for 15 s, 58$^\circ$C for 25 s and 72$^\circ$C for 35 s with data acquisition performed at the end of each cycle. As the assay included a hydrolysis probe, melt-curve analysis was not performed.

Genomic DNA from the \textit{S. pneumoniae} ATCC49619 reference isolate was used as the assay standard. The standard curve was prepared by 1:10 serial dilution (2000ng-200fg) of the genomic DNA in TE. Based on an \textit{S. pneumoniae} genome size of $\sim$2Mb (GenBank AE005672), the standard curve corresponds to 8.92x10$^5$-8.92x10$^1$ \textit{S. pneumoniae} cells (Table 3.2). The assay was linear within this range. All standards, controls and samples were tested in duplicate.

\textbf{Table 3.2: Dynamic range of bacterial load qPCRs.}
All assays were linear within these ranges. TBL = Total bacterial load.
* Based on \textit{S. pneumoniae} reference standard with four ribosomal operons.

<table>
<thead>
<tr>
<th>qPCR</th>
<th>Dynamic range (mass gDNA)</th>
<th>Dynamic range (number of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBL*</td>
<td>2000pg – 200fg</td>
<td>8.92E+05 – 8.92E+01</td>
</tr>
<tr>
<td>\textit{S. pneumoniae}</td>
<td>2000pg – 200fg</td>
<td>8.92E+05 – 8.92E+01</td>
</tr>
<tr>
<td>\textit{M. catarrhalis}</td>
<td>2000pg – 200fg</td>
<td>9.92E+05 – 9.92E+01</td>
</tr>
</tbody>
</table>

\textbf{3.3.8 \textit{H. influenzae} bacterial load qPCR}
\textit{H. influenzae} bacterial load was measured as previously described (Binks et al. 2011a; Wang et al. 2011). The PCR primers (Table 3.1) amplify a 151-bp region
between positions 822-972 of the hpd gene (Wang et al. 2011). The hydrolysis probe was located between positions 896-928. GenBank accession numbers for H. influenzae hpd reference genes are GQ201998-GQ202009 (Wang et al. 2011).

Each 10µL PCR reaction mix included 1X Taqman® Universal Master Mix, 100nM forward primer, 200nM reverse primer, 100nM probe, and 1µL of template DNA. The PCR was done in a Rotor-Gene 6000 real-time thermocycler. The reaction conditions were an initial hold at 50ºC for 2 min followed by incubation at 95ºC for 10 min. This was followed by 40 cycles of 95ºC for 15 s and 60ºC for 60 s with data acquisition performed at the end of each cycle. As the assay included a hydrolysis probe, melt-curve analysis was not performed.

Genomic DNA from the H. influenzae ATCC19418 reference isolate was used as the assay standard. The standard curve was prepared by 1:10 serial dilution from 2000ng-200fg of the genomic DNA with TE. Based on an approximate H. influenzae genome size of 1.8-Mb (GenBank CP000671), this corresponds to 9.92x10⁵-9.92x10¹ H. influenzae cells (Table 3.2). The assay was linear within this range. All standards, controls and samples were tested in duplicate.

### 3.3.9 M. catarrhalis bacterial load qPCR

M. catarrhalis bacterial load was measured as previously described (Greiner et al. 2003; Smith-Vaughan et al. 2006), with modification. The PCR primers (Table 3.1) amplify a 72-bp region between positions 50-121 of the copB gene (Greiner et al. 2003). The GenBank accession number for the M. catarrhalis copB reference gene is U69982 (Greiner et al. 2003).

Each 10µL PCR reaction mix included 1X SensiMix™ SYBR® reagent, 300nM of each primer and 1µL of template DNA. The PCR was done in a Rotor-Gene 6000 real-time thermocycler. The reaction conditions were an initial hold at 50ºC for 2 min followed by incubation at 95ºC for 10 min. This was followed by 40 cycles of 95ºC for 15 s, 58ºC for 25 s and 72ºC for 35 s with data acquisition performed at the end of each cycle. A melt-curve analysis was then done between 75ºC-95ºC with 0.1ºC steps.
Genomic DNA from the *M. catarrhalis* ATCC8176 reference isolate was used as the assay standard. The standard curve was prepared by 1:10 serial dilution (2000ng-200fg) of the genomic DNA with TE. Based on an approximate *M. catarrhalis* genome size of 1.8-Mb (GenBank CP002005), the standard curve corresponds to 9.92x10^5-9.92x10^1 *M. catarrhalis* cells (Table 3.2). The assay was linear within this range. All standards, controls and samples were tested in duplicate.

The assay was done with SYBR® Green instead of a probe (Smith-Vaughan et al. 2006) to improve the PCR efficiency (Binks, 2008; Binks et al. 2011b). Melt-curve analysis was included to differentiate specific and non-specific amplicons. Dissociation temperatures >0.5ºC different to the mean dissociation temperature of the assay standards were considered indicative of non-specific amplification. Additionally, assays were only accepted if any amplification in the negative control was detected at concentrations below that of the lowest standard (D'haene et al. 2010). Assays were repeated if amplification in the negative control occurred at concentrations above the lowest standard. Further, individual specimens were repeated if melt-curve analysis was irreproducible between replicates.

### 3.3.10 Analysis of qPCR raw data

qPCR raw data were analysed using the Corbett Research Rotor-Gene 6000 software (Corbett Research; version 1.7) which generated the standard curve and calculated the PCR efficiency and R² values. The “Auto-find threshold” function was used with default settings to determine the optimal threshold for measuring the cycle at which amplification was detected (Cq). Data were then analysed as described below.

### 3.3.11 Review of negative controls

Negative controls were reviewed to ensure no amplification had occurred. As SYBR®-based assays can detect non-specific amplification, including primer-dimer formation; a melt-curve analysis was included in all SYBR®-based assays to differentiate amplification of the target gene from non-specific amplicons. Melt-curve analysis was performed as described above for the total bacterial load and *M. catarrhalis* qPCRs.
3.3.12 Review of the standard curve
All standards were reviewed to ensure replicates varied by no more than 0.5 cycles. Where standard replicates differed by >0.5 cycles, only the single replicate producing the highest $R^2$ value and assay efficiency was retained in the standard curve. Standard curves were only accepted when the $R^2$ value was at least 0.99. For SYBR®-based assays, the melt-curves of each standard were also reviewed to ensure amplification of a single amplicon.

3.3.13 Review of the samples
Replicates of each sample were accepted if Cq values differed no more than 0.5 cycles. Samples with replicates differing by > 0.5 cycles were repeated.

For the hydrolysis probe-based assays (H. influenzae and S. pneumoniae) samples were considered positive where amplification was detected above the assay’s limit of detection (discussed below). Any samples with amplification detected below the limit of detection were defined as “not detected” and given a value of zero in subsequent quantitative data analyses. For SYBR®-based assays, melt-curve analyses were also reviewed.

For quantification in all assays, amplification was required to occur within the linear range of the curve. Any samples with Cq values below that of the highest standard (i.e. at higher concentration than the highest standard) were diluted 1:10 with TE and testing was repeated.

3.3.14 Determining the limit of detection and limit of quantification for each assay
Limit of detection was used to qualitatively determine the presence of the target gene. This value indicates the lowest number of cells that can be reliably detected by the assay (Holst-Jensen et al. 2003). The limit of detection was defined as the lowest standard concentration at which specific amplification was detected in at least 95% of replicates (Bustin et al. 2009).

The limit of quantification (the lowest number of cells that can be reliably quantified by the assay) was also determined (Holst-Jensen et al. 2003). The limit of quantification was determined using the assay standards and was defined as the
lowest standard concentration at which specific amplification was detected in at least 95% of replicates. Additionally, standard replicates were required to differ by \( \leq 0.5 \) cycles. As primer-dimer formation is expected during the later cycles of SYBR\(^\circledR\)-based assays, primer-dimer dissociation peaks in the melt-curves of low standards were required to be no more than 1/3 of that of the expected amplicon (Buh Gasparic et al. 2008).

Specimens with amplification between the limit of quantification and limit of detection were recorded as “positive below the limit of quantification”. For subsequent analyses, a standardised value was used for specimens positive below the limit of quantification. This value was the median bacterial load of all specimens positive below the limit of quantification for each assay.

### 3.3.15 Further data analysis

qPCR results were exported from the Corbett Research Rotor-Gene 6000 software to a Microsoft\(^\circledR\) Office Access database (Microsoft\(^\circledR\), 2003). An Access query was used to multiply raw qPCR data by dilution factors introduced during DNA extraction to generate the bacterial load per swab. Results in the database were checked to ensure no duplication had occurred. A “10% check” was also done in which 10% of results for each assay were checked against the original Rotor-Gene file to ensure no errors had occurred.

Analyses were done in Stata/IC (StataCorp LP, version 11.2 for Windows). Graphs were prepared using GraphPad Prism (GraphPad Software Inc, version 5.01 for Windows). The primary analysis compared total bacterial load and clinical treatment outcome. Secondary analyses compared the combined pathogen load (\( S. \) pneumoniae, \( H. \) influenzae and \( M. \) catarrhalis load combined) and individual pathogen loads with clinical treatment outcome. Normally distributed datasets were analysed using a logistic regression. The non-parametric Mann Whitney U test was used to analyse data that were not normally distributed. Clinical treatment failure was defined as persistence of ear pain or a bulging tympanic membrane when the child was reviewed 6-11 days after treatment commenced (Morris et al. 2010).
3.4 Results

Swabs from 210 children in the original RCT had consent for further research. Of these, 151 nasopharyngeal and 3 nasal discharge swabs (henceforth collectively termed nasopharyngeal swabs) from 154 children with acute otitis media were available for this analysis (Table 3.3). None of the children had acute otitis media with perforation. The children were between 6 months and 5 years of age. 84 children had bilateral acute otitis media; 60 children had unilateral acute otitis media with the opposite ear presenting with OME (n= 49); a retracted mobile drum (n=5); abnormal tympanic membrane appearance but normal mobility (n=5); or was normal (n=1). 10 children had data recorded for a single ear, all of whom had acute otitis media without perforation. 81 children had been treated with azithromycin and 73 treated with amoxicillin (Table 3.3). Overall, 57/154 children had continued signs or symptoms of acute otitis media at follow-up (Table 3.3).

Microbiological culture results are shown in Table 3.3. 87%, 86% and 94% of children were culture-positive for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, respectively. 75% of children were culture-positive for all three species. Penicillin and azithromycin susceptibility data were available for encapsulated *S. pneumoniae* isolates (Table 3.4). Of the 134 swabs that were culture-positive for *S. pneumoniae*, none contained isolates with intermediate penicillin susceptibility and one contained a penicillin resistant isolate. The penicillin resistant *S. pneumoniae* isolate was obtained from a child in the azithromycin treatment group. Swabs from 11 children were culture-positive for azithromycin non-susceptible pneumococci – 6/11 had intermediate azithromycin susceptibility and five had azithromycin resistant isolates. Six children culture-positive for azithromycin non-susceptible *S. pneumoniae* were treated with azithromycin (Table 3.4) and three of these children had clinical treatment failure (two with intermediate-level resistant isolates; one with a resistant isolate). No swabs contained *S. pneumoniae* isolates resistant to penicillin and azithromycin.

132/154 children were culture-positive for *H. influenzae* (all non-typable *H. influenzae*). 10/132 (8%) *H. influenzae* culture-positive swabs contained β-lactamase producing *H. influenzae* isolates (Table 3.5). No other antibiotic susceptibility data were available for *H. influenzae* isolates.
Table 3.3: Characteristics of nasopharyngeal swabs analysed in Chapter 3.
Data were derived from the original RCT. All culture data describe pre-treatment bacteriology. Note: all *H. influenzae* isolates were non-typable.

<table>
<thead>
<tr>
<th></th>
<th>Azithromycin group</th>
<th>Amoxycillin group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children</td>
<td>81</td>
<td>73</td>
<td>154</td>
</tr>
<tr>
<td>Number Children with Treatment Failure (%)</td>
<td>24 (30)</td>
<td>33 (45)</td>
<td>57 (37)</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number culture-positive children (%)</td>
<td>69 (85)</td>
<td>65 (89)</td>
<td>134 (87)</td>
</tr>
<tr>
<td>Number of children with treatment failure (%)</td>
<td>20 (25)</td>
<td>29 (40)</td>
<td>49 (32)</td>
</tr>
<tr>
<td><strong>H. influenzae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number culture-positive children (%)</td>
<td>71 (88)</td>
<td>61 (84)</td>
<td>132 (86)</td>
</tr>
<tr>
<td>Number of children with treatment failure (%)</td>
<td>23 (28)</td>
<td>27 (37)</td>
<td>50 (32)</td>
</tr>
<tr>
<td><strong>M. catarrhalis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number culture-positive children (%)</td>
<td>77 (95)</td>
<td>68 (93)</td>
<td>145 (94)</td>
</tr>
<tr>
<td>Number of children with treatment failure (%)</td>
<td>23 (28)</td>
<td>32 (43)</td>
<td>55 (36)</td>
</tr>
<tr>
<td><strong>S. pneumoniae, H. influenzae and M. catarrhalis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number culture-positive children (%)</td>
<td>60 (74)</td>
<td>56 (77)</td>
<td>116 (75)</td>
</tr>
<tr>
<td>Number of children with treatment failure (%)</td>
<td>19 (23)</td>
<td>24 (33)</td>
<td>43 (28)</td>
</tr>
</tbody>
</table>
Table 3.4: Number of children with nasopharyngeal swabs culture-positive for antibiotic resistant *S. pneumoniae*.
Data were derived from the original RCT and are described for each analysis group. Penicillin non-susceptibility was defined as intermediate (MIC > 0.12-1µg/mL) or resistant (MIC >2µg/mL) (Clinical Laboratory Standards Institute, 2009). Azithromycin non-susceptibility was defined as intermediate (1µg/mL) or resistant (>2µg/mL) (Clinical Laboratory Standards Institute, 2009).

<table>
<thead>
<tr>
<th></th>
<th>Azithromycin group</th>
<th>Amoxycillin group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children in each treatment group</td>
<td>81</td>
<td>73</td>
<td>154</td>
</tr>
<tr>
<td>Number of <em>S. pneumoniae</em> culture-positive swabs (%)</td>
<td>69 (85)</td>
<td>65 (89)</td>
<td>134 (87)</td>
</tr>
<tr>
<td>Number of <em>S. pneumoniae</em> culture-positive swabs with penicillin non-susceptible <em>S. pneumoniae</em> isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate-level (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Resistant (%)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (&lt;1)</td>
</tr>
<tr>
<td>Number of <em>S. pneumoniae</em> culture-positive swabs with azithromycin non-susceptible <em>S. pneumoniae</em> isolates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intermediate-level (%)</td>
<td>3 (4)</td>
<td>3 (5)</td>
<td>6 (5)</td>
</tr>
<tr>
<td>Resistant (%)</td>
<td>3 (4)</td>
<td>2 (3)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Number of <em>S. pneumoniae</em> culture-positive swabs with penicillin and azithromycin non-susceptible <em>S. pneumoniae</em> isolates (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

Table 3.5: Number of nasopharyngeal swabs culture-positive for *H. influenzae* isolates producing β-lactamase.
Data were derived from the original RCT.

<table>
<thead>
<tr>
<th></th>
<th>Azithromycin Treatment group</th>
<th>Amoxycillin Treatment group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of children</td>
<td>81</td>
<td>73</td>
<td>154</td>
</tr>
<tr>
<td>Number of <em>H. influenzae</em> culture-positive swabs</td>
<td>71 (88)</td>
<td>61 (84)</td>
<td>132 (86)</td>
</tr>
<tr>
<td>Number of <em>H. influenzae</em> culture-positive swabs containing a β-lactamase producing <em>H. influenzae</em> (%)</td>
<td>6 (8)</td>
<td>4 (7)</td>
<td>10 (8)</td>
</tr>
</tbody>
</table>
3.4.1 Bacterial load assay performance

Reaction parameters for each bacterial load assay are described in Table 3.6. For all assays, the R² value of standard curves was greater than 0.99 (range 0.994-0.999). Median reaction efficiency was 0.86, 0.87, 0.94 and 0.88 for the *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and total bacterial load qPCRs, respectively.

The limit of detection and limit of quantification were defined for each assay. For the total bacterial load assay, the limit of detection and limit of quantification were equivalent - both 90 cells assuming four 16S ribosomal operons per genome. As with earlier reports (Nadkarni et al. 2002), a non-specific PCR amplicon dominated the melt-curve of standards tested below this concentration which prevented accurate detection and quantification. As expected (Nadkarni et al. 2002), some amplification was also detected in the total bacterial load assay’s no template control during the final PCR cycles. The Cq of the no template control ranged from 25.9-34.0. This amplification always occurred at a Cq greater than the limit of detection (3.8-8.3 cycles beyond the limit of detection; with concentration equivalent to <10 cells). Additionally, negative control replicate melt-curves were irreproducible, consistent with non-specific amplification.

For the *H. influenzae* and *M. catarrhalis* assays, the limit of detection was 10 cells and the limit of quantification was 100 cells. No amplification was detected in either assay’s negative controls. For the *S. pneumoniae* assay, the limit of detection was nine cells and the limit of quantification was 90 cells. No amplification was detected in the *S. pneumoniae* assay’s negative controls (Table 3.6).

The amount of DNA template tested in each qPCR was equivalent to 1µL of each swab. Thus, for swabs in 1mL of STGGB media, the limit of detection is equivalent to 9x10⁴ cells/swab for the total bacterial load assay; 1x10⁴ cells/swab for the *H. influenzae* and *M. catarrhalis* assays; and 0.9x10⁴ cells/swab for the *S. pneumoniae* assay. Similarly, the limit of quantification was 1x10⁵ cells/swab for the *H. influenzae* and *M. catarrhalis* qPCR assays, and 0.9x10⁵ cells/swab for *S. pneumoniae* and total bacterial load assays (Table 3.6).
Table 3.6: Summary of bacterial load qPCR assay performance.
The limit of detection and limit of quantification reflect the number of genome copies which define these parameters. For the total bacterial load, the limit of detection and limit of quantification are based on genome copies in the *S. pneumoniae* standard which has four 16S ribosomal operons.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Total bacterial load</th>
<th><em>H. influenzae</em> Load</th>
<th><em>S. pneumoniae</em> Load</th>
<th><em>M. catarrhalis</em> Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>R² of standard curves (median)</td>
<td>0.994-0.999 (0.999)</td>
<td>0.999 (0.999)*</td>
<td>0.996-0.999 (0.999)</td>
<td>0.999 (0.999)*</td>
</tr>
<tr>
<td>Efficiency (median)</td>
<td>0.81-0.90 (0.88)</td>
<td>0.86-0.89 (0.86)</td>
<td>0.80-0.90 (0.87)</td>
<td>0.90-0.96 (0.94)</td>
</tr>
<tr>
<td>Cq of no template control</td>
<td>25.87 - 34.0</td>
<td>No amplification</td>
<td>No amplification</td>
<td>No amplification</td>
</tr>
<tr>
<td>Limit of detection (number of cells)</td>
<td>90</td>
<td>10</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Limit of quantification (number of cells)</td>
<td>90</td>
<td>100</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>Limit of detection per swab (cells/swab)</td>
<td>9 x 10⁴</td>
<td>1 x 10⁴</td>
<td>0.9 x 10⁴</td>
<td>1 x 10⁴</td>
</tr>
<tr>
<td>Limit of quantification per swab (cells/swab)</td>
<td>0.9 x 10⁵</td>
<td>1 x 10⁵</td>
<td>0.9 x 10⁵</td>
<td>1 x 10⁵</td>
</tr>
</tbody>
</table>

* R² for all *H. influenzae* and *M. catarrhalis* standard curves was 0.999
3.4.2 qPCR data

Bacteria were detected in all swabs by the total bacterial load qPCR. The total bacterial load ranged from $2.62 \times 10^5$ - $6.29 \times 10^8$ cells/swab (Figure 3.2) and was log-normally distributed. The geometric mean total bacterial load was $1.4 \times 10^7$ cells/swab (95% CI $1.13 \times 10^7$ cells/swab, $1.75 \times 10^7$ cells/swab).

136/154 swabs (88%) were PCR-positive for *S. pneumoniae*. The *S. pneumoniae* bacterial load ranged from 0 – $8.04 \times 10^6$ cells/swab (Figure 3.2) and was not normally distributed. The median *S. pneumoniae* bacterial load was $2.05 \times 10^5$ cells/swab (95% CI $1.51 \times 10^5$ cells/swab, $2.59 \times 10^5$ cells/swab). 37 swabs were PCR-positive below the limit of quantification with a median *S. pneumoniae* load of $3.52 \times 10^4$ cells/swab. The relative abundance of *S. pneumoniae* was 0.1 - 42.3% of the total bacterial load (median 1.3%; 95% CI 0.8, 1.7%) (Figure 3.3).

144/154 swabs (94%) were PCR-positive for *H. influenzae*. The *H. influenzae* bacterial load ranged from 0 - $4.82 \times 10^7$ cells/swab (Figure 3.2) and was not normally distributed. The median *H. influenzae* bacterial load was $5.17 \times 10^5$ cells/swab (95% CI $3.16 \times 10^5$ cells/swab, $7.18 \times 10^5$ cells/swab). 21 swabs were PCR-positive below the limit of quantification with a median *H. influenzae* load of $4.32 \times 10^4$ cells/swab. The relative abundance of *H. influenzae* was 0.1-75.7% of the total bacterial load (median 3.7%; 95% CI 2.5, 4.8%) (Figure 3.3).

149/154 swabs (97%) were PCR-positive for *M. catarrhalis*. The *M. catarrhalis* bacterial load ranged from 0 - $2.76 \times 10^7$ cells/swab (Figure 3.2) and was not normally distributed. The median *M. catarrhalis* bacterial load was $1.44 \times 10^6$ cells/swab (95% CI $9.75 \times 10^5$ cells/swab, $1.90 \times 10^6$ cells/swab). 12 swabs were PCR-positive below the limit of quantification with median *M. catarrhalis* load of $4.29 \times 10^4$ cells/swab. The relative abundance of *M. catarrhalis* was 0.1 - 92% of the total bacterial load (median 9.5%; 95% CI 7.6, 11.3%) (Figure 3.3).

All swabs were PCR-positive for at least one of *S. pneumoniae, H. influenzae* or *M. catarrhalis* and 126/154 (81%) swabs were PCR-positive for all three species. The combined pathogenic bacterial load (combined load of any *S. pneumoniae, H. influenzae* and *M. catarrhalis*) ranged from $4.29 \times 10^4$ - $5.20 \times 10^7$ cells/swab and was log-normally distributed. The geometric mean of the combined pathogen load was
Figure 3.2: Nasopharyngeal total and pathogenic bacterial load in 154 Indigenous children with acute otitis media.
The median of each dataset is indicated by the bar. For *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* the line of dots at the bottom of each dataset reflects the standardised value applied to specimens that were PCR-positive below the limit of quantification. TBL = total bacterial load. Spn = *S. pneumoniae*. Hi = *H. influenzae*. Mc = *M. catarrhalis*.

<table>
<thead>
<tr>
<th></th>
<th>TBL</th>
<th>Spn</th>
<th>Hi</th>
<th>Mc</th>
<th>Spn+Hi+Mc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial load (cells/swab)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0E+04</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1.0E+05</td>
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<td>1.0E+06</td>
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<td>1.0E+07</td>
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<tr>
<td>1.0E+09</td>
<td></td>
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</tbody>
</table>

Figure 3.3: Relative abundance of pathogenic species in nasopharyngeal swabs from 154 Indigenous children with acute otitis media.
Relative abundance indicates the proportion of each pathogen as a percentage of the total bacterial load. Spn = *S. pneumoniae*. Hi = *H. influenzae*. Mc = *M. catarrhalis*. Spn+Hi+Mc = combined *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* load.

<table>
<thead>
<tr>
<th></th>
<th>Spn</th>
<th>Hi</th>
<th>Mc</th>
<th>Spn+Hi+Mc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative abundance (%)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>0</td>
<td></td>
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<tr>
<td>20</td>
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<td>40</td>
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<tr>
<td>60</td>
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<td>80</td>
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<tr>
<td>100</td>
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</tbody>
</table>
2.6x10^6 cells/swab (95% CI 2.1x10^6, 3.3x10^6 cells/swab). The relative abundance of the three pathogens combined ranged from 0.8-98.9% (median 18.1%; 95% CI 14.8, 21.4%) (Figure 3.3) of the total bacterial load.

### 3.4.3 qPCR versus culture

Overall, the qPCR detected bacteria more often than culture; however, some swabs were culture-positive and PCR-negative (Table 3.7). For *S. pneumoniae*, five swabs were culture-positive but PCR-negative and seven were culture-negative but PCR-positive. For *H. influenzae*, five swabs were culture-positive but PCR-negative and 17 were culture-negative but PCR-positive. For *M. catarrhalis*, three swabs were culture-positive but PCR-negative and seven were culture-negative but PCR-positive. Only one swab did not grow any bacteria when cultured. This swab had a total bacterial load of 6.67x10^5 cells/swab as determined by qPCR.

As higher sensitivity was expected for the qPCR assays (Smith-Vaughan et al. 2006), raw data from culture-positive but PCR-negative specimens were reviewed. For *S. pneumoniae* and *H. influenzae*, some amplification was present below the limit of detection in 4/5 and 3/5 culture-positive but PCR-negative specimens, respectively. For *M. catarrhalis*, some amplification was present below the limit of detection in 2/3 culture-positive but PCR-negative specimens. The third had good amplification but the dissociation temperature was >0.5°C different to the average dissociation temperature of the standards, and was therefore recorded as non-specific amplification.

### Table 3.7: Summary of culture and qPCR data.

<table>
<thead>
<tr>
<th></th>
<th>Culture</th>
<th>PCR</th>
<th>Culture-positive and PCR negative</th>
<th>Culture-negative and PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any bacteria (%)</td>
<td>153/154 (99)</td>
<td>154/154 (100)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> (%)</td>
<td>134 (87)</td>
<td>136 (88)</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td><em>H. influenzae</em> (%)</td>
<td>132 (86)</td>
<td>144 (93)</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td><em>M. catarrhalis</em> (%)</td>
<td>145 (94)</td>
<td>149 (97)</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td><em>S. pneumoniae</em>, <em>H. influenzae</em> and <em>M. catarrhalis</em> (%)</td>
<td>116 (75)</td>
<td>126 (81)</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
3.4.4 Total bacterial load and treatment outcome

Bacterial load data were reviewed to determine if an association existed between the baseline nasopharyngeal total bacterial load and the clinical outcome after antibiotic therapy. As the total bacterial load dataset was log-normally distributed, the data were log-transformed (base 10) and analysed by logistic regression. The total bacterial load distribution in children with failed or successful antibiotic treatment is shown in Figure 3.4. No association was observed between the nasopharyngeal total bacterial load and clinical treatment outcome (OR 0.91; 95%CI 0.72, 1.16; p=0.455). Further, no association was observed between the total bacterial load and clinical treatment outcome after adjusting for the treatment group (OR 1.92; 95%CI 0.97, 3.75; p=0.058), although a higher failure rate was observed in children treated with amoxycillin.

Figure 3.4: Nasopharyngeal total bacterial load distribution in children with successful or failed acute otitis media antibiotic treatment.
3.4.5 Pathogenic bacterial load and treatment outcome

*S. pneumoniae, H. influenzae* and *M. catarrhalis* data were then reviewed to determine if an association existed between the baseline nasopharyngeal pathogen loads and clinical outcome after antibiotic treatment. A non-parametric test was used as the datasets were not normally distributed. No significant association was observed between the bacterial load of *S. pneumoniae, H. influenzae* or *M. catarrhalis* and clinical treatment outcome (Table 3.8).

A final analysis was performed to test for an association between the combined pathogen bacterial load (*S. pneumoniae, H. influenzae* and *M. catarrhalis*, combined) and clinical treatment outcome. As this dataset was log-normally distributed, the data were log-transformed (base 10) and logistic regression performed. No association was observed between the nasopharyngeal combined pathogenic bacterial load and clinical treatment outcome (OR 0.95; 95%CI 0.75, 1.20; *p*=0.643).

3.5 Discussion

Nasopharyngeal total and pathogenic bacterial loads are positively associated with otitis media onset (Smith-Vaughan et al. 2008) and severity (Smith-Vaughan et al. 2006) in Indigenous children but have not previously been considered in relation to otitis media treatment outcomes. This study has investigated pre-treatment nasopharyngeal total and pathogenic bacterial loads as potential prognostic indicators of clinical antibiotic treatment outcomes in Indigenous children with acute otitis media. The use of bacterial load measures to predict clinical outcome has previously been reported in clinical studies of sepsis (Kirkbright et al. 2011; Rello et al. 2009; Waterer et al. 2011) and lower airway infections (Kais et al. 2006), but has not been described in relation to acute otitis media.

Total and pathogenic bacterial loads in the study cohort are consistent with earlier studies which have reported geometric mean nasopharyngeal total bacterial loads of \(\sim 10^7\) cells/swab and pathogenic loads of \(\sim 10^5-10^6\) cells/swab in Indigenous children with suppurative or acute otitis media (Binks et al. 2011b; Smith-Vaughan et al. 2006). The data support a role for increased nasopharyngeal bacterial loads in the pathogenesis of acute otitis media in this population.
Table 3.8: Outcome of Mann Whitney U test for association between pathogenic bacterial load and clinical treatment outcome. No significant associations were identified.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment outcome</th>
<th>Median (cells/swab)</th>
<th>95% CI (bootstrap estimate)</th>
<th>Mann-Whitney U test</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>Success</td>
<td>$2.35 \times 10^5$</td>
<td>$1.37 \times 10^5$, $3.32 \times 10^5$</td>
<td>$P=0.11$</td>
</tr>
<tr>
<td></td>
<td>Failure</td>
<td>$1.94 \times 10^5$</td>
<td>$1.06 \times 10^5$, $2.82 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>Success</td>
<td>$5.08 \times 10^5$</td>
<td>$2.31 \times 10^5$, $9.07 \times 10^5$</td>
<td>$P=0.83$</td>
</tr>
<tr>
<td></td>
<td>Failure</td>
<td>$5.69 \times 10^5$</td>
<td>$2.36 \times 10^5$, $7.80 \times 10^5$</td>
<td></td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>Success</td>
<td>$1.54 \times 10^6$</td>
<td>$0.81 \times 10^6$, $1.92 \times 10^6$</td>
<td>$P=0.72$</td>
</tr>
<tr>
<td></td>
<td>Failure</td>
<td>$1.37 \times 10^6$</td>
<td>$0.68 \times 10^6$, $2.40 \times 10^6$</td>
<td></td>
</tr>
</tbody>
</table>
Despite this, no association was observed between nasopharyngeal total or pathogenic bacterial loads and the clinical outcome of antibiotic therapy for acute otitis media in this study. The reasons for this are unclear. Antibiotic resistance in the two primary otopathogens is unlikely to have affected the study outcomes as few children were culture-positive for antibiotic resistant *S. pneumoniae* or β-lactamase producing *H. influenzae*. Most swabs were polymicrobial (81% were PCR-positive for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*); however, the relative abundance of the three pathogens was generally low, and a role for other bacterial species cannot be excluded. It is also possible that antibiotic resistance in other bacterial pathogens may also contribute to recalcitrance of acute otitis media.

An alternative explanation may be that nasopharyngeal bacterial loads are important to acute otitis media onset but middle ear bacterial dynamics determine clinical treatment outcomes. Overall, the absence of an association with clinical outcomes in this study does not support the use of nasopharyngeal total or pathogenic bacterial load as a prognostic indicator of acute otitis media antibiotic treatment outcomes.

### 3.5.1 MIQE guideline compliance

The need for standardisation of molecular methods is increasingly being recognised and guidelines are continually being developed to define the minimum information required for publication. MIQE guidelines have been developed for standardised publication of qPCR data (Bustin et al. 2009) with a focus on gene expression studies. Ideally, guidelines for publication of bacterial load qPCRs are needed as several MIQE criteria are not applicable to the bacterial load assays. In the absence of specific guidelines, efforts to conform to the MIQE criteria will improve standardised reporting of bacterial load data.

qPCR methods in this study were applied as previously described (Binks et al. 2011b; Smith-Vaughan et al. 2006). As the MIQE guidelines were published after commencement of the study, no methodological modifications were made to increase conformity with the MIQE criteria. Despite this, the results reported here are largely consistent with most essential MIQE criteria (Bustin et al. 2010) with the following exceptions. The MIQE guidelines require reporting of the specificity of each assay. This was not done as the specificity of each qPCR assay had been previously
reported (Greiner et al. 2003; Horz et al. 2005; McAvin et al. 2001; Wang et al. 2011). The specificity of probe-based assays was not checked by gel electrophoresis as the qPCR assays were well-established within the institute’s laboratory, and had previously been shown to have good specificity (Binks et al. 2011b). Finally, the qPCRs did not include a check for PCR inhibition. This is a potential limitation of this study which should be addressed in future analyses.

The MIQE criteria require reporting of amplification in negative controls, including the Cq values. In this study, amplification in negative controls was only observed for the total bacterial load assay. The Cq values of such amplification have been reported; however, it is important to note that Cq values can vary between different thermocyclers. To achieve a more objective measure of amplification in the no template control, the number of cycles separating the limit of detection and amplification within the negative control were also examined (D’haene et al. 2010). This measure is recommended for use in future studies.

3.5.2 Other methodological considerations

3.5.2.1 qPCR efficiency

The efficiency of qPCRs used in this study has not previously been reported (Binks et al. 2011b; Smith-Vaughan et al. 2006). Ideally, qPCR efficiency should range from 0.90-1.10 (D’haene et al. 2010) (efficiency >1 is expected in qPCRs affected by primer-dimer formation). The *M. catarrhalis* qPCR was the only assay to achieve efficiency within this range. Reduced efficiency was observed for the total bacterial load assay. This was expected as the total bacterial load PCR product is larger than the 80-150-bp recommended for quantification assays (D’haene et al. 2010). Despite this, the efficiency of the total bacterial load qPCR approached 0.90.

qPCR efficiency below 0.90 was observed in *S. pneumoniae* and *H. influenzae* assays. The reason for this is not clear. The results are comparable to those of Kais et al. (Kais et al. 2006) who reported the efficiency of *S. pneumoniae* and *H. influenzae* qPCRs assays as 0.86 and 0.84, respectively; however, these assays targeted different genes (*S. pneumoniae ply* gene and *H. influenzae frdB* gene). Few other respiratory studies have reported the efficiency of *S. pneumoniae* and *H. influenzae* qPCRs (Binks et al. 2011b; Carrol et al. 2007; Hill et al. 2000; Smith-Vaughan et al. 2006;
Vu et al. 2011). Further assay optimisation may be required to improve the efficiency of the *S. pneumoniae* and *H. influenzae* qPCRs.

### 3.5.2.2 qPCR limit of detection and quantification

As with reaction efficiency, the limit of detection and limit of quantification of the qPCR assays have not been well described. Overall, it is widely accepted that the theoretical lowest limit of detection for qPCR assays is three gene copies (Bustin et al. 2009). Nadkarni et al. (Nadkarni et al. 2002) reported the total bacterial load limit of detection to be 48 *E. coli* cells, and noted quantification was unreliable at high (>1000pg) and low (<0.1pg) DNA concentrations. Wang et al. (Wang et al. 2011) reported the limit of detection for the *H. influenzae hpd* qPCR to be 40 genomes, but did not consider a limit of quantification. McAvin et al. (McAvin et al. 2001) reported the *S. pneumoniae lytA* qPCR limit of detection as 10fg genomic DNA, equivalent to 4.4 cells. This qPCR was also used by Vu et al. (Vu et al. 2011) who reported the limit of detection as 10 cells. Greiner et al. (Greiner et al. 2003) reported the limit of detection for the *M. catarrhalis copB* qPCR to be one cell. The *M. catarrhalis copB* qPCR was also used by Vu et al. (Vu et al. 2011) who reported the limit of detection as 10 cells.

In this study, the limit of detection for the species-specific qPCRs was ~10 cells. The limit of detection was higher for the total bacterial load assay (90 cells) as excessive primer-dimer was evident in the assay standards when fewer cells were tested. The limit of quantification was also considered. For the species-specific assays, the limit of quantification was higher than the limit of detection (90-100 cells) as poor Cq reproducibility was observed below this level. As the limit of detection and quantification differed, a number of specimens generated results that were positive below the limit of quantification in species-specific assays. To account for these specimens in quantitative analyses a standardised bacterial load was applied. Based on the results of the assay standards, all bacterial loads in specimens positive below the limit of quantification range were consistent with ~10^4 cells/swab. As the bacterial load ranged logarithmically, variation in the actual bacterial load of specimens positive below the limit of quantification is unlikely to have affected subsequent data analyses.
The bacterial load of specimens with negative qPCR results were considered to be zero in subsequent quantitative analyses; however, it is important to note that the limit of detection was at least $10^4$ cells/swab for each assay. This value is higher than the theoretical limit of detection of culture ($10^2$ cfu/swab), most likely reflecting the higher volume of specimen sampled for culture. Despite this difference, few swabs were PCR-negative and culture-positive. Thus, zero values in the bacterial load datasets are unlikely to indicate false-negative results.

### 3.5.2.3 Methodological assumptions

qPCR estimates of nasopharyngeal bacterial load include several assumptions. The method assumes that the bacterial load per swab is indicative of the actual nasopharyngeal bacterial burden (Smith-Vaughan et al. 2006). Further, in the absence of weighed swabs, the method assumes consistent volumes of nasal secretions have been sampled (Binks et al. 2011b; Smith-Vaughan et al. 2006). This may not be the case; however, logarithmic differences in the specimen volumes are unlikely. The bacterial load qPCRs also assumed 100% DNA extraction efficiency and have used G:C content to estimate molecular weight (Binks et al. 2011b). Finally, as described above, the total bacterial load assay assumes four 16S ribosomal operons per bacterial cell; however, the actual number of ribosomal operons varies between species from 1-15 copies (Lee et al. 2009).

### 3.5.2.4 Study limitations

This was a retrospective analysis using available specimens from an earlier RCT and possible type 2 errors cannot be excluded. Although limited, retrospective analyses are useful for generating pilot data to address new research questions. Further, retrospective analyses enable exploratory research while minimising the research burden on Indigenous communities, as discussed in Chapter 2.2.1. Specimens used in this study had been stored for approximately five years at -70°C. While degradation of the specimens during this time cannot be excluded, previous studies have demonstrated continued viability of pathogenic species in nasopharyngeal swabs after storage for up to 12 years at -70°C (Hare et al. 2011).

### 3.5.3 Conclusions and recommendations for future studies

This study has found that nasopharyngeal total and pathogenic bacterial load is not a suitable measure for predicting clinical outcomes of antibiotic therapy for acute otitis
media in Indigenous children. Future studies should consider testing changes in nasopharyngeal bacterial load following antibiotic therapy. Additionally, studies of middle ear bacterial load may also be important to understanding clinical antibiotic treatment failure; however, this may be difficult as collection of middle ear fluid in the absence of spontaneous perforation is an invasive procedure.

Almost all specimens analysed in this study were polymicrobial. Additionally, the low relative abundance of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in most swabs indicates the presence of other bacterial species. While this may indicate detection of commensal species, the presence of other pathogens cannot be excluded. Further studies to better understand polymicrobial interactions between nasopharyngeal bacteria in Indigenous children with acute otitis media are warranted.

Future studies should also aim to reach conformity with minimum qPCR reporting standards. The MIQE guidelines should be used until criteria specific to bacterial load analyses are developed. Efforts should also be made to achieve methodological standardisation between laboratories to enable comparisons between studies. This should include consideration of qPCR gene targets and choice of detection method (for example SYBR® Green versus probe-based detection).

Finally, future studies should consider methodological improvements including optimisation of *S. pneumoniae* and *H. influenzae* assays to achieve higher reaction efficiencies. Sampling of larger volumes of each swab, or entire swabs, should also be considered in conjunction with concentration during DNA extraction to reduce the limit of detection per swab.
CHAPTER 4

qPCR of ear discharge from Indigenous Australian children with acute otitis media supports a role for *Alloilococcus otitidis* as a secondary pathogen.
Chapter 4: qPCR of ear discharge from Indigenous children with acute otitis media supports a role for *Alloiococcus otitidis* as a secondary pathogen

4.1 Summary

**Background:** Otitis media is endemic in many Indigenous communities of the Northern Territory of Australia. *Alloiococcus otitidis* is an outer ear commensal and putative middle ear pathogen that has not previously been described in acute otitis media in this population.

**Objective:** To determine if *A. otitidis* is present in Indigenous children with acute otitis media with perforation.

**Methods:** Matched nasopharyngeal and ear discharge swabs from 27 Indigenous children with acute otitis media with perforation were tested by *A. otitidis* qPCR. All *A. otitidis* PCR-positive samples were then cultured, with incubation for 21 days. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and total bacterial load in *A. otitidis*-positive swabs were also determined by qPCR.

**Results:** *A. otitidis* was detected in ear discharge from 10/27 children (37%) but was not detected in nasopharyngeal swabs. It was cultured from 5/11 PCR-positive swabs from four children. All *A. otitidis* PCR-positive samples were culture-positive for other bacteria.

*A. otitidis* bacterial load ranged from 2.2x10^4-1.1x10^8 cells/swab. The relative abundance of *A. otitidis* ranged from 0.01-34% (median 0.7%). In 6/11 PCR-positive swabs the *A. otitidis* relative abundance was <1% and in 5/11 it was between 2-34%. The *A. otitidis* bacterial load and relative abundance measures were comparable to that of *H. influenzae*. Bacterial loads and relative abundance measures of *S. pneumoniae* and *M. catarrhalis* in *A. otitidis*-positive ear discharge swabs were low.

**Conclusion:** *A. otitidis* can be a dominant species in ear discharge of Indigenous children with acute otitis media with perforation. Its absence in nasopharyngeal
swabs suggests the ear canal as the likely primary reservoir. The significance of *A. otitidis* at low relative abundance is unclear; however, at higher relative abundance it may be contributing to the pathology, possibly as a secondary pathogen. Further studies to better understand the role of *A. otitidis* in acute otitis media with perforation and subsequent progression to chronic suppurative otitis media are warranted.

**Acknowledgements:** Ms Jemima Beissbarth assisted with processing of qPCR assays under my supervision.

### 4.2 Introduction

*Alloiococcus otitidis* is a slow-growing, strictly aerobic, Gram-positive coccus which produces small α-haemolytic colonies on blood agar. It was first reported in 1989 as the sole isolate from middle ear fluid (MEF) of 10 children with chronic otitis media with effusion (OME) (Faden et al. 1989). Since then, PCR-based studies have reported *A. otitidis* in MEF from 20-61% of children with chronic OME (Guvenc et al. 2010; Harimaya et al. 2006; Hendolin et al. 1999; Leskinen et al. 2002) and in MEF from 25-50% of children with acute otitis media (Harimaya et al. 2006; Kaur et al. 2010; Leskinen et al. 2004; Neumark et al. 2011). One study of 22 Indigenous and 28 non-Indigenous Australian children aged 1-10 years with chronic OME cultured *A. otitidis* from 45% and 36% of MEF specimens, respectively (Ashhurst-Smith et al. 2007). *A. otitidis* has not previously been considered in acute otitis media affecting Indigenous children.

Despite its detection in MEF, a pathogenic role for *A. otitidis* remains controversial. Data supporting an otopathogenic role include detection of *A. otitidis* as the sole bacterial species in MEF from children with OME (Faden et al. 1989; Guvenc et al. 2010; Kaur et al. 2010) and acute otitis media (Neumark et al. 2011) (as determined by culture or species-specific PCRs) and the capacity of *A. otitidis* to provoke an inflammatory response (Faden et al. 1989; Harimaya et al. 2007; Himi et al. 2000; Kita et al. 2000). In one study of children with acute otitis media, IL-8, IL-1-β and IL-6 levels in MEF solely positive for *A. otitidis* were similar to that of *S. pneumoniae*-positive specimens (Harimaya et al. 2009).
The reservoir of *A. otitidis* associated with otitis media is unclear. *A. otitidis* has been detected in 7-12% of nasopharyngeal swabs from paediatric and adult patients with upper respiratory infection or otitis media (Kaur et al. 2010; Takada et al. 2003; Tano et al. 2008). It has also been detected in 14-83% of ear canal swabs from healthy volunteers (De Baere et al. 2010; Frank et al. 2003; Stroman et al. 2001; Tano et al. 2008) suggesting that it is part of the normal ear canal flora which may contaminate MEF during specimen collection. However, PCR and culture-based studies have reported *A. otitidis* as the sole identified bacterium in MEF collected by tympanocentesis after careful cleaning and disinfection of the canal and tympanic membrane (Ashhurst-Smith et al. 2007; Guvenc et al. 2010; Harimaya et al. 2009; Khoramrooz et al. 2011) suggesting it is infecting the middle ear.

*A. otitidis* can be difficult to culture. It requires prolonged incubation for 5-14 days in an aerobic environment (Ashhurst-Smith et al. 2007; Harimaya et al. 2009; Tano et al. 2008) and thus will not be detected by standard otitis media culture methods. Although Clinical Laboratory Standards Institute (CLSI) minimum inhibitory concentration (MIC) breakpoints have not been defined for *A. otitidis*, MIC levels consistent with macrolide resistance have been detected in 11/20 (55%) (Ashhurst-Smith et al. 2007) and 11/12 (92%) (Bosley et al. 1995) isolates from two studies. As the macrolide azithromycin is an emerging therapy for Indigenous Australian children with acute otitis media, further consideration of the role of *A. otitidis* in these children is warranted. The aims of this study were:

i) to determine the prevalence of *A. otitidis* in nasopharyngeal and ear discharge swabs from Indigenous Australian children with acute otitis media with perforation.

ii) to determine the antibiotic resistance profile of any *A. otitidis* isolates.

iii) to determine the relative abundance of *A. otitidis* in polymicrobial specimens.
4.3 Methods

4.3.1 Clinical specimens

This study used matched nasopharyngeal and ear discharge swabs collected at enrolment of Indigenous children with acute otitis media into a randomised controlled trial (RCT) of amoxycillin or azithromycin (Morris et al. 2010), as described in Chapter 2.2. All swabs were from children with acute otitis media with perforation, defined as the presence of middle ear discharge for less than six weeks, and perforation covering less than 2% of the pars tensa of the tympanic membrane (Morris et al. 2010). Nasopharyngeal and ear discharge swabs were collected and stored as described in Chapter 2.2.2.

Paired nasopharyngeal and ear discharge swabs from 27 Indigenous children aged 6 months to 4 years were available for *A. otitidis* testing. Four children had bilateral perforation. Thus, the sample set included 27 nasopharyngeal and 31 ear discharge swabs (58 swabs in total). As part of the original RCT, 12 children were treated with amoxycillin and 15 received azithromycin. 25/27 children had clinical treatment failure (defined as persistence of ear pain or a bulging tympanic membrane) when reviewed 6-11 days after antibiotic treatment commenced.

4.3.2 DNA extraction

DNA was extracted from swabs and isolates using a QIAamp kit with enzymatic pre-treatment as described in Chapter 2.8.

4.3.3 *A. otitidis* qPCR

The *A. otitidis* qPCR used previously described primers (Hendolin et al. 1997) (5’-CTACGCATTTCACCGCTACAC-3’ and 5’-GGGGAAGAACACGGATAGGA-3’) which amplify a 265-bp *A. otitidis*-specific region of the 16S rRNA gene between positions 437-702, based on *E. coli* numbering (Brosius et al. 1978). The GenBank accession number for the *A. otitidis* reference 16S rRNA gene sequence is NR_026088.

The qPCR was done using a SensiMix™ NoRef Kit (Bioline). Each 10µL PCR included 1µM of each primer, 1X SensiMix™ NoRef reagent, 0.5X SYBR® Green I
solution (Bioline), and 1µL template DNA. The qPCR was done in a Corbett Research Rotor-Gene 6000 real-time thermocycler. The reaction conditions were 95°C for 10 min followed by 35 cycles of 95°C for 15 s, 66°C for 30 s and 72°C for 15 s; then a final extension at 72°C for 1 min. This was followed by 50°C for 2 min and then a melt-curve analysis from 80°C-90°C in 1°C steps. Data acquisition was done at the end of each cycle. Agarose gel electrophoresis was done (as described in Chapter 2.12) to confirm amplification of the expected 265-bp product. qPCR raw data were analysed using the Corbett Research Rotor-Gene 6000 software as described in Chapter 3.3.10.

Genomic DNA from the *A. otitidis* ATCC51267 reference isolate was used as the qPCR standard. The standard curve was prepared by 1:10 serial dilution (100pg-100fg) of the genomic DNA with TE. The assay was linear within this range. The *A. otitidis* whole genome size (Mcmichael et al. 2005) was used to determine genome copies per mass of DNA. Based on an *A. otitidis* genome size of ~1.7-Mb, 100fg of *A. otitidis* genomic DNA is equivalent to ~50 genome copies. All standards, controls and samples were tested in duplicate.

The limit of detection and limit of quantification were determined as described in Chapter 3.3.14. Positive results were consistently obtained when >100fg *A. otitidis* DNA (50 genome copies) was tested, comparable with earlier reports (Hendolin et al. 1997; Kaur et al. 2010; Tano et al. 2008). Thus, the 100fg standard defined the limit of detection and limit of quantification. Positive results were defined as Cq-value less than or equal to that of the 100fg standard with dissociation temperature (as determined by melt-curve analysis) within 0.5°C of the mean dissociation temperature of the assay’s standards.

Data were analysed as described in Chapter 3.3.11 – 3.3.13. Negative results were recorded for samples with i) no amplification; or ii) amplification but dissociation temperature >0.5°C different to the mean dissociation temperature of the standards, consistent with primer-dimer or other non-specific amplicons. Results were considered equivocal when i) replicates gave a positive and a negative result; or ii) Cq-values were greater than the 100fg control but dissociation temperatures were consistent with *A. otitidis*, suggesting detection of <50 cells. Equivocal specimens
were re-tested with 2μL template DNA. Specimens that remained equivocal upon repeat testing were considered to be *A. otitidis* negative.

The qPCR efficiency ranged from 0.87-0.90 and all standard curves had $R^2$ values >0.99. A no template control was included in all assays and was negative in all except two instances in which non-specific amplification was detected below the assay’s limit of detection (Cq 32.9 and 33.2; at least 5.7 cycles below the limit of detection with concentration equivalent to less than one cell, suggesting detection of primer-dimers).

### 4.3.4 *A. otitidis* culture and identification

PCR-positive swabs were thawed on ice and vortexed. 10μL was inoculated onto horse blood agar (Oxoid), and brain-heart-infusion agar (BHI; Oxoid) supplemented with 6.5% NaCl (Crown Scientific). Horse blood agar was selected as it had previously been used to culture *A. otitidis* from middle ear specimens (Ashhurst-Smith et al. 2007). As *A. otitidis* is salt tolerant (Aguirre et al. 1992), BHI agar with 6.5% NaCl was included as a potentially selective medium, as previously proposed by Tano et al. (Tano et al. 2008).

*A. otitidis* reference strain ATCC51267 was used as a positive control. Plates were incubated at 37°C in an ambient atmosphere and were read after 2, 5, 7, 9, 14 and 21 days of incubation. Small, off-white colonies (Ashhurst-Smith et al. 2007) of similar appearance to the *A. otitidis* reference isolate were Gram stained and subcultured on horse blood agar with incubation at 37°C for 48 hrs in an ambient atmosphere. Alpha-haemolytic, catalase-positive, oxidase-negative, Gram-positive cocci were considered presumptive *A. otitidis* (Aguirre et al. 1992). Genomic DNA from presumptive *A. otitidis* colonies was extracted and identification was confirmed using the PCR described above.

### 4.3.5 Antibiotic susceptibility testing

Penicillin, erythromycin and azithromycin MICs were determined using E-Tests® as previously described by Ashhurst-Smith et al. (Ashhurst-Smith et al. 2007). Briefly, isolates were suspended in sterile saline to no. 3 MacFarland standard then inoculated onto horse blood agar plates. After drying, Etest® strips were applied and
the plates incubated at 37°C in an ambient atmosphere for 48 hrs. These conditions are required to support sufficient *A. otitidis* growth to enable MIC determination (Ashhurst-Smith et al. 2007). As CLSI MIC breakpoints have not been determined for *A. otitidis*, the breakpoints of *S. pneumoniae* were applied consistent with previous studies (Ashhurst-Smith et al. 2007; Bosley et al. 1995). Isolates were considered susceptible, of intermediate susceptibility or resistant as follows: penicillin sensitive \( \leq 0.06\mu g/mL \), intermediate susceptibility 0.12-1\( \mu g/mL \), resistant \( \geq 2\mu g/mL \); erythromycin sensitive \( \leq 0.25\mu g/mL \), intermediate susceptibility 0.5\( \mu g/mL \), resistant \( \geq 1\mu g/mL \); azithromycin sensitive \( \leq 0.5\mu g/mL \), intermediate susceptibility 1\( \mu g/mL \), resistant \( \geq 2\mu g/mL \) (Clinical Laboratory Standards Institute, 2009).

### 4.3.6 Culture and qPCR for other respiratory pathogens

All swabs had been cultured during the original RCT (Morris et al. 2010), as described in Chapter 2.4. Overgrowth by *Proteus* sp. may have prevented culture-based detection of *M. catarrhalis* and *P. aeruginosa* from some swabs, and in these instances culture data are not available. Culture was limited to 18-24 hrs incubation.

*S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and total bacterial load in *A. otitidis*-PCR-positive specimens was determined by qPCR as described in Chapter 3.

### 4.4 Results

#### 4.4.1 *A. otitidis* detected in ear discharge swabs

*A. otitidis* was detected in ear discharge from 10/27 children (37%), including one child with bilateral detection (11 positive swabs in total). In the remaining three children with bilateral perforation, two were positive for *A. otitidis* in one ear only; and one was *A. otitidis* negative. All nasopharyngeal swabs were negative (0/27).

#### 4.4.2 Culture of *A. otitidis* PCR-positive swabs

All *A. otitidis* PCR-positive swabs were cultured on horse blood agar and BHI agar with 6.5% NaCl for 21 days. *A. otitidis* was isolated from 5/11 PCR-positive ear discharge swabs from 4/10 children after 2-14 days incubation (Table 4.1).
Table 4.1: *A. otitidis* isolates from ear discharge swabs.
Culture and antibiotic susceptibility results for five isolates from four children (designated child A-D). All cultures were positive for other bacteria after two days incubation. MIC values are in µg/mL. Isolates were considered susceptible, of intermediate susceptibility or resistant as follows: penicillin sensitive $\leq 0.06$µg/mL, intermediate susceptibility 0.12-1µg/mL, resistant $\geq 2$µg/mL; erythromycin sensitive $\leq 0.25$µg/mL, intermediate susceptibility 0.5µg/mL, resistant $\geq 1$µg/mL; azithromycin sensitive $\leq 0.5$µg/mL, intermediate susceptibility 1µg/mL, resistant $\geq 2$µg/mL. HBA = Horse blood agar.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days of culture until <em>A. otitidis</em> was isolated</th>
<th>Penicillin MIC</th>
<th>Erythromycin MIC</th>
<th>Azithromycin MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBA agar</td>
<td>BHI agar with 6.5% NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Child A (Left ear)</td>
<td>5</td>
<td>7</td>
<td>0.19</td>
<td>4</td>
</tr>
<tr>
<td>Child A (Right ear)</td>
<td>2</td>
<td>5</td>
<td>0.125</td>
<td>3</td>
</tr>
<tr>
<td>Child B</td>
<td>14</td>
<td>5</td>
<td>0.006</td>
<td>$&gt;256$</td>
</tr>
<tr>
<td>Child C</td>
<td>Not detected*</td>
<td>9</td>
<td>0.19</td>
<td>$&gt;256$</td>
</tr>
<tr>
<td>Child D</td>
<td>7</td>
<td>Not detected</td>
<td>0.032</td>
<td>96</td>
</tr>
</tbody>
</table>

*This plate was overgrown by a *Proteus* sp.*
As *A. otitidis* is salt tolerant (Aguirre et al. 1992), it was initially hoped the BHI agar with 6.5% NaCl would act as a selective medium (Tano et al. 2008); however, all cultures grew other bacterial species after two days incubation, resulting in substantial overgrowth before day 21. This may have obscured the small *A. otitidis* colonies preventing isolation in the 6/11 PCR-positive but culture-negative samples.

### 4.4.3 Susceptibility of *A. otitidis* isolates

Five isolates were available for antibiotic susceptibility testing (Table 4.1). Erythromycin MICs ranged from 4 - >256µg/mL. Azithromycin MICs ranged from 1.5 - >256µg/mL. Penicillin MICs ranged from 0.006 – 0.19µg/mL.

CLSI MIC breakpoints for *A. otitidis* have not been determined. If *S. pneumoniae* MIC breakpoints (Clinical Laboratory Standards Institute, 2009) are applied, as done previously (Ashhurst-Smith et al. 2007; Bosley et al. 1995), all isolates would be resistant to erythromycin and 4/5 would be resistant to azithromycin. If *S. pneumoniae* CLSI breakpoints for oral penicillin V are applied, 3/5 isolates would have intermediate penicillin susceptibility.

### 4.4.4 Other bacteria cultured from *A. otitidis*-positive ear discharge swabs

In the 11 *A. otitidis* PCR-positive swabs, the middle ear pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were cultured from two, four and one swabs respectively (Table 4.2). *Pseudomonas* sp., *Proteus* sp. and β-haemolytic streptococci were cultured from two, three and one swab/s respectively (Table 4.2). 10/11 *A. otitidis* PCR-positive swabs grew *Staphylococcus* sp. and 9/11 grew other species that were not identified. All *A. otitidis*-positive swabs were polymicrobial with at least 2-5 other species detected (Table 4.2). In one sample *A. otitidis*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *Staphylococcus* sp. and other unidentified bacteria were all detected.

### 4.4.5 *A. otitidis* bacterial load and relative abundance in ear discharge specimens

As all *A. otitidis*-positive swabs were positive for at least two other species, bacterial load measures were used to determine if *A. otitidis* was a minor or dominant
A. otitidis relative abundance was then determined by expressing its load as a percentage of the total bacterial load (Table 4.3). The relative abundance of A. otitidis ranged from 0.01-34% (median 0.7%). In 6/11 PCR-positive swabs the A. otitidis relative abundance was <1%. The A. otitidis load in these swabs ranged from 2.2x10^4 - 1.1x10^8 cells/swab and only one swab was positive by culture. In the remaining five swabs the relative abundance ranged from 2-34% of the total bacterial load (median 9%). These samples had the highest A. otitidis loads (5.9x10^6 - 1.1x10^8 cells/swab) and 4/5 were A. otitidis-positive by culture.

4.4.6 Bacterial load and relative abundance of A. otitidis compared to recognised otopathogens

A. otitidis bacterial load and relative abundance estimates were compared to those of S. pneumoniae, H. influenzae and M. catarrhalis to determine if A. otitidis was achieving loads similar to that of the recognised otopathogens (Figure 4.1). 10/11 A. otitidis-positive swabs were also PCR-positive for H. influenzae. Overall, H. influenzae and A. otitidis bacterial loads and relative abundances were similar (Table 4.3). The bacterial load ranged from 4.3x10^4-1.2x10^7 cells/swab for H. influenzae (median 7.4x10^5 cells/swab) and 2.2x10^4-1.1x10^8 cells/swab for A. otitidis (median 1.8x10^5 cells/swab). Maximum relative abundance was 27% for H. influenzae versus 34% for A. otitidis. Relative abundance was <1% in 5/10 H. influenzae PCR-positive swabs and 6/11 A. otitidis PCR-positive swabs. In 5/10 H. influenzae PCR-positive swabs the A. otitidis bacterial load was higher than that of H. influenzae.

S. pneumoniae and M. catarrhalis were detected in fewer A. otitidis-positive swabs (3/11 and 4/11, respectively) and were only present at <1% relative abundance (Table 4.3). S. pneumoniae bacterial load ranged from 3.5x10^2-1.8x10^5 cells/swab. M. catarrhalis bacterial load ranged from 4.3x10^4-5.9x10^5 cells/swab.
Table 4.2: Other bacteria previously cultured from *A. otitidis*-positive ear discharge specimens.
These data were derived from the original RCT database. Culture data for *M. catarrhalis* and *P. aeruginosa* were not available for some swabs due to overgrowth by *Proteus* species (n/a = not available).

<table>
<thead>
<tr>
<th>Swab</th>
<th><em>S. pneumoniae</em></th>
<th><em>H. influenzae</em></th>
<th><em>M. catarrhalis</em></th>
<th>β-haemolytic streptococi</th>
<th>Staphylococcus sp.</th>
<th><em>P. aeruginosa</em></th>
<th><em>Proteus</em> sp.</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child A (Left ear)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Child A (Right ear)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Child B</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Child C</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Child D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Child E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Child F</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Child G</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Child H</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Child I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Child J</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 4.3: *A. otitidis*, *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* in 11 *A. otitidis* PCR-positive ear discharge (ED) swabs and 10 nasopharyngeal (NP) swabs from 10 Indigenous children diagnosed with acute otitis media with perforation.

<table>
<thead>
<tr>
<th></th>
<th><em>A. otitidis</em></th>
<th><em>H. influenzae</em></th>
<th><em>S. pneumoniae</em></th>
<th><em>M. catarrhalis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In <em>A. otitidis</em>-positive ED swabs (n=11)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR-positive swabs</td>
<td>11</td>
<td>10</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Culture-positive swabs</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Bacterial load range (cells/swab)</td>
<td>$2.2 \times 10^4$-$1.1 \times 10^8$</td>
<td>$4.3 \times 10^4$-$1.2 \times 10^7$</td>
<td>$3.5 \times 10^4$-$1.8 \times 10^5$</td>
<td>$4.3 \times 10^4$-$5.9 \times 10^5$</td>
</tr>
<tr>
<td>Bacterial load &gt; $1 \times 10^5$ cells/swab</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Culture-positive and load &gt; $1 \times 10^5$ cells/swab</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Relative abundance &lt;1%</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Relative abundance range (%)</td>
<td>0.01-0.70</td>
<td>0.01-0.79</td>
<td>0.01-0.68</td>
<td>0.01-0.89</td>
</tr>
<tr>
<td><strong>In matched NP swabs (n=10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCR-positive swabs</td>
<td>0</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Culture-positive swabs</td>
<td>Not done*</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

* *A. otitidis* culture was only performed for PCR-positive swabs*
Figure 4.1: Total and pathogenic bacterial loads in 11 *A. otitidis*-positive swabs. Species-specific data points are only shown for PCR-positive specimens. *A. otitidis*, *H. influenzae* and total bacterial load median values are indicated with a bar. TBL = Total bacterial load. Ao = *A. otitidis*. Hi = *H. influenzae*. Spn = *S. pneumoniae*. Mc = *M. catarrhalis*
4.5 Discussion

4.5.1 *A. otitidis* is present in the ear discharge of Indigenous children with acute otitis media with perforation

In this study, *A. otitidis* was detected by PCR in 37% of 31 ear discharge swabs from 27 children. This rate is consistent with PCR-based acute otitis media studies from Finland, the USA and Japan (25%, 32% and 50% respectively) (Harimaya et al. 2006; Kaur et al. 2010; Leskinen et al. 2004), and a culture-based study of OME in Indigenous and non-Indigenous Australian children (45% and 36% respectively) (Ashhurst-Smith et al. 2007) which tested MEF collected by tympanocentesis.

4.5.2 *A. otitidis* isolation from polymicrobial swabs

*A. otitidis* was cultured from 5/11 PCR-positive swabs. Published incubation times for *A. otitidis* isolation vary from 5-14 days (Harimaya et al. 2009; Tano et al. 2008). In this study, ear discharge swabs were cultured for 21 days, but all isolates were obtained by 14 days. BHI agar with 6.5% NaCl was trialled as an *A. otitidis* selective media, but was unsuccessful due to heavy overgrowth by other species after two days incubation, despite the high salt concentration. Until a suitable *A. otitidis* selective medium is identified, isolation from polymicrobial specimens is likely to remain difficult.

Several factors may have contributed to the low *A. otitidis* isolation rate. Firstly, PCR-positive but culture-negative results may indicate detection of non-viable bacteria. This has been considered in other otitis media studies and it is generally accepted that DNA is quickly cleared from the middle ear (Aul et al. 1998; Bakaletz, 2009; Post et al. 1996) suggesting that a positive PCR is indicative of viable cells. Secondly, *A. otitidis* viability may have been affected by storage at -70ºC for up to five years; however, viability of *S. pneumoniae* and *H. influenzae* has been demonstrated in swabs stored in STGGB after up to 12 years at -70ºC (Hare et al. 2011) suggesting good overall bacterial survival despite the prolonged storage. Finally, overgrowth by other bacterial species may have affected *A. otitidis* isolation. All cultures grew other species after two days incubation, which potentially obscured the small *A. otitidis* colonies. Overall, the results are consistent with the lower
sensitivity of culture compared to PCR that has been documented for several pathogens in middle ear samples (Hall-Stoodley et al. 2006).

Consistent with earlier reports (Ashhurst-Smith et al. 2007; Bosley et al. 1995), macrolide resistance was detected in most A. otitidis isolates. Although A. otitidis CLSI breakpoints have not been established, the observed MIC values for erythromycin and azithromycin were higher than breakpoints describing resistance in other species. If S. pneumoniae CLSI breakpoints are applied (Ashhurst-Smith et al. 2007; Bosley et al. 1995) 3/5 A. otitidis isolates had intermediate level penicillin resistance; however, the validity of this approach remains to be determined, especially as atypical culture conditions are required to achieve sufficient growth for MIC determination (Ashhurst-Smith et al. 2007).

4.5.3 Is A. otitidis contributing to middle ear pathology?

The clinical significance of A. otitidis detected in ear discharge swabs is unclear. Several studies have found A. otitidis in the ear canal of healthy volunteers indicating that it is part of the normal canal flora (De Baere et al. 2010; Frank et al. 2003; Stroman et al. 2001; Tano et al. 2008). Other studies using culture or species-specific PCRs have detected A. otitidis as the sole bacterial species identified in MEF from children with otitis media collected after careful disinfection of the canal and tympanic membrane suggesting it is infecting the middle ear (Ashhurst-Smith et al. 2007; Khoramrooz et al. 2011).

In this study, A. otitidis was not detected in any nasopharyngeal swabs. This was unexpected as A. otitidis has previously been reported in 7-12% of nasopharyngeal swabs (Kaur et al. 2010; Takada et al. 2003; Tano et al. 2008) in other populations. As the study was limited to 27 children, it is possible that A. otitidis carriage may be detected if a larger cohort were tested; however, failure to detect A. otitidis in nasopharyngeal swabs from any of the children with positive ear discharge swabs suggests it is unlikely to be a primary otopathogen in this population. In light of this, it was important to determine if A. otitidis in ear discharge swabs was indicative of middle ear infection or contamination by canal flora.

Quantification of A. otitidis bacterial load has not previously been reported. In this study, bacterial load and relative abundance measures were used to better understand
the significance of *A. otitidis* in the polymicrobial ear discharge swabs. *A. otitidis* bacterial load and relative abundance measures were similar to that of the otopathogen *H. influenzae*. For both species, relative abundance <1% was associated with bacterial load ≤10⁵ cells/swab and most samples were PCR-positive and culture-negative. These data suggest a minor role for these species in these specimens, and may be indicative of canal flora (*H. influenzae* has also been reported in normal canal flora from healthy volunteers (De Baere et al. 2010)). For the remaining specimens, relative abundance ranged from 2-34% and 4-27% for *A. otitidis* and *H. influenzae*, respectively. The significance of this finding is unclear as there are no data describing relative abundance thresholds; however, a relative abundance of 34% is clearly suggestive of a dominant species. *A. otitidis* was present at relative abundance greater than that of *H. influenzae* in 5/10 swabs positive for both species. Based on these data, I hypothesise that *A. otitidis* can secondarily infect the middle ear following tympanic membrane perforation. A similar hypothesis was proposed by De Baere et al. (De Baere et al. 2010) who reported substantially higher rates of *A. otitidis* in canal rather than nasopharyngeal swabs (83% versus 8%) and concluded that *A. otitidis* in MEF from children with OME most likely reflected either secondary infection following perforation or specimen contamination by canal flora.

### 4.5.4 Study limitations

The study focused on Indigenous Australian children who are at high-risk of progression to CSOM, and thus the findings may not be generalisable to other populations. The small study size (27 children) also limits generalisation of the findings. Despite these limitations, it appears that *A. otitidis* is often present in the ear discharge of young Indigenous children with AOM with perforation (37% of our cohort).

The qPCR described in this study is a modification of a previously published qualitative *A. otitidis* PCR (Hendolin et al. 1997). The qPCR had efficiency of 0.87-0.90, slightly below the ideal range of 0.9-1.10 (D'haene et al. 2010). This most likely reflects the relatively large PCR product size which is approximately twice that recommended for qPCR (D'haene et al. 2010). This limitation was accepted as there are few data describing alternative *A. otitidis*-specific PCR targets. Further, this
limitation is common to other qPCR measures of bacterial load. For example, the total bacterial load method proposed by Nadkarni et al. (Nadkarni et al. 2002) which amplifies a 466-bp product but provides a useful target for universal 16S rRNA gene amplification (Horz et al. 2005). Further data are required for design of alternative A. otitidis-specific qPCR targets to facilitate better reaction efficiency.

The study was also limited by the ear discharge sampling technique. Ear discharge swabs in this study had been collected from children with acute otitis media with perforation affecting <2% of the pars tensa of the tympanic membrane for up to six weeks. Paired canal swabs were not collected. Canals were cleaned prior to sampling, but disinfection was not done. As the size of the perforations prevented middle ear sampling, it is likely that some degree of contamination from canal flora will be present in the swabs. This limitation prevents definitive differentiation of canal flora from middle ear pathogens.

4.5.5 Conclusions and recommendations

This study used PCR to show that A. otitidis is present in ear discharge of Indigenous children with acute otitis media with perforation. The absence of A. otitidis in nasopharyngeal swabs, together with high bacterial load and relative abundance in a subset of ear discharge swabs, suggests A. otitidis may have secondarily infected the middle ear following tympanic membrane perforation.

Further studies are required to test this hypothesis. Such studies should be PCR-based and include culture to assess antibiotic susceptibility. Bacterial load and relative abundance estimates should be considered when investigating the significance of A. otitidis in polymicrobial contexts, particularly in children with existing perforations. Where collection of MEF by tympanocentesis is not feasible, matched middle ear and canal swabs may be helpful in distinguishing bacteria from the outer and middle ear. Additional studies investigating a role for A. otitidis as a secondary pathogen in chronic suppurative otitis media are also warranted in populations with a high prevalence of spontaneous tympanic membrane perforation.

Finally, all A. otitidis-positive swabs contained polymicrobial bacterial communities. Bacterial community profiling of nasopharyngeal and ear discharge swabs is now
required to better understand how polymicrobial factors may contribute to otitis media pathogenesis and severity.
CHAPTER 5

Optimisation and validation of T-RFLP for application to upper respiratory specimens
Chapter 5: Optimisation and validation of T-RFLP for application to upper respiratory specimens

5.1 Summary

**Background:** A range of bacterial community finger-printing techniques are available to characterise polymicrobial specimens. Terminal restriction fragment length polymorphism (T-RFLP) profiling is a bacterial community finger-printing method that has previously been used to characterise lower airway infections. As T-RFLP has not been described for otitis media, the aim of this study was to optimise and validate the method for upper respiratory specimens.

**Methods:** T-RFLP previously published for lower respiratory infections was optimised and validated using a laboratory-prepared multi-template control containing equal amounts of DNA from 13 common respiratory bacteria. Optimisation included assessment of primers; enzymes; reaction conditions; and signal:noise algorithms.

**Results:** T-RFLP of the multi-template control revealed differential PCR amplification biases. Two fragments were detected for some species, including *Moraxella catarrhalis*. Comparison of signal:noise algorithms identified the constant baseline threshold method as optimal for differentiation of signal and noise. The control was also used to show that specific restriction enzymes could differentiate common respiratory pathogens from closely-related commensal species.

**Conclusion:** T-RFLP of the multi-template control identified PCR amplification biases; profiling characteristics of important species; and optimal signal:noise algorithm. Primer selection and PCR conditions were critical to T-RFLP detection of all species in the control. A T-RFLP method for otitis media specimens was determined and validation with clinical specimens is now required.

**Acknowledgements:** Mr Peter Christensen assisted with PCR and fragment analysis processing under my supervision.
5.2 Introduction

Terminal restriction fragment length polymorphism (T-RFLP) is a culture-independent bacterial community profiling tool. It was first described in 1997 (Liu et al. 1997) and since then has been used extensively to characterise bacterial communities in marine and terrestrial environments (Lanfranconi et al. 2010; Sagova-Mareckova et al. 2011). It has also been used to characterise bacterial communities in agricultural contexts (Torok et al. 2011) and forensic applications (Macdonald et al. 2011). In humans, T-RFLP has been used to study bacterial communities associated with mucosal disease in the gut (Andoh et al. 2011), female genital tract (Thies et al. 2007), oral cavity (Jakobsson et al. 2010; Montagner et al. 2010), lower airways (Rogers et al. 2006; Stressmann et al. 2010; Tunney et al. 2011) and sinuses (Stressmann et al. 2011). It is an attractive tool for bacterial community analyses as it is a relatively low-cost and potentially high-throughput method.

5.2.1 How does T-RFLP work?

T-RFLP is a multi-step laboratory process which estimates bacterial diversity by using a restriction digestion of 16S rRNA genes amplified from a specimen (Kim et al. 2004; Schutte et al. 2008; Stres, 2006). The analysis is done using DNA extracted directly from clinical specimens. Fluorescently-labelled universal primers are used to amplify the 16S rRNA gene from all bacteria in the specimen. The resulting PCR product is then digested with one or more restriction enzymes to generate fluorescently-labelled terminal fragments. These terminal fragments vary in size due to species-specific sequence variation in the 16S rRNA gene. Capillary electrophoresis is then used to size-separate the terminal fragments. The fluorescent signal of each terminal fragment in the specimen is then plotted against size in base-pairs. This generates an electropherogram containing a series of peaks which describe the bacterial community profile of the specimen (Figure 5.1). Each peak in the electropherogram indicates a T-RFLP fragment which is defined by its size (in base-pairs); peak height and the peak area. The number of fragments in each profile provides an estimate of bacterial richness, whereas the height or area of each peak can be used to estimate relative abundance.
Figure 5.1: Experimental stages in T-RFLP analyses.
DNA is extracted directly from clinical specimens and fluorescently-labelled universal primers are used to amplify the 16S rRNA gene. Restriction digestion is then used to generate species-specific fragments of different sizes. The fragments are separated by capillary electrophoresis. Fluorescence is then plotted against fragment size to generate an electropherogram which defines the T-RFLP profile.
T-RFLP can be applied to any gene of interest. It has previously been used to target metabolic genes associated with ammonia oxidation (Yao et al. 2011); methane oxidation (Krause et al. 2010) and nitrogen cycling (Abell et al. 2010); however, it is most commonly used to profile bacterial diversity by targeting the 16S rRNA gene.

5.2.2 Why target the 16S rRNA gene?

The 16S rRNA gene is ubiquitous in bacteria and has long been recognised as being phylogenetically informative (Woese, 1987). The gene contains a combination of conserved regions common to all bacteria, and hypervariable regions with sequence variation unique to specific taxa. Seminal work by Pace et al. (Pace et al. 1985; Lane et al. 1985) during the 1980’s showed that the conserved regions of the 16S rRNA gene could be targeted with universal PCR primers such that the gene could be simultaneously amplified from all bacteria in a specimen. Sequencing of the hypervariable regions could then be used to identify specific taxa and subsequently provide a profile of the entire bacterial community. In T-RFLP, species-specific sequence variations in the 16S rRNA gene hypervariable regions are detected by restriction digestion.

5.2.3 Important methodological considerations in T-RFLP

Like all ecological measures of bacterial diversity, T-RFLP analyses can be subject to experimental bias. To minimise T-RFLP biases, careful consideration must be given to the experimental conditions. There is little consensus in the literature regarding optimal T-RFLP conditions. This applies to all stages of the experimental process as well as the data processing and analysis (as reviewed by Stres, 2006). Further, as methodological differences can affect the bacterial community profile, it is critical that experimental and analytical methods be applied consistently if valid comparative analyses are to be achieved (Kim et al. 2004). The following sections briefly review key methodological factors that require standardisation for robust comparative T-RFLP analyses.
5.2.4 T-RFLP experimental parameters

5.2.4.1 DNA extraction
Ideally, DNA should be extracted evenly from all species in polymicrobial specimens; however, this can be difficult to achieve (Rantakokko-Jalava et al. 2002) particularly with regards to differences in the cell wall of Gram-positive and Gram-negative bacteria (Kowalchuk et al. 2008). Inefficient DNA extraction potentially biases community profiling because of underestimation of the bacterial richness (Kowalchuk et al. 2008). It is important that a single method is used within a study to control for potential DNA extraction biases. DNA extraction methods should also minimise shearing and other degradation as these can lead to chimera formation during PCR (Frey et al. 2006), as discussed below.

5.2.4.2 PCR
PCR chemistry and cycle number also need to be applied consistently. As the PCR is a multi-template amplification, hetero-duplexes and chimeras can occur (Acinas et al. 2005). Chimeras are hybrid PCR amplicons arising from hetero-duplexes which form when genes from different species combine during PCR. Chimera formation can be reduced by using no more than 30 PCR cycles or applying a reconditioning PCR (Thompson et al. 2002). It is important that chimera formation is minimised as these amplicons can result in false-positive T-RFLP peaks and subsequent overestimation of bacterial richness (Haas et al. 2011).

Primer choice is also an important consideration. Ideally, the universal 16S rRNA gene primers should amplify evenly from as many species as possible. Additionally, the primers should be specific to conserved regions of the gene that have a low degree of secondary structure (Kim et al. 2004). Researchers must also decide if one or both primers will be fluorescently-labelled (Kim et al. 2004).

5.2.4.3 Choice of restriction enzymes
T-RFLP requires optimal selection of primers and restriction enzymes to achieve the maximum resolution of bacterial richness (Kim et al. 2004). Theoretically, restriction digestion of 16S rRNA gene amplicons generates species-specific terminal fragments; however, some species produce terminal fragments of identical size such that a single peak in the T-RFLP profile may be indicative of multiple species (reviewed by Osborne et al. 2006). As T-RFLP uses peak number to determine
bacterial richness, this may lead to underestimation of the bacterial community complexity. To address this, data from multiple restriction digests can be combined to increase the T-RFLP resolution of bacterial richness (Kim et al. 2004). Freeware programs are available which identify optimal T-RFLP restriction enzymes. One example is REPK (Collins et al. 2007) which performs an *in silico* restriction digestion of 16S rRNA gene sequences to identify enzymes which will resolve maximum bacterial richness.

### 5.2.4.4 Removal of salts from PCR amplicons and digests

Restriction digestions require a balance of salts optimised to provide maximum enzymatic activity. It is critical that residual salts from the PCR amplification be removed prior to restriction digestion. Likewise, residual salts from the restriction digestion can adversely affect capillary electrophoresis such that terminal-restriction fragments (T-RFs) are incorrectly sized (Moeseneder et al. 1999) and, thus, digests should be purified prior to fragment analysis.

Precipitation and silica column-based methods are commonly used to purify PCR products and restriction digests for down-stream applications. Although precipitation methods are arguably the gold-standard, they are labour intensive and may not remove unincorporated primers. Kit-based silica columns have been developed to facilitate high-throughput purification of nucleic acids. These methods remove unwanted salts and unincorporated primers, but may have a size exclusion limit, commonly ~100-bp. Modified buffers are required when silica columns are used to remove salt from restriction digests if fragments below the size exclusion limit are expected.

### 5.2.4.5 Minimising contamination and photobleaching

16S rRNA gene PCRs are notoriously prone to contamination and it is critical that good laboratory practices are employed to minimise specimen and PCR contamination. A degree of contamination is, however, expected in all 16S rRNA gene amplifications because of the general difficulty eradicating sources of bacterial DNA. One example of this is the presence of remnant bacterial DNA in silica columns used for DNA extraction (van der Zee et al. 2002). Additionally, remnant bacterial DNA in PCR reagents can be a source of contamination (Corless et al. 2000). To overcome this, high purity molecular grade reagents should be used.
wherever possible. Further, it is critical that negative controls be used extensively during T-RFLP to monitor for contamination.

Good laboratory practice should also be employed to minimise photobleaching. Excessive photobleaching reduces the fluorophore signal in T-RFLP profiles and potentially reduces the detection of less abundant species which are expected to give small peaks.

5.2.5 T-RFLP data analysis

There is little consensus regarding T-RFLP data analysis methods (Stres, 2006). Perhaps most importantly, this relates to signal:noise algorithms which are used to differentiate true T-RFLP peaks from false “noise”. The simplest method to differentiate signal and noise is to apply an arbitrarily selected fixed threshold, for example 100FU; however, this may result in inaccurate peak detection (reviewed by Schutte et al. 2008). For example, thresholds set too low may fail to exclude false peaks resulting in overestimation of bacterial richness. Conversely, thresholds set too high may underestimate bacterial richness by excluding true peaks (Dunbar et al. 2001). Additionally, this method does not address inter-specimen variation in the DNA mass loaded onto the capillary which can also affect formation of false peaks (Dunbar et al. 2001; Sait et al. 2003).

A range of proportional measures have been proposed to address these limitations (reviewed by Stres, 2006). This includes the constant baseline threshold method (Dunbar et al. 2001); constant percentage threshold method (Sait et al. 2003); and the variable percentage threshold method (Osborne et al. 2006). Statistical methods have also been developed (Abdo et al. 2006) and have been incorporated into freeware programs, such as T-REX (Culman et al. 2009). Few studies have compared these methods to determine optimal signal:noise algorithms (Stres, 2006). Additionally, T-RFLP signal:noise algorithms have not been validated with well-defined controls (Abdo et al. 2006; Osborne et al. 2006), and thus it is difficult to gauge the true effectiveness of each method.
5.2.6 T-RFLP of respiratory environments

At the time of writing, no reports have described T-RFLP in the context of otitis media; however, the method has been used to describe bacterial communities in the lower airways of patients with cystic fibrosis (Rogers et al. 2004) and bronchopulmonary dysplasia (Stressmann et al. 2010). The method has also been recently used to describe bacterial communities in mucus and nasal biopsy specimens from patients with chronic rhinosinusitis (Stressmann et al. 2011). T-RFLP of nasopharyngeal swabs has been described in one methodological paper (Kwambana et al. 2011).

T-RFLP provides an improved measure of lower airway bacterial richness than traditional culture-based methods (Rogers et al. 2009); however, there has been little investigation of potential methodological biases. Furthermore, a lack of consensus in published T-RFLP methodologies for respiratory specimens limits comparison of findings between studies. For example, Rogers et al. (Rogers et al. 2003) performed T-RFLP of lower respiratory specimens using 16S rRNA gene primers 8F[FAM] and 926R together with the restriction enzyme CfoI. Tunney et al. (Tunney et al. 2011) also used these primers, but performed the restriction digest with the enzymes CfoI and HaeIII. In contrast, Kwambana et al. (Kwambana et al. 2011) used 16S rRNA gene primers 338F and 1046R together with the restriction enzyme AluI. A range of signal:noise algorithms have also been used. Kwambana et al. (Kwambana et al. 2011) arbitrarily applied a fixed signal:noise threshold of 100FU, whereas Rogers et al. (Rogers et al. 2003) used a signal threshold of 0.01%, and Tunney et al. (Tunney et al. 2011) did not describe which method was used. These differences highlight the need for critical review and validation of T-RFLP protocols to determine an optimal method for analysis of otitis media specimens.

5.2.7 Aims of this study

An optimised T-RFLP for otitis media has not been described. It is also unclear if previously described T-RFLP methods for respiratory specimens can differentiate the major otitis media pathogens *H. influenzae* and *S. pneumoniae* from closely-related commensal species. The objective of this study was to review and optimise the T-RFLP method of Rogers et al. (Rogers et al. 2003) to achieve maximum resolution of the bacterial communities in nasopharyngeal and middle ear specimens from
Indigenous children with otitis media. The methods of Kwambana et al. (Kwambana et al. 2011) and Tunney et al. (Tunney et al. 2011) were not considered as these were not published until 2011.

The aims of the study were:

i) to use in silico analyses to identify restriction enzymes capable of differentiating otitis media pathogens from closely-related commensal species.

ii) to assess bias and optimise T-RFLP experimental conditions for otitis media specimens using a laboratory-prepared multi-template control containing genomic DNA from potential respiratory bacteria.

iii) to use data from the multi-template control to compare signal:noise algorithms.

5.3 Methods

5.3.1 Datasets used for in silico analyses

Reference 16S rRNA gene sequences were downloaded from public databases for in silico analyses. Unless otherwise specified, sequences were sourced from the Ribosomal Database Project (RDP) collection (RDP 10.4; http://rdp.cme.msu.edu/; accessed October 2008). The RDP collection was filtered to provide good quality sequences that were derived from isolates and >1200-bp in length. Where suitable sequences were not available in the RDP collection, sequences were sourced from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) as indicated in the relevant tables. All sequences were downloaded in FASTA format. For inclusion in the study, sequences were required to be at least 1400-bp long and commence within 30-bp of E. coli position 8. Sequences were aligned and trimmed against E. coli J01695 using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Alignments were saved as FASTA files for subsequent in silico analyses.

Three reference 16S rRNA gene sequence datasets were used for in silico analyses - a S. pneumoniae reference set containing S. pneumoniae and the closely-related S. pseudopneumoniae and S. mitis (Table 5.1); a H. influenzae reference set containing
*H. influenzae* and five closely-related *Haemophilus* sp. (Table 5.2); and a general reference set containing sequences from 89 potential respiratory species. The general reference set included recognised acute and chronic otitis media pathogens (Rovers et al. 2004; Stuart et al. 2003), atypical otitis media bacteria (Clark et al. 2006; Gomez-Garces et al. 2004), upper respiratory commensals (Brook, 2005; Wilson, 2008), ear canal flora (Frank et al. 2003), lower airway pathogens (Wilson, 2008), oral flora (Wilson, 2008), and a selection of anaerobic species (Murray et al. 2003). For some genera, additional species were included to provide a selection of closely-related sequences. *E. coli* sequence J01695 was used as the 16S rRNA reference gene (Brosius et al. 1978). A full description of the general reference set is provided in Appendix B.

### Table 5.1: 16S rRNA gene sequences in the *S. pneumoniae* reference set.

* = 16S rRNA gene sequences obtained from whole genome sequence.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em> R6*</td>
<td>GenBank</td>
<td>AE007317</td>
</tr>
<tr>
<td><em>S. pneumoniae</em> TIGR4 *</td>
<td>GenBank</td>
<td>AE005672.3</td>
</tr>
<tr>
<td><em>S. pseudopneumoniae</em></td>
<td>RDP</td>
<td>S0004214981</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>RDP</td>
<td>S00061787044</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>RDP</td>
<td>S00061788244</td>
</tr>
</tbody>
</table>

### Table 5.2: 16S rRNA gene sequences included in the *H. influenzae* reference set.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. influenzae</em></td>
<td>RDP</td>
<td>S000573548</td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>RDP</td>
<td>S000436674</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>RDP</td>
<td>S000903366</td>
</tr>
<tr>
<td><em>H. parahaemolyticus</em></td>
<td>RDP</td>
<td>S000436702</td>
</tr>
<tr>
<td><em>H. aphrophilus</em></td>
<td>RDP</td>
<td>S000436711</td>
</tr>
<tr>
<td><em>H. segnis</em></td>
<td>RDP</td>
<td>S0004366726</td>
</tr>
</tbody>
</table>
5.3.2 In silico analyses

5.3.2.1 REPK analysis to predict T-RFLP fragment size
REPK (Collins et al. 2007) is a freeware program which identifies restriction sites in DNA sequence data (http://rocaplab.ocean.washington.edu/tools/repk). The program is designed to specifically identify terminal fragments generated by virtual restriction digestion of input DNA sequences. The program contains 192 reference restriction enzymes which can be screened to identify optimal enzymes for specific T-RFLP analyses.

In this study, REPK (Collins et al. 2007) was used to predict expected terminal fragments for individual bacterial species. This was done by analysis of reference sequences using the REPK default parameters. Reference DNA sequences were trimmed as required using ClustalW2, prior to REPK analysis.

5.3.2.2 ISPaR virtual T-RFLP analyses
ISPaR freeware (Shyu et al. 2007) (http://mica.ibest.uidaho.edu/digest.php) was used to perform virtual T-RFLP analyses. This program requires users to input T-RFLP primers and enzymes, and then performs a “virtual digest” of large 16S rRNA gene databases. The ISPaR analysis quickly provides an indication of the potential usefulness of primers and restriction enzymes to be used in T-RFLP analyses.

In this study, ISPaR analyses were used to predict the overall resolution of bacterial richness that could be achieved with each selected restriction enzyme. Specific primers and restriction enzymes were input as required. Unless otherwise specified, no mismatches were permitted between primers and target sequences. Default settings were used for all other parameters. ISPaR analyses were performed in October 2008 using the SILVA RefSSU 16S rRNA database or the GreenGenes database, as indicated in the results.

5.3.3 Reference isolates
Bacterial reference isolates used in this study are listed in Table 5.3. An additional collection of nine M. catarrhalis isolates collected during a study of paediatric bronchiectasis was kindly donated by Ms Kim Hare (Menzies School of Health Research). The isolates had been phenotypically identified as M. catarrhalis based on Gram stain, oxidase production and tributyrin hydrolysis (Murray et al. 2003). All
strains were from different children and had originated from several geographic regions (Darwin, Central Australia and Brisbane).

Table 5.3: Reference isolates used to prepare the multi-template control.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. otitidis</em></td>
<td>ATCC51267</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>ATCC23745</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>MSHR E. coli</td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>MSHR5164</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>ATCC19418</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>ATCC7901</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>MSHR110128</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>ATCC8176</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>ATCC12453</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>NCTC10662</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>MSHR_SCC1177</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>ATCC6249</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>ATCC49619</td>
</tr>
</tbody>
</table>

5.3.4 Preparation of a multi-template control

A multi-template control was prepared to provide a defined bacterial community for T-RFLP validation and optimisation. The control contained DNA from 13 species representing common respiratory pathogens and a selection of respiratory tract commensal species (Table 5.3). Gram-positive and Gram-negative species were included. *Bacteroides fragilis* was also included as a representative anaerobic species. The control contained an equal quantity of genomic DNA from each of the 13 species at a final concentration of 20-30ng/µL. DNA was extracted from each isolate as described in Chapter 2.8.1. DNA concentration was determined using PicoGreen® reagent as described in Chapter 2.9.
5.3.5 Primers

Primers used in this study are described in Table 5.4. 8F[FAM] was used at the forward primer in all PCRs. This primer contained a 6-Carboxyfluorescein ([FAM]) fluorescent label. Three unlabelled reverse primers were tested – 926R (consistent with the method of Rogers et al. 2003), 1113R and 1512R (Table 5.4). Reverse primer 1492R was also used in some in silico analyses.

Table 5.4: Primers used in Chapter 5.
Primer 1492R was as described in the ISPaR freeware program (http://mica.ibest.uidaho.edu/digest.php). Redundancies are indicated using the standard annotations - M (A and C) and Y (T and C).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>926R</td>
<td>5’ CCCTCAATTCTCTTTAGTTT 3’</td>
<td>Rogers et al. 2003 Schutte et al. 2008</td>
</tr>
<tr>
<td>1113R</td>
<td>5’ GGGTTGCGCTCGTTG 3’</td>
<td>Schutte et al. 2008</td>
</tr>
<tr>
<td>1492R</td>
<td>5’ GYTACCTTGTTACGACTT 3’</td>
<td>As per ISPaR freeware</td>
</tr>
<tr>
<td>1512R</td>
<td>5’ACGGYTACCTTGTTACGACTT 3’</td>
<td>Li et al. 2007</td>
</tr>
</tbody>
</table>

5.3.6 PCR

PCR analyses were initially done based on previously published methods (Li et al. 2007; Rogers et al. 2003). Additional optimisation was done as required to improve T-RFLP resolution of the multi-template control, as indicated below. All experimental manipulations were done in a light-reduced environment to prevent photobleaching, as described in Chapter 2.16. All PCR analyses were prepared as described in Chapter 2.15 to minimise 16S rRNA gene contamination.

5.3.6.1 Negative controls

Multiple negative controls were used in all PCR analyses to monitor for 16S rRNA gene contamination. All PCR analyses included a no template control consisting solely of the PCR mix. Additional negative controls using sterile water as template were included after every 10th tube. Following PCR, negative controls were analysed by agarose gel electrophoresis (Chapter 2.12). If no product was observed, the negative controls were pooled and included as a single sample in all subsequent
analyses. Where contamination was detected in negative controls all reagents were discarded and the reactions repeated with new reagents.

An additional negative control was included to exclude non-specific amplification of human DNA (Frank et al. 2003). This control contained 10ng/µL Taqman® Control Human Genomic DNA (Applied Biosystems).

### 5.3.6.2 PCR conditions

PCRs were done in 0.2mL tubes (Axygen) in a CG-1 96 thermocycler (Corbett Research). Unless otherwise specified, PCRs were done using 1X QIAGEN Taq buffer (QIAGEN); 0.1mM dNTPs (Roche); 0.2µM forward primer; 0.2µM reverse primer; and 0.5mg/mL molecular-grade bovine serum albumin (Roche); 1U Taq DNA polymerase (QIAGEN); and 25ng DNA template. Reactions were done at a final volume of 50µL. Unless otherwise specified, PCR cycling conditions were 95°C for 3 min followed by 30 cycles of 95°C for 30 s; 53°C for 30 s; and 72°C 60 s. A final extension was done at 72°C for 7 min and reactions were then held at 4°C. Two to four reactions were done per sample to provide sufficient PCR amplicons for subsequent restriction digestion and to minimise formation of PCR artefacts (Acinas et al. 2005). Replicates were pooled prior to agarose gel electrophoresis.

Optimisation of the PCR was also done as required to improve T-RFLP resolution of the multi-template control. PCR parameters considered in optimisation analyses were: DNA template concentration (2.5-200ng); annealing temperature (50°C, 53°C and 56°C); number of cycles (20, 25 and 30); and inclusion of the PCR additives 20mM tetramethylammonium chloride (Sigma®); 5% DMSO (Sigma®) and 0.02% Triton X-100 (Sigma®) (Dethlefsen et al. 2008).

### 5.3.7 Restriction digestion

Restriction digestions of 100ng of cleaned PCR products were done in 0.2mL tubes and were incubated in a CG-1 96 thermocycler. All digestions were prepared to a final volume of 50µL with sterile water. Samples were stored at 4°C following restriction digestion. Unless otherwise specified, the experimental conditions for each restriction digest were as given below. Optimisation of the restriction digest was also done where required to improve T-RFLP resolution of the multi-template control. Experimental parameters optimised were the mass of PCR products digested.
(100-500ng), the number of enzymatic units used (1-5U) and the incubation time (3-6 hrs).

**5.3.7.1 CfoI**

*CfoI* was sourced from Sigma®. *CfoI* digestions were done with 1X *CfoI* buffer (Sigma®) and 1U of restriction enzyme. Digests were incubated at 37°C for 3 hrs then heat inactivated by incubation at 65°C for 20 min.

**5.3.7.2 NlaIV**

*NlaIV* was sourced from New England Biolabs. *NlaIV* digestions were done with 1X NEBL buffer B (New England Biolabs); 1U of restriction enzyme; and 1X bovine serum albumin (New England Biolabs; as supplied with the enzyme). Digests were incubated at 37°C for 3 hrs then heat inactivated by incubation at 65°C for 20 min.

**5.3.7.3 BaeGI**

*BaeGI* was sourced from New England Biolabs. *BaeGI* digestions were done with 1X NEBL buffer A (New England Biolabs) and 1U of restriction enzyme. Digests were incubated at 37°C for 3 hrs then heat inactivated by incubation at 65°C for 20 min.

**5.3.8 Nucleic acid purification**

Residual salts and primers were removed from PCR amplicons and restriction digests using QIAquick PCR Purification and QIAquick Nucleotide Removal kits, respectively, as described in Chapter 2.13 and 2.14.

**5.3.9 Fragment analysis**

Fragment analysis was done by capillary-electrophoresis in an ABI3130XL GeneAnalyzer (Applied Biosystems) located at the Bioscience North Australia facility. Fragment sizing was done with a GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems). The size standard was prepared by adding 2µL of the standard to 170µl of Hi-Di™ Formamide (Applied Biosystems) for every 16 samples to be analysed. 10µL was then added to the required number of wells in a 96-well plate. 1.2µL of sample, containing 1-3ng DNA, was then added. The plate was briefly centrifuged to remove air bubbles; incubated at 95°C for 3 min; then put on ice for ~3 min before being loaded into the GeneAnalyzer. Fragment analysis was then
performed using the standard Bioscience North Australia protocol. Sample injection was done at 1.6kV for 15 s. The run time was 4000 s and the run voltage was 12kV. All fragment analyses were done in duplicate or triplicate.

5.3.10 Preparation of T-RFLP raw data

T-RFLP electropherograms were prepared using GeneMapper software (Applied Biosystems, version 4.0). Sizing was done using the Local Southern size-calling method. Fragment sizing was performed between 40-1040-bp as sizing of fragments outside this range was unreliable. Unless otherwise specified, the peak detection threshold was 25FU. The binning window was 0.5-bp, consistent with the observed capillary sizing error (0.4±0.01 bases) and previous T-RFLP analyses (Dunbar et al. 2001). Data from electropherograms was tabulated in GeneMapper and exported as a .csv file for downstream analyses. Binning of replicate T-RFLP profiles was performed according to the method of Dunbar et al. (Dunbar et al. 2001). Further alignment of the replicate profiles was not required as the multi-template control profile contained a defined bacterial community and inter-specimen comparative analyses were not required.

5.3.11 T-RFLP signal:noise analysis

Four previously published algorithms for distinguishing signal and noise in T-RFLP electropherograms were compared using data obtained from the multi-template control. The four algorithms compared were the constant baseline threshold method (Dunbar et al. 2001); the constant percentage threshold method (Sait et al. 2003); the variable percentage threshold method (Osborne et al. 2006); and a statistical method available as the freeware program T-REX (Culman et al. 2009). The number of peaks detected by each method was then tabulated and compared to the expected number of true peaks as determined by in silico analysis and experimental testing of each species included in the multi-template control. With the exception of T-REX analyses, signal:noise algorithms were performed using a combination of Stata/IC (StataCorp LP, version 11.2 for Windows) and Microsoft® Office Access and Excel (Microsoft®, 2003).
5.4 Results

The T-RFLP method of Rogers et al. (Rogers et al. 2003) was reviewed and optimised for application to upper respiratory specimens from patients with otitis media. The study was performed in four parts using a combination of *in silico* analyses and experimental validations as described below:

i) *In silico* analyses to determine optimal T-RFLP restriction enzymes for application to upper respiratory specimens (5.4.1).

ii) T-RFLP analysis of individual species for confirmation of *in silico* predicted fragments (5.4.2).

iii) T-RFLP validation and optimisation using the multi-template control (5.4.3 and 5.4.5).

iv) Comparison of T-RFLP signal:noise algorithms (5.4.4).

5.4.1 *In silico* analysis of optimal T-RFLP restriction enzymes

5.4.1.1 *Does CfoI differentiate S. pneumoniae and H. influenzae from closely-related commensal species?*

The T-RFLP method published by Rogers et al. (Rogers et al. 2003) used the restriction enzyme *CfoI* but did not describe the ability of this enzyme to differentiate *S. pneumoniae* and *H. influenzae* from closely-related commensal species which may also colonise the upper respiratory tract. A REPK analysis of the *S. pneumoniae* and *H. influenzae* reference sets was performed using sequences trimmed to *E. coli* positions 8-926, consistent with the primers used by Rogers et al. (Rogers et al. 2003). As shown in Table 5.5, the REPK analysis found *CfoI* did not differentiate the *S. pneumoniae* reference set, and would not differentiate *H. influenzae* from 4 of the 5 closely-related species that were tested.
Table 5.5: Predicted T-RFLP fragments expected from CfoI digest of *S. pneumoniae*, *H. influenzae* and closely-related commensal species.
Predicted fragment sizes were determined by REPK analysis of reference sequences trimmed to *E. coli* positions 8-926. The *H. parahaemolyticus* sequence lacked a CfoI restriction site within this region (Uncut).

<table>
<thead>
<tr>
<th>Species</th>
<th>Predicted CfoI fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>579</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>579</td>
</tr>
<tr>
<td><em>S. pseudopneumoniae</em></td>
<td>579</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>364</td>
</tr>
<tr>
<td><em>H. haemolyticus</em></td>
<td>364</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>364</td>
</tr>
<tr>
<td><em>H. parahaemolyticus</em></td>
<td>Uncut</td>
</tr>
<tr>
<td><em>H. aphrophilus</em></td>
<td>364</td>
</tr>
<tr>
<td><em>H. segnis</em></td>
<td>364</td>
</tr>
</tbody>
</table>

### 5.4.1.2 Identifying restriction enzymes to differentiate *S. pneumoniae* from closely-related commensal species

A further REPK analysis was done to determine if other restriction enzymes could differentiate *S. pneumoniae* from closely-related commensal species. The analysis was done using the *S. pneumoniae* reference set. The reference set was trimmed to *E. coli* positions 8-1400 to provide data across the entire 16S rRNA gene. The REPK analysis predicted that the restriction enzymes *BaeGI*, *Bsp1286I* and *Hpy8I* would differentiate *S. pneumoniae* from *S. mitis* and *S. pseudopneumoniae* (Table 5.6).

A further REPK analysis was done to determine if the identified enzymes would also differentiate *S. pneumoniae* from other potential upper respiratory flora. This analysis was done using the general reference set trimmed to *E. coli* positions 8-1400. Best resolution was obtained with *BaeGI* which was predicted to generate no additional fragments within 10-20 bases of *S. pneumoniae*.

An ISPaR virtual T-RFLP digest was then done to provide data from a larger number of *S. pneumoniae* and *S. mitis* sequences. The ISPaR analysis was done using *BaeGI* and the eubacterial primers 8F[FAM] and 1113R against the SILVA RefSSU 16S
rRNA database. The reverse primer 1113R, was used instead of 926R, to provide sufficient sequence length for \textit{BaeGI} differentiation of \textit{S. pneumoniae}. This analysis identified 46 \textit{S. pneumoniae} sequences and 16 \textit{S. mitis} sequences which were all differentiated by \textit{BaeGI}, as predicted by REPK. Thus, \textit{BaeGI} was chosen for inclusion in the T-RFLP.

Table 5.6: Restriction enzymes predicted to differentiate \textit{S. pneumoniae} from \textit{S. pseudopneumoniae} and \textit{S. mitis}.
Predicted fragment sizes as determined by REPK analysis of the \textit{S. pneumoniae} reference set trimmed to \textit{E. coli} positions 8-1400. The table shows the fragment size predicted to be generated by each enzyme.

<table>
<thead>
<tr>
<th></th>
<th>\textit{BaeGI} (bp)</th>
<th>\textit{Bsp1286I} (bp)</th>
<th>\textit{Hpy8I} (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. pneumoniae}</td>
<td>208</td>
<td>208</td>
<td>206</td>
</tr>
<tr>
<td>\textit{S. pseudopneumoniae}</td>
<td>929</td>
<td>929</td>
<td>470</td>
</tr>
<tr>
<td>\textit{S. mitis}</td>
<td>929</td>
<td>929</td>
<td>470</td>
</tr>
</tbody>
</table>

5.4.1.3 Identifying restriction enzymes to differentiate \textit{H. influenzae} from closely-related commensals
REPK analysis was also performed to identify restriction enzymes which could differentiate \textit{H. influenzae} from closely-related commensal species. The analysis was done using the \textit{H. influenzae} reference set trimmed to \textit{E. coli} positions 8-1400. This analysis failed to identify a restriction enzyme that would differentiate \textit{H. influenzae} from all other \textit{Haemophilus} species in the reference set. Best resolution was achieved with \textit{NlaIV} and \textit{MaeIII} (Table 5.7).

These enzymes were then tested against the general reference set to understand their capacity to differentiate \textit{H. influenzae} from other potential upper respiratory flora. Good resolution was achieved with \textit{NlaIV}. Although this enzyme would not differentiate \textit{H. influenzae} from \textit{H. parahaemolyticus}, fragments from all other tested species were predicted to be at least 10-70-bp different. An ISPaR virtual T-RFLP digest using \textit{NlaIV} and the eubacterial primers 8F[FAM] and 1113R against the SILVA RefSSU 16S rRNA database gave the expected fragment sizes for 355 of 362 \textit{H. influenzae} and 3 of 3 \textit{H. parainfluenzae} sequences.
In contrast, poor resolution was obtained with MaeIII. REPK analysis predicted 79 of 89 species in the general reference set would generate fragments between 55-bp and 117-bp. This included a theoretically continual peak representing 53 species between 99-bp and 112-bp. Additionally, an ISPaR virtual digest with MaeIII using primers 8F[FAM] and 1113R against the SILVA RefSSU 16S rRNA database found 75% of 65,000 predicted fragments occurred between 55-bp and 120-bp.

Based on these data, NlaIV was selected for inclusion in the T-RFLP to achieve highest possible resolution of H. influenzae from commensal Haemophilus species. MaeIII was considered unsuitable due to its low-level of resolution, and consequently the T-RFLP is not expected to differentiate H. influenzae from H. parahaemolyticus.

Thus, further T-RFLP in this study was performed with CfoI to maintain consistency with the method of Rogers et al. (Rogers et al. 2003), and with BaeGI and NlaIV to improve resolution of the major otitis media pathogens S. pneumoniae and H. influenzae.

Table 5.7: Restriction enzymes achieving best differentiation of the Haemophilus species.
Predicted fragment sizes as determined by REPK analysis of the H. influenzae reference set trimmed to E. coli positions 8-1400.

<table>
<thead>
<tr>
<th></th>
<th>NlaIV fragment (bp)</th>
<th>MaeIII fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae</td>
<td>375</td>
<td>639</td>
</tr>
<tr>
<td>H. haemolyticus</td>
<td>197</td>
<td>639</td>
</tr>
<tr>
<td>H. parainfluenza</td>
<td>197</td>
<td>61</td>
</tr>
<tr>
<td>H. parahaemolyticus</td>
<td>375</td>
<td>87</td>
</tr>
<tr>
<td>H. aphrophilus</td>
<td>197</td>
<td>61</td>
</tr>
<tr>
<td>H. segnis</td>
<td>197</td>
<td>61</td>
</tr>
</tbody>
</table>
5.4.1.4 In silico analysis to predict overall T-RFLP bacterial community resolution

An ISPaR virtual digest was then performed to estimate the overall capacity of each restriction enzyme to resolve mixed bacterial communities. The ISPaR analysis was done using each restriction enzyme with primers 8F[FAM] and 1492R against the GreenGenes database. Primer 1492R was selected to determine if longer sequences would enable better T-RFLP resolution. Two ISPaR analyses were done - i) with no primer mismatches permitted, and ii) with up to three mismatches permitted within 15 bases of the 5’ end of each primer (Table 5.8).

The number of fragments resolved by each restriction enzyme was comparable in both analyses with 573-611 fragments predicted when no primer mismatches were allowed, and 640-691 fragments predicted when up to three mismatches were permitted. Although the highest number of fragments in both analyses were predicted for BaeGI, almost half of the fragments predicted for this enzyme were >900-bp long and ~15% were >1200-bp long. As capillary-based fragment sizing can be unreliable beyond 900-bp (Kim et al. 2004), this may limit the usefulness of this enzyme.

CfoI and NlaIV produced comparable levels of resolution. 96-99% of fragments resolved by CfoI and NlaIV were <1200-bp in length, supporting use of PCR primers amplifying no more than 1200-bp of the gene. Therefore, it was decided to proceed with the T-RFLP using the reverse primer 1113R as it would achieve a good balance between amplicon size and the expected level of bacterial community resolution, while also enabling BaeGI differentiation of S. pneumoniae from closely-related commensal species.
Table 5.8: 16S rRNA gene T-RFLP fragments as predicted by ISPaR virtual digest using primers 8F[FAM] and 1492R with restriction enzymes *CfoI*, *NlaIV* and *BaeGI* and the GreenGenes database (October 2008).

a) Fragments predicted when no mismatches between primers and target sequences were permitted.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Number of sequences analysed</th>
<th>Total number of fragments predicted</th>
<th>Number of fragments &lt;900-bp (%)</th>
<th>Number of fragments &lt;1200-bp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CfoI</em></td>
<td>11687</td>
<td>586</td>
<td>483 (82)</td>
<td>578 (98)</td>
</tr>
<tr>
<td><em>NlaIV</em></td>
<td>11687</td>
<td>573</td>
<td>518 (90)</td>
<td>568 (99)</td>
</tr>
<tr>
<td><em>BaeGI</em></td>
<td>11687</td>
<td>611</td>
<td>341 (55)</td>
<td>530 (86)</td>
</tr>
</tbody>
</table>

b) Fragments predicted when up to three mismatches between primers and target sequences were permitted within 15 bases of the 5’ end of each primer.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Number of sequences analysed</th>
<th>Total number of fragments predicted</th>
<th>Number of fragments &lt;900-bp (%)</th>
<th>Number of fragments &lt;1200-bp (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CfoI</em></td>
<td>15506</td>
<td>654</td>
<td>533 (81)</td>
<td>642 (98)</td>
</tr>
<tr>
<td><em>NlaIV</em></td>
<td>15506</td>
<td>640</td>
<td>556 (87)</td>
<td>615 (96)</td>
</tr>
<tr>
<td><em>BaeGI</em></td>
<td>15506</td>
<td>691</td>
<td>402 (58)</td>
<td>602 (87)</td>
</tr>
</tbody>
</table>
5.4.2 T-RFLP of individual bacterial species

T-RFLP analysis of individual bacterial species was then done to confirm that fragments predicted in silico would also be obtained experimentally. The analysis was done for each of the 13 species to be included in the multi-template control. Data from the REPK analysis of the general reference set was used to determine the predicted T-RFLP fragment size for each species. Genomic DNA from each species was then tested by T-RFLP using primers 8F[FAM] and 1113R and each of the three restriction enzymes.

The experimental results largely confirmed the in silico findings (Table 5.9). Overall, experimentally-derived fragments were within 2-7 bases of the in silico predicted fragment size when the Local Southern size-calling method was used. Alternative sizing algorithms were tried, but did not improve correlation between predicted and actual fragment sizes. The observed variation most likely reflects electrophoretic drift inherent to capillary electrophoresis (Schutte et al. 2008; Stres, 2006). No amplification or T-RFLP fragments were detected in the no template control or human DNA control.

The experimentally-derived results also confirmed that BaeGI would differentiate S. pneumoniae from S. mitis, and that NlaIV would differentiate H. influenzae from H. parainfluenzae. This differentiation was not possible with CfoI alone (Table 5.9). The analysis also detected three instances of multiple peaks from individual species, which were not predicted by the in silico analysis (Table 5.9). The T-RFLP produced two peaks for M. catarrhalis and Klebsiella pneumoniae when digested with CfoI, and two peaks for E. coli when digested with BaeGI. Repeat testing of these species using a second genomic DNA preparation generated from a single, well-isolated, pure colony gave the same results.
Table 5.9: T-RFLP of the 13 species to be included in the multi-template control.
This table shows T-RFs for each species based on \textit{in silico} analyses (predicted) and experimental data (actual). T-RFLP was done using primers 8F[FAM] and 1113R with restriction enzymes \textit{CfoI}, \textit{NlaIV} and \textit{BaeGI}.

<table>
<thead>
<tr>
<th>Species</th>
<th>\textit{CfoI}</th>
<th>\textit{NlaIV}</th>
<th>\textit{BaeGI}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>Actual</td>
<td>Predicted</td>
</tr>
<tr>
<td>\textit{A. otitidis}</td>
<td>238</td>
<td>234</td>
<td>483</td>
</tr>
<tr>
<td>\textit{B. fragilis}</td>
<td>102</td>
<td>97</td>
<td>304</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>373</td>
<td>369</td>
<td>197</td>
</tr>
<tr>
<td>Group A Streptococcus</td>
<td>581</td>
<td>575</td>
<td>542</td>
</tr>
<tr>
<td>\textit{H. influenzae}</td>
<td>364</td>
<td>361</td>
<td>375</td>
</tr>
<tr>
<td>\textit{H. parainfluenzae}</td>
<td>364</td>
<td>361</td>
<td>197</td>
</tr>
<tr>
<td>\textit{K. pneumoniae}</td>
<td>371</td>
<td>366 and 560</td>
<td>195</td>
</tr>
<tr>
<td>\textit{M. catarrhalis}</td>
<td>558</td>
<td>555 and 557</td>
<td>909</td>
</tr>
<tr>
<td>\textit{P. mirabilis}</td>
<td>367</td>
<td>370</td>
<td>722</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>154</td>
<td>151</td>
<td>824</td>
</tr>
<tr>
<td>\textit{S. mitis}</td>
<td>579</td>
<td>574</td>
<td>540</td>
</tr>
<tr>
<td>\textit{S. pneumoniae}</td>
<td>579</td>
<td>574</td>
<td>540</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>238</td>
<td>234</td>
<td>232</td>
</tr>
</tbody>
</table>
This phenomenon was further explored by testing an additional nine *M. catarrhalis* isolates. The T-RFLP identified two *M. catarrhalis* populations – the first population (6/9 isolates) consistently gave two peaks when digested by *CfoI*; the second population (3/9 isolates) was not cut by *CfoI*. These findings suggest detection of multiple peaks in some species reflects heterogeneity between 16S rRNA gene copies, as discussed further below.

### 5.4.3 T-RFLP of the multi-template control

The multi-template control was prepared such that it contained equivalent concentrations of genomic DNA from each of the 13 species described in Table 5.9. T-RFLP of the multi-template control was done to confirm that results obtained when bacteria were tested individually would also be obtained from polymicrobial specimens. Initial analyses were limited to *CfoI* to achieve “proof-of-principle” before extending testing to the additional enzymes.

Figure 5.2 shows a representative multi-template control T-RFLP electropherogram as generated by primers 8F[FAM] and 1113R with a *CfoI* digest. Comparison of the multi-template control profile to a manually-prepared ladder describing the expected profile shows that some expected peaks were not detected. One example is *P. aeruginosa*, an important chronic suppurative otitis media and lower airway pathogen, which was absent from the multi-template control profile despite reacting well when tested individually. Additionally, the peaks detected in the multi-template control profile were not evenly represented. As the degree of unevenness was not accounted for by differences in 16S ribosomal operon number, this suggests a PCR amplification bias is present which may affect T-RFLP profiling of clinical specimens.

The multi-template control profile also contained several small peaks, including some unexpected peaks, and thus, a signal:noise algorithm was required to differentiate true peaks from noise. As there are few data describing optimal signal:noise algorithms, replicate profiles of the multi-template control were used to compare methods. This was done prior to any further assay optimisation as accurate signal:noise differentiation is critical to T-RFLP profiling.
Figure 5.2: T-RFLP of the multi-template control as generated by primers 8F[FAM] and 1113R with a CfoI digest. A manually-prepared ladder based on results obtained when bacteria were tested individually is provided for comparison. Peak positions consistent with expected fragments are numbered as follows: 1 = *B. fragilis*; 2 = *P. aeruginosa*; 3 = *A. otitidis* and *S. aureus*; 4 = *H. influenzae* and *H. parainfluenzae*; 5 = *K. pneumoniae* (peak 1); 6 = *E. coli*; 7 = *P. mirabilis*; 8 and 9 = *M. catarrhalis*; 10 = *K. pneumoniae* (peak 2); 11 = *S. pneumoniae* and *S. mitis*; 12 = Group A Streptococcus.
5.4.4 Comparison of T-RFLP signal:noise algorithms

Four published signal:noise algorithms were compared using data from 22 replicate profiles of the multi-template control generated using primers 8F[FAM] and 1113R with CfoI digestion. Each signal:noise algorithm was applied and the number of fragments identified as true peaks were counted. As 8/12 expected peaks were evident in the multi-template control raw data, an optimal signal:noise algorithm was expected to identify eight true peaks per profile.

Signal from eight peaks was identified in 22/22 replicates using the constant baseline threshold method (Dunbar et al. 2001) and 19/22 replicates using the constant percentage threshold method (Sait et al. 2003). The variable percentage threshold method (Osborne et al. 2006) overestimated the peak number in 21/22 replicates with a median of seven additional peaks detected - almost doubling the estimate of bacterial richness. Analysis with the statistically-based T-REX freeware (Culman et al. 2009) was limited to 13 replicates as high background fluorescence in nine profiles prevented data upload. T-REX of the 13 uploaded profiles overestimated the peak number with a minimum of 19 and maximum of 48 peaks reported, equivalent to an approximately 2 to 6 fold overestimation of bacterial richness. As best results were obtained with the constant baseline threshold method (Dunbar et al. 2001), this method was used for all subsequent analyses.

5.4.5 T-RFLP optimisation to improve characterisation of the multi-template control

Following selection of a signal:noise algorithm, the T-RFLP experimental parameters were reviewed to determine if alternative conditions would improve T-RFLP profiling of the multi-template control. At the initial PCR stage, template concentration (2.5-200ng/µL); annealing temperature (50°C, 53°C, 56°C); number of PCR cycles (20, 25 and 30) and inclusion of PCR additives (20mM tetramethylammonium chloride; 5% DMSO and 0.02% Triton X-100 (Dethlefsen et al. 2008)) were all investigated. Additionally, the mass of PCR amplicons digested (100-500ng); the number of enzymatic units used for the restriction digestion (1-5U); the duration of the digestion (3-6 hrs); and the amount of digested product loaded onto the capillary (1-3ng) were also investigated. Modification of any of these...
parameters, either individually or in combination, did not improve T-RFLP characterisation of the multi-template control. Regardless of the reaction parameters used, peaks from some species were consistently not detected by the T-RFLP (Figure 5.3). For example, the peak consistent with *P. aeruginosa* was absent from all profiles generated with this T-RFLP (peak 2 in Figure 5.3). Although small peaks consistent with some expected fragments were evident in the T-RFLP raw data (for example, *B. fragilis* fragment in Figure 5.3c), these peaks did not pass signal:noise thresholds, and thus were considered not detected.

A selection of alternative reverse primers were then tested to determine if primer-specificity was adversely affecting profiling of the multi-template control. Two additional reverse primers were tested – 926R consistent with the method of Rogers et al. (Rogers et al. 2003); and 1512R which has been widely used in environmental T-RFLP analyses (Li et al. 2007).

As shown in Figure 5.4, these alternative reverse primers affected which expected peaks were detected and the profile evenness. For example, the peak consistent with *B. fragilis* (peak 1 in Figure 5.4) was not detected when reverse primer 1113R was used, but was present in profiles generated with other reverse primers. Additionally, the evenness of this peak varied dependent on the reverse primer. For example, the *B. fragilis* peak was present as 11% of the total signal in the profile generated with reverse primer 926R but only 4% in profiles generated with 1512R. Similarly, the peak consistent with *Haemophilus* species was more abundant in profiles generated with reverse primers 1113R and 1512R (40% and 32% of the total signal, respectively) than in the profile generated with 926R (4% of the total signal). None of the tested primer combinations resulted in detection of all expected peaks in the multi-template control profiles.
Figure 5.3: T-RFLP electropherograms generated by primers 8F[FAM] and 1113R with CfoI digest.
Electropherograms are shown at the same scale without normalisation. Note: Similar profiles were obtained regardless of changes in the experimental parameters. Profile A shows the ladder indicating expected fragments and profiles B-F show experimentally derived profiles obtained with the following PCR reaction parameters:

B = 25ng DNA; PCR for 25 cycles; annealing temperature 53°C; no PCR additives.
C = 25ng DNA; PCR for 25 cycles; annealing temperature 50°C; no PCR additives.
D = 25ng DNA; PCR for 30 cycles; annealing temperature 53°C; no PCR additives.
E = 200ng DNA; PCR for 25 cycles; annealing temperature 53°C; no PCR additives.
F = 25ng DNA; PCR for 25 cycles; annealing temperature 53°C; with PCR additives.

Peak positions consistent with expected fragments are numbered as follows:
1 = B. fragilis
2 = P. aeruginosa
3 = A. otitidis and S. aureus;
4 = H. influenzae and H. parainfluenzae;
5 = K. pneumoniae (peak 1);
6 = E. coli;
7 = P. mirabilis;
8 / 9 = M. catarrhalis;
10 = K. pneumoniae (peak 2);
11 = S. pneumoniae and S. mitis;
12 = Group A Streptococcus.
Figure 5.4: T-RFLP electropherograms generated by primers 8F[FAM] and 1113R, 926R, 1512R and 1512R with PCR additives.

All profiles were generated with CfoI digest. Electropherograms are shown at the same scale without normalisation. Peaks 4 and 12 in electropherograms 1113R and 926R have been trimmed to accommodate this scale. The T-RFLP electropherograms are aligned against a manually prepared ladder indicating expected fragments.

Peak positions consistent with expected fragments are numbered as follows:

1 = B. fragilis
2 = P. aeruginosa
3 = A. otitidis and S. aureus;
4 = H. influenzae and H. parainfluenzae;
5 = K. pneumoniae (peak 1);
6 = E. coli;
7 = P. mirabilis;
8 / 9 = M. catarrhalis;
10 = K. pneumoniae (peak 2);
11 = S. pneumoniae and S. mitis;
12 = Group A Streptococcus.
The 16S rRNA gene also possesses a high-degree of secondary structure (Behrens et al. 2003; Brosius et al. 1978) which may adversely affect primer-binding. To minimise any potential effects of secondary structure on primer binding, T-RFLP of the multi-template control was repeated with 20mM tetramethylammonium chloride; 5% DMSO and 0.02% Triton X-100 (Dethlefsen et al. 2008) added to the PCRs. When these PCR additives were included all expected peaks were present in profiles generated with the reverse primer 1512R (Figure 5.4); however, unevenness of the profile remained. Inclusion of PCR additives did not alter profiles obtained with reverse primers 1113R and 926R. Collectively, these data indicate a PCR amplification bias which adversely affects T-RFLP profiling of the multi-template control.

Subsequent analyses in this study were done using the primers 8F[FAM] and 1512R. The PCR was done with the additives 20mM tetramethylammonium chloride, 5% DMSO and 0.02% Triton X-100; 2.5U Taq DNA polymerase; annealing temperature of 53ºC; and 25 cycles. Restriction digestion was done using 100ng PCR products, 1U of enzyme and 3 hrs incubation. Fragment analysis was performed using 1.2µL of cleaned restriction digests, and signal:noise and data normalisation was performed using the constant baseline threshold method.

5.4.6 Do the restriction enzymes NlaIV and BaeGI accurately profile the multi-template control?

T-RFLP with the restriction enzymes NlaIV and BaeGI was then performed using the optimised experimental parameters described above.

With the exception of E. coli, all expected peaks were detected in the NlaIV profile of the multi-template control (Table 5.10). The analysis confirmed NlaIV could differentiate H. influenzae from H. parainfluenzae in this polymicrobial specimen. Additionally, the profile also differentiated S. aureus and A. otitidis which are not resolved by CfoI. Two unexpected peaks were also present. These peaks occurred above the signal:noise threshold and thus are indicative of false-positive fragments in this profile.
Table 5.10: T-RFLP profiles of the multi-template control obtained by NlaIV restriction digestion of PCR products generated by primers 8F[FAM] and 1512R with PCR additives.

The table shows the expected fragment size for each species in the control (as determined by testing each species individually) and the corresponding fragments present in the multi-template control. A peak consistent with *E. coli* was not detected. Two unexpected peaks were also detected in the multi-template control (480-bp and 840-bp; not shown).

<table>
<thead>
<tr>
<th>Species</th>
<th>Expected fragment (bp)</th>
<th>Fragment in multi-template control (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em></td>
<td>190</td>
<td>190</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>192</td>
<td>-</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td>195</td>
<td>195</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>228</td>
<td>228</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>371</td>
<td>372</td>
</tr>
<tr>
<td><em>A. otitidis</em></td>
<td>477</td>
<td>478</td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>535</td>
<td>535</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>721</td>
<td>721</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>820</td>
<td>820</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>907</td>
<td>907</td>
</tr>
</tbody>
</table>
Table 5.11: T-RFLP profiles of the multi-template control obtained by *BaeGI* restriction digestion of PCR products generated by primers 8F[FAM] and 1512R with PCR additives.
The table shows the expected fragment size for each species in the control (as determined by testing each species individually) and the corresponding fragments present in the multi-template control.

<table>
<thead>
<tr>
<th>Species</th>
<th>Expected fragment (bp)</th>
<th>Fragment in multi-template control (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. pneumoniae</em></td>
<td>204</td>
<td>205</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>220</td>
<td>221</td>
</tr>
<tr>
<td><em>E. coli</em> (peak 1)</td>
<td>222</td>
<td>221</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>468</td>
<td>469</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>568</td>
<td>569</td>
</tr>
<tr>
<td><em>H. parainfluenzae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> (peak 2)</td>
<td>825</td>
<td>-</td>
</tr>
<tr>
<td><em>M. catarrhalis</em></td>
<td>911</td>
<td>913</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>914</td>
<td>913</td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>918</td>
<td>920</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>920</td>
<td>923</td>
</tr>
<tr>
<td><em>S. mitis</em></td>
<td>927</td>
<td>930</td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>928</td>
<td>930</td>
</tr>
<tr>
<td><em>A. otitidis</em></td>
<td>1106</td>
<td>1132</td>
</tr>
</tbody>
</table>
Similar results were obtained with *BaeGI* (Table 5.11). This analysis confirmed *BaeGI* could differentiate *S. pneumoniae* from *S. mitis* in this polymicrobial specimen. As with *NlaIV*, the *BaeGI* profile also differentiated *S. aureus* and *A. otitidis*. Two peaks were expected for *E. coli* in the *BaeGI* profile; however, only one peak consistent with *E. coli* was present (221-bp) and this peak may equally indicate *K. pneumoniae*. The absence of the second *E. coli* peak suggests this species has not been detected, as was also shown in the *NlaIV* profile. Five peaks in the *BaeGI* profile were >900-bp and therefore may not be sized accurately. Although a 913-bp peak was detected, it is unclear if this indicates detection of *M. catarrhalis*, *P. aeruginosa* or both species.

### 5.5 Discussion

T-RFLP has previously been used to characterise bacterial communities in respiratory specimens, but there are few data describing assessment of methodological biases. Validation of the method has been limited to assessment of freezing conditions on T-RFLP profiling (Kwambana et al. 2011). No studies of respiratory specimens, and few studies across the wider literature, have used well-defined controls to assess the strengths and limitations of T-RFLP.

In this study, a combination of *in silico* analyses and experimental validations with reference isolates were used to investigate the ability of T-RFLP to accurately profile bacterial communities potentially present in the upper respiratory tract. Several key findings emerged which affect the choice of experimental parameters and have implications for data analysis, as described below.

#### 5.5.1 Optimal restriction enzymes for T-RFLP of upper respiratory specimens

*In silico* analyses demonstrated that *CfoI* is a suitable enzyme for measuring overall bacterial richness, but does not differentiate the major otitis media pathogens *S. pneumoniae* and *H. influenzae* from closely-related upper respiratory commensal species. Despite this limitation, *CfoI* was included in the T-RFLP to maintain consistency with the previously published method of Rogers et al. (Rogers et al. 2003). Subsequent to this study, two additional respiratory T-RFLP methods were published which used the restriction enzymes *AluI* (Kwambana et al. 2011) and
Like CfoI, these restriction enzymes do not differentiate *S. pneumoniae* and *H. influenzae* from closely-related commensal species.

*BaeGI* and *NlaIV* were added to the T-RFLP to improve resolution of *S. pneumoniae* and *H. influenzae*. *In silico* and experimental data suggest that these enzymes provide a high-level of differentiation of these pathogens from closely-related commensals; however, it cannot be excluded that other closely-related species may generate identical fragments. This is an inherent limitation of T-RFLP (Osborne et al. 2006) which must be considered when assessing bacterial richness.

### 5.5.2 T-RFLP may detect multiple peaks from single species

Although not predicted *in silico*, three species gave two peaks in their T-RFLP profile. For *M. catarrhalis*, subsequent analyses with freshly extracted DNA obtained from well-isolated single colonies demonstrated that this was not due to specimen contamination due to multiple species being present. Contamination would also be expected if multiple peaks were observed regardless of the restriction enzyme used; however, in all instances multiple peaks were only demonstrated with one enzyme. For example, the *M. catarrhalis* reference isolate consistently gave two peaks when digested with *CfoI*, but gave a single peak when digested with *NlaIV* or *BaeGI*. The occurrence of multiple peaks was investigated further by testing a small collection of *M. catarrhalis* clinical isolates. Although this confirmed the occurrence of multiple peaks, it also identified an additional genotype which lacked a *CfoI* restriction site.

The reasons for multiple peak formation are unclear, but likely reflect heterogeneity between 16S rRNA gene copies within an individual bacterial strain (Pei et al. 2010). Multiple peaks may reflect formation of pseudo-T-RFs during restriction digestion (Liesack et al. 2004); however, this is unlikely to explain the results as multiple peaks were not generated by all restriction enzymes. Regardless of the cause, duplicate peaks from single species results in overestimation of bacterial richness based on peak number. Identification of two *M. catarrhalis* populations also emphasises the difficulty associated with achieving consistent T-RFLP profiling of this common otitis media pathogen. This is an important consideration in T-RFLP of upper respiratory specimens from Indigenous Australian children from the Northern
Territory as *M. catarrhalis* carriage rates of ~70% have been documented in this population (Mackenzie et al. 2010).

### 5.5.3 The multi-template control demonstrates the effect of primer choice on the T-RFLP measures of bacterial diversity

In this study, only T-RFLP done with primers 8F[FAM] and 1512R resulted in detection of all expected peaks in the multi-template control, and then, only when tetramethylammonium chloride, Triton X-100 and DMSO were added to the PCR. Despite extensive investigation of a range of other experimental conditions, several peaks were consistently absent when other primer pairs were used. Importantly, of all the primer pairs trialled, those previously used to study lower airway infections (Rogers et al. 2003; Tunney et al. 2011) detected the fewest expected peaks in the multi-template control when digested with *CfoI*. This primer pair did not detect *M. catarrhalis* or *P. aeruginosa*. This finding is in contrast to T-RFLP studies of lower airway specimens in which peaks consistent with *P. aeruginosa* have been detected (Rogers et al. 2009). The reasons for this are unclear but may reflect competitive PCR interactions due to variation in primer specificity and secondary structure of the 16S rRNA gene which prevent even amplification of all species. This effect has previously been described (Hong et al. 2009; Sipos et al. 2007) and is a general limitation of T-RFLP which restricts its use to broad characterisation of bacterial communities.

While this limitation has been accepted for studies of complex environmental communities, it has implications for clinical analyses. Culture-based studies have clearly demonstrated the importance of several bacterial pathogens in respiratory tract infections. The presence or absence of such pathogens is often an important consideration in clinical analyses of respiratory mucosal infections. For example, the presence or absence of *P. aeruginosa* in the lower airways of cystic fibrosis patients has important clinical and therapeutic implications (Rogers et al. 2011). PCR amplification biases identified in this study and by others (Hong et al. 2009; Sipos et al. 2007) limit the application of T-RFLP to broad characterisation of bacterial community richness. Caution must be applied when considering the presence or absence of peaks consistent with recognised pathogens in T-RFLP profiles. This does not diminish the usefulness of restriction enzymes which resolve closely-related
pathogenic and commensal species. On the contrary, all experimental conditions which improve resolution of bacterial richness should be included.

All T-RFLP profiles of the multi-template control also showed a degree of unevenness that could not be accounted for by differences in 16S ribosomal operon copy number. This likely reflects the PCR amplification bias as different primer combinations affected the evenness of specific T-RFs. This bias prevents assessment of the relative abundance of individual peaks in T-RFLP profiles (Frey et al. 2006).

5.5.4 Would better results be obtained with other primers?

The findings of this study need to be balanced against the limited number of primer pairs tested. This analysis was limited to investigation of a single forward primer to ensure T-RFs would be consistent with the method of Rogers et al. (Rogers et al. 2003). Clear differences were observed for each of the reverse primers tested with best results obtained with reverse primer 1512R when PCR additives were used. This variation from the method of Rogers et al. (Rogers et al. 2003) was accepted to improve the overall resolution of the T-RFLP. It is possible that better results may be obtained with alternative primer combinations, especially alternative forward primers, but this has not been investigated further in this study.

5.5.5 Is the control a suitable surrogate for clinical specimens?

Testing of the multi-template control has provided insight into important limitations of T-RFLP when applied to upper respiratory bacteria; however, the control is an artificial construct and results may not be generalisable to clinical specimens.

The multi-template control was prepared to contain equal amounts of genomic DNA from each of the 13 species. Such equivalence is unlikely to be present in clinical specimens which may be dominated by a single species. In such cases, dominant species may have a competitive advantage in PCR and thus be detected in the T-RFLP. This scenario is supported by the results from T-RFLP of individual species. Expected T-RFs were detected when all individual species were tested, including those that were difficult to detect in the multi-template control. Competitive PCR biases may also explain why *P. aeruginosa* was detected in clinical studies by Rogers et al. (Rogers et al. 2010a), but has been difficult to detect in the multi-
template control. Thus, while the control has been a useful tool in understanding the strengths and weaknesses of the method, testing of clinical specimens should now be done to provide a ‘real-world’ context. Comparison with culture results may also prove useful in determining if the optimised T-RFLP detects important pathogens in clinical specimens.

5.5.6 Optimal signal:noise algorithms
Testing of a well-defined multi-template control allowed objective investigation of signal:noise algorithms. In this study, data from 22 replicates of the multi-template control digested with \textit{CfoI} were used to test which method most accurately differentiated signal from noise. As the composition of the control was known, the expected number of peaks could be determined with certainty based on \textit{in silico} and experimentally-derived data. Of the algorithms tested, only the constant baseline threshold method (Dunbar et al. 2001) correctly identified the expected number of peaks in the T-RFLP profile of all replicates. The constant percentage method (Sait et al. 2003) also worked well but over-estimated richness in 2/22 replicates and underestimated richness in one replicate. The variable percentage threshold method (Osborne et al. 2006) and T-REX freeware (Culman et al. 2009) both overestimated bacterial richness by at least 2-fold in most replicates, emphasising the need to use well-defined specimens when comparing signal:noise algorithms. The constant baseline threshold method is recommended for T-RFLP of upper respiratory specimens.

5.5.7 Recommended use of multi-template control as an inter-run reference sample
Another strength of the multi-template control is that it can provide an objective measure of inter-run variability. It is also likely to provide a reference point for the constant baseline threshold signal:noise algorithm. Dunbar et al. (Dunbar et al. 2001) proposed the method be applied such that the signal from all T-RFLP profiles in each series of fragment analyses be normalised to that of the specimen giving the lowest signal. However, this approach may be complicated by results from outlier specimens providing substantially lower signal than other samples. The multi-
template control could provide a more objective reference point for signal normalisation and this should be considered when applying this algorithm.

5.5.8 Conclusions

The success of bacterial community profiling is dependent upon reproducibility at the sampling, testing and analysis levels. From the moment of specimen collection, biases may be accumulated. Regardless of the care taken by researchers, a certain amount of experimental error and bias are unavoidable. To achieve meaningful data, researchers must be acutely aware of methodological limitations and take care to minimise introduction of biases.

This study has shown that T-RFLP estimates of bacterial diversity are adversely affected by primer biases inherent to universal 16S rRNA gene amplification. While the method provided a profile of bacterial diversity when applied to the multi-template control, it required optimisation of primer choice and inclusion of PCR additives for all expected peaks to be detected. Further testing of clinical specimens is now required to determine if the optimised method provides equivalent or greater characterisation of bacterial communities in upper respiratory specimens compared with culture.
CHAPTER 6

T-RFLP of nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation
6.1 Summary

**Background:** In Chapter 5, conditions for T-RFLP analysis of upper respiratory specimens were optimised using a multi-template control consisting of 13 potential respiratory bacteria. Limitations of the method were identified, most importantly, a PCR amplification bias which adversely affected the bacterial community profiling. The control, however, is an artificial construct and it is unclear if the identified PCR biases will also adversely affect bacterial community profiling of clinical specimens.

**Aims:** The aims of the study were i) to determine whether the T-RFLP can be used to profile bacterial communities in paediatric upper respiratory specimens, ii) to profile bacterial communities in nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation, and iii) to determine whether fragments consistent with pathogenic species previously cultured from the swabs are present in the T-RFLP profiles.

**Methods:** T-RFLP analysis of matched nasopharyngeal and ear discharge swabs from 10 Indigenous children with acute otitis media with perforation was performed. All swabs had previously been characterised by culture and PCR.

**Results:** Insufficient DNA yield was obtained from nasopharyngeal and ear discharge swabs to permit T-RFLP with the 8F[FAM]-1512R PCR. This primer combination was replaced with 8F[FAM]-1113R PCR to achieve a balance between PCR yield and the measure of bacterial richness. Following this amendment, three swabs continued to generate insufficient PCR amplicons for T-RFLP analysis, and were excluded from further analysis.

T-RFLP of the remaining 17 swabs identified a high level of bacterial richness with 47 different fragments detected overall, and 2-12 fragments present in each swab. Non-metric multi-dimensional scaling and hierarchical group-average clustering
analysis showed site-specific clustering with T-RFLP profiles from ear discharge swabs showing higher similarity to other ear discharge specimens than to their matched nasopharyngeal swabs. This suggests that the nasopharyngeal and middle ear bacterial communities in these children are different. While the T-RFLP provided a broad description of bacterial communities in each swab, important otopathogens, including *S. pneumoniae*, were absent from profiles of some culture-positive swabs.

**Conclusions:** T-RFLP may be useful for estimating broad differences in bacterial communities; however, it may not detect important otopathogenic species. Careful consideration of primer selection is required, particularly for analysis of low biomass specimens. Analysis of ear discharge and nasopharyngeal swabs supports the hypothesis that microbiomic differences exist at these sites. More sensitive bacterial community analyses using a 16S rRNA gene microarray or deep sequencing are now required to better understand the microbiomic richness in middle ear specimens. Such studies may provide better understanding of bacterial dynamics in the pathogenesis of acute otitis media with perforation in Indigenous children.

**Acknowledgements:** Comparative statistical analyses were selected by myself but were performed by Dr Mirjam Kaestli.

**6.2 Introduction**

The ultimate aim of T-RFLP analyses is to reveal maximum bacterial diversity in polymicrobial specimens. In the preceding chapter, a T-RFLP method was developed for analysis of upper respiratory specimens from otitis media patients. The method was optimised using a well-defined multi-template control containing equivalent concentrations of genomic DNA from 13 respiratory bacteria. The analysis revealed a PCR primer bias which adversely affected T-RFLP profiling of the control. Only one of the tested primer combinations resulted in T-RFLP detection of all expected fragments from the multi-template control, and this only occurred when a cocktail of PCR additives were included.

As the multi-template control is an artificial construct, these results may not be generalisable to clinical specimens. For example, studies using T-RFLP with primers 8F[FAM] and 926R have reported detection of fragments consistent with *P.*
aeruginosa in sputum specimens obtained from cystic fibrosis patients (Rogers et al. 2010a); whereas the expected P. aeruginosa peak was consistently absent from T-RFLP profiles of the multi-template control when analysed with these primers, despite reacting well when tested individually. This suggests that the PCR biases identified with the control may be affected by bacterial community evenness. While the control is highly even in that it contains equivalent concentrations of all species, such evenness is unlikely to occur in biological specimens. Further testing is now required to determine if the T-RFLP optimised in the preceding chapter can be used to characterise upper respiratory bacterial communities, and to determine if the method will detect important otopathogens in clinical specimens.

Further testing is also required to determine if sufficient DNA can be obtained from upper respiratory swabs to enable T-RFLP analysis. Published respiratory T-RFLP studies have used 20-100ng of template DNA per PCR (Rogers et al. 2003; Rogers et al. 2009; Tunney et al. 2011). Such high template concentrations may be difficult to achieve with low volume, and potentially low biomass, paediatric upper respiratory swabs.

This chapter reports results of a pilot T-RFLP analysis of matched nasopharyngeal and ear discharge swabs from 10 Indigenous children with acute otitis media with perforation. The specific aims of the study were:

i) to determine whether the T-RFLP can be used to profile bacterial communities in paediatric upper respiratory specimens,

ii) to profile bacterial communities in nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation, and

iii) to determine whether fragments consistent with pathogenic species previously cultured from the swabs are present in the T-RFLP profiles.
6.3 Methods

6.3.1 Clinical specimens

6.3.1.1 Nasopharyngeal and ear discharge swabs
This study used matched nasopharyngeal and ear discharge swabs collected at enrolment of Indigenous children with acute otitis media into a randomised controlled trial (RCT) of amoxycillin or azithromycin (Morris et al. 2010), as described in Chapter 2.2. Matched nasopharyngeal and ear discharge swabs from 10 Indigenous children with acute otitis media with perforation were randomly selected using Research Randomizer (Urbaniak, 2011) (version 3.0; www.randomizer.org).

Metadata describing the swabs, including culture and qPCR data, are described in Tables 6.1 and 6.2. Culture data were derived from the original RCT, with the exception of A. otitidis data. A. otitidis culture and PCR data were derived from the data presented in Chapter 4. PCR-based detection of S. pneumoniae, H. influenzae, and M. catarrhalis was done using PCR assays described in Chapter 3. Total bacterial load was determined as described in Chapter 3.

6.3.1.2 Nasal discharge swabs used for methodological optimisation
A separate collection of seven nasal discharge swabs was used, where required, for methodological optimisation. This was done to avoid exhaustion of DNA from the study specimens. The nasal discharge swabs were sourced from a previously completed study comparing nose blowing and nasal swabs for detection of upper respiratory pneumococcal carriage (Leach et al. 2008c). Each swab contained nasal discharge which had been sampled after a child’s nose was blown into a tissue (Leach et al. 2008c). DNA had previously been extracted from 1mL of each nasal swab media using QIAtamp DNA extraction kits with enzymatic pre-treatment as described in Chapter 2.8.2. DNA concentration was determined using PicoGreen® reagent as described in Chapter 2.9.

6.3.2 Controls
Positive and negative controls were included through all stages of the T-RFLP. The multi-template control described in Chapter 5.3.4. was used a positive control. A specimen of sterile STGGB media (the media all swabs had been stored in) was used as a negative control. The STGGB negative control was included through all
experimental stages, including DNA extraction. Additional negative controls were included in PCR analyses. 1µL of sterile water was tested as a no template control. 10ng of commercially sourced human DNA (Taqman® Control Genomic DNA – Human; Applied Biosystems) was also tested as a negative control.

### 6.3.3 DNA extraction and quantification

Consistent with previous respiratory T-RFLP methods (Kwambana et al. 2011; Rogers et al. 2003; Tunney et al. 2011), DNA from nasopharyngeal and ear discharge swabs was extracted using a bead-beating protocol which physically disrupts bacterial cell walls. Bead-beating is routinely used to extract DNA for bacterial community profiling analyses as it can achieve higher DNA yield and improve diversity measurements (de Boer et al. 2010; Li et al. 2007; Rantakokko-Jalava et al. 2002).

For this study, DNA was extracted from 200µL of each nasopharyngeal and ear discharge swab using the bead-beating method described in Chapter 2.8.2.2. DNA was eluted with 50µL of Buffer AE to achieve maximum concentration. The eluted DNA was separated into two 20µL aliquots. One aliquot was used in this study, and the other was stored at -20ºC for subsequent microbiomic analyses. DNA was quantified using PicoGreen® reagent as described in Chapter 2.9.

### 6.3.4 T-RFLP

#### 6.3.4.1 General

All experimental manipulations were done in a light-reduced environment to prevent photobleaching, as described in Chapter 2.16. All PCR analyses were prepared as described in Chapter 2.15 to minimise 16S rRNA gene contamination.
Table 6.1: Metadata describing characteristics of nasopharyngeal swabs used Chapter 6.
“Other” indicates instances where other bacterial colonies were detected but not identified.

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<thead>
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<th>Patient identifier</th>
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<th>30155</th>
<th>30162</th>
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<td>2.2</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><em>H. influenzae</em></td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>-</td>
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</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td><em>P. aeruginosa</em></td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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</table>
Table 6.2: Metadata describing characteristics of ear discharge swabs used in Chapter 6.
Culture data for *M. catarrhalis* and *P. aeruginosa* in some swabs could not be determined due to overgrowth by *Proteus* species (indicated by n/a).

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<tr>
<td><em>P. aeruginosa</em></td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>Proteus</em> sp.</td>
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<td>+</td>
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<tr>
<td>β-haemolytic streptococci</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Other</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>DNA yield (ng/µL)</td>
<td>13.6</td>
<td>5.2</td>
<td>10.6</td>
<td>20.6</td>
<td>0</td>
<td>1.2</td>
<td>16.9</td>
<td>5.4</td>
<td>1.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Total bacterial load (cells/swab)</td>
<td>2.59x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.73x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.80x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>5.96x10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>9.44x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.55x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.86x10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>6.48x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>2.55x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.49x10&lt;sup&gt;8&lt;/sup&gt;</td>
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<tr>
<td>Pathogen detection by PCR</td>
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<tr>
<td><em>S. pneumoniae</em></td>
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<td>+</td>
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<td>+</td>
<td>-</td>
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<tr>
<td><em>M. catarrhalis</em></td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td><em>A. otitidis</em></td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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</table>
6.3.4.2 16S rRNA gene PCR

Initial PCR amplification of the 16S rRNA gene was done using fluorescently-labelled forward primer 8F[FAM] and reverse primer 1512R (Table 6.3). Where required, additional PCR analyses were performed with reverse primers 926R and 1113R (Table 6.3).

Each 50µL PCR contained 1X QIAGEN Taq buffer; 0.1mM dNTPs, 0.2µM of each primer; 2.5U Taq DNA polymerase; and 4µL template DNA. Amplifications with reverse primer 1512R also included the PCR additives 5% DMSO, 0.02% Triton-X 100, and 20mM tetramethylammonium chloride, as described in Chapter 5. The cycling conditions were 95ºC for 3 min followed by 25 cycles of 95ºC for 30 s, 53ºC for 30 s and 72ºC for 2 min; then a final extension at 72ºC for 7 min. Completed reactions were held at 4ºC. Agarose gel electrophoresis was done as described in Chapter 2.12 to confirm amplification of the expected products.

To maximise PCR yield and minimise random amplification errors (Stres, 2006), four replicate PCRs were prepared for each specimen. Replicates were pooled prior to agarose gel electrophoresis.

6.3.4.3 Purification of PCR products

Pooled PCR products from each swab were purified as described in Chapter 2.13. The PCR products were eluted with 30µL Buffer EB and visualised by agarose gel electrophoresis. The concentration of purified PCR products was determined using PicoGreen® reagent as described in Chapter 2.9.

6.3.4.4 Restriction digestion

Restriction digestion of purified PCR products was done with the enzymes CfoI, NlaIV and BaeGI. Each restriction digest contained 100ng of purified PCR products and 1U of the restriction enzyme. Digests were incubated at 37ºC for 3 hrs, then heat inactivated by incubation at 65ºC for 20 min. Completed digests were stored at -20ºC prior to purification and fragment analysis.
Table 6.3: Primers used in Chapter 6.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>926R</td>
<td>5’ CCGTCAATTCCCTTTRAGTTT 3’</td>
<td>Rogers, 2003 Schutte, 2008</td>
</tr>
<tr>
<td>1113R</td>
<td>5’ GGGTTGCAGCTGTGG 3’</td>
<td>Schutte, 2008</td>
</tr>
<tr>
<td>1512R</td>
<td>5’ACGGYTACCTTGTACGACTT 3’</td>
<td>Li, 2007</td>
</tr>
</tbody>
</table>

6.3.4.5 Purification of restriction digests
Restriction digests were purified as described in Chapter 2.14. The concentration of purified restriction digests was then measured using PicoGreen® reagent as described in Chapter 2.9.

6.3.4.6 Fragment analysis
Fragment analysis was done by capillary-electrophoresis in an ABI3130XL GeneAnalyzer as described in Chapter 5.3.9. Each specimen was analysed in triplicate. Raw data were prepared using GeneMapper software as described in Chapter 5.3.10. Profiles were compiled in the GeneMapper Genotype table and exported as .csv files for further analysis.

6.3.5 Data analysis
T-RFLP data analyses were essentially as described in Chapter 5; however, application to clinical specimens required further consideration of fragment binning (categorising fragments from different profiles into size-defined groups called “bins”) and alignment as described below.

The T-RFLP raw data were binned and normalised using the multi-step method of Dunbar et al. (Dunbar et al. 2001). Briefly, replicate profiles were first binned. The maximum permitted bin-width was 0.5-bp for fragments ≤900-bp (Dunbar et al. 2001; Holland et al. 2008). The maximum permitted bin-width for fragments >900-bp was increased to 1-bp as sizing of fragments in this range can be unreliable (Kim...
et al. 2004). The maximum number of fragments per bin was equal to the number of profiles analysed to ensure differentiation of reproducible fragments differing by less than 0.5-bp (Dunbar et al. 2001). The minimum fragment size in each bin defined the bin’s starting position. Fragments more than 0.5-bp larger than the bin’s minimum fragment size were placed into a new bin.

Replicate profiles from each swab were then normalised using the constant baseline threshold method (Dunbar et al. 2001) (intra-sample normalisation). A consensus profile was then generated for each swab by taking the average values of each bin in the normalised replicate profiles. Consensus profiles generated by each restriction enzyme were then compiled into individual datasets. Each dataset was then normalised using the constant baseline threshold method (Dunbar et al. 2001) (inter-sample normalisation). The normalised datasets were then binned and aligned as described above. The normalised and aligned profiles from all datasets were then combined to produce a matrix describing the peak area of each fragment in the normalised T-RFLP profile of each swab (Sessitsch et al. 2002).

Comparison of T-RFLP profiles between samples was done using the Bray-Curtis index for quantitative comparisons of fragment sizes and peak areas (Stres, 2006) and the Sørenson Index for binary analyses of fragment sizes only. Based on these resemblance matrices, non-metric multi-dimensional scaling (NMDS) ordination plots were created and hierarchical group-average clustering analysis (HCAN) was performed to show the relatedness of T-RFLP profiles (Stres, 2006). The analyses were done using PRIMER 6 software (PRIMER-E Ltd, version 6.1.9).

6.4 Results

6.4.1 DNA yield from upper respiratory specimens

The DNA yield from nasopharyngeal and ear discharge swabs ranged from 0-20.6ng/µL (median 1.9ng/µL). DNA extractions from one ear discharge and four nasopharyngeal swabs had DNA concentrations of 0ng/µL; however, all of these swabs had bacteria detected by the total bacterial load qPCR (total bacterial loads 6.43x10^5-9.44x10^7 cells/swab). Higher DNA yields were obtained from ear discharge swabs (range 0-20.6ng/µL; median 5.3ng/µL) than nasopharyngeal swabs (range 0-12.3ng/µL; median 0.9ng/µL).
Published respiratory T-RFLP studies have used 20-100ng of template DNA per PCR (Rogers et al. 2003; Rogers et al. 2009; Tunney et al. 2011). Only 7/20 swabs had sufficient DNA to achieve a minimum of 20ng in 4µL, and no swabs had sufficient DNA to achieve 100ng in 4µL.

6.4.2 Confirming T-RFLP can be performed on specimens with low DNA concentrations

As only 7/20 swabs met the minimum DNA requirements previously used for T-RFLP of respiratory specimens, two additional validation steps were performed prior to testing the nasopharyngeal and ear discharge swabs. Firstly, the multi-template control was used to determine the lower limit of detection of the 8F[FAM]-1512R PCR, and thus, the minimum amount of DNA required for the T-RFLP. A 1:10 serial dilution of the multi-template control showed that PCR products were obtained when at least 1ng DNA template was amplified (Figure 6.1).

As the multi-template control is an artificial construct and does not contain human DNA (which could theoretically add further amplification bias), a second validation was also done to confirm PCR amplification from clinical samples. An additional collection of nasal discharge swabs was used for this validation to avoid exhaustion of the nasopharyngeal and ear discharge specimens. The DNA concentration of the nasal discharge swabs (range 0-1.4ng/µL) was comparable to the lowest concentrations in the nasopharyngeal and ear discharge swabs. 1µL of DNA from each nasal discharge swab was tested with the 8F[FAM]-1512R PCR. Amplicons were detected from only 3/7 nasal discharge swabs and the bands were very faint (Figure 6.2a). Further, the PCR yield from specimens that did amplify was insufficient for the three T-RFLP restriction digests which each require at least 100ng of PCR products.

6.4.3 Primer choice affects yield from clinical specimens

1µL of DNA from each nasal discharge swab was then amplified with the forward primer 8F[FAM] and an alternative reverse primer (either 926R or 1113R) to determine if the PCR yield could be improved. As shown in Figure 6.2b and 6.2c, both additional primer combinations provided substantially higher yield than the 8F[FAM]-1512R PCR. These results suggest inefficient reaction kinetics in the
8F[FAM]-1512R PCR which prevent amplification from specimens with concentrations <2ng/µL. As 6/10 nasopharyngeal swabs and 4/10 ear discharge swabs had DNA concentrations <2ng/µL, the 8F[FAM]-1512R PCR was unsuitable for analysis of these specimens.

Figure 6.1: Sensitivity of the 8F[FAM]-1512R PCR as determined by serial dilution of the multi-template control.
Results show the amount of PCR amplicon obtained from the 8F[FAM]-1512R PCR when 10-0.01ng of the multi-template control DNA was tested in a 25µL reaction.

M = 2-log Ladder
N = No template control
1 = 10ng template
2 = 1ng template
3 = 0.1ng template
4 = 0.01ng template
Figure 6.2: PCR yield obtained from nasal discharge swabs tested with three primer combinations.
a) 8F[FAM]-1512R; b) 8F[FAM]-1113R; and c) 8F[FAM]-926R. The best PCR yield was obtained with reverse primer 1113R. Two nasal discharge swabs failed to amplify with any tested primer combination. Note: the division in these gels reflects removal of lanes unrelated to this study. M = 2-log Ladder. N = no template control. H = human DNA. C = Multi-template control. 1-7 = nasal discharge swabs.

a) PCR yield using primers 8F[FAM] and 1512R

b) PCR yield using primers 8F[FAM] and 1113R

c) PCR yield using primers 8F[FAM] and 926R
The PCR data were reviewed to select an alternative reverse primer which could be used for T-RFLP of the nasopharyngeal and ear discharge swabs. Reverse primer 1113R was chosen as it provided good PCR yield from low concentration DNA specimens. In addition, it detected more fragments in the multi-template control than 8F[FAM]-926R (as described in Chapter 5) and provided sufficient amplicon length to enable BaeGI differentiation of S. pneumoniae from closely-related commensal species.

6.4.4 T-RFLP of paired nasopharyngeal and ear discharge swabs
T-RFLP of the nasopharyngeal and ear discharge swabs was then done using the 8F[FAM]-1113R PCR. The T-RFLP was performed as described in Chapter 5.

6.4.4.1 16S rRNA gene amplification with 8F[FAM]-1113R PCR
The 8F[FAM]-1113R PCR yield from the nasopharyngeal and ear discharge swabs varied (Figure 6.3). No amplicons were obtained from 2/20 specimens (one nasopharyngeal swab and one ear discharge swab from different children) and these swabs were excluded from further analysis. One additional nasopharyngeal swab failed to generate sufficient amplicons for the restriction digestion (<300ng PCR product), and this specimen was also excluded from further analysis. Thus, paired nasopharyngeal and ear discharge swabs from seven children had sufficient PCR amplicons for T-RFLP profiling. The individual nasopharyngeal (n=1) or ear discharge swabs (n=2) from the remaining three children were also included in the T-RFLP analysis. No amplification was observed in negative controls or from human DNA (Figure 6.3).
Figure 6.3: Variable PCR yield obtained from nasopharyngeal and ear discharge swabs amplified with 8F[FAM]-1113R PCR. The gel shows amplicons present in 2μL of PCR products after QIAquick purification.

M = 2-log Marker  
N1 = No template control  
C = Multi-template control  
N = Nasopharyngeal swab  
E = Ear discharge swab  
1-10 = Indicating the 10 study children  
H = 10ng Human DNA  
N2 = STGGB negative control
6.4.4.2 Normalisation of T-RFLP profiles
The T-RFLP profiles were normalised and aligned according to the method of Dunbar et al. (Dunbar et al. 2001; Schutte et al. 2008) which incorporates the constant baseline threshold algorithm for distinguishing signal and noise. The constant baseline threshold algorithm involves an iterative process where each profile in a dataset is normalised to the specimen with the lowest total signal (calculated by summing the peak height of fragments in each profile). For profiles generated with BaeGI this was not possible as normalisation of the dataset to the specimen with the lowest sum peak height could not be resolved. Additionally, normalisation of this dataset reduced signal from the multi-template control such that expected fragments evident in the raw data were excluded by the signal:noise algorithm. Low signal profiles were sequentially excluded from the BaeGI dataset until the signal:noise algorithm identified all expected fragments in the multi-template control. This process identified BaeGI profiles from four swabs as having insufficient signal for accurate T-RFLP profiling. Each of these swabs had sum peak height values <1000FU (range 440-966FU), suggesting a minimum of 1000FU is required for normalisation using the constant baseline threshold algorithm. No swabs in the CfoI or NlaIV datasets had sum peak height values below 1000FU and normalisation of these datasets was resolved within three iterative cycles. Additionally, normalisation of the CfoI and NlaIV datasets resulted in detection of all expected fragments in the multi-template control. As the BaeGI dataset was incomplete, profiles generated by this enzyme were not included in subsequent bacterial richness analyses (6.4.4.3), but were reviewed when investigating T-RFLP detection of S. pneumoniae (6.4.4.5).

6.4.4.3 Bacterial richness in T-RFLP profiles
The bacterial richness (the number of different bacteria) in each swab was estimated by counting the number of fragments in each profile. A total of 47 and 48 different fragments were present in the CfoI and NlaIV datasets, respectively. The number of fragments per swab varied from 2-11 (median 7 fragments) in the CfoI dataset and 2-12 (median 6 fragments) in the NlaIV dataset. Nasopharyngeal profiles generated by CfoI or NlaIV contained 4-7 fragments (median 6 fragments). For ear discharge swabs, 2-11 fragments (median 8 fragments) were present in each CfoI profile and 2-12 fragments (median 6 fragments) were present in each NlaIV profile. Data from
matched nasopharyngeal and ear discharge swabs were available for seven children. In 4/7 children the ear discharge swabs contained higher bacterial richness than the matched nasopharyngeal swab.

6.4.4.4 Estimating similarity between T-RFLP profiles
Non-metric multi-dimensional scaling plots (NMDS) and hierarchical group-average clustering analysis (HCAN) were used to compare nasopharyngeal and ear discharge profiles. Significant site-specific clustering was observed for ear discharge and nasopharyngeal profiles (Figure 6.4). This clustering was evident in NMDS analyses based on binary presence/absence data using the Sørenson similarity index (Analysis of similarities (ANOSIM): global R 0.62; P=0.001) and square root transformed abundance data (the peak area of each fragment in the normalised T-RFLP profile of each swab) using the Bray-Curtis similarity index (ANOSIM global R=0.558; P=0.001). Clustering of nasopharyngeal and ear discharge profiles was also evident in hierarchical group-average clustering analyses based on binary or abundance metrics (Figures 6.5 and 6.6). Dissimilarity was observed between T-RFLP profiles from matched nasopharyngeal and ear discharge swabs in NMDS and HCAN analyses (Figure 6.7). Collectively, these data demonstrate higher similarity between swabs from the same site than between swabs collected from a single child, suggesting that the nasopharyngeal and ear discharge bacterial communities are different.

6.4.4.5 Did the T-RFLP detect previously cultured species?
Culture data obtained from each swab were reviewed and compared to the T-RFLP profiles (Figure 6.8). Fragments consistent with *H. influenzae*, *M. catarrhalis*, and β-haemolytic streptococci were evident in the T-RFLP profiles of all swabs culture-positive for these species. This included a fragment consistent with *H. influenzae* in profiles generated by *NlaIV* which is required to differentiate *H. influenzae* from most commensal *Haemophilus* sp.
Figure 6.4: Non-metric Multi-Dimensional Scaling (NMDS) analysis of similarity between nasopharyngeal and ear discharge T-RFLP profiles.

a) NMDS based on the Sørenson similarity distance matrix demonstrating significant differences between nasopharyngeal and ear discharge T-RFLP profiles (R=0.62; P=0.001). ED = ear discharge swab. NP = nasopharyngeal swab.

b) NMDS based on the Bray-Curtis similarity distance matrix demonstrating significant differences between nasopharyngeal and ear discharge T-RFLP profiles (R=0.558; P=0.001). ED = ear discharge swab. NP = nasopharyngeal swab.
Figure 6.5: Hierarchical group-average clustering analysis (HCAN) of nasopharyngeal and ear discharge T-RFLP profiles based on Sørenson similarity distance matrix (binary metric).

Black lines indicate significant subgroup structures at 1% significance level. Red lines indicate no significant dissimilarity.
Figure 6.6: Hierarchical group-average clustering analysis (HCAN) of nasopharyngeal and ear discharge T-RFLP profiles based on Bray-Curtis similarity distance matrix (abundance metric).
Black lines indicate significant subgroup structures at 1% significance level. Red lines indicate no significant dissimilarity.

Transform: Square root
Resemblance: S17 Bray Curtis similarity
ED=1; NP=0

Samples

0 20 40 60 80 100
Similarity
Figure 6.7: Non-metric Multi-Dimensional Scaling (NMDS) analysis demonstrating dissimilarity between matched nasopharyngeal and ear discharge.

Note: these NMDS are identical to those shown in Figure 6.4, but have been relabelled to indicate matched nasopharyngeal and ear discharge swabs.

a) NMDS based on the Sørenson similarity distance matrix (binary data).

b) NMDS based on the Bray-Curtis similarity distance matrix (abundance data).
Figure 6.8: Detection of otitis media bacteria by culture and T-RFLP.
This graph shows combined results from nasopharyngeal and ear discharge swabs. T-RFLP data were derived from analysis of all restriction enzyme profiles. Fragments consistent with a species were required to be present in the \textit{CfoI}, \textit{NlaIV} and \textit{BaeGI} profiles to be considered T-RFLP positive.
Fragments consistent with other previously cultured species were underrepresented in the T-RFLP data. *S. pneumoniae* was cultured from 11/17 swabs, whereas fragments consistent with *S. pneumoniae* were only present in three swabs. This included detection in profiles generated by *BaeGI* which differentiates *S. pneumoniae* from closely-related commensal species. Similarly, fragments consistent with *P. aeruginosa* and *Proteus* sp. were detected in T-RFLP profiles from 1/4 and 3/4 culture-positive swabs, respectively. Fragments consistent with staphylococcal sp. or *A. otitidis* were not present in any T-RFLP profiles.

The data were also reviewed to determine if fragments consistent with species sought by culture were evident in T-RFLP profiles of culture-negative swabs. For *H. influenzae*, 4/6 culture-negative swabs had a fragment consistent with *H. influenzae* in T-RFLP profiles generated with *CfoI* and *NlaIV*. Each of these four swabs was PCR-positive for *H. influenzae*. There was also one swab which contained a fragment consistent with *P. aeruginosa* in *CfoI* and *NlaIV* profiles but was culture-negative (P. aeruginosa PCR data were not available).

### 6.5 Discussion

T-RFLP of nasopharyngeal and ear discharge swabs demonstrated polymicrobial bacterial communities in each specimen with at least two and as many as 12 fragments detected. Despite methodological differences, the observed richness is comparable to that described by Kwambana et al. (Kwambana et al. 2011) who reported between 0-15 fragments in T-RFLP profiles of nasopharyngeal swabs from 12 children. A similar level of richness has also been described in T-RFLP analyses of lower airway specimens from cystic fibrosis patients (Rogers et al. 2003; Rogers et al. 2005; Rogers et al. 2009). The largest of these studies tested 71 sputum specimens from 17 adult cystic fibrosis patients and detected 2-37 fragments per T-RFLP profile (mean 13.3 fragments per profile) (Rogers et al. 2004). There are no other studies describing T-RFLP analysis of middle ear specimens.

Comparative analyses demonstrated significant clustering specific to each anatomic site, suggesting that the ear discharge and nasopharyngeal bacterial communities, or microbiomes, are different. In addition, dissimilarity was observed between matched nasopharyngeal and ear discharge swabs. These findings must be interpreted with
caution, as the sample size is small and experimental biases are present (as discussed below). However, the T-RFLP was applied consistently to all samples and it was expected that similar bacterial communities would be similarly affected by the experimental biases. Thus, a degree of clustering was expected in the NMDS and HCAN analyses if the matched nasopharyngeal and ear discharge microbiomes were not dissimilar. The absence of such clustering compared to the significant clustering observed between profiles from the same site supports the hypothesis that the nasopharyngeal and ear discharge microbiomes in these children are different. Site-specific microbiomic differences were also observed in a study of nasal and oropharyngeal microbiomes. Lemon et al. (Lemon et al. 2010) used sequencing of a 16S rRNA gene clone library and a 16S rRNA gene microarray (PhyloChip™) to characterise the nostril and oropharyngeal bacterial communities of seven healthy adult volunteers. This small study demonstrated different bacterial communities at each site with distinct phylum-level community structures. Such site-specific microbiomic differences suggest that environmental pressures, including homeostatic mechanisms, exert a selective force which defines the microbiomic niches at different anatomic sites.

The clinical implications of the T-RFLP data are unclear. The T-RFLP has demonstrated the presence of complex bacterial communities in some ear discharge swabs; however, as discussed in Chapter 4, it is unclear if this reflects polymicrobial middle ear infection or specimen contamination by canal flora. Further studies using more sensitive microbiomic methods which will provide taxonomic identification are required to better understand if these bacterial communities are consistent with canal flora.

6.5.1 Methodological considerations

6.5.1.1 Underestimation of bacterial richness due to PCR amplification biases

The observed bacterial richness in nasopharyngeal and ear discharge swabs is likely an underestimate due to PCR amplification biases (which prevent detection of fragments from some species as described in Chapter 5) and because different species can produce identical T-RFLP fragments (Schutte et al. 2008).
Analyses presented in the previous chapter demonstrated that PCR primer choice affected characterisation of the multi-template control. The only tested primer combination that detected all expected fragments from the multi-template control was 8F[FAM]-1512R in the presence of PCR additives. This primer combination was initially selected for analysis of upper respiratory specimens; however, the PCR failed to produce sufficient amplification from specimens with low DNA concentration. The reason for this has not been investigated, but likely reflects low PCR efficiency, possibly due to secondary structure in the 16S rRNA gene. Although the 8F[FAM]-1512R PCR provided the best characterisation of the multi-template control, the low PCR yield from clinical specimens made it unsuitable for T-RFLP of the nasopharyngeal and ear discharge swabs.

To overcome this, the 8F[FAM]-1113R PCR was used. This PCR achieves a balance between bacterial community resolution and 16S rRNA gene amplification from clinical specimens with low DNA concentrations. However, this PCR is also expected to underestimate bacterial community richness, as previously demonstrated with the multi-template control (Chapter 5). The 8F[FAM]-926R PCR, which has previously been used for most respiratory T-RFLP studies (Rogers et al. 2003; Tunney et al. 2011), was not used as it provided lower resolution of the multi-template control than the 8F[FAM]-1113R PCR. The 8F[FAM]-1113R is recommended for future upper respiratory T-RFLP analyses; however, alternative primer combinations, including those using a different forward primer, should also be considered (as discussed in Chapter 5).

6.5.2 Important otopathogens may not be detected by this T-RFLP

An important application of microbiomic analyses is assessment of changes in mucosal bacterial communities in response to colonisation by pathogenic species (Lemon et al. 2010). The T-RFLP described here will not be useful in monitoring changes in upper respiratory flora following pathogen colonisation as it failed to detect important pathogenic species from some specimens. Most notably, the T-RFLP failed to detect *S. pneumoniae* from 8/11 culture-positive swabs and did not detect any staphylococcal species. Other studies have also reported low T-RFLP detection of some species. For example, Tunney et al. (Tunney et al. 2011) used T-RFLP with the 8F[FAM]-926R PCR to characterise bacterial communities in sputum
from cystic-fibrosis patients. This T-RFLP did not detect staphylococcal sp. in 19/20 culture-positive specimens and did not consistently detect anaerobic species. Environmental studies have also examined profiling biases in T-RFLP analyses and concluded that the method provides a useful relative measure of bacterial richness but lacks the sensitivity required to more deeply characterise complex bacterial communities (Orcutt et al. 2009). This limitation of T-RFLP must be considered in comparative analyses of upper respiratory bacterial communities.

### 6.5.3 Optimal enzymes for T-RFLP of upper respiratory specimens

As described in Chapter 5, the T-RFLP used the restriction enzyme \textit{CfoI} to maintain methodological consistency with previously published methods (Rogers et al. 2003) and also included \textit{NlaIV} and \textit{BaeGI} to achieve improved resolution of \textit{H. influenzae} and \textit{S. pneumoniae} from closely-related commensal species. \textit{CfoI} and \textit{NlaIV} provided comparable measures of bacterial richness. Additionally, where \textit{CfoI} profiles contained a fragment consistent with \textit{Haemophilus} sp., the expected fragment consistent with \textit{H. influenzae} was also present in the corresponding \textit{NlaIV} profile. This supports inclusion of \textit{CfoI} and \textit{NlaIV} in future respiratory T-RFLP studies.

Profiling with \textit{BaeGI} was unsuccessful for 4/17 specimens due to low total signal detection. The reason for the low signal from \textit{BaeGI} profiles is not clear. It is unlikely to indicate photo-bleaching as the \textit{BaeGI} digests were tested concurrently with the \textit{CfoI} and \textit{NlaIV} digests. As the \textit{BaeGI} dataset was incomplete, these profiles were not included in subsequent comparative analyses. The \textit{BaeGI} profiles were useful in determining if fragments consistent with streptococcal sp. in \textit{CfoI} profiles were likely to be \textit{S. pneumoniae}; however, the overall benefit of including a \textit{BaeGI} digest was minimised by the low overall detection of fragments consistent with \textit{S. pneumoniae} (only 3/11 culture-positive swabs). In light of this, it may be more cost-effective for future studies to limit \textit{BaeGI} profiling to specimens containing fragments consistent with streptococcal sp. when profiled with other enzymes.

### 6.5.4 Differentiating signal:noise in unknown specimens

Based on data from Chapter 5, the constant baseline method (Dunbar et al. 2001) was used to differentiate signal:noise in T-RFLP raw data. Analysis of the \textit{BaeGI} dataset
showed that the constant baseline threshold algorithm could not be resolved if profiles with sum peak height values <1000FU were included in the dataset. Minimum total signal values for normalisation of T-RFLP data have not previously been described (Dunbar et al. 2001; Osborne et al. 2006). Importantly, the multi-template control was critical to determining the minimum total signal required for resolution of the constant baseline threshold algorithm. Inclusion of a multi-template control is strongly recommended for future respiratory T-RFLP studies to aid validation of signal:noise algorithms, as well as providing an objective measure of reproducibility. To date, use of a multi-template control has not been described in published respiratory T-RFLP studies (Kwambana et al. 2011; Rogers et al. 2003; Rogers et al. 2010c; Tunney et al. 2011).

### 6.5.5  Binning and aligning T-RFLP data from unknown specimens

Objective and reproducible T-RFLP profiling of unknown specimens requires consistent binning and alignment of detected fragments. Although several methods for binning T-RFLP data have been described, each has its own limitations and there is little consensus regarding which method is best (Schutte et al. 2008; Stres, 2006). Most published respiratory T-RFLP studies have not described how data were binned or aligned (Rogers et al. 2003; Rogers et al. 2010c; Tunney et al. 2011). The exception is the report of Kwambana et al. (Kwambana et al. 2011) which describes using a bin-width of +/- 1-bp but does not indicate how the bins were aligned.

In this study, data were binned according to the method of Dunbar et al. (Dunbar et al. 2001). This method uses a bin-width of 0.5-bp and limits the number of fragments per bin to the number of replicates analysed to achieve resolution of reproducible bins differing by <0.5-bp (bins differing by <0.5-bp can occur in T-RFLP data due to sequence variations which can subtly affect electrophoresis of fragments containing an identical number of bases (Schutte et al. 2008)). Most bins in the nasopharyngeal and ear discharge profiles contained fragments differing by <0.5-bp and were separated from subsequent bins by at least 2-bp, and thus, could be aligned without difficulty. Where bins contained fragments differing by >0.5-bp the starting point of the first bin was defined by the smallest fragment size occurring within that bin. Any fragments >0.5-bp above the starting point were placed into the next bin until all...
fragments within the region had been aligned. This is a modification of the method of Dunbar et al. (Dunbar et al. 2001) which used an unspecified clustering algorithm.

Several other clustering algorithms have been proposed for aligning T-RFLP data (reviewed by Schutte et al. 2008); however, these are not without limitation and, in general, have not been well validated and can be difficult to reproduce. Freeware programs have also been developed for alignment of T-RFLP data. One example is the freeware program T-ALIGN (Smith et al. 2005) which aligns using a moving average procedure but will only allow upload of two replicate profiles per specimen. Triplicate profiles were generated for nasopharyngeal and ear discharge swabs in this study as earlier analyses (Chapter 5) demonstrated that at least three replicates were required for accurate signal:noise determination with the constant baseline threshold method, and thus, T-ALIGN was not suitable for aligning the T-RFLP data. Overall, alignment of T-RFLP profiles remains challenging and it is recommended that future studies use the method described in section 6.3.5 until an optimal alignment algorithm is identified.

6.5.6 Minimum sample volumes for T-RFLP of upper respiratory swabs

Low DNA yield from the nasopharyngeal and ear discharge swabs prevented T-RFLP analysis of some specimens. Kwambana et al. (Kwambana et al. 2011) also reported difficulty obtaining sufficient PCR amplification from upper respiratory specimens with 7/24 nasopharyngeal swabs failing to amplify. Low DNA yield has also been identified as a limiting factor in deep-sequencing analysis of nasopharyngeal flora (Bogaert et al. 2011) and in lower airway specimens from healthy controls (Huang et al. 2011).

Previous respiratory T-RFLP analyses have used 20-100ng of template DNA per PCR (Rogers et al. 2003; Tunney et al. 2011). Thus, the initial approach taken in this study was to use at least 20ng of DNA per PCR. As several specimens contained less than 20ng DNA, PCR amplification was performed using a defined volume of DNA, as has been previously described for T-RFLP of gut microbiomes (Jakobsson et al. 2010). Unexpectedly, this resulted in amplification from three specimens with DNA concentration of 0ng/µL, as determined using PicoGreen® reagent. This possibly reflects a minimum bacterial load having been met. Each of these specimens had a
total bacterial load of at least $1 \times 10^7$ cells/swab, whereas the two swabs that failed to amplify had loads $<10^6$ cells/swab. Further, the total bacterial load in the swab that amplified but had insufficient PCR product for the restriction digests was $5 \times 10^6$ cells/swab. As the volume of DNA template used was equivalent to 1µL of each swab, this suggests that each PCR will require at least $10^4$ bacterial cells to obtain sufficient yield for T-RFLP profiling.

These data also suggest that total bacterial load may be a more useful measure than DNA concentration in determining the suitability of specimens for T-RFLP analyses. Total bacterial load qPCR may also provide a more objective measure as it does not detect human DNA. Overall, the sensitivity of the PicoGreen® method should be considered when reviewing DNA concentration data. For example, the PicoGreen® method used in this study has a lower limit of detection equivalent to 2ng/µL. After accounting for dilution factors used to prepare DNA samples for quantification, this is equivalent to a lower detection limit of 100ng, and thus the PicoGreen® method is unlikely to accurately quantify low concentration specimens. Based on this, it is recommended that future studies consider using DNA quantification by PicoGreen® (or similar reagent) together with total bacterial load to determine the suitability of specimens for T-RFLP.

### 6.5.7 Potential study limitations

As noted above this is a small study and further research is required to confirm the study findings. In particular, further analysis is required to confirm that site-specific microbiomic differences exist in the middle ear and nasopharynx of Indigenous children with acute otitis media with perforation. The study may also be limited by the sampling methods which collected ear discharge from Indigenous children with acute otitis media with perforation for up to six weeks. Although specimen contamination by canal flora cannot be excluded, better understanding of the composition of ear discharge bacterial communities may identify potential secondary middle ear pathogens associated with progression to CSOM in this high-risk population.

The study was a retrospective analysis of swabs that had been stored at -70°C for up to five years. Kwambana et al. (Kwambana et al. 2011) described a “modest effect”
of freezing on T-RFLP profiling of paediatric nasopharyngeal swabs, with significant changes observed for <10% of fragments after freezing. In contrast, Lauber et al. (Lauber et al. 2010) analysed human skin swabs and faeces using a deep sequencing microbiomic method and demonstrated no significant difference in the bacterial communities after freezing. These conflicting findings may reflect methodological limitations of the T-RFLP method used by Kwambana et al. especially as an arbitrary threshold of 100FU was used to differentiate signal and noise. Additionally, the data were not normalised to account for differences in the amount of DNA used in the fragment analysis and, thus, observed differences in the T-RFLP profiles may not be reliable. In this study, fresh specimens were not available for comparison, and an effect from freezing on the T-RFLP profiles cannot be excluded; however, any such effect is unlikely to have significantly affected the study findings.

6.5.8 Conclusions and recommendations

This study has found T-RFLP can be used to estimate bacterial richness in nasopharyngeal and ear discharge swabs but may not detect important respiratory pathogens. This is likely due to the PCR amplification biases identified by analysis of the multi-template control in the preceding chapter. Despite this limitation, the T-RFLP revealed the presence of complex bacterial communities in some nasopharyngeal and ear discharge swabs. Together, these data suggest that T-RFLP can be used to profile bacterial communities in upper respiratory swabs; however, such profiles should be considered estimates only as failure to detect previously cultured species demonstrates that the T-RFLP did not detect the true richness in these specimens.

The usefulness of T-RFLP to future upper respiratory studies will depend upon the research question being addressed. For example, the method may be useful in studies aiming to measure broad microbiomic changes in response to antibiotic therapies, but may not be useful in monitoring changes in upper respiratory flora following vaccination. Researchers must also consider the large quantities of DNA and minimum bacterial loads required for T-RFLP analysis, which may be difficult to achieve from low volume paediatric upper respiratory specimens.
Overall, the results from this study suggest microbiomic differences exist between the nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media with perforation. Further analysis with a more sensitive microbiomic method is now required to better understand the composition of polymicrobial bacterial communities in the ear discharge swabs. Such data may also help to determine if the polymicrobial ear discharge communities are indicative of canal or upper respiratory flora.
CHAPTER 7

Pilot PhyloChip characterisation of ear discharge microbiomes in Indigenous children with acute otitis media with perforation
Chapter 7: Pilot PhyloChip™ characterisation of ear discharge microbiomes in Indigenous children with acute otitis media with perforation

7.1 Summary

Background: T-RFLP of ear discharge swabs detected multiple taxa suggesting a high level of bacterial richness; however, T-RFLP has limited sensitivity and provides little data about the identity of detected bacteria. Better resolution of the bacterial communities may be achieved using PhyloChip™ - a 16S rRNA gene microarray which contains probes for over 50,000 bacterial taxa. PhyloChip™ analysis of lower respiratory specimens has consistently demonstrated higher sensitivity than clone library analysis, and comparable results to deep sequencing of the 16S rRNA gene. To date, PhyloChip™ analysis of otitis media specimens has not been reported.

Aims: The aims of this study were: i) to provide “proof-of-principle” demonstrating that PhyloChip™ can be used to characterise bacterial communities; ii) to describe the microbiome in ear discharge swabs from Indigenous children with acute otitis media; and iii) to determine if PhyloChip™ detects operational taxonomic units (OTUs) consistent with species previously cultured from the swabs.

Methods: Five ear discharge swabs from Indigenous children with acute otitis media were characterised using the PhyloChip™ microarray.

Results: A high degree of bacterial richness was observed with 67 phyla detected from the five ear discharge swabs. The PhyloChip™ microarray also detected species previously cultured or detected by PCR from the swabs, and demonstrated higher sensitivity than T-RFLP. The core microbiome of taxa common to all swabs contained 12 phyla and 26 families. Twenty OTUs consistent with at least 14 bacterial species were common to all swabs. Families and OTUs in the core microbiome were typical of common otitis media pathogens, skin flora, gut flora and environmental bacteria. PhyloChip™ analysis also detected numerous phyla and
families not previously considered in otitis media, including Archaea and anaerobic species, which would not be detected using standard culture methods.

**Conclusions:** PhyloChip™ analysis provided high-level characterisation of the ear discharge microbiomes. This pilot study has found that ear discharge from Indigenous children with small perforations of less than six weeks duration had a more complex microbiology than previously revealed by culture. The pilot data demonstrate that PhyloChip™ can be used for otitis media microbiomic analyses, but further studies are required to determine if changes in the ear discharge microbiome are associated with clinical presentation and disease progression. Future studies to better understand the role of canal flora in clinical progression to chronic suppurative otitis media are indicated.

**Acknowledgements:** Data summaries and statistical analyses were prepared with the assistance of Dr Mirjam Kaestli and Ms Linda Ward. Microbiomic comparative analyses were selected by myself, but were generated by Dr Mirjam Kaestli.

### 7.2 Introduction

T-RFLP analyses in Chapter 6 suggest the presence of polymicrobial bacterial communities, also called microbiomes, in ear discharge swabs from Indigenous children with acute otitis media with perforation. While the T-RFLP provided a crude measure of overall bacterial richness in ear discharge, it lacked sensitivity as demonstrated by its failure to detect clinically important species such as *S. pneumoniae* and *Staphylococcus* sp. from culture-positive swabs. Further, while the ear discharge T-RFLP profiles contained multiple fragments, accurate identification of the corresponding bacteria was not possible (Harris et al. 2007). These limitations may be addressed by analysis with more sensitive microbiomic tools.

#### 7.2.1 PhyloChip™ microbiomic characterisation

A range of microbiomic methods targeting the 16S rRNA gene have emerged in recent years for characterisation of complex bacterial communities. These methods have demonstrated previously unsuspected bacterial diversity in many environmental contexts. One method is PhyloChip™, a high-density 16S rRNA gene microarray
which provides family-level resolution of bacterial communities with preliminary identification of constituent taxa.

PhyloChip™ is an attractive microbiomic technology as it provides a more comprehensive measure of bacterial richness than clone library sequencing with a higher level of taxonomic identification than T-RFLP. Its cost per sample is comparable or less than that of deep sequencing technologies (Lemon et al. 2010) and less than the cost of sequencing large clone libraries. The hybridisation approach ensures detection of low abundance species which may not be detected by less sensitive methods (Lemon et al. 2010). Additionally, the array provides a level of standardisation between assays, which can be difficult to achieve with sequencing methods (Orcutt et al. 2009).

PhyloChip™ has been used to characterise microbiomes in a range of terrestrial and marine environments including soil (Brodie et al. 2006), ocean water associated with deep-sea oil spills (Hazen et al. 2010); and space craft assembly rooms (Vaishampayan et al. 2010). It has also been used to characterise human microbiomes in the gut (Maldonado-Contreras et al. 2010) and respiratory tract (Cox et al. 2010; Lemon et al. 2010), as discussed further below.

### 7.2.2 How does PhyloChip™ work?

The PhyloChip™ microarray uses hybridisation between fragmented 16S rRNA gene amplicons and a range of specific probe sets to determine the richness of Bacteria and Archaea in environmental specimens (Hazen et al. 2010). The first PhyloChip™ microarray was described in 2006 (Brodie et al. 2006) and contained probes for 8,935 taxa, referred to as operational taxonomic units (OTUs). Since then, the microarray has been refined such that the current PhyloChip™ (version G3) contains over 1,000,000 probes representing 2 domains; 147 phyla; 1,123 classes; 1,219 orders; 1,464 families; 10,993 sub-families; and 59,959 OTUs (Hazen et al. 2010). The microarray is designed to provide robust family-level identification (Cox et al. 2010).
7.2.3 How does PhyloChip™ compare to other microbiomic methods?

Microbiomic analyses can largely be considered as “open” or “closed” systems (Zhou, 2008). Open-systems are those which can potentially detect an unlimited number of taxa as well as previously undescribed taxa. Sequencing-based microbiomic methods are examples of open-systems. PhyloChip™ is an example of a closed-system in that it will only detect a limited number of pre-defined taxa. Despite this seeming limitation, comparative studies have found PhyloChip™ analyses to be consistent with those obtained using sequencing-based microbiomic methods.

Deep sequencing of 16S rRNA gene amplicons is commonly used to characterise bacterial communities. The method has been applied widely in human contexts, including analyses done as part of the Human Microbiome Project (Peterson et al. 2009). As with other 16S rRNA gene microarrays (van den Bogert et al. 2011), PhyloChip™ analyses have demonstrated comparable findings to deep sequencing. For example, Saulnier et al. (Saulnier et al. 2011) used PhyloChip™ to confirm findings of a deep sequencing measure of bacterial diversity in paediatric gut flora.

PhyloChip™ analyses have also been confirmed by clone library sequencing. Overall, PhyloChip™ detects higher levels of sub-family richness than clone library sequencing, even when several hundred clones are analysed (Brodie et al. 2006; DeSantis et al. 2007; Huang et al. 2011). For example, Flanagan et al. (Flanagan et al. 2007) detected 30 times more bacterial phylotypes with PhyloChip™ compared to sequencing of 192 clones, consistent with more sensitive detection of low-abundance taxa. As with all microarray technologies, there is potential for cross-hybridisation resulting in false-positive detection of OTUs; however, this is overcome by performing analyses at higher taxonomic levels where more robust identification is achieved (Cox et al. 2010). PCR has also been used to confirm the presence of OTUs present in PhyloChip™ data (DeSantis et al. 2007; Huang et al. 2010; Huang et al. 2011) and regression analysis has been used to demonstrate significant correlation between qPCR estimates of bacterial load and PhyloChip™-derived abundance data (Huang et al. 2010).

PhyloChip™ (version G3) has also been validated by testing mock communities prepared using a semi-randomised Latin Square structure (Hazen et al. 2010), where
each mock community contained 26 bacterial species at different abundances. This study showed good correlation between the actual composition of each community and the composition determined by the array with mean coefficient of variation of 0.097. A significant correlation was also reported between the abundance of each species and its corresponding PhyloChip™ HybScore (Hazen et al. 2010). It is important to note that these findings relate to the abundance of individual OTUs. While PhyloChip™ provides robust data for inter-specimen comparisons of OTU abundance (the differences in abundance of an OTU between specimens), intra-specimen measures of relative abundance (comparing the abundance of different OTUs within a specimen) are not possible as hybridisation affinities can vary substantially across the microarray (Todd DeSantis, Second Genome, personal communication).

7.2.4 PhyloChip™ analysis of respiratory specimens

PhyloChip™ has been used to characterise bacterial communities in respiratory specimens. This includes analysis of nostril and oropharyngeal swabs from healthy adults (Lemon et al. 2010) and studies of lower airway infection in cystic fibrosis (Cox et al. 2010), chronic obstructive pulmonary disease (COPD) (Huang et al. 2010) and asthma (Huang et al. 2011). These studies have consistently detected a high level of bacterial richness in respiratory specimens. For example, in a study of throat swabs and sputum from cystic fibrosis patients, Cox et al. (Cox et al. 2010) detected a total of 43 phyla represented by 1837 OTUs from 63 specimens, with 78-1012 OTUs detected per specimen. Similarly high levels of richness have been reported in studies of COPD and asthma. For example, in a study of eight endotracheal specimens from intubated COPD patients, Huang et al. (Huang et al. 2010) detected a total of 38 phyla and 140 distinct families. Another study by the same authors compared the bacteriology in bronchial brushings from 65 asthmatic patients and 10 healthy controls (Huang et al. 2011) and detected a total of 161 families, with 48-1240 OTUs detected per specimen in the asthmatic group, and 200-1121 OTUs detected in each control specimen. High levels of bacterial richness have also been demonstrated in the nose. Lemon et al. (Lemon et al. 2010) detected a total of 34 phyla in seven nostril swabs from healthy adults, with 125-778 OTUs detected per swab.
Collectively, these studies demonstrate previously unrecognised bacterial richness in respiratory specimens, but may still have underestimated the total richness as the data were generated using an early PhyloChip™ formulation (version G2) which contained probes for 8,935 OTUs. It remains to be seen if higher levels of richness will be demonstrated in respiratory specimens tested with PhyloChip™ version G3 which contains ~59,000 OTUs.

7.2.5 Aims of this study
Overall, PhyloChip™ studies have reported high bacterial richness in respiratory specimens. To date, PhyloChip™ analysis of otitis media specimens has not been reported. This chapter provides results of a cross-sectional pilot study using PhyloChip™ to characterise the microbiome of ear discharge swabs collected from Indigenous children with acute otitis media with perforation. The aims of the study were:

i) to provide “proof-of-principle” demonstrating PhyloChip™ can be used to characterise bacterial communities in ear discharge swabs.

ii) to describe the microbiome in ear discharge swabs from five Indigenous children diagnosed with acute otitis media with perforation.

iii) to determine if PhyloChip™ detects OTUs consistent with species previously cultured from the swabs.

Although this is a small pilot study, preliminary analysis of a core ear discharge microbiome will also be described.

7.3 Methods

7.3.1 Clinical specimens
Ear discharge swabs from five Indigenous children with acute otitis media with perforation were tested. All swabs had been collected as described in Chapter 2.2.2. As discussed earlier, a degree of specimen contamination by canal flora could not be excluded.
The study specimens were selected from amongst those tested by T-RFLP (Chapter 6, Table 6.1). Specimens with the highest DNA concentrations were selected to ensure all possessed the minimum DNA mass required for PhyloChip™ analysis (at least 100ng in 10µL). Metadata describing the PhyloChip™ sample set is provided in Table 7.1. All samples had been previously cultured as described in Chapter 6.3.1.1. Overgrowth by Proteus sp. may have prevented culture-based detection of M. catarrhalis and P. aeruginosa in some swabs, and in these instances culture data are not available (Table 7.1). Species-specific PCR data are as determined in Chapters 3 and 4. T-RFLP data are as described for CfoI analyses in Chapter 6.

7.3.2 Negative control specimen

A sample of STGGB, the media all swabs had been stored in, was also tested as a negative control. This control was processed identically to ear discharge swabs through all laboratory manipulations - from DNA extraction through to PhyloChip™ analysis.

7.3.3 DNA extraction and quantification

DNA was extracted with bead-beating pre-treatment as described in Chapter 6.3.3, and quantified with PicoGreen® reagent as described in Chapter 2.9. A dedicated DNA aliquot was available for PhyloChip™ analysis. This dedicated aliquot had been stored at -20°C immediately following extraction to prevent specimen contamination prior to microbiomic analysis.

7.3.4 Specimen shipment

PhyloChip™ testing is only available as a commercial service from a company called Second Genome which is based in the United States. DNA was shipped to Second Genome on dry ice to maintain specimen integrity. Additional dry ice was added during transportation to ensure the DNA did not thaw. All samples remained frozen when received by Second Genome.
Table 7.1: Metadata describing characteristics of ear discharge specimens included in the PhyloChip™ pilot study.
All swabs were from Indigenous children with acute otitis media with perforation. Overgrowth by *Proteus* sp. may have prevented culture-based detection of *M. catarrhalis* and *P. aeruginosa* in some swabs, and in these instances culture data are not available (n/a = not available).

<table>
<thead>
<tr>
<th>Ear Discharge Swab</th>
<th>Laboratory number</th>
<th>PhyloChip™ Identifier</th>
<th>Age (years)</th>
<th>Culture</th>
<th>DNA yield (ng/µL)</th>
<th>qPCR estimate of total bacterial load (cells/swab)</th>
<th>Pathogen detection by PCR</th>
<th>Number T-RFLP peaks (CfoI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30072</td>
<td>ED270</td>
<td>2.2</td>
<td>+ - - - +</td>
<td>13.6</td>
<td>2.59x10^7</td>
<td>H. influenzae</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>30155</td>
<td>ED271</td>
<td>0.8</td>
<td>- - - + -</td>
<td>10.6</td>
<td>6.8x10^7</td>
<td>S. pneumoniae</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>30162</td>
<td>ED272</td>
<td>1.2</td>
<td>- - + - -</td>
<td>20.6</td>
<td>5.96x10^8</td>
<td>M. catarrhalis</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>31072</td>
<td>ED273</td>
<td>2.0</td>
<td>- - - + -</td>
<td>16.9</td>
<td>1.86x10^8</td>
<td>A. otitidis</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>31163</td>
<td>ED274</td>
<td>1.2</td>
<td>- - + - +</td>
<td>5.4</td>
<td>6.48x10^6</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>
7.3.5 PhyloChip™ analysis

PhyloChip™ testing and preliminary data analysis were done by Second Genome using standardised protocols as previously described (Hazen et al. 2010; supplemental information). Briefly, the 16S rRNA gene was amplified using universal primers 27F.1 and 1492R.jgi (Table 7.2) with PCR for 40 cycles, consistent with current PhyloChip™ protocols. The PCR cycle number was selected by Second Genome based on in-house validation demonstrating that 40 cycles accommodates low-mass specimens and achieves improved overall data quality (Janet Warrington, Second Genome, personal communication).

PCR products were purified and concentrated, then quantified by electrophoresis in an Agilent 2100 Bioanalyzer®. The PhyloChip™ control mix (Hazen et al. 2010) was then added to each specimen to control experimental variation in fragmentation and hybridisation (Cox et al. 2010), and to provide a scaling and normalisation reference for quantitative analyses. 500ng of PCR product was then fragmented, biotinylated, and hybridised to the PhyloChip™ microarray (version G3). Each PhyloChip™ microarray was washed and stained then scanned using a GeneArray® scanner (Affymetrix). Each scan was captured using standard Affymetrix software (GeneChip® Microarray Analysis Suite).

Table 7.2: Primers used to amplify the 16S rRNA gene for PhyloChip™ analysis. Redundancies are indicated using the standard annotations - R (A and G) and M (A and C).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>27F.1</td>
<td>5’-AGRGTTTGATCMTGGCTCAG-3’</td>
</tr>
<tr>
<td>1492R.jgi</td>
<td>5’-GGTTACCTTGTTACGACTT-3’</td>
</tr>
</tbody>
</table>
7.3.6 PhyloChip™ raw data analysis

PhyloChip™ raw data were prepared by specialist bioinformaticians at Second Genome as previously described (Hazen et al. 2010; supplemental information). Briefly, the summary intensity of each array feature was calculated by ranking the fluorescent intensity of the central nine pixels of each probe and taking the 75th percentile. The summary intensity of each probe was then background subtracted and scaled to the PhyloChip™ control mix. The hybridisation score (HybScore) for each OTU was then calculated as the mean intensity of perfectly matching probes exclusive of the maximum and minimum. The algorithm of Hazen et al. (Hazen et al. 2010) was used to determine the presence or absence of each OTU. An OTU was defined by a group of highly similar 16S rRNA gene sequences, with most probes for each OTU possessing >99% similarity (Hazen et al. 2010).

The resulting data were tabulated to show HybScore values for each detected OTU (HybScore of zero indicates an OTU was not detected). Taxonomic description of each OTU to the sub-family taxonomic level (includes domain, phylum, sub-phylum, class, order, family and sub-family levels) was made based on the GreenGenes annotation. GenBank accession numbers describing each OTU were also provided.

7.3.7 Other data analysis

Further data analyses and summaries were prepared using a combination of Stata/IC (StataCorp LP; version 11.2 for Windows); Microsoft® Office Access and Excel (Microsoft®, 2003); and PRIMER 6 software (PRIMER-E Ltd, version 6.1.9).

With the exception of the Bray-Curtis Index, all descriptions and analyses were based on binary data describing the presence or absence of each OTU. As described above, previous studies have demonstrated robust and biologically meaningful differences in the inter-specimen abundance of each OTU (Hazen et al. 2010); however, as the hybridisation affinity of each OTU can vary substantially across the PhyloChip™ microarray (Todd DeSantis, Second Genome, personal communication), intra-specimen measures of relative abundance were not done.

Non-metric multi-dimensional scaling (NMDS) and hierarchical group-average clustering analysis were done to compare the ear discharge microbiomes. The
analyses were done using binary data describing the presence and absence of each OTU (Sørenson Index) and abundance data (Bray-Curtis Index based on HybScore). A similarity profile test (SIMPROF) with 999 permutations was performed on the hierarchical clustering analysis to test the null-hypothesis that there was no difference in the multivariate structure of the ear discharge microbiomes.

7.4 Results

7.4.1 OTUs in the negative control

A negative control containing only STGGB media was processed through all stages of the study from DNA extraction to PhyloChip™ analysis. This control was used to detect contaminating OTUs introduced to the microbiomic profile during experimental procedures. Two OTUs were detected in the negative control - a member of the Micrococcaceae family (*Nesterenkonia* sp.; OTU 49100) and a member of the Pseudomonadaceae family (*Pseudomonas* sp.; OTU 24520) which were also present in 5/5 and 3/5 ear discharge swabs, respectively (Figure 7.1). These OTUs are indicative of specimen or reagent contamination and were excluded from all subsequent analyses.

It is important to note that exclusion of these OTUs does not preclude detection of these genera, as other OTU probe-sets representing each genus are also present on the PhyloChip™ microarray. For example, exclusion of OTU 24520 does not exclude overall detection of *Pseudomonas* sp. as several hundred other OTUs representing this genus, including *P. aeruginosa*, are also present. Similarly, other *Nesterenkonia* sp. OTUs are included on the microarray.

7.4.2 Did PhyloChip™ detect previously identified species?

PhyloChip™ data from each swab were reviewed to determine if bacteria previously cultured or detected by PCR were also detected by the microarray. The analysis was initially done at the family-level as PhyloChip™ is not designed to provide robust species-level identification (Cox et al. 2010).
Figure 7.1: Presence and abundance of negative control OTUs in the ear 
discharge specimens.
Two OTUs were detected in the negative control. a) OTU 49100 representing a
_Nesterenkonia_ sp. was present in all ear discharge swabs. b) OTU 24520 representing
_a Pseudomonas_ sp. was present in 3/5 ear discharge swabs. OTU 24520 data includes
two instances where abundance data is shown but the OTU was not present. This
reflects hybridisation to a minority of probes associated with this OTU below the
threshold indicative of the taxon’s presence.

a) OTU 49100: Actinobacteria; Actinobacteridae; Actinomycetales;
Micrococcinaceae; _Nesterenkonia_. 275_Ncont = negative control.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Abundance</th>
<th>Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>275_NCont</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>274_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>273_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>272_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>271_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>270_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
</tbody>
</table>

b) OTU 24520: Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; _Pseudomonas_. 275_Ncont = negative control.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Abundance</th>
<th>Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>275_NCont</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>274_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>273_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>272_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>271_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
<tr>
<td>270_ED</td>
<td>[ ]</td>
<td>X</td>
</tr>
</tbody>
</table>
Families consistent with each species sought by culture were present in PhyloChip™ data from all swabs (Table 7.3). For 10/12 culture-positive results, OTUs consistent with cultured bacteria were also detected by PhyloChip™. The exceptions were two swabs which were culture and PCR-positive for *H. influenzae*. In these instances, the PhyloChip™ result was based on detection of a single OTU specific to *H. influenzae* and *H. haemolyticus*. Although this OTU was only detected in 2/5 swabs, OTUs with a high level of sequence similarity to *H. influenzae* were detected in all swabs. For example, 3/29 Pasteurellaceae OTUs were present in all swabs (OTUs 18788, 18560 and 18466). BLAST analysis showed that these OTUs possess >94% similarity with the *H. influenzae* 16S rRNA gene over ~1400-bp.

100% concordance was observed between PCR and PhyloChip™ detection of *S. pneumoniae*. While the OTU corresponding to *S. pneumoniae* may also detect *S. oralis* and *S. infantis*, no false-positive detection was observed in the two swabs negative for *S. pneumoniae* by culture and PCR.

Higher detection of *P. aeruginosa*, *S. aureus* and *Proteus* sp. was observed with PhyloChip™ than culture. These species were cultured from 3/5, 2/5 and 3/5 swabs, respectively, but were present in PhyloChip™ data from every specimen. *A. otitidis* was detected by PCR in 2/5 swabs but only 1/5 by PhyloChip™; however, the family Aerococcaceae was present in all swabs. *M. catarrhalis* and group A streptococcus were not detected in any swabs by any of the tested methods.

### 7.4.3 Microbiomic richness in ear discharge swabs

The OTUs detected in each specimen are listed in Appendix C. Overall, a total of 6258 OTUs representing 489 families and 67 phyla were detected across the five ear discharge swabs (Table 7.4). 3108 OTUs (50%), 189 families (39%); and 17 phyla (25%) were only present in a single swab (Table 7.5). The percentage of OTUs, families and phyla unique to each swab ranged from 18-33%, 12-25% and 0-11%, respectively (Table 7.5). The number of OTUs detected per swab represented only 0.2-7% of all OTUs present on the PhyloChip™ microarray (Table 7.4).
**Table 7.3: Detection of bacteria by culture, PCR and PhyloChip™.**

n/a = data not available due to overgrowth by a *Proteus* sp. Staphylococcaceae OTU 9164 represents *S. aureus*. Two *Proteus* sp. OTUs are shown to represent *P. mirabilis* and *P. vulgaris* which were detected in 4/5 and 3/5 swabs, respectively.

<table>
<thead>
<tr>
<th>Family name</th>
<th><em>H. influenzae</em></th>
<th><em>S. pneumoniae</em></th>
<th><em>A. otitidis</em></th>
<th><em>Staphylococcus</em> sp.</th>
<th><em>P. aeruginosa</em></th>
<th><em>Proteus</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhyloChip™ OTU number</td>
<td>Pasteurellaceae</td>
<td>Streptococcaceae</td>
<td>Aerococcaceae*</td>
<td>Staphylococcaceae</td>
<td>Pseudomonadaceae</td>
<td>Enterobacteriaceae</td>
</tr>
<tr>
<td>ED270 Culture</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PhyloChip™ family-level</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PhyloChip™ OTU</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ED271 Culture</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>PCR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PhyloChip™ OTU</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ED272 Culture</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>PCR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PhyloChip™ family-level</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PhyloChip™ OTU</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ED273 Culture</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>PhyloChip™ family-level</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>PhyloChip™ OTU</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ED274 Culture</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<td>n/a</td>
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<tr>
<td>PhyloChip™ family-level</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>PhyloChip™ OTU</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Consistent with previous taxonomy, the *A. otitidis* OTU 11520 is described as family Aerococcaceae in PhyloChip™ data (Appendix C). More recent taxonomic descriptions include *A. otitidis* in the family Carnobacteriaceae (GreenGenes Taxonomy, September 2011).
Table 7.4: Bacterial and Archaeal richness per ear discharge swab.  
This table describes the number of taxa detected in each swab from taxonomic levels phylum to OTU. Total = combined data from all swabs. n/a = not applicable.

<table>
<thead>
<tr>
<th>Taxonomic level</th>
<th>Total</th>
<th>ED270</th>
<th>ED271</th>
<th>ED272</th>
<th>ED273</th>
<th>ED274</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phylum</td>
<td>57</td>
<td>28</td>
<td>31</td>
<td>39</td>
<td>41</td>
<td>37</td>
<td>28 - 41</td>
</tr>
<tr>
<td>Class</td>
<td>376</td>
<td>43</td>
<td>89</td>
<td>87</td>
<td>102</td>
<td>79</td>
<td>43 – 102</td>
</tr>
<tr>
<td>Order</td>
<td>400</td>
<td>68</td>
<td>197</td>
<td>212</td>
<td>255</td>
<td>188</td>
<td>68 – 255</td>
</tr>
<tr>
<td>Family</td>
<td>472</td>
<td>73</td>
<td>228</td>
<td>245</td>
<td>298</td>
<td>216</td>
<td>73 – 298</td>
</tr>
<tr>
<td>Subfamily</td>
<td>830</td>
<td>82</td>
<td>341</td>
<td>319</td>
<td>427</td>
<td>299</td>
<td>82 – 427</td>
</tr>
<tr>
<td>OTU</td>
<td>6233</td>
<td>161</td>
<td>2431</td>
<td>3252</td>
<td>4034</td>
<td>1721</td>
<td>161 - 4034</td>
</tr>
<tr>
<td><strong>Archaea</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phylum</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>1 – 6</td>
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<tr>
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<td>1</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>1 – 8</td>
</tr>
<tr>
<td>Order</td>
<td>16</td>
<td>1</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>1 – 8</td>
</tr>
<tr>
<td>Family</td>
<td>17</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>1</td>
<td>1 – 8</td>
</tr>
<tr>
<td>Subfamily</td>
<td>21</td>
<td>2</td>
<td>6</td>
<td>8</td>
<td>9</td>
<td>2</td>
<td>2 – 9</td>
</tr>
<tr>
<td>OTU</td>
<td>25</td>
<td>2</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>2</td>
<td>2 - 10</td>
</tr>
<tr>
<td><strong>Total OTU per swab</strong></td>
<td>6258</td>
<td>163</td>
<td>2438</td>
<td>3262</td>
<td>4043</td>
<td>1723</td>
<td>163-4043</td>
</tr>
<tr>
<td>% of OTUs on the chip that were detected</td>
<td>10.4%</td>
<td>0.2%</td>
<td>4%</td>
<td>5%</td>
<td>7%</td>
<td>3%</td>
<td>0.2 – 7%</td>
</tr>
</tbody>
</table>

Table 7.5: Number of phyla, families and OTUs only present in a single swab.  
Percentage data indicates the number of each as a proportion of the total microbiome in each swab. The final column shows total values for combined data from all swabs.

<table>
<thead>
<tr>
<th></th>
<th>ED270</th>
<th>ED271</th>
<th>ED272</th>
<th>ED273</th>
<th>ED274</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phyla (%)</strong></td>
<td>2 (7)</td>
<td>4 (11)</td>
<td>3 (7)</td>
<td>8 (17)</td>
<td>0 (0)</td>
<td>17 (25)</td>
</tr>
<tr>
<td><strong>Families (%)</strong></td>
<td>16 (22)</td>
<td>34 (15)</td>
<td>38 (15)</td>
<td>76 (25)</td>
<td>25 (12)</td>
<td>189 (39)</td>
</tr>
<tr>
<td><strong>OTUs (%)</strong></td>
<td>46 (28)</td>
<td>430 (18)</td>
<td>824 (25)</td>
<td>1342 (33)</td>
<td>466 (27)</td>
<td>3108 (50)</td>
</tr>
</tbody>
</table>
A high level of richness was present in all microbiomes with 163-4043 OTUs representing 74-306 families from 29-47 phyla detected per swab (Table 7.4). Both Bacteria and Archaea (the third domain of life, previously called Archaeabacteria (Woese, 1987)) were present in all swabs. Overall, 472 bacterial families from 57 phyla, and 17 archaeal families from 10 phyla were detected (Table 7.4). Bacteria dominated with 73-298 families from 28-41 phyla present in each swab, compared to 1-8 archaeal families from 1-6 phyla (Figure 7.2). Lowest richness was observed for ear discharge specimen ED270 which contained only 163 OTUs - at least 10-fold fewer OTUs than the other swabs which had 1723-4043 OTUs.

Bacterial richness in each swab was investigated by plotting the proportion of OTUs classified at the phylum, order and family levels (Figures 7.3-7.5). It is important to note that this provides an overview of the number of taxa detected, but is not indicative of relative abundance. Overall, Proteobacteria, Firmicutes, Actinobacteria and Bacteriodetes were the richest phyla. The profile of ED270 was different to the other swabs, with highest order-level richness observed for Lactobacillales, Staphylococcaceae and Enterobacteriales. All Lactobacillales in this specimen were of the family Streptococcaceae and included the OTU for S. pneumoniae which was also detected in this swab by culture and PCR. Highest order-level richness in the remaining specimens was observed for the Pseudomonadaceae, Enterobacteriales, Arthrobacter, Streptomycineae and Clostridiales. The Pseudomonadaceae dominated as the richest order in each of these specimens.

7.4.4 Microbiomic similarity between ear discharge swabs

Non-metric multi-dimensional scaling (NMDS) and hierarchical group-average clustering analysis identified a distinct cluster of three ear discharge swabs. As shown in Figure 7.6, this cluster was evident in NMDS analyses based on binary presence/absence data (Sørenson similarity index) and square root transformation of abundance data (Bray-Curtis similarity index). Hierarchical clustering analysis of Bray-Curtis similarity data also demonstrated significant subgroup structures between ED270 and ED274 (Figure 7.7). The remaining swabs formed a distinct cluster (Figure 7.7) with no dissimilarity observed between ED272 and ED273 (SIMPROF permutation test at 1% significance level; p<0.01).
Figure 7.2: Bacterial and Archaeal richness in ear discharge swabs. The graphs demonstrate phylum (a) and family (b) level Bacterial and Archaeal richness in the negative control (N); each swab (ED270-274); and the average of all swabs (AV).

a) Phylum-level Bacterial and Archaeal richness.

b) Family-level Bacterial and Archaeal richness.
Figure 7.3: Phylum-level richness in the ear discharge swabs.
These data show the phylum-level proportion of OTUs present in each swab. Note: The figure demonstrates the richness in each swab and is not indicative of relative abundance.
Figure 7.4: Order-level richness in the ear discharge swabs.
These data show the order-level proportion of OTUs present in each swab. Note: The figure demonstrates the richness in each swab and is not indicative of relative abundance. The legend is limited to the 10 richest orders. A full list of orders detected in each swab is provided in Appendix C.
Figure 7.5: Family-level richness in the ear discharge swabs.
These data show the family-level proportion of OTUs present in each swab. Note: The figure demonstrates the richness in each swab and is not indicative of relative abundance. The legend is limited to the 15 richest families. A full list of families detected in each swab is provided in Appendix C.
Figure 7.6: Non-metric Multi-Dimensional Scaling (NMDS) analysis of similarity between ear discharge microbiomes. This figure shows NMDS plots based on (a) Sørenson similarity distance matrix (binary metric) and (b) Bray-Curtis similarity distance matrix (abundance metric).

a) NMDS based on the Sørenson similarity distance matrix.

b) NMDS based on the Bray-Curtis similarity distance matrix.
Figure 7.7: Hierarchical group-average clustering analysis of ear discharge microbiomes based on Bray-Curtis similarity distance matrix. Black lines indicate significant subgroup structures at 1% significance level. Red lines indicate no significant dissimilarity.
7.4.5 Core microbiome in the ear discharge swabs

The core microbiome was defined as taxa present in all swabs. A second analysis was done excluding ED270 which was dissimilar to all other ear discharge swabs in that it contained fewer OTUs and less overall richness, despite having a similar total bacterial load (Table 7.1). Core microbiome analyses were done at three taxonomic levels – phylum, family and OTU.

7.4.5.1 Phylum- and family-level core microbiome

12 phyla were common to all ear discharge swabs (Table 7.6) and all were of the domain Bacteria. Seven of the 12 core phyla did not contain families common to all swabs. This reflects instances where each swab contained at least one family belonging to the core phyla; however, these families were not common to all other swabs. Overall, 26 families were common to the five ear discharge swabs. This included families consistent with otitis media bacteria. For example, the families Streptococcaceae, Pasteurellaceae, Moraxellaceae, Staphylococcaceae and Pseudomonadaceae. Families consistent with environmental bacteria were also present in the core microbiome. For example, Shewanellaceae, Janthinobacterium, Ruminococcus and Anaerolineae.

The core microbiome in four swabs (excluding the outlier specimen ED270) was also reviewed. A further eight phyla and 53 families were common to the four swabs (Table 7.7). This included families consistent with normal flora in the ear canal, skin, gut, oral cavity or upper respiratory tract. For example Propionibacteriaceae, Enterobacteriaceae, Porphyromonadaceae, Fusobacteriaceae and Mycoplasmataceae.

7.4.5.2 OTU-level core microbiome

20 OTUs representing 10 families from three phyla were common to all swabs (Table 7.8). Although PhyloChip™ does not provide robust identity below the family-level, BLAST analysis of the OTU sequences was done to provide preliminary genus- or species-level identification. As shown in Table 7.8, OTUs consistent with the recognised otopathogens P. aeruginosa, S. aureus and H. influenzae, but not S. pneumoniae or M. catarrhalis, were present in all swabs. OTUs from the families Enterobacteriaceae and Clostridiaceae consistent with E. coli and
Table 7.6: Core phylum and families present in all ear discharge swabs.
12 phyla were common to all swabs; however, seven phyla did not contain a family common to all swabs (indicated by a dash in the family column). FM = family.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacteria</td>
<td>Arthrobacter_FM</td>
</tr>
<tr>
<td></td>
<td>Gordoniaceae</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Anaerolineae</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>Aerococcaceae</td>
</tr>
<tr>
<td></td>
<td>Catabacter_FM</td>
</tr>
<tr>
<td></td>
<td>Clostridiaceae</td>
</tr>
<tr>
<td></td>
<td>Lactobacillaceae</td>
</tr>
<tr>
<td></td>
<td>Paenibacillaceae</td>
</tr>
<tr>
<td></td>
<td>Peptostreptococcaceae</td>
</tr>
<tr>
<td></td>
<td>Planococcaceae</td>
</tr>
<tr>
<td></td>
<td>Ruminococcus_FM</td>
</tr>
<tr>
<td></td>
<td>Staphylococcaceae</td>
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<tr>
<td></td>
<td>Streptococcaceae</td>
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</tr>
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<td>Enterobacteriaceae</td>
</tr>
<tr>
<td></td>
<td>Janthinobacterium</td>
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<tr>
<td></td>
<td>Moraxellaceae</td>
</tr>
<tr>
<td></td>
<td>Oleomonas</td>
</tr>
<tr>
<td></td>
<td>Pasteurellaceae</td>
</tr>
<tr>
<td></td>
<td>Pseudomonadaceae</td>
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<tr>
<td></td>
<td>Shewanellaceae</td>
</tr>
<tr>
<td></td>
<td>Vibrionaceae</td>
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<tr>
<td>Verrucomicrobia</td>
<td>-</td>
</tr>
<tr>
<td>WS6</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 7.7: Core phyla and families present in four ear discharge swabs (excluding the outlier specimen ED270).
Note: these phyla and families are in addition to those presented in Table 7.6. Two phyla were present in the four swabs but did not contain any families common to all swabs (indicated by a dash in the family column). FM = family. * Indicates phyla also present in ED270.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Families</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidobacteria</td>
<td>Holophagaceae</td>
</tr>
<tr>
<td>Actinobacteria*</td>
<td>Actinomycineae, Frankineae, Leucobacter_FM, Propionibacterineae, Pseudonocardiaceae, Streptomycineae, Unclassified (2 families)</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Bacteroidaceae, Porphyromonadaceae, Saprospiraceae, Unclassified (1 family)</td>
</tr>
<tr>
<td>Chloroflexi*</td>
<td>Unclassified (1 family)</td>
</tr>
<tr>
<td>Fibrobacteres</td>
<td>Unclassified (1 family)</td>
</tr>
<tr>
<td>Firmicutes*</td>
<td>Coprococcus_FM, Faecalibacterium_FM, Leuconostoc_FM, Mycoplasmataceae, Unclassified (4 families)</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>Fusobactriaceae</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>Unclassified (1 family)</td>
</tr>
<tr>
<td>OP11</td>
<td>Unclassified (1 family)</td>
</tr>
<tr>
<td>Proteobacteria*</td>
<td>Aeromonadaceae, Alcaligenaceae, Alcanivoracaceae, Burkholderiaceae, Caedibacteraceae, Chromatiaceae, Colwelliaceae, Comamonadaceae, Desulfovibrionaceae, Frateria_FM, Halomonadaceae, Lysobacter_FM, Oxalobactereaceae, Pseudoalteromonadaceae, Ralstoniaceae, Thiomonas_FM, Unclassified (12 families)</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>-</td>
</tr>
<tr>
<td>TM7</td>
<td>-</td>
</tr>
</tbody>
</table>

* Indicates phyla also present in ED270.
Table 7.8: Core microbiome in all ear discharge swabs.
Preliminary identification of taxa consistent with each core OTU was determined by BLAST search of the corresponding GenBank accession number (Presumptive identity).

<table>
<thead>
<tr>
<th>OTU</th>
<th>Phylum</th>
<th>Family</th>
<th>GenBank</th>
<th>Presumptive identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>49642</td>
<td>Actinobacteria</td>
<td>Arthrobacter</td>
<td>DQ125642.1</td>
<td>Arthrobacter sp.</td>
</tr>
<tr>
<td>48304</td>
<td>Actinobacteria</td>
<td>Gordoniaceae</td>
<td>AB239927.1</td>
<td>Mycobacterium arupense</td>
</tr>
<tr>
<td>48810</td>
<td>Actinobacteria</td>
<td>Gordoniaceae</td>
<td>EU911931.1</td>
<td>Corynebacterium auriscanis</td>
</tr>
<tr>
<td>10162</td>
<td>Firmicutes</td>
<td>Clostridiaceae</td>
<td>EU473362.1</td>
<td>Clostridium sp.</td>
</tr>
<tr>
<td>4914</td>
<td>Firmicutes</td>
<td>Ruminococcus</td>
<td>EU459631.1</td>
<td>Blautia sp.</td>
</tr>
<tr>
<td>6124</td>
<td>Firmicutes</td>
<td>Unclassified</td>
<td>EU508200.1</td>
<td>Lachnospiraceae bacterium</td>
</tr>
<tr>
<td>6920</td>
<td>Firmicutes</td>
<td>Unclassified</td>
<td>EU506823.1</td>
<td>Lachnospiraceae bacterium</td>
</tr>
<tr>
<td>9164</td>
<td>Firmicutes</td>
<td>Staphylococcaceae</td>
<td>EU515208.1</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>9405</td>
<td>Firmicutes</td>
<td>Staphylococcaceae</td>
<td>DQ532199.1</td>
<td>Staphylococcus sp.</td>
</tr>
<tr>
<td>28102</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>DQ819019.1</td>
<td>E. coli</td>
</tr>
<tr>
<td>29197</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>EU459209.1</td>
<td>E. coli,</td>
</tr>
<tr>
<td>28496</td>
<td>Proteobacteria</td>
<td>Enterobacteriaceae</td>
<td>EU024331.1</td>
<td>Rahnella sp.</td>
</tr>
<tr>
<td>20523</td>
<td>Proteobacteria</td>
<td>Janthinobacterium</td>
<td>AY959205.1</td>
<td>Janthinobacterium sp.</td>
</tr>
<tr>
<td>22100</td>
<td>Proteobacteria</td>
<td>Unclassified</td>
<td>AY221066.1</td>
<td>Unclassified</td>
</tr>
<tr>
<td>18560</td>
<td>Proteobacteria</td>
<td>Pasteurellaceae</td>
<td>EF511973.1</td>
<td>Aggregatibacter aphrophilus*</td>
</tr>
<tr>
<td>18466</td>
<td>Proteobacteria</td>
<td>Pasteurellaceae</td>
<td>M75041.1</td>
<td>Aggregatibacter aphrophilus*</td>
</tr>
<tr>
<td>18788</td>
<td>Proteobacteria</td>
<td>Pasteurellaceae</td>
<td>AJ534673.1</td>
<td>Haemophilus sp*.</td>
</tr>
<tr>
<td>27008</td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td>EF510903.1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>27047</td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td>AY486371.1</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>32061</td>
<td>Proteobacteria</td>
<td>Pseudomonadaceae</td>
<td>AM111015.1</td>
<td>Pseudomonas sp.</td>
</tr>
</tbody>
</table>

* These sequences also showed 94% similarity with H. influenzae over ~1400-bp.
Clostridium sp., respectively, were also present in all swabs. The core microbiome also contained OTUs consistent with ear canal flora or environmental bacteria, for example, Arthrobacter sp. and Janthinobacterium sp. Interestingly, OTUs consistent with strict aerobic and strict anaerobic taxa were present in all swabs, for example, P. aeruginosa and Clostridium sp.

The core microbiome in four swabs (excluding the outlier specimen ED270) contained an additional 441 OTUs (Appendix D). This list includes OTUs belonging to families consistent with upper respiratory flora, oral flora and environmental bacteria.

7.4.6 Were other potential otitis media bacteria detected?

The PhyloChip™ data were also reviewed to determine the presence of atypical bacteria previously identified in otitis media studies using extended culture methods (Kononen et al. 2003; Stuart et al. 2003) (these bacteria are additional to those described in Table 7.3.). The data were also reviewed to determine if families and OTUs consistent with lower airway pathogens were also present.

As shown in Table 7.9, families and OTUs consistent with atypical otitis media bacteria, potential respiratory pathogens and upper respiratory commensals were present in most swabs. Families and OTUs consistent with the anaerobic genera Fusobacterium, Prevotella, Porphyromonas and Peptostreptococcus were present in at least three swabs. OTUs consistent with Bordetella sp. were also present in three swabs. This included OTU 20087 which represents B. pertussis on the PhyloChip™ microarray; however, this finding requires further confirmation as taxonomic identification below the family-level may not be reliable.

7.5 Discussion

This pilot study demonstrates that PhyloChip™ can be used for microbiomic analysis of ear discharge swabs from children with acute otitis media with perforation. The PhyloChip™ microarray detected most species previously detected in the swabs by culture or species-specific PCR. Importantly, false-positive results were not observed in any instances of PCR-negative results. This included M. catarrhalis which was not detected in these specimens by culture, PCR or PhyloChip™.
Table 7.9: Presence of atypical otitis media bacteria and other respiratory pathogens in the ear discharge microbiomes.
Bacteria described in this table are additional to those considered in Table 7.3 above. The table shows the number of swabs that contained families and OTUs consistent with each of the listed bacteria.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>OTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Achromobacter sp.</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Bordetella sp.*</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Chlamydia sp.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Fusobacterium sp.</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Pasteurella multicida</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Peptostreptococcus sp.</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Porphyromonas sp.</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Prevotella sp.</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Propionibacterium sp</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

* This included OTU 20087 which represents Bordetella pertussis in the PhyloChip™ microarray. This OTU was detected in three swabs.
7.5.1 Study limitations

The study was limited to five ear discharge swabs, as is appropriate for initial microbiomic analyses of mucosal bacterial communities (Lemon et al. 2010; Liu et al. 2011). As PhyloChip™ analysis costs ~$900 per specimen it was necessary to demonstrate the suitability of the method before committing to a larger prospective study.

The study was potentially limited by sampling biases. As discussed in preceding chapters, the study used ear discharge swabs which may potentially be contaminated by canal flora during specimen collection (discussed further, below). A bias may also have been introduced by selection of specimens with the highest DNA yields. Difficulty obtaining sufficient DNA for PhyloChip™ analysis has previously been described for low biomass lower airway specimens. For example, Huang et al. (Huang et al. 2011) reported insufficient DNA for PhyloChip™ analysis was obtained from protected bronchial brushings from 23/65 asthmatic patients and 5/10 healthy controls, possibly due to a low overall sample volume or low bacterial load. Further testing is needed to determine if ear discharge specimens with DNA yields below that of swabs in the current analysis (5.4-20.6ng/µL) will be suitable for PhyloChip™ analysis. Total bacterial load may also prove useful in determining which samples are suitable for PhyloChip™ analysis, as discussed for T-RFLP analyses in Chapter 6. All specimens in this study had total bacterial loads >10⁶ cells/swab. Further testing is required to determine if PhyloChip™ analysis can be performed for specimens with total bacterial loads <10⁶ cells/swab.

While the potential sample selection bias may limit the generalisability of the results, the study provides good pilot data to guide future microbiomic research of the polymicrobial complexity in ear discharge from Indigenous children with acute otitis media with perforation, as discussed below.

7.5.2 Methodological considerations

7.5.2.1 Controls

PhyloChip™ (version G3) has previously been well validated by testing mock communities (Hazen et al. 2010) and, thus, a multi-template control was not included in this study. Additionally, culture and PCR data were available for all swabs, thus
providing an external confirmation regarding detection of species associated with otitis media.

A negative control, containing swab media only, was included in all laboratory analyses, including DNA extraction. PhyloChip™ detected two OTUs in the negative control. Although the exact origin of this contamination cannot be identified, these OTUs most likely reflect reagent contamination with remnant bacterial DNA. Universal 16S rRNA gene amplification commonly detects remnant bacterial DNA in otherwise sterile reagents (Corless et al. 2000). This includes contamination of DNA extraction columns, which, although sterile, are not guaranteed to be DNA-free. Two reports have documented remnant bacterial DNA from *Legionella* species (Evans et al. 2003; van der Zee et al. 2002) in QIAGEN DNA extraction columns. Although *Legionella* species were not detected in the negative control, the potential for remnant bacterial DNA contamination highlights the importance of including negative controls through all stages of specimen preparation for microbiomic analysis.

### 7.5.2.2 PhyloChip™ laboratory processes

PhyloChip™ analysis is only available as a commercial service from Second Genome in the United States. Specific methodological parameters were selected by Second Genome, consistent with their current standard operating procedures. This included 40 cycles of PCR which Second Genome routinely use for low-biomass specimens, and as previously described for low-biomass lower respiratory specimens (Huang et al. 2011). As discussed in earlier chapters, chimera formation can occur when a high number of PCR cycles is used (Thompson et al. 2002). Second Genome report that PhyloChip™ analysis is optimised to minimise detection of chimeric amplicons. Ideally, however, PhyloChip™ analyses should be complemented by a sequence-based microbiomic method to provide an indication of chimera formation. This was beyond the scope of the current pilot study, but should be considered in any future PhyloChip™ analyses.

Another important difference between current and previously published PhyloChip™ methods is the algorithm used to determine the presence/absence of each OTU. Earlier PhyloChip™ respiratory papers used a positive-fraction, or pf-value, of $>0.90$ to indicate the presence of specific OTUs (Cox et al. 2010; Lemon et al. 2010;
Vaishampayan et al. 2010). This method required signal from at least 90% of the perfectly-matching probes before an OTU was detected. PhyloChip™ version G3 no longer uses pf-values to define OTU presence. Instead, a complex algorithm analysing signal from perfectly-matching probes as a continuous variable is used (Hazen et al. 2010). According to this algorithm, only OTU probe-sets with signal above the 75th percentile will be detected.

Two technical limitations must also be considered when reviewing PhyloChip™ data. Firstly, although analysis of mock communities has demonstrated good correlation between the actual and observed abundance of each OTU between specimens (Hazen et al. 2010), hybridisation variation across the microarray prevents accurate intra-specimen measures of relative abundance. Hybridisation variation has not been considered in previously published PhyloChip™ respiratory studies describing intra-specimen relative abundance (Klepac-Ceraj et al. 2010; Lemon et al. 2010). Again, this limitation may be addressed in future studies by complementing PhyloChip™ analysis with another microbiomic measure. For example, combined microbiomic analysis with PhyloChip™ and 16S rRNA gene deep sequencing would provide a complementary analysis achieving a balance between taxonomic identification (PhyloChip™) and relative abundance measures (deep sequencing).

Cross-hybridisation between different OTUs must also be considered. As with all microarrays, cross-hybridisation can result in false-positive OTU detection. This limitation is addressed by performing analyses at higher taxonomic levels (Cox et al. 2010). For example, Rigsbee et al. (Rigsbee et al. 2011) investigated factors affecting OTU detection by a gut flora-specific microarray, and identified cross-hybridisation in 1.58% of signal at the genus level, but only 0.19% at higher taxonomic levels. Similarly for PhyloChip™, taxonomic identification is robust at the family and higher taxonomic levels; however, detection of specific OTUs can be affected by cross-hybridisation. Thus, OTU-level findings should be cautiously interpreted and detection of rare or unexpected OTUs requires confirmation.
7.5.3 **Bacterial richness in the ear discharge microbiome**

PhyloChip™ demonstrated more sensitive bacterial detection than culture, and revealed a high degree of bacterial richness in each swab. Inter-individual variation was observed as has been described for human gut and salivary microbiomes (Dethlefsen et al. 2007; Lazarevic et al. 2010; Ley et al. 2006). Despite this variation, a high degree of richness was observed in the core microbiome at both the phylum and family levels. Additionally, NMDS and hierarchical group-average clustering analysis revealed a distinct cluster of three ear discharge swabs, suggesting microbiomic similarity between these specimens. Further analysis of a larger number of specimens is required to better understand microbiomic differences between individuals and the clinical significance of these findings (discussed further below).

7.5.4 **Comparison with other published microbiomic data**

There are currently few studies describing middle ear microbiomes, and no reports describing PhyloChip™ analysis in relation to otitis media. No studies to date have described middle ear microbiomes in patients with acute otitis media (with or without perforation). Two studies have reported culture-independent analysis of the middle ear microbiome in OME. Beswick et al. (Beswick et al. 1999) used a clone-and-sequence method to identify bacteria in culture-negative middle ear fluid surgically collected from 12 children with OME. Low bacterial richness was reported in this study, with a maximum of three species detected per specimen; however, analysis was limited to 20 clones per specimen. More recently, Liu et al. (Liu et al. 2011) used Roche GS-FLX 454 deep sequencing of the 16S rRNA gene to describe the microbiome in surgically collected middle ear fluid from one child with OME. This study detected nine bacterial families in the middle ear fluid; however, analysis was limited to 214 sequences.

Higher levels of bacterial richness have been reported in PhyloChip™ analyses of respiratory specimens. For example, PhyloChip™ analysis of sputum and throat swabs from 51 cystic fibrosis patients detected 78 - 1012 OTUs per specimen representing a total of 43 phyla (Cox et al. 2010). Another study of endotracheal aspirates from eight intubated patients with chronic obstructive pulmonary disease detected a total of 38 phyla from 140 families, with a mean of 411±246 OTUs per specimen (Huang et al. 2010). Similarly, a total of 911 OTUs from 34 phyla were
detected in seven nose swabs from healthy adult volunteers (Lemon et al. 2010). These measures of bacterial richness are less than those detected in the current study, but were all generated with PhyloChip™ version G2 which only contained probes for 8,935 OTUs.

Overall, this study describes a higher level of bacterial richness than previous otitis media and upper respiratory microbiomic studies.

7.5.5 Microbiomic differences between ear discharge swabs

Although this study was limited to a small number of specimens, distinct clustering was observed in NMDS and hierarchical group-average clustering analysis. The clustering was observed for both binary (presence/absence) and abundance (HybScore) data. While the small study size requires cautious data interpretation, the results support further studies using clustering algorithms to better define microbiomic signatures associated with clinical otitis media presentations.

In this pilot data, the microbiome of one swab, ED270, was dissimilar to all other swabs in terms of bacterial richness, the overall number of OTUs detected, and its position in cluster analyses. This swab also had low richness in T-RFLP data with only two fragments detected when digested by CfoI. ED270 showed increased richness of Lactobacillales of the Streptococcaceae family, and was culture and PCR-positive for S. pneumoniae.

In contrast, higher bacterial richness was observed in all other swabs which contained at least 10-fold more OTUs and 3 to 4 fold more families than ED270. This difference is unlikely to reflect a sampling bias, as the total bacterial load of ED270 was comparable to the other swabs. ED270 also differed from the other swabs in that it had lower Pseudomonadaceae and Enterobacteriales richness. These families include P. aeruginosa and Proteus sp. which are associated with chronic suppurative otitis media (CSOM) in Indigenous children (Leach et al. 2008a), prompting further consideration of the otitis media diagnosis in these children.

Consistent with current guidelines (Morris et al. 2001), the diagnostic criteria used in this study defined acute otitis media with perforation as perforation for less than six weeks (Morris et al. 2010). In comparison, the World Health Organisation has
suggested children with perforation for more than two weeks be considered as having CSOM (World Health Organisation, 2004). Data describing duration of perforation in the study children is not available beyond “less than six weeks”, and it is possible that swabs from some children may have been collected between 2-6 weeks after perforation. As the ear canals were cleaned to remove any visible pus prior to ear discharge sampling, it is unlikely that the observed bacterial richness reflects overgrowth in the exudate following perforation. Future studies should consider testing of paired ear canal and discharge swabs to confirm this interpretation.

Based on the results of this pilot study, I hypothesise that following perforation the middle ear is secondarily infected by ear canal and environmental flora, with subsequent increase in microbiomic richness which contributes to progression to CSOM. Longitudinal studies to test this hypothesis are now required. These studies should also consider testing of matched nasopharyngeal and canal swabs to better understand the origin of species in the ear discharge. Cluster analyses should be used to identify microbiomic signatures associated with progression to CSOM. Such data is likely to inform future therapeutic interventions to prevent progression to CSOM.

### 7.5.6 The core microbiome in five ear discharge swabs

The core microbiome was considered at three taxonomic levels - phylum, family and OTU. A high-level of richness was observed in the core microbiome at all taxonomic levels. Phyla and families detected in the core microbiome were consistent with previous microbiomic analyses of middle ear fluid from patients with OME (Liu et al. 2011), upper respiratory flora (Bogaert et al. 2011), ear canal flora from healthy volunteers (Frank et al. 2003), and environmental bacteria. As discussed below, the clinical significance of this complexity is unclear.

OTU-level data were reviewed to provide preliminary insight into taxa common to all swabs. Only 20 of 6058 detected OTUs were common to all specimens, suggesting a high degree of inter-individual variation. OTUs consistent with recognised otitis media pathogens, such as *H. influenzae*, *S. aureus* and *P. aeruginosa*, were present in all specimens. OTUs consistent with anaerobic species, such as *Clostridium* species, were also present in all swabs. The significance of
concurrent detection of strict aerobic and strict anaerobic species is not clear, but is suggestive of a complex microbial niche.

7.5.7  Does the observed bacterial richness reflect ear canal flora?

As discussed in earlier chapters, this study analysed ear discharge swabs collected from spontaneously perforated ears. Thus, it is possible that swabs may have been contaminated by ear canal flora during sampling. This is supported by detection of families previously observed in canal flora from healthy individuals. For example, the families Aerococcaceae, Peptostreptococcaceae, Staphylococcaceae, Streptococcaceae, Enterobacteriaceae, Pasteurellaceae and Pseudomonadaceae have all been described in normal canal flora (Frank et al. 2003; Stroman et al. 2001) and were detected in all ear discharge swabs.

While this suggests a degree of canal flora contamination in all swabs, these families (and OTUs) are also consistent with recognised otopathogens such as *H. influenzae*, *S. pneumoniae*, *S. aureus* and *P. aeruginosa*. Additionally, taxa reported in the canal flora of healthy volunteers have also been reported as part of normal upper respiratory flora. For example, Proteobacteria, Firmicutes, Actinobacteria and Fusobacteria have been reported in ear canal and nasopharyngeal microbiomic studies, with overlap also observed at lower taxonomic ranks (Bogaert et al. 2011; Frank et al. 2003; Lemon et al. 2010; Liu et al. 2011; Stroman et al. 2001). These findings highlight the difficulty associated with determining the origin of taxa based on their identity alone.

While it is possible that the observed richness in the ear discharge swabs reflects canal contamination, secondary infection of the middle ear following perforation cannot be excluded, as discussed in earlier chapters. It is also possible that middle ear pus may have drained into the canal and become overgrown by canal flora. This potential contamination or overgrowth by canal flora complicates interpretation of the microbiomic richness observed in this study; however, at a minimum, the PhyloChip™ data describe a vast reservoir of species in the canal flora with potential to secondarily infect the middle ear. Further studies are required to better understand the role of the canal flora in secondary middle ear infection and disease progression.
from acute otitis media to CSOM, particularly as regards polymicrobial secondary infection.

Future prospective studies should also carefully consider specimen collection methods which may be critical to accurate differentiation of canal and middle ear microbiomes. While analysis of paired canal and middle ear specimens may be useful, differentiation will remain difficult if middle ear pus has drained into the canal. Studies of normal canal flora in Indigenous children at high risk of progression to CSOM are also warranted. Better understanding of the canal flora in children with no recent history of perforation will aid interpretation of data from ear discharge swabs.

### 7.5.8 Archaea

Archaea, previously called Archaebacteria (Woese, 1987) but now accepted as the third domain of life (Horz et al. 2010), were also, unexpectedly, detected in all swabs. Archaea have not previously been described in respiratory specimens. This includes earlier PhyloChip™ studies, although this may reflect methodological differences between the microarray versions G2 and G3. The significance of Archaea to human infections, in general, is not clear (Horz et al. 2010). The Archaea detected in the ear discharge swabs included the Methanobrevibacter family which has previously been identified in the human gut (Horz et al. 2010); however, no Archaeal OTUs were common to all swabs and no Archaeal phyla were common to more than three swabs. These findings are suggestive of environmental contamination of the ear canal; however, a role for Archaea in the complex polymicrobial community present in these swabs cannot be excluded. For example, Zhang et al. (Zhang et al. 2009) postulated a metabolic relationship between hydrogen-producing Prevotellaceae and hydrogen-utilizing-Archaea in the human gut. Future ear discharge microbiomic studies should continue to use universal primers targeting Bacteria and Archaea until the role of Archaea in human mucosal infections is better understood.

### 7.5.9 Conclusions and recommendations

This pilot study has successfully addressed its aims and shown that PhyloChip™ can be used to describe the ear discharge microbiomes associated with acute otitis media with perforation in Indigenous children. The study has also provided a unique dataset
which will guide future microbiomic studies to better understand the microbiology underlying progression to chronic suppurative otitis media in Indigenous children.

A high level of bacterial richness was observed in all swabs. Increased richness of Pseudomonadaceae and Enterobacteriales in most tested swabs is suggestive of CSOM. Further studies are required to better understand microbiomic signatures associated with progression to chronic suppurative otitis media. Ideally, prospective longitudinal studies are required to describe the canal flora prior to perforation and subsequent microbiomic changes associated with progression from acute otitis media with perforation to chronic suppurative otitis media.

The pilot data suggest that clustering analyses may be useful tools for correlating microbiomic signatures with clinical parameters. Clustering analyses of ear discharge from children with recent and prolonged perforations may reveal microbiomic signatures associated with the disease progression.

Future studies should also consider complementary PhyloChip™ and deep sequencing methods to describe ear discharge microbiomes. This approach will enable good taxonomic identification as well as relative abundance and rarefaction data. Additionally, PhyloChip™ is a closed-system in that it will only detect OTUs included on the microarray, and it is possible that further richness may be revealed by deep sequencing methods.
CHAPTER 8

Final discussion
8.1 Background

In 1979, Moran et al. (Moran et al. 1979) reported findings of a national survey of otitis media in 21,988 Indigenous and 15,450 non-Indigenous Australian children up to nine years of age. This study reported the prevalence of otitis media in Indigenous children to be ~10-fold higher than that observed in non-Indigenous children leading the authors to conclude that:

“It should be a national objective to reduce the incidence of chronic respiratory disease in Australian Aboriginals to acceptable levels. When this has been achieved, the prevalence of otitis media may also be acceptable.” (Moran et al. 1979)

Despite significant and ongoing research efforts since then, chronic respiratory disease and otitis media remain endemic in many remote Indigenous communities. Otitis media in Indigenous children living in remote communities begins in the first weeks and months of life (Leach et al. 1994) and often progresses to perforation and chronic suppurative otitis media (Leach et al. 2008a). In the absence of improved housing and environmental health, research efforts have focused on identifying preventive and therapeutic interventions to reduce the burden of otitis media in Indigenous communities. Antibiotic therapies have shown limited benefit as clinical treatment failure has been documented in ~50% of Indigenous children with acute otitis media (Morris et al. 2010), and ~70% of children with chronic suppurative otitis media (Leach et al. 2008a).

It has been hypothesised that the high clinical failure rate following antibiotic treatment is due to the dense, polymicrobial nature of otitis media in this population (Smith-Vaughan et al. 2006). Culture-based methods optimised for recovery of the major otitis media pathogens \textit{S. pneumoniae}, \textit{H. influenzae} and \textit{M. catarrhalis} have been used to investigate the bacteriology of acute otitis media in Indigenous children and have demonstrated a multiplicity of species and strains in nasopharyngeal and ear discharge swabs (Smith-Vaughan et al. 1996). However, standard otitis media
This thesis presents a culture-independent analysis of the bacteriology associated with acute otitis media in Indigenous children from the Northern Territory. The objective of the study was to use culture-independent methods to better understand the bacteriology underlying acute otitis media in a population at high-risk of progression to chronic suppurative otitis media. The study aimed to address three key research questions:

1. Can nasopharyngeal total or pathogenic bacterial load be used as a prognostic indicator of clinical outcomes following antibiotic treatment of acute otitis media in Indigenous children?

2. Is the atypical otopathogen *Alloiococcus otitidis* present in nasopharyngeal or ear discharge swabs collected from Indigenous children with acute otitis media with perforation?

3. Are polymicrobial bacterial communities present in nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media with perforation?

### 8.2 Principle outcomes of the study

This study used a combination of qPCR and bacterial profiling methods to investigate the bacteriology of nasopharyngeal and ear discharge swabs collected from Indigenous children with acute otitis media.

Nasopharyngeal bacteriology is important to acute otitis media pathogenesis as the upper respiratory tract is the primary reservoir of otitis media pathogens. Earlier studies have demonstrated increased nasopharyngeal pathogenic and total bacterial loads to be positively correlated with onset (Smith-Vaughan et al. 2008) and severity (Smith-Vaughan et al. 2006) of otitis media in Indigenous children, but have not described bacterial load in relation to clinical treatment outcomes. In this study, nasopharyngeal total and pathogenic bacterial loads were investigated as possible prognostic indicators of the clinical outcome of antibiotic treatment of acute otitis
media in Indigenous children. The study confirmed the presence of increased nasopharyngeal bacterial loads in Indigenous children with acute otitis media, but did not find any association between pre-treatment total or pathogenic bacterial load and the clinical outcome of antibiotic therapy. The reason for the absence of an association is unclear. I hypothesise that nasopharyngeal bacterial loads are important to acute otitis media onset, but middle ear bacterial dynamics determine the clinical outcome of antibiotic therapy. For children with acute otitis media without perforation, testing of this hypothesis would require collection of middle ear fluid by tympanocentesis (an invasive procedure) which may not be ethically justifiable.

Subsequent studies reported in this thesis focused on acute otitis media with perforation as this enables investigation of the middle ear microbiology.

qPCR was used to detect and quantify *Alloiococcus otitidis* in nasopharyngeal and ear discharge swabs from Indigenous children with acute otitis media with perforation. *A. otitidis* was investigated as it has previously been detected in Indigenous children with OME (Ashhurst-Smith et al. 2007) but has not been described in relation to acute otitis media in this population. Furthermore, earlier work has demonstrated macrolide resistance in *A. otitidis* (Ashhurst-Smith et al. 2007; Bosley et al. 1995). As the macrolide azithromycin is an emerging therapy for acute otitis media in Indigenous children, it was important to determine if *A. otitidis* was present. Interestingly, *A. otitidis* was found in ear discharge swabs but not nasopharyngeal swabs, suggesting the ear canal as the likely reservoir of this species. Five *A. otitidis* isolates were cultured from PCR-positive swabs, all of which had minimum inhibitory concentrations consistent with macrolide resistance. The clinical significance of this resistance is difficult to determine as only a small number of isolates were obtained and all *A. otitidis*-positive swabs were polymicrobial. Relative abundance measures were used to better understand if *A. otitidis* was a dominant constituent of the polymicrobial ear discharge bacterial communities. This is the first time qPCR has been used to understand *A. otitidis* in a polymicrobial context. In most swabs, *A. otitidis* was present at very low relative abundance; however, higher relative abundances were observed in a subset of swabs suggesting that *A. otitidis* may have a role as a secondary middle ear pathogen. This finding highlights the need
to better understand the ear canal as a reservoir of potential secondary pathogens and also prompts consideration of a role for *A. otitidis* in chronic suppurative otitis media.

Community profiling analyses were then done to better understand the complexity of bacterial richness in nasopharyngeal and ear discharge swabs. T-RFLP analysis demonstrated significant site-specific clustering of ear discharge and nasopharyngeal swabs, suggesting microbiomic differences at these two sites. I hypothesise that this difference reflects divergence of the middle ear microbiome following secondary infection by canal flora. Bacterial richness in the ear discharge swabs varied from 2-12 fragments; however, in the absence of taxonomic identification it was not possible to determine if this reflected upper respiratory or canal flora.

Subsequent PhyloChip™ analysis confirmed that a high degree of bacterial richness was present in the ear discharge swabs. The PhyloChip™ pilot study identified bacterial families consistent with upper respiratory, canal and environmental flora in the ear discharge microbiome of five Indigenous children with acute otitis media with perforation. The core microbiome contained both strictly anaerobic and strictly aerobic species, indicating the presence of complex ecological parameters within the ear discharge. As with the T-RFLP analysis, bacterial richness varied. One of 5 swabs contained significantly lower richness and was dominated by streptococcal and staphylococcal taxa. In contrast, 4 of 5 swabs showed increased Pseudomonadales and Enterobacteriales richness, which may suggest progression to chronic suppurative otitis media in these children. Based on these preliminary data, I hypothesise that bacterial richness in the middle ear increases following secondary infection by canal flora and subsequently contributes to the pathogenesis of chronic suppurative otitis media. Further studies are now required to test this hypothesis. Ideally, prospective longitudinal studies are required to better understand the canal flora before perforation, the microbiology of acute otitis media immediately following perforation, and microbiomic changes associated with prolonged perforation. Clustering analyses may prove useful to identifying specific microbiomic signatures within complex polymicrobial communities that are associated with progression to chronic suppurative otitis media.
A strength of this thesis is the focus on methodological optimisation and standardisation. Methodological optimisation and standardisation is critical to accurate estimation of bacterial community richness and structure. In this thesis, a laboratory-prepared multi-template control was used to validate T-RFLP methods and also provide an inter-assay measure of reproducibility. Such controls are strongly recommended for future culture-independent analyses. Additionally, continued efforts to reduce culture-independent experimental biases are required and will be aided by inclusion of well-define multi-template controls.

8.3 Future research directions and challenges

Preventing acute otitis media in Indigenous children from progressing to CSOM remains challenging with current therapeutic options showing limited benefit. This thesis has provided pilot data describing polymicrobial communities in ear discharge obtained following spontaneous tympanic membrane perforation in Indigenous children with acute otitis media which suggests bacterial richness may contribute to CSOM pathogenesis. These pilot data have been obtained from small cohorts and larger prospective studies are now required to confirm the study findings and provide better understanding of polymicrobial bacterial communities in otitis media pathogenesis and treatment failure.

This thesis has focused on the bacteriology of acute otitis media; however, future studies should also consider other micro-ecological factors which may contribute to otitis media pathogenesis and chronicity in Indigenous children. Bacterial analyses should be complemented by viral and fungal analyses in conjunction with host factors, particularly mucosal immunological and inflammatory processes associated with chronic infections. Such studies will be challenging, particularly as regards low volume paediatric middle ear specimens. This will be especially challenging to microbiomic analyses which can require nanogram quantities of DNA. The limitations of low volume specimens may be overcome with whole specimen amplification techniques, for example multiple displacement amplification; however, such methods will require rigorous review of potential biases before being applied.
8.4 Conclusion

This thesis has used culture-independent methods to explore the bacteriology associated with acute otitis media in Indigenous children. Overall, the study has shown that microbiomic methods may be useful to understanding the complex bacteriology associated with chronic middle ear infections in Indigenous children. It is my hope that a better understanding of the bacteriology of chronic otitis media in Indigenous children will inform development of effective interventions to treat and prevent the condition, such that future generations of Indigenous children will no longer be at high risk of CSOM.

Abdo, Z; Schuette, UM; Bent, SJ; Williams, CJ; Forney, LJ; Joyce, P. 2006. Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Environ Microbiol. 8(5): 929-938.

Abell, GC; Revill, AT; Smith, C; Bissett, AP; Volkman, JK; Robert, SS. 2010. Archaeal ammonia oxidizers and nirS-type denitrifiers dominate sediment nitrifying and denitrifying populations in a subtropical macrotidal estuary. ISME J. 4(2): 286-300.

Access Economics Pty Ltd. 2009. "The cost burden of otitis media in Australia".


Andoh, A; Imaeda, H; Aomatsu, T; Inatomi, O; Bamba, S; Sasaki, M; Saito, Y; Tsujikawa, T; Fujiyama, Y. 2011. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. J Gastroenterol. 46(4): 479-486.


Behrens, S; Fuchs, BM; Mueller, F; Amann, R. 2003. Is the *in situ* accessibility of the 16S rRNA of *Escherichia coli* for Cy3-labeled oligonucleotide probes predicted by a three-dimensional structure model of the 30S ribosomal subunit? Appl Environ Microbiol. 69(8): 4935-4941.

Bell, SM; Pham, JN; Fisher, GT. 2009. Antibiotic susceptibility testing by the CDS method. A manual for Medical and Veterinary Laboratories. 5th Edition. The Antibiotic Reference Laboratory, Department of Microbiology, The Prince of Wales Hospital. Sydney, Australia.


Binks, M; Kirkham, LA; Wiertsema, SP; Dunne, E; Richmond, P; Leach, AJ; Smith-Vaughan, H. 2011a."Comparison of PCR methods for differentiation of *Haemophilus influenzae* and *Haemophilus haemolyticus". Presented at the 10th International Symposium of Recent Advances in Otitis Media, New Orleans, USA.


Brimblecombe, FS; Cruikshank, R; Masters, PL; Reid, DD; Stewart, GT. 1958. Family studies of respiratory infections. Br Med J. 1 119-128.

Brodie, EL; DeSantis, TZ; Joyner, DC; Baek, SM; Larsen, JT; Andersen, GL; Hazen, TC; Richardson, PM; Herman, DJ; Tokunaga, TK; Wan, JM; Firestone, MK. 2006. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Appl Environ Microbiol. 72(9): 6288-6298.


Carrol, ED; Guiver, M; Nkhoma, S; Mankhambo, LA; Marsh, J; Balmer, P; Banda, DL; Jeffers, G; White, SA; Molyneux, EM; Molyneux, ME; Smyth, RL; Hart, CA. 2007. High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease. Pediatr Infect Dis J. 26(5): 416-422.

Carvalho, M; Pimenta, FC; Jackson, D; Roundtree, A; Ahmad, Y; Millar, EV; O'Brien, KL; Whitney, CG; Cohen, AL; Beall, BW. 2010. Revisiting pneumococcal carriage by use of broth enrichment and PCR techniques for enhanced detection of carriage and serotypes. J Clin Microbiol. 48(5): 1611-1618.

Carvalho, M; Tondella, ML; McCaustland, K; Weidlich, L; McGee, L; Mayer, LW; Steigerwalt, A; Whaley, M; Facklam, RR; Fields, B; Carlone, G; Ades, EW; Dagan, R; Sampson, JS. 2007. Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. J Clin Microbiol. 45(8): 2460-2466.


Charlson, ES; Chen, J; Custers-Allen, R; Bittinger, K; Li, H; Sinha, R; Hwang, J; Bushman, FD; Collman, RG. 2010. Disordered microbial communities in the upper respiratory tract of cigarette smokers. PLoS ONE. 5(12): e15216.


Clark, LL; Dajcs, JJ; McLean, CH; Bartell, JG; Stroman, DW. 2006. Pseudomonas otitidis sp. nov., isolated from patients with otic infections. Int J Syst Evol Microbiol. 56(Pt 4): 709-714.


Cox, MJ; Allgaier, M; Taylor, B; Baek, MS; Huang, YJ; Daly, RA; Karaoz, U; Andersen, GL; Brown, R; Fujimura, KE; Wu, B; Tran, D; Koff, J; Kleinhenz, ME; Nielson, D; Brodie, EL; Lynch, SV. 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PLoS ONE. 5(6): e11044.


de Boer, R; Peters, R; Gierveld, S; Schuurman, T; Kooistra-Smid, M; Savelkoul, P. 2010. Improved detection of microbial DNA after bead-beating before DNA isolation. J Microbiol Meth. 80(2): 209-211.


DeSantis, TZ; Brodie, EL; Moberg, JP; Zubieta, IX; Piceno, YM; Andersen, GL. 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microb Ecol. 53(3): 371-383.


Ehrlich, GD; Ahmed, A; Earl, J; Hiller, NL; Costerton, JW; Stoodley, P; Post, JC; DeMeo, P; Hu, FZ. 2010. The distributed genome hypothesis as a rubric for understanding evolution in situ during chronic bacterial biofilm infectious processes. FEMS Immunol Med Microbiol. 59(3): 269-279.

Erb-Downward, JR; Thompson, DL; Han, MK; Freeman, CM; McCloskey, L; Schmidt, LA; Young, VB; Toews, GB; Curtis, JL; Sundaram, B; Martinez, FJ; Huffnagle, GB. 2011. Analysis of the lung microbiome in the "healthy" smoker and in COPD. PLoS ONE. 6(2): e16384.

Evans, GE; Murdoch, DR; Anderson, TP; Potter, HC; George, PM; Chambers, ST. 2003. Contamination of Qiagen DNA extraction kits with Legionella DNA. J Clin Microbiol. 41(7): 3452-3453.


Gardner, SN; Jaing, CJ; McLoughlin, KS; Slezak, TR. 2010. A microbial detection array (MDA) for viral and bacterial detection. BMC Genomics. 11:668. 668.


Gibney, KB; Morris, PS; Carapetis, JR; Skull, SA; Smith-Vaughan, HC; Stubbs, E; Leach, AJ. 2005. The clinical course of acute otitis media in high-risk Australian Aboriginal children: a longitudinal study. BMC Pediatr. 5(1): 16.

Goldstein-Daruech, N; Cope, EK; Zhao, KQ; Vukovic, K; Kofonow, JM; Doghramji, L; Gonzalez, B; Chiu, AG; Kennedy, DW; Palmer, JN; Leid, JG; Kreindler, JL; Cohen, NA. 2011. Tobacco smoke mediated induction of sinonasal microbial biofilms. PLoS ONE. 6(1): e15700.


Haas, BJ; Gevers, D; Earl, AM; Feldgarden, M; Ward, DV; Giannoukos, G; Ciulla, D; Tabbaa, D; Highlander, SK; Sodergren, E; Methe, B; DeSantis, TZ; The Human Microbiome Consortium; Petrosino, JF; Knight, R; Birren, BW. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res. 21(3): 494-504.
Hall-Stoodley, L; Hu, FZ; Gieseke, A; Nistico, L; Nguyen, D; Hayes, J; Forbes, M; Greenberg, DP; Dice, B; Burrows, A; Wackym, PA; Stoodley, P; Post, JC; Ehrlich, GD; Kerschner, JE. 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. JAMA. 296(2): 202-211.


Hare, KM; Smith-Vaughan, HC; Leach, AJ. 2011. Viability of respiratory pathogens cultured from nasopharyngeal swabs stored for up to 12 years at −70 degrees in skim milk tryptone glucose glycerol broth. J Microbiol Meth. 86(3): 364-367.


Harris, JK; De Groote, MA; Sagel, SD; Zemanick, ET; Kapsner, R; Penvari, C; Kaess, H; Deterding, RR; Accurso, FJ; Pace, NR. 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc Natl Acad Sci USA. 104(51): 20529-20533.
Hazen, TC; Dubinsky, EA; DeSantis, TZ; Andersen, GL; Piceno, YM; Singh, N; Jansson, JK; Probst, A; Borglin, SE; Fortney, JL; Stringfellow, WT; Bill, M; Conrad, ME; Tom, LM; Chavarria, KL; Alusi, TR; Lamendella, R; Joyner, DC; Spier, C; Baelum, J; Auer, M; Zenla, ML; Chakraborty, R; Sonnenthal, EL; D’haeseleer, P; Holman, HY; Osman, S; Lu, Z; Van Nostrand, JD; Deng, Y; Zhou, J; Mason, O. 2010. Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science. 330(6001): 204-208.


Hilty, M; Burke, C; Pedro, H; Cardenas, P; Bush, A; Bossley, C; Davies, J; Ervine, A; Poulter, L; Pachter, L; Moffatt, MF; Cookson, WO. 2010. Disordered microbial communities in asthmatic airways. PLoS ONE. 5(1): e8578.


Hoberman, A; Greenberg, DP; Paradise, JL; Rockette, HE; Lave, JR; Kearney, DH; Colborn, DK; Kurs-Lasky, M; Haralam, MA; Byers, CJ; Zoffel, LM; Fabian, IA; Bernard, BS; Kerr, JD. 2003. Effectiveness of inactivated influenza vaccine in preventing acute otitis media in young children: a randomized controlled trial. JAMA. 290(12): 1608-1616.

Hoberman, A; Paradise, JL; Rockette, HE; Shaikh, N; Wald, ER; Kearney, DH; Colborn, DK; Kurs-Lasky, M; Bhatnagar, S; Haralam, MA; Zoffel, LM; Jenkins, C; Pope, MA; Balentine, TL; Barbadora, KA. 2011. Treatment of acute otitis media in children under 2 years of age. New Engl J Med. 364(2): 105-115.


Holst-Jensen, A; Ronning, SB; Lovseth, A; Berdal, KG. 2003. PCR technology for screening and quantification of genetically modified organisms (GMOs). Anal Bioanal Chem. 375(8): 985-993.


Hong, S; Bunge, J; Leslin, C; Jeon, S; Epstein, SS. 2009. Polymerase chain reaction primers miss half of rRNA microbial diversity. ISME J. 3(12): 1365-1373.


Hotomi, M; Arai, J; Billal, DS; Takei, S; Ikeda, Y; Ogami, M; Kono, M; Beder, LB; Toya, K; Kimura, M; Yamanaka, N. 2010. Nontypeable *Haemophilus influenzae* isolated from intractable acute otitis media internalized into cultured human epithelial cells. Auris Nasus Larynx. 37(2): 137-144.


Huang, YJ; Nelson, CE; Brodie, EL; DeSantis, TZ; Baek, MS; Liu, J; Woyke, T; Allgaier, M; Bristow, J; Wiener-Kronish, JP; Sutherland, ER; King, TS; Icitovic, N; Martin, RJ; Calhoun, WJ; Castro, M; Denlinger, LC; DiMango, E; Kraft, M; Peters, SP; Wasserman, SI; Wechsler, ME; Boushey, HA; Lynch, SV. 2011. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. J Allergy Clin Immun. 127(2): 372-381.


Jacoby, P; Watson, K; Bowman, J; Taylor, A; Riley, TV; Smith, DW; Lehmann, D; Team, KOMR. 2007. Modelling the co-occurrence of *Streptococcus pneumoniae* with other bacterial and viral pathogens in the upper respiratory tract. Vaccine. 25(13): 2458-2464.

Jacoby, PA; Coates, HL; Arumugaswamy, A; Elsbury, D; Stokes, A; Monck, R; Finucane, JM; Weeks, SA; Lehmann, D. 2008. The effect of passive smoking on the risk of otitis media in Aboriginal and non-Aboriginal children in the Kalgoorlie-Boulder region of Western Australia. Med J Aust. 188(10): 599-603.


Khoramrooz, SS; Mirsalehian, A; Emaneini, M; Jabalameli, F; Aligholi, M; Saedi, B; Bazargani, A; Taherikalani, M; Borghaei, P; Razmpa, E. 2012. Frequency of Alloiococcus otitidis, Streptococcus pneumoniae, Moraxella catarrhalis and Haemophilus influenzae in children with otitis media with effusion (OME) in Iranian patients. Auris Nasus Larynx. 39(4):369-73.


Kirkham, LA; Wiertsema, SP; Mowe, EN; Bowman, JM; Riley, TV; Richmond, PC. 2010. Nasopharyngeal carriage of *Haemophilus haemolyticus* in otitis-prone and healthy children. J Clin Microbiol. 48(7): 2557-2559.


Klepac-Ceraj, V; Lemon, KP; Martin, TR; Allgaier, M; Kembel, SW; Knapp, AA; Lory, S; Brodie, EL; Lynch, SV; Bohannan, BJ; Green, JL; Maurer, BA; Kolter, R. 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. Environ Microbiol. 12(5): 1293-1303.


Kulekci, G; Ciftci, S; Keskin, F; Kilic.O.; Turkoglu, S; Badur, S; Develioglu, ON; Leblebicioglu, B; Kulekci, M. 2001. PCR analysis of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Fusobacterium nucleatum* in middle ear effusion. Anaerobe. 7(5): 241-246.


Lane, DJ; Pace, B; Olsen, GJ; Stahl, DA; Sogin, ML; Pace, NR. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc Natl Acad Sci USA. 82(20): 6955-6959.


Leach, AJ; Morris, PS; Mathews, JD. 2008b. Compared to placebo, long-term antibiotics resolve otitis media with effusion (OME) and prevent acute otitis media with perforation (AOMwiP) in a high-risk population: a randomized controlled trial. BMC Pediatr. 8: 23.

Leach, A; Morris, P; McCallum, G; Wilson, C; Stubbs, L; Beissbarth, J; Jacups, S; Hare, K; Smith-Vaughan, H. 2009. Emerging pneumococcal carriage serotypes in a high-risk population receiving universal 7-valent pneumococcal conjugate vaccine and 23-valent polysaccharide vaccine since 2001. BMC Infect Dis. 9(1): 121.


Lee, ZM-P; Bussema, C; Schmidt, TM. 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucl Acids Res. 37(suppl 1): D489-D493.
Lehmann, D; Willis, J; Moore, HC; Giele, C; Murphy, D; Keil, AD; Harrison, C; Bayley, K; Watson, M; Richmond, P. 2010. The changing epidemiology of invasive pneumococcal disease in aboriginal and non-aboriginal western Australians from 1997 through 2007 and emergence of nonvaccine serotypes. Clin Infect Dis. 50(11): 1477-1486.

Lehmann, D; Arumugaswamy, A; Elsbury, D; Finucane, J; Stokes, A; Monck, R; Jeffries-Stokes, C; McAullay, D; Coates, H; Stanley, FJ. 2008a. The Kalgoorlie Otitis Media Research Project: rationale, methods, population characteristics and ethical considerations. Paediatr Perinat Ep. 22(1): 60-71.

Lehmann, D; Tennant, MT; Silva, DT; McAullay, D; Lannigan, F; Coates, H; Coates, H; Stanley, FJ. 2003. Benefits of swimming pools in two remote Aboriginal communities in Western Australia: intervention study. BMJ. 327(7412): 415-419.

Lehmann, D; Weeks, S; Jacoby, P; Elsbury, D; Finucane, J; Stokes, A; Monck, R; Coates, H; the Kalgoorlie Otitis Media Research Project Team. 2008b. Absent otoacoustic emissions predict otitis media in young Aboriginal children: A birth cohort study in Aboriginal and non-Aboriginal children in an arid zone of Western Australia. BMC Pediatr. 8(1): 32.

Leibovitz, E; Greenberg, D; Piglansky, L; Raiz, S; Porat, N; Press, J; Leiberman, A; Dagan, R. 2003. Recurrent acute otitis media occurring within one month from completion of antibiotic therapy: relationship to the original pathogen. Pediatr Infect Dis J. 22(3): 209-216.


Lu, J; Huang, Z; Yang, T; Li, Y; Mei, L; Xiang, M; Chai, Y; Li, X; Li, L; Yao, G; Wang, Y; Shen, X; Wu, H. 2011. Screening for delayed-onset hearing loss in preschool children who previously passed the newborn hearing screening. Int J Pediatr Otorhi. 75(8): 1045-1049.


Marsh, R; Smith-Vaughan, H; Hare, KM; Binks, M; Kong, F; Warning, J; Gilbert, GL; Morris, P; Leach, AJ. 2010. The nonserotypeable pneumococcus: phenotypic dynamics in the era of anticapsular vaccines. J Clin Microbiol. 48(3): 831-835.
Marsh, RL; Smith-Vaughan, H; Beissbarth, J; Hare, K; Kennedy, M; Wigger, C; Mellon, G; Stubbs, E; Gadil, JR; Pettit, A; Mackenzie, G; Tipakalippa, P; Morris, PS; Leach, AJ. 2007. Molecular characterisation of pneumococcal serotype 16F: Established predominant carriage and otitis media serotype in the 7vPCV era. Vaccine. 25(13): 2434-2436.

McAvin, JC; Reilly, PA; Roudabush, RM; Barnes, WJ; Salmen, A; Jackson, GW; Beninga, KK; Astorga, A; McCleskey, FK; Huff, WB; Niemeyer, D; Lohman, KL. 2001. Sensitive and specific method for rapid identification Streptococcus pneumoniae using real-time fluorescence PCR. J Clin Microbiol. 39(10): 3446-3451.

McCormick, DP; Grady, JJ; Diego, A; Matalon, R; Revai, K; Patel, JA; Han, Y; Chonnaitree, T. 2011. Acute otitis media severity: association with cytokine gene polymorphisms and other risk factors. Int J Pediatr Otorhi. 75(5): 708-712.


Mcmichael, JC; Zagursky, RJ; Fletcher, LD. 2005. Alloiococcus otitidis open reading frames (orfs) encoding polypeptide antigens, immunogenic compositions and uses thereof. Patent: 20050203280, USA.


Morey, P; Cano, V; Marti-Lliterals, P; Lopez-Gomez, A; Regueiro, V; Saus, C; Bengoechea, JA; Garmendia, J. 2011. Evidence for a non-replicative intracellular stage of nontypable Haemophilus influenzae in epithelial cells. Microbiology. 157(Pt 1): 234-250.

Morris, P; Ballinger, D; Leach, AJ; Koops, H; Hayhurst, B; Stubbs, L; Scott, J; Anand, A; Daby, J; Paterson, B; Yonovitz, A. 2001. "Recommendations for clinical care guidelines on the management of otitis media in Aboriginal and Torres Strait Islander populations". Canberra, Australia.


Morris, PS; Leach, AJ; Halpin, S; Mellon, G; Gadil, G; Wigger, C; Mackenzie, G; Wilson, C; Gadil, E; Torzillo, P. 2007. An overview of acute otitis media in Australian Aboriginal children living in remote communities. Vaccine. 25(13): 2389-2393.


Nakamura, S; Yang, CS; Sakon, N; Ueda, M; Tougan, T; Yamashita, A; Goto, N; Takahashi, K; Yasunaga, T; Ikuta, K; Mizutani, T; Okamoto, Y; Tagami, M; Morita, R; Maeda, N; Kawai, J; Hayashizaki, Y; Nagai, Y; Horii, T; Iida, T; Nakaya, T. 2009. Direct metagenomic detection of viral pathogens in nasal and fecal specimens using an unbiased high-throughput sequencing approach. PLoS ONE. 4(1): e4219.


Nistico, L; Kreft, R; Gieseke, A; Coticchia, JM; Burrows, A; Khampang, P; Liu, Y; Kerschner, JE; Post, JC; Lonergan, S; Sampath, R; Hu, FZ; Ehrlich, GD; Stoodley, P; Hall-Stoodley, L. 2011. Adenoid reservoir for pathogenic biofilm bacteria. J Clin Microbiol. 49(4): 1411-1420.


Pace, NR; Stahl, DA; Lane, DJ; Olsen, GJ. 1985. The analysis of natural microbial populations by ribosomal RNA sequences. Amer Soc Microbiol News. 51 4-12.

Palaci, M; Dietze, R; Hadad, DJ; Ribeiro, FKC; Peres, RL; Vinhas, SA; Maciel, ELN; do Valle Dettoni, V; Horder, L; Boom, WH; Johnson, JL; Eisenach, KD. 2007. Cavitary disease and quantitative sputum bacillary load in cases of pulmonary tuberculosis. J Clin Microbiol. 45(12): 4064-4066.


Parra, MM; Aguilar, GM; Echaniz-Aviles, G; Rionda, RG; Estrada, ML; Cervantes, Y; Pircon, JY; Van Dyke, MK; Colindres, RE; Hausdorff, WP. 2011. Bacterial etiology and serotypes of acute otitis media in Mexican children. Vaccine. 29(33): 5544-5549.

Pei, AY; Oberdorff, WE; Nossa, CW; Agarwal, A; Chokshi, P; Gerz, EA; Jin, Z; Lee, P; Yang, L; Poles, M; Brown, SM; Sotero, S; DeSantis, T; Brodie, E; Nelson, K; Pei, Z. 2010. Diversity of 16S rRNA genes within individual prokaryotic genomes. Appl Environ Microbiol. 76(12): 3886-3897.

Peterson, J; Garges, S; Giovanni, M; McInnes, P; Wang, L; Schloss, JA; Bonazzi, V; McEwen, JE; Wetterstrand, KA; Deal, C; Baker, CC; Di, F; V; Howcroft, TK; Karp, RW; Lunsford, RD; Wellington, CR; Belachew, T; Wright, M; Giblin, C; David, H; Mills, M; Salomon, R; Mullins, C; Akolkar, B; Begg, L; Davis, C; Grandison, L; Humble, M; Khalsa, J; Little, AR; Peavy, H; Pontzer, C; Portnoy, M; Sayre, MH; Starke-Reed, P; Zakhari, S; Read, J; Watson, B; Guyer, M. 2009. The NIH Human Microbiome Project. Genome Res. 19(12): 2317-2323.


Post, JC; Aul, JJ; White, GJ; Wadowsky, RM; Zavoral, T; Tabari, R; Kerber, B; Doyle, WJ; Ehrlich, GD. 1996. PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media. Am J Otolaryngol. 17(2): 106-111.

Prymula, R; Kriz, P; Kaliskova, E; Pascal, T; Poolman, J; Schuerman, L. 2009. Effect of vaccination with pneumococcal capsular polysaccharides conjugated to Haemophilus influenzae-derived protein D on nasopharyngeal carriage of Streptococcus pneumoniae and H. influenzae in children under 2 years of age. Vaccine. 28(1): 71-78.

Prymula, R; Peeters, P; Chrobok, V; Kriz, P; Novakova, E; Kaliskova, E; Kohl, I; Lommel, P; Poolman, J; Prieels, JP; Schuerman, L. 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. Lancet. 367(9512): 740-748.


Rogers, G; Russell, L; Preston, P; Marsh, P; Collins, J; Saunders, J; Sutton, J; Fine, D; Bruce, K; Wright, M. 2010a. Characterisation of bacteria in ascites – reporting the potential of culture-independent, molecular analysis. Eur J Clin Microbiol. 29(5): 533-541.


Rogers, GB; Carroll, MP; Serisier, DJ; Hockey, PM; Jones, G; Kehagia, V; Connett, GJ; Bruce, KD. 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. J Clin Microbiol. 44(7): 2601-2604.

Rogers, GB; Carroll, MP; Serisier, DJ; Hockey, PM; Kehagia, V; Jones, GR; Bruce, KD. 2005. Bacterial activity in cystic fibrosis lung infections. Respir Res. 6:49. 49.

Rogers, GB; Hart, CA; Mason, JR; Hughes, M; Walshaw, MJ; Bruce, KD. 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 41(8): 3548-3558.

Rogers, GB; Marsh, P; Stressmann, AF; Allen, CE; Daniels, TV; Carroll, MP; Bruce, KD. 2010b. The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. Clin Microbiol Infect. 16(11): 1656-1658.

Rogers, G; Daniels, T; Tuck, A; Carroll, M; Connett, G; David, G; Bruce, K. 2009. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. BMC Pulm Med. 9(1): 14.

Rogers, GB; Skelton, S; Serisier, DJ; van der Gast, CJ; Bruce, KD. 2010c. Determining cystic fibrosis-affected lung microbiology: comparison of spontaneous and serially induced sputum samples by use of terminal restriction fragment length polymorphism profiling. J Clin Microbiol. 48(1): 78-86.


Rovers, MM; Glasziou, P; Appelman, CL; Burke, P; McCormick, DP; Damoiseaux, RA; Gaboury, I; Little, P; Hoes, AW. 2006. Antibiotics for acute otitis media: a meta-analysis with individual patient data. The Lancet. 368(9545): 1429-1435.


Rye, MS; Wiertsema, SP; Scaman, ES; Oommen, J; Sun, W; Francis, RW; Ang, W; Pennell, CE; Burgner, D; Richmond, P; Vijayasekaran, S; Coates, HL; Brown, SD; Blackwell, JM; Jamieson, SE. 2011. FBXO11, a regulator of the TGF-β pathway, is associated with severe otitis media in Western Australian children. Genes Immun. 12(5): 352-359.


Saulnier, DM; Riehle, K; Mistretta, T; Diaz, MA; Mandal, D; Raza, S; Weidler, EM; Qin, X; Coarfa, C; Milosavljevic, A; Petrosino, JF; Highlander, S; Gibbs, R; Lynch, SV; Shulman, RJ; Versalovic, J. 2011. Gastrointestinal microbiome signatures of pediatric patients with irritable bowel syndrome. Gastroenterology. 141(5): 1782-1791.


Schutte, UM; Abdo, Z; Bent, SJ; Shyu, C; Williams, CJ; Pierson, JD; Forney, LJ. 2008. Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. Appl Microbiol Biotechnol. 80(3): 365-380.


Singleton, RJ; Hennessy, TW; Bulkow, LR; Hammit, LL; Zulz, T; Hurlburt, DA; Butler, JC; Rudolph, K; Parkinson, A. 2007. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaskan native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA. 297(16): 1784-1792.


Smith, CJ; Danilowicz, BS; Clear, AK; Costello, FJ; Wilson, B; Meijer, WG. 2005. T-Align, a web-based tool for comparison of multiple terminal restriction fragment length polymorphism profiles. FEMS Microbiol Ecol. 54(3): 375-380.


Smith-Vaughan, HC; Leach, AJ; Shelby-James, TM; Kemp, K; Kemp, DJ; Mathews, JD. 1996. Carriage of multiple ribotypes of non-encapsulated Haemophilus influenzae in aboriginal infants with otitis media. Epidemiol Infect. 116(2): 177-183.


Smith-Vaughan, HC; Sriprakash, KS; Leach, AJ; Mathews, JD; Kemp, DJ. 1998. Low genetic diversity of Haemophilus influenzae type b compared to nonencapsulated H. influenzae in a population in which H. influenzae is highly endemic. Infect Immun. 66(7): 3403-3409.


Stephen, A; Leach, AJ; Morris, PS; Boyle, L; Hare, K; Hampton, V. 2011."A randomised controlled trial of swimming versus no swimming on resolution of ear discharge associated with chronic suppurative otitis media". Presented at the 10th International Symposium of Recent Advances in Otitis Media, New Orleans, USA.

Stressmann, FA; Connett, GJ; Goss, K; Kollamparambil, TG; Patel, N; Payne, MS; Puddy, V; Legg, J; Bruce, KD; Rogers, GB. 2010. The use of culture-independent tools to characterize bacteria in endo-tracheal aspirates from pre-term infants at risk of bronchopulmonary dysplasia. J Perinat Med. 38(3): 333-337.

Stressmann, FA; Rogers, GB; Chan, SW; Howarth, PH; Harries, PG; Bruce, KD; Salib, RJ. 2011. Characterization of bacterial community diversity in chronic rhinosinusitis infections using novel culture-independent techniques. Am J Rhinol Allergy. 25(4): e133-e140.


Thompson, JR; Marcelino, LA; Polz, MF. 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucl Acids Res. 30(9): 2083-2088.

Thornton, RB; Rigby, PJ; Wiertsema, SP; Filion, P; Langlands, J; Coates, HL; Vijayasekaran, S; Keil, AD; Richmond, PC. 2011. Multi-species bacterial biofilm and intracellular infection in otitis media. BMC Pediatr. 11:94.


Tunney, MM; Klem, ER; Fodor, AA; Gilpin, DF; Moriarty, TF; McGrath, SJ; Muhlebach, MS; Boucher, RC; Cardwell, C; Doering, G; Elborn, JS; Wolfgang, MC. 2011. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax. 66(7): 579-584.


van der Zee, A; Peeters, M; de Jong, C; Verbakel, H; Crielaard, JW; Claas, ECJ; Templeton, KE. 2002. Qiagen DNA extraction kits for sample preparation for Legionella PCR are not suitable for diagnostic purposes. J Clin Microbiol. 40(3): 1126.

Vergison, A; Dagan, R; Arguedas, A; Bonhoeffer, J; Cohen, R; DHooge, I; Hoberman, A; Liese, J; Marchisio, P; Palmu, AA; Ray, GT; Sanders, EA; Simoes, EA; Uhari, M; van Eldere, J; Pelton, SI. 2010. Otitis media and its consequences: beyond the earache. Lancet Infect Dis. 10(3): 195-203.


Wang, X; Mair, R; Hatcher, C; Theodore, MJ; Edmond, K; Wu, HM; Harcourt, BH; Carvalho, MdG; Pimenta, F; Nymadawa, P; Altantsetseg, D; Kirsch, M; Satola, SW; Cohn, A; Messonnier, NE; Mayer, LW. 2011. Detection of bacterial pathogens in Mongolia meningitis surveillance with a new real-time PCR assay to detect Haemophilus influenzae. Int J Med Microbiol. 301(4): 303-309.


Watson, K; Carville, K; Bowman, J; Jacoby, P; Riley, TV; Leach, AJ; Lehmann, D. 2006. Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. Pediatr Infect Dis J. 25(9): 782-790.

Weckwerth, PH; de Magalhaes Lopes, CA; Duarte, MA; Weckwerth, AC; Martins, CH; Neto, DL; de Aguiar, HF. 2009. Chronic suppurative otitis media in cleft palate: microorganism etiology and susceptibilities. Cleft Palate Craniofac J. 46(5): 461-467.


Williams, CJ; Coates, HL; Pascoe, EM; Axford, Y; Nannup, I. 2009a. Middle ear
disease in Aboriginal children in Perth: analysis of hearing screening data, 1998-

Williams, CJ; Jacobs, AM. 2009b. The impact of otitis media on cognitive and

Blackwell Publishing Ltd. Malden, USA.

Winther, B; Gwaltney, JM, Jr.; Phillips, CD; Hendley, JO. 2005. Radiopaque
contrast dye in nasopharynx reaches the middle ear during swallowing and/or

Winther, B; Alper, CM; Mandel, EM; Doyle, WJ; Hendley, JO. 2007. Temporal
relationships between colds, upper respiratory viruses detected by polymerase chain
reaction, and otitis media in young children followed through a typical cold season.


Xie, J; He, Z; Liu, X; Liu, X; Van Nostrand, JD; Deng, Y; Wu, L; Zhou, J; Qiu,
77(3): 991-999.

Xu, L; McLennan, SV; Lo, L; Natfaji, A; Bolton, T; Liu, Y; Twigg, SM; Yue,

Yao, H; Gao, Y; Nicol, GW; Campbell, CD; Prosser, JI; Zhang, L; Han, W;
Singh, BK. 2011. Links between ammonia oxidizer community structure, abundance,

Zemanick, ET; Wagner, BD; Sagel, SD; Stevens, MJ; Accurso, FJ; Harris, JK.
2010. Reliability of quantitative real-time PCR for bacterial detection in cystic

Zhang, H; DiBaise, JK; Zuccolo, A; Kudrna, D; Braidotti, M; Yu, Y;
Parameswaran, P; Crowell, MD; Wing, R; Rittmann, BE; Krajmalnik-Brown,
R. 2009. Human gut microbiota in obesity and after gastric bypass. Proceedings of
the National Academy of Sciences. 106(7): 2365-2370.

Zhang, W; Case, S; Bowler, RP; Martin, RJ; Jiang, D; Chu, HW. 2011.
Cigarette smoke modulates PGE(2) and host defence against Moraxella catarrhalis
infection in human airway epithelial cells. Respirology. 16(3): 508-516.

Zhou, J. 2008."Functional Gene Arrays". Presented at the 12th International
Symposium on Microbial Ecology, Cairns, Australia.
Zhou, Y; Lin, P; Li, Q; Han, L; Zheng, H; Wei, Y; Cui, Z; Ni, Y; Guo, X. 2010. Analysis of the microbiota of sputum samples from patients with lower respiratory tract infections. Acta Biochim Biophys Sin (Shanghai). 42(10): 754-761.

Appendix A
Appendix A: Media and Solutions

MEDIA
Culture media were purchased from Oxoid. Additional media were prepared as described below.

Skim milk tryptone glucose glycerol broth (STGGB)
- 30g/L Tryptone Soy Broth (Oxoid)
- 5g/L Glucose (Ajax)
- 20g/L Skim milk powder (Oxoid)
- 10% v/v Glycerol (Merck)

Brain-heart-infusion (BHI) agar with 6.5% NaCl
- 47g/L Brain Heart Infusion agar (Oxoid)
- 6.5% w/v NaCl (Crown Scientific)

SOLUTIONS
Agarose gel loading dye
- 312.5 mM Tris-Cl, pH 6.8
- 50% Glycerol
- 0.05% Bromophenol Blue (Acros)

Enzymatic lysis buffer
- 36.25 mM Phosphate buffer, pH 6.7
- 1 mg/mL Lysozyme (Sigma®)
- 0.75 mg/mL Mutanolysin (Sigma®)
- 2 mg/mL Proteinase K (QIAGEN)

50mM Phosphate buffer, pH 6.7
- 2.04 mL 1M Na₂HPO₄ (Merck)
- 2.96 mL 1M NaH₂PO₄ (BDH AnalAR®)
- 95 mL Sterile water

1xTAE
- 4.84 mg/mL Tris base (Merck)
- 0.11% v/v Glacial Acetic Acid (BDH AnalAR®)
- 1 mM EDTA (BDH AnalAR®)

Tris-Cl
- 121.1 g/L Tris base
- 157.6 g/L Trizma-HCl (Sigma®)
- Adjust to the desired pH with HCl.

1 x Tris-EDTA (TE).
- 10mM Tris-Cl, pH 8.0
- 1mM EDTA, pH 8.0
Appendix B: 16S rRNA gene reference set used for *in silico* analyses

This table describes the general reference set used for Chapter 5 *in silico* analyses. Sequences in this reference set were downloaded from the Ribosomal Database Project as described in Chapter 5.3.1.

* = 16S rRNA gene sequences obtained from whole genome sequence.
RDP = Ribosomal Database Project.

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Appendix C and D
Appendices C and D: OTUs detected by PhyloChip™ analysis of ear discharge swabs from Indigenous children with acute otitis media with perforation.

Appendix C and D are available as .pdf files on the accompanying CD (attached below).