

# **The microbiome of otitis media with effusion and the influence of *Alloiococcus otitidis* on *Haemophilus influenzae* in polymicrobial biofilm**

**Chun L Chan**

**Bachelor of Radiography and Medical Imaging (Honours)**

**Bachelor of Medicine, Bachelor of Surgery**

---

Department of Otolaryngology, Head and Neck Surgery

University of Adelaide, Adelaide, Australia

Submitted for the title of Doctor of Philosophy

November 2016

This thesis is dedicated to those who have sacrificed  
the most during my scientific endeavours

My amazing family  
Flora, Aidan and Benjamin

## Table of Contents

TABLE OF CONTENTS .....	III
THESIS DECLARATION.....	VII
ACKNOWLEDGEMENTS.....	VIII
THESIS SUMMARY.....	X
PUBLICATIONS ARISING FROM THIS THESIS.....	XII
PRESENTATIONS ARISING FROM THIS THESIS.....	XIII
ABBREVIATIONS.....	XV
LIST OF TABLES.....	XVII
LIST OF FIGURES .....	XVIII
<b>CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW .....</b>	<b>1</b>
1.1 OTITIS MEDIA WITH EFFUSION .....	2
1.1.1 <i>Background</i> .....	2
1.1.2 <i>Epidemiology and natural history</i> .....	3
1.1.3 <i>Aetiology and pathogenesis</i> .....	3
1.1.3 <i>Clinical symptoms and diagnosis of OME</i> .....	13
1.1.4 <i>Management of otitis media with effusion</i> .....	14
1.2 THE ROLE OF BACTERIA IN OTITIS MEDIA.....	23
1.2.1 <i>Detection of bacteria and its advances</i> .....	23
1.2.2 <i>The microbiota of the middle ear</i> .....	31
1.2.3 <i>The nasopharynx as a reservoir for otitis media</i> .....	33
1.2.4 <i>The external ear canal as a potential reservoir for otitis media</i> .....	35
1.2.5 <i>Bacteria associated with otitis media</i> .....	37

1.2.6	<i>The role of biofilm in otitis media</i> .....	51
1.3	SUMMARY OF LITERATURE REVIEW .....	55
1.4	AIMS .....	57
<b>CHAPTER 2: THE MICROBIOME OF OTITIS MEDIA WITH EFFUSION</b> .....		<b>58</b>
2.1	ABSTRACT .....	61
2.2	INTRODUCTION .....	62
2.3	MATERIALS AND METHODS .....	63
2.3.1	<i>Selection Criteria</i> .....	63
2.3.2	<i>Sample collection</i> .....	63
2.3.3	<i>DNA extraction</i> .....	64
2.3.4	<i>PCR amplification of the 16S rRNA gene and sequencing</i> .....	64
2.3.5	<i>Bioinformatics pipeline</i> .....	65
2.3.6	<i>Biostatistical analysis</i> .....	65
2.4	RESULTS .....	67
2.4.1	<i>Demographic data:</i> .....	67
2.4.2	<i>Middle ear microbiome</i> .....	67
2.4.3	<i>Adenoid microbiome versus middle ear microbiome</i> .....	72
2.4.4	<i>MEF adenoid microbiome versus control</i> .....	74
2.5	DISCUSSION .....	77
2.6	CONCLUSION .....	81
<b>CHAPTER 3: THE EXTERNAL EAR CANAL AS A RESERVOIR FOR THE MIDDLE EAR: A 16S</b>		
<b>PHYLOGENETIC ANALYSIS</b> .....		<b>82</b>
3.1	ABSTRACT .....	85
3.2	INTRODUCTION .....	86

3.3	METHODS .....	87
3.3.1	<i>Study group</i> .....	87
3.3.2	<i>Sample collection</i> .....	87
3.3.3	<i>DNA extraction</i> .....	88
3.3.4	<i>PCR amplification of the 16S rRNA gene and sequencing</i> .....	88
3.3.5	<i>Bioinformatics pipeline</i> .....	88
3.3.6	<i>Biostatistical analysis</i> .....	89
3.4	RESULTS .....	91
3.4.1	<i>Microbiome of middle ear with effusion</i> .....	91
3.4.2	<i>Microbiome of the external ear canal</i> .....	91
3.4.3	<i>Comparing the microbiome of the middle ear to the external canal</i> .....	94
3.4.4	<i>Bilateral effusions</i> .....	95
3.4.5	<i>Combined with previous data set</i> .....	95
3.5	DISCUSSION .....	98
3.6	CONCLUSION.....	102

**CHAPTER 4: INDIRECT PATHOGENICITY OF ALLOIOCOCCUS OTITIDIS IN MULTISPECIES BIOFILM WITH HAEMOPHILUS INFLUENZAE: EFFECTS ON ANTIBIOTIC SENSITIVITY AND PERSISTENCE ..... 103**

4.1	ABSTRACT.....	106
4.2	BACKGROUND .....	107
4.3	METHODS .....	109
4.3.1	<i>Bacterial strains and handling</i> .....	109
4.3.2	<i>Planktonic cell assays</i> .....	109
4.3.3	<i>Biofilm assays</i> .....	110
4.3.4	<i>Crystal Violet Assay</i> .....	111

4.3.5	<i>Antibiotic susceptibility testing</i> .....	112
4.3.6	<i>Statistical Analysis</i> .....	114
4.4	RESULTS .....	114
4.4.1	<i>A. otitidis planktonic growth and biofilm formation</i> .....	114
4.4.2	<i>Co-culture of H. influenzae and A. otitidis and formation of polymicrobial biofilm</i> ...	116
4.4.3	<i>Antibiotic susceptibility</i> .....	122
4.5	DISCUSSION .....	126
4.6	CONCLUSION.....	131
<b>CHAPTER 5: FINAL DISCUSSION</b> .....		<b>132</b>
5.1	THESIS SYNOPSIS.....	133
5.2	FUTURE DIRECTIONS.....	136
5.3	CONCLUSION.....	138
<b>BIBLIOGRAPHY</b> .....		<b>139</b>

## *Thesis declaration*

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time

Chun Ling Chan

## *Acknowledgements*

I really could not have completed this PhD without the support of many others, and it is difficult to find the words to express the extent of my gratitude to the people below.

First of all, thank you to Professor PJ Wormald for offering me the opportunity to undertake this higher degree. You are the founding father and leader of our phenomenal department, which continues to be at the cutting edge in the field of not only otolaryngology research, but also in otolaryngology surgery. It has truly been a pleasure to be part of this research family for the last three years.

To Associate Professor Alkis Psaltis, who has been a mentor, an inspiration and above all, a friend during my time in the department. Your commitment to excellence, both surgically, and academically is an inspiration. Thank you for expecting of me what you would expect from yourself, it has truly been a great source of encouragement.

To Dr Sarah Vreugde, thank you for your support and counsel, especially as I grappled with laboratory life. Your guidance and encouragement were invaluable and helped me sharpen my focus to bring this work to fruition.

To my colleagues and friends from the Queen Elizabeth Hospital ENT Department, thank you for making the last three years so memorable. In particular, to Dr Jae Murphy and Dr Sakiko Oue who started this PhD journey together with me, thank you for your camaraderie, perspective and friendship throughout.

To my friends in the laboratory: Clare Cooksley, Dijana Miljkovic and Kati Richter, thank you for sharing your patience, enthusiasm and love for science with me. Thank you to my collaborators and co-authors, especially Dr Ahmed Bassiouni and Dr Jake Jervis Bardy who ventured out into the

uncharted waters of otologic microbiome research with me. Special thanks to Lyn Martin who is the cornerstone of the department.

To all of the ENT surgery staff at the Women's and Children's Hospital for putting up with my pestering and for playing an integral role in helping me collect tissue. In particular, thank you to Dr David Wabnitz, Connie Fong and all of the ENT registrars who swabbed throats and suctioned ears. Without your help, this research would not have been possible. I also owe a great debt of gratitude to our patients and their families, who graciously consent to involvement in much of this research, in the hope that we will be able to improve the lives of our future patients.

To my amazing wife Flora, your belief and love have been the foundation for my achievements, without you none of this would have been possible. You have offered unwavering support every step of the way in this journey to achieve my ambitions, often at the detriment of your own, thank you for believing in me. You have raised our boys, often in my absence, for which, I cannot thank you enough. To my two boys, Aidan and Benjamin, you are both my greatest source of joy and pride and have been my inspiration to complete this work. And finally, to my parents, Bill and Jenny, thank you for giving me the gift of education. You have given me the confidence to follow my ambitions, without ever applying pressure or criticism – thank you.

## *Thesis Summary*

The research described herein follows an extensive review of the literature on the role of bacteria in the aetiology of otitis media with effusion. Otitis media and otitis media with effusion have been classically associated with three main bacteria, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*. However, with advancements in culture-independent molecular techniques, previously un-culturable bacteria have been identified. Moreover, using these technologies researchers have been able to characterise the entire microbial populations of many body sites, which has allowed for greater insight into the host-pathogen relationship.

The initial investigation focused on characterising the microbiome of the middle ear in children with otitis media with effusion and is detailed in chapter two. This study formed the foundation for the thesis and was critical in confirming whether the adenoid pad is a bacterial reservoir for the middle ear. We collected middle ear fluid and adenoid pad swabs from children undergoing ventilation tube insertion at the Women's and Children's Hospital, Adelaide, Australia and used state of the art 16S rRNA molecular gene sequencing to characterise the microbiome of the middle ear in these patients. We found that the microbial communities within the middle ear and on the adenoid pad shared some common bacteria; however, we also discovered the presence of bacteria not found in the adenoid pad. Namely, these bacteria were *Alloiococcus*, *Corynebacterium spp.* and *Staphylococcus spp.* These bacteria are more commonly associated with colonisation of the outer ear canal, which lead us to believe that the external auditory canal could play a part in seeding the middle ear with bacteria.

The follow-on study is detailed in chapter three and addresses the question of whether or not the external auditory canal was a reservoir for the middle ear. We again returned to the Women's and Children's Hospital to collect samples. For this study, we collected middle ear fluid and also external auditory canal lavages. Upon comparisons of the microbiome, we found that both the external ear

canal and adenoid pad contribute to the microbiome of the middle ear. Additionally, upon analysis of the bacterial relationships within the middle ear effusion, we recognised that there was an inverse correlation relationship between *H. influenzae* and *A. otitidis*.

The final study addressed the question of the nature of the relationship between *H. influenzae* and *A. otitidis* and is the subject of chapter 4. Otitis media with effusion has been suggested to be a biofilm driven disease. Therefore, we sought to discover whether *A. otitidis* forms biofilm. We found that not only does *A. otitidis* form single species biofilm, but that it also forms polymicrobial biofilm with *H. influenzae*. The subsequent polymicrobial biofilm allowed the bacteria to express a new phenotype, that altered the persistence and antibiotic resistance profile of these bacteria. The findings of this study suggest that *A. otitidis* plays a previously undescribed role in the pathogenesis of otitis media with effusion.

*Publications arising from this thesis*

**The microbiome of otitis media with effusion**

**Chan C**, Wabnitz, D, Jervis-Bardy J, Bassiouni A, Vreugde, S, Wormald PJ, Psaltis AJ

The Laryngoscope, published online, 23/06/16

**The external ear canal as a reservoir for the middle ear: a 16S analysis**

**Chan C**, Wabnitz, D, Bassiouni A, Vreugde, S, Wormald PJ, Psaltis AJ

JAMA Otolaryngology, Head and Neck Surgery, accepted for publication 16/08/16 (in press)

**Indirect pathogenicity of *Alloiococcus otitidis* in multispecies biofilm with *Haemophilus influenzae*:  
effects on antibiotic sensitivity and persistence**

**Chan C**, Richter K, Wormald PJ, Psaltis AJ, Vreugde, S,

Submitted PLOS Pathogens 28/10/16 (in review)

*Presentations arising from this thesis*

**The probiotic effects of commensal bacteria on *Staphylococcus aureus***

The Australian Society of Otolaryngology Head & Neck Surgery (ASOHNS) Annual Scientific Meeting,  
Sydney, March 2015

**The microbiome of otitis media with effusion and the evolving role of *Alloiococcus otitidis***

The Australian Society of Otolaryngology Head & Neck Surgery (ASOHNS) Annual Scientific Meeting,  
Melbourne, March 2016

***Alloiococcus otitidis* forms multispecies biofilm with *Haemophilus influenzae*: effects on antibiotic sensitivity and persistence**

The Australian Society of Otolaryngology Head & Neck Surgery (ASOHNS) Annual Scientific Meeting,  
Adelaide, March 2017



## Abbreviations

<b>A. Otitidis</b>	<i>Alloiococcus otitidis</i>
<b>AO</b>	<i>Alloiococcus otitidis</i>
<b>AGRF</b>	Australian Genomics Research Foundation
<b>ANOVA</b>	Analysis of variance
<b>AOM</b>	Acute otitis media
<b>AR</b>	Allergic rhinitis
<b>ASOHNS</b>	The Australian Society of Otolaryngology Head & Neck Surgery
<b>ATCC</b>	American Type Culture Collection
<b>BHI</b>	Brain heart infusion
<b>BHib</b>	Brain heart infusion supplemented 5% sheep blood
<b>BHIs</b>	Brain heart infusion supplemented with factor V and X
<b>BLAST</b>	Basic Local Alignment Search Tool
<b>BLNAR</b>	$\beta$ -lactamase negative, ampicillin resistance
<b>BLPACR</b>	$\beta$ -lactamase positive, ampicillin and ampicillin/clavulanate resistant
<b>CSLM</b>	Confocal scanning laser microscopy
<b>CSOM</b>	Chronic suppurative otitis media
<b>DNA</b>	Deoxyribonucleic acid
<b>EAC</b>	External auditory canal
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>EPS</b>	Extracellular polysaccharide
<b>ET</b>	Eustachian tube
<b>Factor V</b>	Hemin
<b>Factor X</b>	Nicotinamide adenine dinucleotide
<b>FISH</b>	Fluorescence in situ hybridization
<b><i>H. influenzae</i></b>	<i>Haemophilus influenzae</i>
<b>HI</b>	<i>Haemophilus influenzae</i>
<b>HiB</b>	<i>Haemophilus influenzae</i> type B
<b><i>M. catarrhalis</i></b>	<i>Moraxella catarrhalis</i>
<b>LPR</b>	Laryngopharyngeal reflux
<b>MBC</b>	Minimum inhibitory concentration
<b>MEM</b>	Middle ear mucosa

<b>MIC</b>	Minimum bactericidal concentration
<b>MSSA</b>	Methicillin-sensitive <i>S. aureus</i>
<b>MRSA</b>	Methicillin-resistant <i>S. aureus</i>
<b>NCBI</b>	National Centre for Biotechnology Information
<b>NP</b>	Nasopharyngeal
<b>NT</b>	Non-typeable
<b>NTHi</b>	Non-typeable <i>Haemophilus influenzae</i>
<b>OM</b>	Otitis media
<b>OME</b>	Otitis media with effusion
<b>OTU</b>	Operational taxonomic unit
<b><i>P. aeruginosa</i></b>	<i>Pseudomonas aeruginosa</i>
<b>PEAR</b>	Paired-End reAd merger
<b>PERMANOVA</b>	Permutational analysis of variance
<b>PCR</b>	Polymerase chain reaction
<b>PCV</b>	Pneumococcal conjugate vaccine
<b>PCV 7</b>	7 valent (heptavalent) pneumococcal conjugate vaccine
<b>PCV13</b>	13 valent pneumococcal conjugate vaccine
<b>PPV</b>	Pneumococcal polysaccharide vaccine
<b><i>r</i></b>	Pearson's co-efficient of correlation
<b>rAOM</b>	Recurrent acute otitis media
<b>rRNA</b>	Ribosomal ribonucleic acid
<b><i>S. aureus</i></b>	<i>Staphylococcus aureus</i>
<b>SD</b>	Standard deviation
<b><i>S. pneumoniae</i></b>	<i>Streptococcus pneumoniae</i>
<b>SCFU</b>	Single colony forming units
<b>SEM</b>	Scanning electron microscopy

## List of Tables

Table 1-1	<b>Studies reporting the incidence of <i>A. otitidis</i> in otitis media with effusion using polymerase chain reaction</b>	38
Table 1-2	<b>Studies reporting the incidence of <i>A. otitidis</i> in acute otitis media using polymerase chain reaction</b>	39
Table 1-3	<b>Studies reporting the incidence of <i>A. otitidis</i> from samples of the nasopharynx and external auditory canal using polymerase chain reaction</b>	40
Table 2-1	<b>The bacteria found on the adenoid pad and from middle ear effusion</b>	69
Table 2-2	<b>Bacterial correlations in MEF</b>	75
Table 3-1	<b>Distribution of bacteria from the adenoid pad, external ear canal and middle ear fluid</b>	93
Table 4-1	<b>Minimum inhibitory concentrations and minimum bactericidal concentration for <i>Alloicoccus otitidis</i> in planktonic and biofilm antibiotic susceptibility</b>	123
Table 4-2	<b>Minimum inhibitory concentrations and minimum bactericidal concentration for <i>H. influenzae</i> strains in planktonic and biofilm antibiotic susceptibility</b>	125

## List of Figures

Figure 1-1	<b>16S rRNA gene</b>	26
Figure 1-2	<b>Descriptions of microbiome data</b>	28
Figure 2-1	<b>The microbiome of otitis media with effusion.</b>	68
Figure 2-2	<b>Bacterial correlations within middle ear fluid:</b>	70
Figure 2-3	<b>Cluster analysis of the similarities between MEF samples</b>	71
Figure 2-4	<b>Microbiome of bilateral MEF</b>	72
Figure 2-5	<b>Principal Co-ordinates Analysis of the microbiota of the adenoid and MEF</b>	73
Figure 2-6	<b>Co-occurrence comparisons of the adenoid pad and MEF</b>	74
Figure 2-7	<b>Bacterial correlations on the adenoid pad</b>	76
Figure 3-1	<b>Comparing the microbiome of the adenoid, middle ear and external ear canal</b>	92
Figure 3-2	<b>Bacterial correlations within the middle ear and external canal</b>	94
Figure 3-3	<b>Microbiome of bilateral ear effusions</b>	96
Figure 3-4	<b>Microbiome of the middle ear over time</b>	97
Figure 4-1	<b><i>A. otitidis</i> growth in BHIs and BHlb</b>	115
Figure 4-2	<b>Confocal laser scanning microscopy image of <i>A. otitidis</i> biofilm</b>	116
Figure 4-3	<b>Confocal scanning laser microscopy images of <i>A. otitidis</i> and <i>H. influenzae</i> polymicrobial biofilm</b>	118
Figure 4-4	<b>Scanning electron microscopy image of <i>A. otitidis</i> biofilm</b>	118
Figure 4-5	<b>Crystal violet assay of mono and polymicrobial biofilm</b>	119
Figure 4-6	<b><i>H. influenzae</i> growth alone and in combination with <i>A. otitidis</i> in reduced media</b>	120
Figure 4-7	<b><i>H. influenzae</i> growth alone and in combination with <i>A. otitidis</i> at 30°C</b>	121

## *Chapter 1: Introduction and literature review*

## 1.1 *Otitis media with effusion*

### 1.1.1 *Background*

It is likely that humans have always suffered from infections of the middle ear and its associated sequelae. Evidence of perforations of the tympanic membrane and destruction of the mastoid were identified in 2600 year old Egyptian mummies<sup>1</sup>. Today, otitis media continues to be a worldwide problem for children. Otitis media (OM) refers to a spectrum of disease involving the middle ear; however, its nomenclature and classification have changed over the years.

Acute otitis media (AOM) represents acute suppurative inflammation of the middle ear. It manifests with local symptoms of otalgia and otorrhea and can be associated with systemic symptoms including fever, irritability, anorexia, nausea, vomiting and even diarrhoea. In AOM without perforation, the tympanic membrane bulges into the external auditory canal and often appears opaque, with reduced mobility of the tympanic membrane upon pneumatic otoscopy. Recurrent AOM (rAOM) is defined as multiple discreet episodes of AOM, with greater than three episodes in 6 months, or greater than 4 in 12 months<sup>2</sup>. rAOM is further distinguished into two categories: acute infection with total resolution of middle ear fluid between attacks and those with AOM with underlying persistent otitis media with effusion (OME). Also, acute otitis media can result in a perforation of the tympanic membrane in up to 30% of cases<sup>3</sup>. AOM with perforation describes AOM resulting in the rupture tympanic membrane, with subsequent discharge from the middle ear within 7 days of infection<sup>2</sup>. Furthermore, for patients with existing tympanostomy tubes (also known as “grommets” or ventilation tubes), AOM resulting in otorrhea is also classified as AOM with perforation. If purulent otorrhea persists for greater than 2 weeks, then this is further defined as chronic suppurative otitis media (CSOM).

Otitis media with effusion, in contrast to AOM, is defined as fluid in the middle ear without local or systemic illness. OME has been synonymously referred to as “glue ear” and “serous otitis media” in the past. However, despite the absence of the acute ear, OME is associated with significant sequelae. It is the commonest cause of deafness<sup>4</sup> and balance disorder in childhood<sup>5</sup>, and may result in development delays and behaviour changes. Examination of the middle ear with pneumatic otoscopy may reveal a retracted tympanic membrane with impaired mobility, but there are a spectrum of changes, including visualisation of fluid itself or air fluid levels and bubbles.

### *1.1.2 Epidemiology and natural history*

Otitis media with effusion (OME) is predominantly a disease of childhood with its incidence peaking at two age groups<sup>6</sup>: between the ages of 6 and 24 months, which coincides with infants weaning and being exposed to environmental conditions; and a smaller secondary peak at age 4–5 years, when children enter child care or kindergarten. The rates then decrease to an incidence of around 8% at 8 years of age<sup>7</sup>.

It has been estimated that the point prevalence of OME at age two is 1 in 5 children, with 80 % of children affected at least temporarily by the age of 10 years<sup>8</sup>. In Australia, 73% of infants will suffer at least one episode of otitis media in the first 12 months of life. Furthermore, it is estimated that for the children born in 2008, that they would suffer a total of 1.2 million episodes of OM collectively by the age of five. Whilst these figures represent episodes of AOM, it has been reported that 45% of children with AOM have a persistent middle ear effusion at 1 month and 10% at 3 months<sup>6</sup>. This suggests that in Australia, close to a quarter of a million children will have OME each year.

### *1.1.3 Aetiology and pathogenesis*

The pathogenesis of otitis media with effusion is thought to be multifactorial and involves a combination of host and environmental factors. Regardless of the mechanism, the resulting middle

ear effusions are composed of water, cells and cell debris, electrolytes and other high molecular weight compounds, which include mucins, proteins, lipids and DNA<sup>4</sup>. In addition, bacterial products, immunoglobulins, lysozyme, lactoferrin, complement components, leukotrienes, and cytokines have also been identified<sup>4</sup>.

### ***1.1.2.1 Host factors***

There are a number of host factors that contribute to the formation of OME. These include inheritable factors, anatomical variation, and birth factors, such as low birth weight and being born at low gestational age<sup>9</sup>.

#### ***1.1.2.1.1 Genetic factors***

As discussed above, the incidence of OME is associated with age. In addition, gender plays a role, with a higher preponderance for boys to suffer from otitis media<sup>6</sup>. Furthermore, genetic factors are associated with otitis media: in a Norwegian study of twin pairs, it was demonstrated that otitis media could be inherited, with a 74% heritability of OM in girls and 45% in boys<sup>10</sup>. Likewise, the *HLA-A2* gene has been noted to be associated with rAOM, despite not being found to be the case in OME<sup>11</sup>.

Another inherited factor that may predispose to OME is in the form of the genes regulating mucin production. Mucins, secreted by goblet cells from the epithelium of the middle ear, play a significant role in otitis media by influencing the physical properties of the effusion. Mucins are large complex glycoproteins that make up 8-32% of non-dialysable solids within effusions samples<sup>4</sup> and are responsible for the viscous and gel-like nature of middle ear effusions. Higher concentrations of mucins have been shown to correlate with increased viscosity<sup>12</sup>, which can subsequently lead to impaired effusion clearance. At least two different molecules of mucin are associated with middle ear effusions; respiratory mucin MUC 5AC and MUC 5B encoded at chromosome 11p15<sup>4</sup>.

In addition, heritable diseases that affect mucociliary clearance can lead to OME. For instance, patients with primary ciliary dyskinesia, a hereditary defect in the ultrastructure of ciliated epithelium, almost universally suffer from OME, due to poor ciliary motility and thus clearance of effusion from the middle ear<sup>13,14</sup>.

#### *1.1.2.1.2 Eustachian tube dysfunction*

Eustachian tube (ET) dysfunction has long been regarded as a major underlying pathophysiological event that leads to the development of OME. The ET is a muscular tube that connects the middle ear to the nasopharynx and shares a common embryological origin with the middle ear from the first branchial pocket<sup>15</sup>. It is lined with respiratory epithelium, and its role is to help ventilate the ear via direct clearance of middle ear fluid and to also maintain the pressure gradient between the outer and middle ears. Compared with adults, the orientation of the ET in children is more horizontal, shorter and with reduced rigidity, which has been thought to increase the risk of OM<sup>16</sup>. This is reflected by the fact that when ET function normalises with age (~3-7 years) that it correlates with a decrease in the prevalence of OME.

In 1867, Pulitzer first described his “hydrops ex vacuo” theory to explain the mechanism leading to OM, which suggests that physical obstruction (i.e. adenoid tissue, nasopharyngeal lesion, etc.) of the ET in the nasopharynx leads to a negative pressure in the middle ear<sup>16,17</sup>. As a result, oxygen within the middle ear is absorbed and not replaced, with a transudate of fluid instead filling the middle ear due to the negative pressure. However, this theory has some flaws, including that the effusion within the middle ear is often an actively secreted exudate and that the model does not adequately explain the inflammation and metaplasia associated with OME<sup>18</sup>.

More recent beliefs centre around dysfunction of the ET, rather than merely ET obstruction that contributes to OME. Children with poorer active ET function (i.e. the ability to equilibrate pressure

differences between the nasopharynx and middle ear on swallowing) have been shown to be more likely to develop OME<sup>19,20</sup>.

However, there is still some controversy as to whether ET dysfunction is the primary cause of OME or whether it is a sequela of prolonged inflammation. Animal studies have suggested that occlusion of the ET, while creating a negative middle ear pressure, does not produce an effusion in the absence of bacterial superinfection<sup>21</sup>. Also, there is some debate as to whether adenoidectomy for children with recurrent OME is effective because of the removal of potential obstruction, or rather due to the removal of a source of ascending infection<sup>22-24</sup>. Therefore, it is likely that a combination of ET dysfunction and infection occurs, which results in the formation of otitis media with effusion.

#### **1.1.2.1.3 Cleft palate**

Otitis media with effusion affects 92-97% of children with cleft palate in their first year of life and persists in 70% of these children at age 4<sup>25,26</sup>. The mechanism behind this high incidence is again thought to be ET dysfunction. The tensor and levator palatine muscles originate from the cartilaginous ET and insert into the soft palate. However, the midline raphe are deficient in cleft palate, which subsequently results in the ineffective opening of the ET<sup>27</sup>. In addition, there is likely an impaired middle ear immune defence system as children with cleft palate have effusions with higher levels of TNF-alpha and IL-8<sup>28</sup> and a greater chance of bacteria (55%) being cultured from middle ear aspirates than those without (32%) cleft palate<sup>29</sup>.

#### **1.1.2.1.4 Laryngopharyngeal reflux**

Laryngopharyngeal reflux has been suggested to play a role in OME as the middle ear is continuous with the upper aerodigestive tract. As discussed above, due to ET angulation and functional immaturity in paediatric patients, it is possible that laryngopharyngeal reflux (LPR) can result in gastric contents translocating into the middle ear, via the nasopharynx. Pepsin and pepsinogen are

digestive enzymes of the stomach and have been used as an indirect marker of reflux of gastric material into the middle ear, with both found in high concentration from the effusions of some middle ear aspirates<sup>30</sup>. Also, *Helicobacter pylori* DNA, derived from bacteria exclusive to the stomach and duodenum, has been reported to be found in 6-18% of middle ear effusions using polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA)<sup>31</sup>. However, despite these findings, Kariya et al., in their review of the literature, concluded that the significance of LPR in the pathogenesis remains unclear due to inconsistent findings<sup>32</sup>.

### 1.1.2.2 Environmental factors

A number of environmental factors have been implicated in increasing the risk of otitis media and otitis media with effusion. Many of these factors do not exclusively impact otitis media, but are general public health issues, and as such have been the subject of interest to policy makers and public health advocates. Examples of this include advocating for breastfeeding<sup>33</sup> and reduction of tobacco smoking and childhood exposure<sup>34,35</sup>.

#### 1.1.2.2.1 Bacterial and Viral Infection

The bacterial microbiology of OME will be discussed in depth in section 1.2; however, bacterial infection has been implicated to play a key role in the aetiology of OM and OME. As an example, there is a seasonal variation in the incidence of OME, with this winter preponderance attributed to the increased exposure to upper respiratory tract infections in this period<sup>36</sup>.

The bacterium most frequently identified in patients with AOM is *Streptococcus pneumoniae* (*S. pneumoniae*), followed closely by nontypeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* (*M. catarrhalis*). *S. pneumoniae* is identified in 27.8% of AOM cases, *Haemophilus influenzae* (*H. influenzae*) in 23.1% and *M. catarrhalis* in 7.0%. In contrast, NTHi is more commonly identified in patients with recurrent AOM and AOM with tympanic membrane perforation (22.8% NTHi vs 18.6% for *S. pneumoniae* and 4.1% *M. catarrhalis*)<sup>37</sup>. In the setting of OME, bacteria are less likely to be identified, with approximately half the average frequency compared to AOM. Despite this, NTHi is still considered the most common pathogen identified (11.6%), followed by *S. pneumoniae* (6.5%)<sup>37</sup>. However, with the increased use of culture-independent techniques, *Alloiococcus otitidis* (*A. otitidis*) has since emerged as a bacteria that is commonly demonstrated in middle ear aspirates from OME (up to 60%)<sup>38</sup>.

In addition, viruses are also a cause of acute otitis media and subsequently predispose to OME<sup>39</sup>. Viral infection is often found to precede bacterial super-infection<sup>40</sup>, but can also be the sole causative agent<sup>41</sup>. The rates of co-infections have varied between 5 to 66%<sup>42</sup>. In co-infection, influenzae A has been shown to promote nasopharyngeal colonisation and dissemination of *S. pneumoniae*<sup>43</sup>. Additionally, the presence of rhinovirus infection, has been shown to significantly change the microbiota of the nasopharynx compared to controls<sup>44</sup>. All respiratory viruses can contribute to this process, but the most common viruses identified from middle ear effusions are respiratory syncytial virus, influenza A, rhinovirus and adenovirus<sup>45-47</sup>.

The classical proposed mechanism for bacterial and viral infection as causative events of OME is as follows: inflammation, whether by viral or bacterial upper respiratory tract infection, results in inflammation and immunomodulation of the mucosa in the nasal cavities and nasopharynx, causing functional obstruction of the ET. A negative pressure develops within the middle ear (as discussed above) and microbe-containing secretions from the upper airway mucosa are drawn into the middle ear owing to the pressure differential. After seeding the middle ear, bacterial replication and infection may subsequently result<sup>48,49</sup>.

#### 1.1.2.2.2 Allergy

Infants with allergy have a 2-4.5-fold increased risk of incidence of OME compared to non-allergic people<sup>50,51</sup>. Specifically, allergic rhinitis (AR) has been implicated in the development of OME, yet, this association is still under debate. One cohort study from birth by Kreiner-Moller et al.<sup>52</sup> showed an association between OME and AR with an odds ratio of 3.36. In addition, patients with AR have been demonstrated to have a higher prevalence of OME than controls<sup>53</sup> and that 50% of children with OME have co-morbid AR<sup>54</sup>.

A likely mechanism of OME in patients with AR is that allergic inflammation of the respiratory epithelium that lines the ET and middle ear, resulting in mucosal swelling, ET dysfunction, reduced mucociliary efficacy and delayed middle ear fluid clearance<sup>55,56</sup>. However, as suggested by the unified upper airway concept, there is evidence that the middle ear mucosa itself also contributes to OME via an allergic reaction, with the middle ear effusions of patients showing a higher level of eosinophils, T lymphocytes, IL-4 and IL-5<sup>57</sup>. Furthermore, patients with AR have an increased level of mast cells in the nasal cavity and from the adenoid pad, with higher levels associated with a greater risk of developing a chronic OME<sup>58,59</sup>. Also, allergy can contribute to adenoid hypertrophy and thus potential ET obstruction. Quaranta et al.<sup>60</sup> found that 70% of patients with adenoidal hypertrophy presented with chronic rhinitis and 64% of them had simultaneous OME.

#### *1.1.2.2.3 Child care attendance*

Studies have shown that children who attend day-care centres have a two- to three- fold increased risk of OME<sup>61,62</sup>. Also, one in five children in full-time day-care has ventilation tubes in situ<sup>63</sup>. This phenomenon may be reflected in a threefold increase risk of upper respiratory tract infection for these children<sup>63</sup>. The reason for this increased incidence is suggested to be related to the density of children in day care and the greater exposure to respiratory pathogens, bacterial and viral. These are also the same factors that are seen from overcrowding in homes and for children with older siblings<sup>61,64</sup>.

#### *1.1.2.2.4 Tobacco smoke exposure*

Otitis media is more common in children exposed to cigarette smoke; however, a causal relationship has not yet been established. Passive cigarette smoking increases the risk of OME, with an Australian study showing that there was a 66% higher risk of recurrent acute otitis media and chronic OME in the presence of parental smoking<sup>65</sup>.

Patients with OME that have been exposed to cigarette smoke had a significantly reduced ciliary beat frequency compared to those who had not<sup>66</sup>. A mechanism behind this is thought to be due to cotinine, a major metabolite of nicotine, which has been shown to reduce the ciliary beat frequency of ciliated cells *in vitro*<sup>67</sup>. Also, the measurement of cotinine concentration in the urine or serum, has been proposed as a reliable marker for exposure to cigarette smoke<sup>68</sup>; with high levels correlating with increased incidence of AOM and longer duration of middle ear effusion after acute infection<sup>68</sup>. In addition, in laboratory conditions, buccal epithelial cells exposed to cigarette smoke showed greater adherence of bacteria, including *Pneumococci*, *Haemophilus* strains, and *Moraxella*<sup>69</sup>. Furthermore, smoke-exposed children were found to have a greater colonisation of pathogenic bacteria in their nasopharynx<sup>70</sup> compared with controls.

However, despite these findings, smoking has never been shown to lead to a middle ear effusion in animals<sup>71</sup>, nor does cigarette smoke affect the length of time for resolution of OME<sup>72</sup>. Similarly, the clinical course of otitis media is no different in smoke-exposed children versus controls<sup>73</sup>.

#### **1.1.2.2.5 Breastfeeding and pacifier use**

Breastfeeding has been reported to confer a protective effect against the development of middle ear disease over formula and bottle feeding; however, the mechanisms for this protective effect remains unclear. Proposed explanations have included that breastfeeding: places the child in a beneficial head position during feeds, improves nutrition, exposes infants to different microorganisms, and has antibacterial and immunological benefits<sup>74</sup>.

Feeding for a period of just three months has been shown to be strongly associated with a decreased risk for AOM during the first year of life<sup>75</sup>. In contrast, bottle feeding or even only partial breastfeeding did not provide these benefits<sup>76</sup>. The evidence suggests that it is the constituents of

breast milk which, are not found in formula milk, that confers protection<sup>77,78</sup>. Recent studies have focussed on the role of human milk polysaccharides in breast milk, which has been proposed to reduce infection via antiadhesive, antimicrobial effects, modulating the immune system of the infant and altering the gut microbiome<sup>79</sup>.

Also, the use of a pacifier after the age of 11 months is associated with otitis media, with an increase in risk of 24%<sup>75</sup>

#### *1.1.2.2.6 Prone sleeping position*

Sleeping prone results in decreased swallowing compared to a supine sleeping position<sup>80</sup>. This subsequently impacts upon the carriage of bacteria in the upper airways. Infants who sleep prone have a higher incidence of otitis media<sup>80</sup> and have been reported to carry a greater number and variety of bacterial species in nasal secretions<sup>81</sup>.

#### *1.1.2.3 Ear disease in the Indigenous Australian population*

Independent of other factors, Indigenous status in Australia significantly increases the risk of middle ear disease in urban, rural and remote locations. The World Health Organisation regards a prevalence of chronic OM above 4 % as an emergency, yet, conservative estimates of chronic otitis media in some aboriginal communities have been estimated to be above 10%<sup>82</sup>. Among Aboriginal Australians, OM develops at a younger age with the prevalence of OM and its associated complications higher than in non-Aboriginal children<sup>83</sup>. It has been reported that up to 73% of indigenous children will have suffered otitis media in the first year of life and within some indigenous communities the prevalence of otitis media with effusion is 50%<sup>84</sup>. In a cross-sectional study by Morris et al.<sup>82</sup> they found that in their examination of 698 children from aboriginal communities, 91% had otitis media, with 80% of these children suffering bilateral ear disease. In addition, this population has a 15 times higher rate of chronic supportive otitis media and

perforation than non-aboriginal children<sup>85</sup> (up to 50%<sup>86</sup>). It has been reported that by 14 years of age, these children will have suffered 8 times the amount of time with disease than their non-aboriginal counterparts (24 months vs 3 months)<sup>87</sup>. This discrepancy, perhaps unsurprisingly, is also mirrored by the length of time that indigenous children experience hearing loss (32 months on average versus 3 months in non-indigenous children)<sup>2</sup>.

It is thought that the increased occurrence of OM is in part due to the higher carriage of pathogenic bacteria in their upper airway and early colonisation in Aboriginal children.<sup>83,88</sup> Watson et al.<sup>88</sup> reported that overall carriage rates of the major middle ear pathogens in Indigenous children < 2 years were 49% for *S. pneumoniae*, 50% for *M. catarrhalis* and 41% for *H. influenzae*. In comparison, for non-Indigenous children from the same area, the carriage rates were 25% and 11% respectively. Furthermore, carriage of more than one pathogen has been associated with a greater risk of OM when compared to the carriage of a single pathogen (33-fold increase)<sup>83</sup>.

Other factors that impact the rates of OM in Indigenous children include poorer social conditions, such as poverty, overcrowding, higher rates of tobacco smoke exposure, poor nutrition, poor access to medical services and lack of running water<sup>89,90</sup>.

### ***1.1.3 Clinical symptoms and diagnosis of OME***

Unlike acute otitis media, which is associated with rapid onset of otalgia, otorrhea, and fever, OME is often asymptomatic and can persist for many months. Some children report fullness or a pressure sensation in the affected ear<sup>91</sup>, however, the leading symptom is hearing loss. Subsequently, speech and language development can potentially be impacted, especially in cases of bilateral OME, with the greatest effect when repeated or persistent episodes occur during early childhood<sup>92</sup>. In this regard, younger children with OME tend to present with a delay in language development and older

children often show a deficit in attention in school<sup>93</sup>. In addition, OME is the commonest cause of balance disorder in childhood, with 50% of children having OME with balance disturbances<sup>93</sup>.

The gold standard of diagnosis of OME is confirmation of middle ear effusion via tympanocentesis; however, tympanocentesis is invasive and difficult to carry out in an outpatient setting. In practice, pneumatic otoscopy is the most robust means for diagnosis, which combines visualisation of the tympanic membrane and assessment of its mobility and has a sensitivity of 94% and specificity of 80% in diagnosing OME<sup>94</sup>.

In addition, tympanometry is a frequently used as an adjunct for diagnosis, which provides a quantitative measure of tympanic membrane mobility and middle ear function. Tympanometry has a similar sensitivity (94%) to pneumatic otoscopy, but a lower specificity (64%)<sup>94</sup>.

The hearing loss associated with OME ranges from normal hearing to moderate hearing loss (0-55 dB)<sup>95</sup> and is a reflection of the volume, but not viscosity, of the effusion<sup>96</sup>. Most children with OME demonstrate mild hearing impairment, with mean three-frequency pure-tone average hearing loss of 25 dB, while a lesser proportion (approximately 20%) exceed 35-dB hearing loss<sup>97</sup>. In addition, there is a poor correlation with parental reporting of hearing loss and the presence or absence of OME<sup>98,99</sup>, therefore formal audiological assessment is essential.

#### ***1.1.4 Management of otitis media with effusion***

All patients presenting with OME should undergo age-appropriate assessment of their hearing to establish a baseline. For the youngest patients, aged 8 months to 2.5 years, visual response audiometry is typically used to assess hearing, which when performed by audiologists has been shown to provide reliable results in infants as young as 6 months<sup>100</sup>. In children, aged 2.5 to 4, play audiometry is best suited to assess hearing, where children perform a task (e.g. placing a toy in a

container) in response to a stimulus tone<sup>92</sup>. For children greater than 4 years of age, a formal audiogram, audiologist work-up and frequency-specific auditory-evoked potentials are the gold standard<sup>101</sup>.

#### ***1.1.4.1 Watchful waiting***

The American Academy of Otolaryngology- Head and Neck Surgery recommend a period of watchful waiting in children presenting with OME of 3 months from the date of effusion onset (if known) or from the date of diagnosis (if date of onset unknown), unless they are at high risk of speech, language or learning problems (e.g. children with severe vision impairment, craniofacial abnormalities or other factors impacting cognitive and linguistic development)<sup>92</sup>. The reason for this is that the majority of OME cases resolve spontaneously without treatment, with the likelihood of spontaneous resolution determined by the cause and duration of the effusion<sup>102</sup>. For instance, 75% to 90% of patients who develop OME after an episode of AOM have a resolution of the effusion by 3 months<sup>6</sup>. In contrast, in patients with OME of unknown duration, spontaneous resolution of OME is lower at 28% after 3 months, 42% at 6 months and 59% by 9 months as defined by tympanometric criteria (type B to type A/C1, peak pressure > -200mm water)<sup>102</sup>. In addition, for children with bilateral OME of 3 months' duration or longer, only 30% will resolve spontaneously by 6 to 12 months<sup>102</sup>. Therefore, decisions to commence therapy for OME is appropriate only if significant benefits can be achieved beyond that of waiting for spontaneous resolution.

During the period of watchful waiting, strategies for improving hearing should be put in place such as listening strategies, preferential seating in the classroom, and counselling to monitoring for an increase in hearing loss.

#### ***1.1.4.2 Antibiotics in otitis media with effusion***

In a 2016 Cochrane review<sup>103</sup>, the use of antibiotics in the treatment of OME showed a mild benefit for complete resolution of the effusion. However, antibiotic use was associated with a greater incidence of side effects such as experiencing diarrhoea, vomiting or skin rash and did not improve hearing loss nor rates of ventilation tube insertions. As a result, the authors concluded that the small benefits of early OME resolution did not outweigh the potential risks of accelerating bacterial resistance.

Similarly, studies looking at antibiotic prophylaxis to prevent OME following AOM have shown only a marginal improvement (4%) compared to no treatment for children at low risk. In contrast, for children at high risk, such as Indigenous children, prophylactic antibiotics significantly reduce the risks of tympanic perforation (14%), showing increased return of normal middle ear function (9.6%) and reduced pneumococcal carriage (12%)<sup>83</sup>. Again, the benefits of prophylaxis may be outweighed by the side effects and accelerating bacterial resistance. Therefore antibiotic prescribing is at the discretion of the health provider after assessing the individual patients benefit versus risk profile<sup>92</sup>.

#### ***1.1.4.3 Oral and topical steroids***

Steroids have been shown to have minimal benefit in the treatment of OME and are associated with significant side effects (behavioural changes, weight gain, idiosyncratic reactions). In their review of the literature, Berkman et al.<sup>104</sup> found that oral steroids did not have a significant impact on the resolution of effusion, nor in the hearing levels of children with OME.

Topical intranasal corticosteroids are associated with fewer side effects than oral steroids, however, again evidence of its efficacy is limited and may depend on co-morbid factors. For example, in a double-blinded placebo controlled trial of children aged 4 to 11 years, no difference was demonstrated in the resolution of effusion, or hearing loss, over 3 months between children treated with intranasal corticosteroid or placebo. However, for children with adenoidal hypertrophy there

may be a short-term benefit, albeit small, and may require higher than recommended dosing<sup>105,106</sup>.

In addition, in patients with simultaneous OME and allergic rhinitis, there may be a role for topical intranasal steroids to reduce inflammation caused by allergy<sup>55</sup>.

#### ***1.1.4.4 Antihistamines and decongestants***

There is no evidence to support a role for antihistamines and decongestants in the management of OME. In a systematic review by Venekamp et al., children treated with antihistamines and/or decongestants did not reduce the length of OME resolution, nor improvement in hearing levels<sup>103</sup>.

#### ***1.1.4.5 Surgery***

After a period of 3 months of watchful waiting, should OME persist, there is an indication for surgical intervention<sup>92</sup>. The aims of surgical management are to improve hearing and to prevent relapse of OME. Techniques include myringotomy without and without ventilation (tympanostomy) tube placement, adenoidectomy and tonsillectomy (or in combination). However, there remains some debate over the timing and technique employed.

##### ***1.1.4.5.1 Ventilation tube insertion***

In a meta-analysis by Browning et al.<sup>107</sup> and subsequent analysis of this and other studies<sup>104</sup>, the efficacy of ventilation tube insertion compared with conservative clinical observation was investigated. The insertion of ventilation tubes reduced the duration of tympanic effusion by 32% compared with watchful waiting. In addition, randomised control trials showed an average of 62% relative decrease in effusion prevalence, with an absolute decrease of 128 effusion days per child during the following year<sup>108</sup>

The insertion of ventilation tubes may also provide some alleviation of hearing loss; however, this is still under debate. After ventilation tube insertion, there have been reports of improved auditory

threshold by 4.2-10dB in children with surgical treatment compared with conservative therapy at 6-9 months<sup>107</sup>, however, this hearing benefit was lost after 1 year.

Furthermore, despite measurable improvements in hearing threshold, the impact of ventilation tube insertion on language development remains inconclusive. There are a number of trials that have shown no improvement in language development between surgical and conservative therapies<sup>104,107</sup>. Yet, in a randomised control trial, Maw et al.<sup>109</sup> found that tympanostomy tubes provided mild benefits for children with bilateral OME and hearing loss. Moreover, following a systematic review by Kuo et al.<sup>110</sup>, the authors concluded that the insertion of tympanostomy tubes result in improved speech and language development outcomes for patients with cleft palate.

There are a number of complications associated with ventilation tube insertion. The most common sequela is otorrhea, which is seen in 16% of children at 4 weeks and has an overall rate of 26% whilst a ventilation tube is in situ<sup>111</sup>. Other complications include: obstruction of the lumen in 7% of cases, premature extrusion (4%) and tube displacement into the middle ear (0.5%)<sup>111</sup>. In addition, persistent perforation occurs in about 2% to 3% of children following ventilation tube placement<sup>112</sup>. Tympanic perforation may require further surgery, such as myringoplasty or tympanoplasty to repair the deficit.

#### *1.1.4.5.2 Adenoidectomy*

The American Academy of Otolaryngology-Head and Neck Surgery guidelines recommend decision making around adenoidectomy based on the age of the patient. The aims of adenoidectomy are to reduce failure rates, reduce time with middle ear effusion, and decrease the need for repeat ventilation tube insertion.

In children under 4 years of age undergoing a primary ventilation tube insertion, adenoidectomy is not recommended unless there is a clear indication (for example, nasal obstruction or chronic adenoiditis) that exists other than OME<sup>92</sup>. The rationale behind this is that the surgical risks of adenoidectomy do not outweigh the limited, short term benefit in children aged 3 years or older without prior ventilation tube insertion<sup>113</sup>. These risks (for children of any age) include: prolonged anaesthetic time and intervention (i.e. intubation, intravenous fluids), a small potential for haemorrhage from the nasopharynx, and a longer recovery period (24 to 48 hours). In addition, velopharyngeal insufficiency can result after adenoidectomy, though only rarely.

In children older than 4 years of age, there is evidence that adenoidectomy at the time of initial ventilation tube insertion can be beneficial, especially for those with adenoid tissue abutting the opening of the ET and torus tuaris<sup>114</sup>. Boonacker et al.<sup>115</sup> found that children undergoing both techniques as primary therapy: spent 50 fewer days with OME in the following 12 months, had lower failure rates (51% vs 70% without adenoidectomy), and a lower rate of future surgery (2% vs 19%). Similarly, in a review of literature by Mikkals and Brigger<sup>116</sup>, adenoidectomy reduced the rate of repeat tympanostomy tube insertion for children under the age of four only (from 36% to 17%), with no significant effect for younger children.

In addition, there is evidence that adenoidectomy and myringotomy only (without insertion of ventilation tubes), has outcomes comparable to ventilation tube insertion, with less otorrhea and tympanic membrane sequelae<sup>108</sup>. However, as discussed above, adenoidectomy has inherent complications that need to be considered.

#### **1.1.4.6 Vaccination**

OM and OME is a polymicrobial disease, with four pathogens predominating: *S. pneumoniae*, *M. catarrhalis*, *H. influenzae* and Respiratory Syncytial Virus. As such, there have been attempts to

produce vaccines to target these pathogens. Currently, there are only vaccines available against *S. pneumoniae* and *H. influenzae type B* (HiB).

In Australia, there are two different types of pneumococcal vaccines available, the pneumococcal conjugate vaccine (PCV) and pneumococcal polysaccharide vaccine (PPV). PCV formulations vary in the number of pneumococcal serotypes that they have coverage for and in the conjugating proteins used. Furthermore, the PCV-type of vaccines has been found to be effective producing an immune memory response. In contrast, whilst containing more serotypes, the 23 valent PPC is poorly immunogenic for most serotypes in infants (children aged <2 years) and does not induce immune memory. Therefore, the 13 valent pneumococcal conjugate vaccine (PCV13) is recommended for infants at 2, 4, and 6 months<sup>117</sup>.

The first pneumococcal conjugate vaccine was introduced in Australia in 2002 as a heptavalent (PCV 7) vaccine, later being replaced by the PCV 13 in 2011. The PCV7 contains polysaccharides of the capsular antigens for pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Since its introduction, two higher-valent pneumococcal conjugate vaccines have become widely available, the PCV 10 and the PCV 13. These two vaccines differ from each other in the number of serotypes and carrier proteins used for their conjugation. PCV10, in addition to the serotypes present in PCV7, extended the vaccination cover to serotypes 1, 5, and 7F, with the PCV 13 further broadening this coverage to include serotypes 3, 6A and 19A<sup>118</sup>.

The majority of the literature has focused on the effect of the introduction of the PCV7 on the incidence of AOM, with research still accumulating on the impact of the higher valent vaccines<sup>119</sup>. In randomised trials, administration of the PCV7 beginning at age two months was associated with a modest reduction in AOM (6 to 8 percent)<sup>120-122</sup>. Also, children who received PCV7 during infancy had a 34 percent lower risk of tympanostomy tube insertion at age two through five years compared

to children who were not vaccinated<sup>123</sup>. However, AOM caused by *S. pneumoniae* gradually rose in incidence following PCV7 administration, due to replacement pneumococcal strains expressing serotypes not contained in PCV7, in particular, phenotype 19A<sup>119</sup>. In addition, the pneumococcal conjugate vaccines were not found to be effective if administered after the development of recurrent otitis media<sup>119,124</sup>.

In the Australian Aboriginal population, the introduction of the PCV 7 and 10 have shown to impact the bacterial aetiology of OM. The dissemination of the PCV 7 reduced vaccine-serotype invasive pneumococcal disease significantly (74% decrease in both indigenous and non-indigenous Australian<sup>125</sup>) and has had indirect protective effects by reducing the carriage of pathogenic bacteria in the upper airway<sup>126</sup>. At the introduction of the PCV 10, rates of suppurative otitis media further decreased by 12%. However, ongoing surveillance showed a reciprocal increase in the rates of OME (10%) and the emergence of increased incidence of non-PCV serotype invasive pneumococcal disease<sup>125</sup>.

Since the widespread dissemination of the PCV, non-typeable (non-encapsulated) *Haemophilus influenzae* (NTHi) has become the most common cause of otitis media<sup>127,128</sup>. Unfortunately, there is currently no effective vaccine to combat this group of bacteria. While vaccines against type B (encapsulated) *Haemophilus influenzae* (HiB) have been effective in eliminating infection by this encapsulated strain, these conjugate vaccines show no effect against NTHi. There is ongoing research in the development of such a vaccine, with a number of promising surface antigens as potential targets<sup>127,128</sup>. Likewise, there remains no commercially available vaccine for *M. catarrhalis*<sup>129</sup>.

Similarly, there are limited vaccines against respiratory viruses. In Australia, yearly influenza vaccine administration is recommended for all children greater than 6 months. Whilst Influenza virus does not have as strong an association with OM as other respiratory viruses such as respiratory syncytial virus, parainfluenza and human metapneumovirus, there is evidence that influenza vaccine administration reduces the rates of AOM, though only modestly. In a 2015 meta-analysis of five randomised trials by Norhayati and Azman, which included 4736 children aged six months to six years, influenza vaccine administration reduced the risk of at least one episode of AOM over six months of follow-up by 4%. The authors also noted a decrease in antibiotic prescribing for AOM by 15% in vaccinated children. However, it is unclear whether this reduction was due to influenza vaccine or increased frequency of “watchful waiting” for AOM to avoid overuse of antibiotics.

## *1.2 The role of bacteria in otitis media*

OM is a multi-factorial disease, with many associated risk factors, both host and environmental. Despite this complexity, bacterial pathogens have been strongly associated with OM development. In the past, identification of bacteria was limited due to the restraints inherent with traditional culture techniques, however, with advancements in culture-independent techniques such as polymerase chain reaction and 16S rRNA pyrosequencing, bacteria are almost universally found in aspirates of the middle ear. The following section discusses some of the recent advances in the detection of bacteria and summarises the role of bacteria in OM, with a focus on OME.

### *1.2.1 Detection of bacteria and its advances*

Only three decades ago, OME was assumed to be a sterile condition that was mainly due to the obstruction of the Eustachian tube. However, with conventional culture methods, bacteria can be identified in 40% of cases of OME<sup>38</sup>, with those results forming the foundation for our classical understanding of the bacteria associated with OM and OME. However, culture-dependent techniques are inherently limited. They rely on bacterial growth, and it is difficult to use traditional culture methods to isolate more than fraction of micro-organisms known to be present in any environment<sup>130</sup>. In addition, isolation of bacteria has an inherent selection bias, due to the often specific and fastidious nature of bacteria and their growth requirements (growth media, atmospheric conditions, temperature etc.).

During the past two decades, there has been a shift towards the identification of bacteria that does not require bacteria to be grown in media. Conventional culture-independent techniques such as PCR, FISH, mass spectrometry and DNA microarray have all been used to identify bacteria. For example, targeted PCR is able to detect bacteria from middle ear aspirates of OME in 80% of cases<sup>38</sup>. These culture-independent techniques have allowed for detection of previously unidentified micro-

organisms, leading to a wider spectrum of organisms involved in OM to be described. With the identification of these new bacterial species, there has been a greater appreciation for the complexity of the bacterial interactions within the middle ear. However, whilst these techniques do not rely on culture to identify microbes, an inherent selection bias remains, as utilisation of these technologies requires the investigator to first target the bacteria they wish to investigate prior to identifying the organism.

Recently, culture-independent molecular methods involving the use of 16S rRNA sequences have allowed non-selective species identification by means of phylogenetic sequence analysis. 16S rRNA sequencing was the first molecular-based technology to be generally applied to the human microbiota. It provides a non-targeted, quantitative description of the bacteria present from a sample, allowing investigation of whole communities and the identification of their constituent members. At the time of writing, two studies have utilised this technology to identify the bacterial population in the middle ear aspirate of patients with OME. Liu et al.<sup>131</sup> described the microbiota of middle ear fluid from one patient with OME and Jervis Bardy et al.<sup>132</sup> characterised the microbiota in 11 Australian Aboriginal Children. The following section will provide an overview of 16S rRNA sequencing technology and discuss some of the potential applications of this technology in the context of otitis media.

#### ***1.2.1.1 Culture-independent bacterial identification and 16S rRNA gene sequencing***

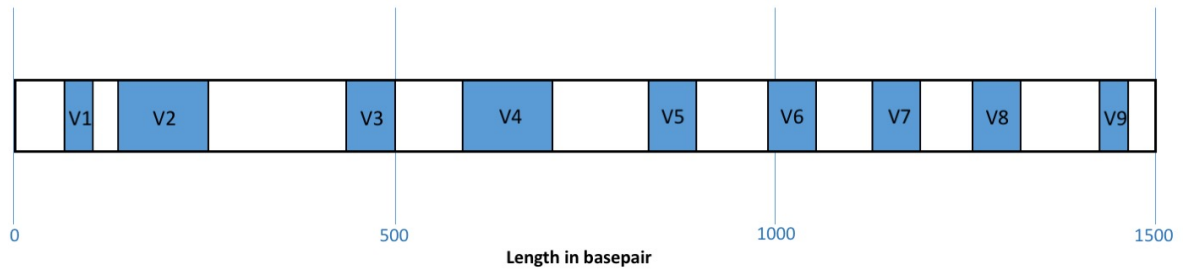
The development of PCR as a technology has been one of the cornerstones of conventional molecular science and biomedical research. Once DNA is extracted from a sample, PCR allows for the amplification of specific regions of DNA more than a billion-fold, by utilising the enzyme DNA polymerase. Following amplification of the DNA sequences, the gene and their products can be analysed or manipulated<sup>133</sup>. In the past PCR has played a critical role in many molecular discoveries,

including in the sequencing of the human genome<sup>134</sup>, and is the foundation by which 16S rRNA sequencing is possible.

In the setting of OM, it had previously been unknown whether a PCR product from bacterial DNA collected from MEF represented viable organisms or not. However, Rayner et al.<sup>135</sup> demonstrated clear evidence that the presence of bacterial DNA suggested the presence of viable, metabolically active, intact organisms even in culture-negative otitis media. Subsequently, the use of PCR targeting the 16S rRNA gene from MEF became more commonplace.

#### *1.2.1.2 The 16S rRNA gene*

The 16S rRNA gene is a small subunit ribosomal gene and is a highly conserved component of the transcriptional processes inherent in all DNA-based life-forms. While, all life forms harbour the 16S rRNA gene, the gene sequences of organisms of the same species and genus are highly conserved (i.e. between bacteria), whereas they significantly differ between organisms of other species and genera (i.e. comparing human rRNA with bacterial rRNA)<sup>136</sup>. It is this inherent difference that is exploited to identify bacteria from human samples, with PCR amplification designed to target these regions. Furthermore, hypervariable regions exist between the conserved regions. These hypervariable regions demonstrate significant sequence diversity between different bacterial species and thus allow for bacterial species differentiation and identification<sup>137</sup>. There are nine recognised hypervariable regions of the RNA component of the 16S ribosome, designated V1 to V9 (figure 1-1). The most commonly targeted regions are V1-3, which allows identification of bacteria in human microbiome samples with sufficient sensitivity<sup>138</sup>.



**Figure 1-1 16S rRNA gene.** Approximately 1500 basepair 16S rRNA gene of *E. coli* showing the nine variable regions that allow the 16S gene to be an ideal phylogenetic marker gene. Adapted from Cox et al.<sup>130</sup>

### 1.2.1.3 Operational taxonomic units and taxonomic assignment

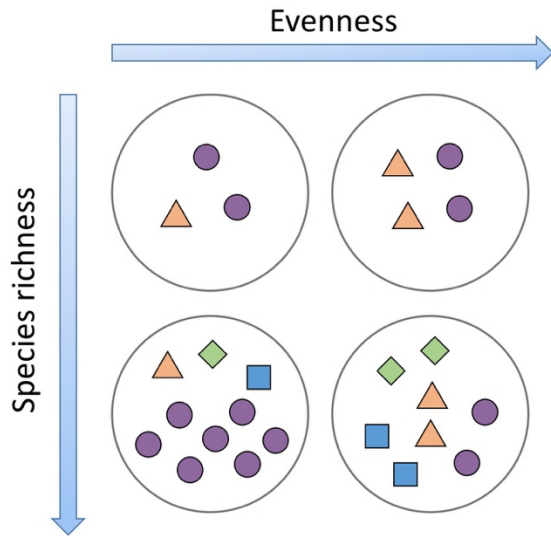
Once the sequences are amplified by PCR, analysis of the resulting data relies upon the clustering of related sequences. This allows for the identification and counting of the representatives of each cluster. The clusters of similar sequences are referred to as operational taxonomic units (OTUs). OTU counts are summarised in a table of relative abundances for each organism in a sample and are used for downstream analysis.

These OTUs are then compared to reference databases based on similarity for taxonomic assignment. A level of 97% similarity of the sequence identity is often chosen as being representative of a species and 95% for a genus when using partial 16rRNA gene sequences<sup>130</sup>. The accuracy of identification is dependent on the reference database that is chosen. Examples of available libraries include: Greengenes<sup>139</sup>, SILVA<sup>140</sup> and The Ribosomal Database Project<sup>141</sup>. However, these databases themselves contain erroneous 16S rRNA sequences<sup>142</sup>, which can lead to decreased sensitivity. Bioinformatic 'pipelines', or algorithms, are utilised to compare datasets to these libraries. Commonly used pipelines include: MG-RAST<sup>143</sup>, QIIME<sup>144</sup> and Mothur<sup>145</sup>. However, there is yet to be a standardised method of applying these pipelines to datasets<sup>130</sup>.

#### *1.2.1.4 Interpreting 16S rRNA sequencing*

16S rRNA gene sequencing allows for several descriptive assessments of a taxonomic composition and phylogenetic diversity of a microbiome. Once a microbiome sequence is obtained, and OTUs defined, the community characteristics of the microbiome are described by their characteristic parameters. Descriptions of these parameters include community richness, which is the number of unique taxonomic units detected in a sample, and evenness, which is the relative number of taxonomic units in a sample (figure 1-2). Furthermore, reporting diversity, such as alpha and beta diversities, are valuable descriptors of the community. Alpha diversity (e.g. Shannon Diversity Index<sup>146</sup>), is a measure of the richness and evenness within a sample, whilst beta diversity (e.g. Bray-Curtis<sup>147</sup> or UniFrac<sup>148</sup>) represents the similarity of the bacterial composition between samples.

By comparing sequences of the 16S rRNA gene in clinical samples to pre-curated databases containing known 16S rRNA sequences, this technology allows for a non-targeted approach to the identification of polymicrobial communities present in clinical specimens, thus facilitating the characterisation of the microbiome of a sample.



**Figure 1-2 Descriptions of microbiome data.** A diagram demonstrating species richness and evenness. Each shape represents an individual bacteria and differences in colour/nature of the shape represents a different type of organism. An increased number of different types of organism is described as increased species richness. When there is no single dominant organism, the community is described as even. Adapted from Cox et al.<sup>130</sup>

#### 1.2.1.5 *The microbiome and the concept of commensals and dysbiosis:*

A microbiome refers to the ecosystem comprising of all the micro-organisms, genetic material and environmental interactions living on and in an individual. The micro-organisms making up the microbiome are themselves referred to as the microbiota (microbial community) and can include bacteria, archaea, viruses, phage, fungi and other microbial eukarya. The study of the microbiota and their relationship to the human has resulted in a paradigm shift. Humans are no longer viewed just as a 'host' to the microbiota, but humans are instead involved in a shared microbial ecosystem. Of these microbes, the bacterial microbiota has been the most well studied. This is likely due to our historical perceptions of bacteria as the cause of disease and the increasing incidence of antimicrobial resistance driving the need to find novel management approaches.

The bacterial microbiome has been characterised at many body sites. The microbial communities have been shown to contribute essential functionalities to human physiology and are considered essential for the maintenance of human health<sup>149</sup>. For instance, in the gastrointestinal tract, the gut microbiome has been implicated in the development of the immune system, promoting intestinal angiogenesis and assisting in digestion<sup>150,151</sup>. In health, the commensal bacteria maintain homeostasis and also exert a biological pressure to inhibit colonisation by pathogens, in a phenomenon referred to as colonisation resistance<sup>152</sup>.

However, it has been consistently shown that there is also high interpersonal variability within a cohort of healthy individuals<sup>153,154</sup>. Within these populations, some body sites show a more stable composition than others, both between individuals in a cohort and also within individuals over time<sup>153,155,156</sup>. Zhou et al.<sup>153</sup> reported that the oral microbiota are more even between individuals but more stable within individuals compared with other body sites. In addition, Li et al.<sup>154</sup> found that the number of “core taxa” (i.e. taxa consistently observed between individuals) varied between body sites. They observed an increasing number of core taxa across the body regions in the ascending order of the vagina, skin, stool, and oral cavity.

These findings support the notion that the microbiome of an ecological niche or particular body site is distinctly different to that of other sites, even those that are anatomically adjacent<sup>153,155</sup>. As an example, Segata et al.<sup>157</sup> sampled the oral cavity at seven different sites within the mouth (the buccal mucosa, hard palate, gingivae, tongue, saliva, and supra- and sub-gingival plaque) and also the throat and tonsils. They analysed their samples with 16S rRNA techniques and found that three distinct bacterial communities existed within these sites; the buccal mucosa, gingivae and hard palate had similar microbiota, while the saliva, tongue, tonsils and throat, and the supra- and sub-gingival plaque each had distinctive communities. This finding “supports the idea of a strong local

selective pressure on community membership even in the absence of disease”<sup>157</sup>. However, despite this variation discussed above, Zhou et al.<sup>153</sup> found that habitats also follow a general rule; one or several genera predominate a body site, but are accompanied by a long tail list of less abundant organisms.

While the microbiota in health has been reported to have a high degree of richness and variability, in a disease state, often the converse is true. During times of disease, one bacteria (considered the etiological pathogen) emerges as the dominant strain either from within the pool of commensal bacteria or exogeneously. This results in the flattening of the richness of the microbiota in the niche, representing a dysbiotic microbiota. For example, this phenomenon has been reported in a number of studies looking at the microbiome of chronic rhinosinusitis. In these individuals with chronic disease, there is a dysbiotic microbiome of the sinuses, with the emergence of *Staphylococcus* in high abundance. There is a resulting decrease in the richness, most noticeably in the reciprocal reduction in *Corynebacterium* and *Propionibacterium* when compared to controls<sup>158</sup>. This has lead researchers to investigate whether *Corynebacterium* and *Propionibacterium* play a health promoting or probiotic role in CRS. A similar finding is found from the nasopharyngeal sampling of patients with OM. Patients with OM had an increase in pathogenic bacteria (often *S. pneumoniae*, *H. influenzae* or *M. catarrhalis*), with a decreased carriage of *Dolosigranulum* and *Corynebacterium* species<sup>159</sup> compared with controls.

However, there is evidence that a dysbiotic microbiome can be affected in a way so that it reverts into one of health. The most striking example is in the treatment of *Clostridium difficile* infection of the gastrointestinal tract. *C. difficile* infection is brought about due to the extended use of antibiotics. As a result, there is a decrease in richness of the microbiome of the gut commensals, allowing *C. difficile* to emerge as the dominant bacterium, resulting in disease. However, faecal

microbiota transplantation; the infusion of a faecal suspension from a healthy individual into the gut of the person suffering from *C difficile infection*, has been shown to be effective in the treatment of this disease<sup>160,161</sup>. This successful treatment modality highlights the potential for novel treatments of disease; however, the initial step is first to characterise and elucidate the interactions within the microbiota in health and disease.

### 1.2.2 *The microbiota of the middle ear*

Up until 2016, studies suggested that the healthy human middle ear and mastoid cavities were sterile. Westerberg et al. recruited patients (13 children, 9 adults) undergoing Cochlear implant surgery and lavaged the middle ear cavity intraoperatively and found no evidence of bacteria using standard culture and PCR techniques. In addition, Hall Stoodley et al.<sup>162</sup> found that bacterial biofilm was not detected on the middle ear mucosal surfaces of control patients. However, a recent study by Neeff et al.<sup>163</sup> demonstrated bacteria in the middle ear clefts and mastoid cavities of 45% of their control patients (undergoing Cochlear implant or benign brain tumour removal), using 16S rRNA techniques. The bacterial genera included *Novosphingobium*, *Staphylococcus*, *Streptococcus*, *Escherichia-Shigella*, and *Burkholderia*.

There have been two studies that have utilised 16S rRNA gene sequencing to characterise the microbiota in OME. Liu et al.<sup>131</sup> characterised the microbiota present in one patient with OME. They found a high abundance of the bacterial family Pseudomonadaceae, which predominated the sample of MEF (82.7%). They also identified the families of common ear pathogens: *Streptococcaceae*, *Pastuerellaceae* (*Haemophilus*) and *Moraxellaceae*, however, genus level differentiation and relative abundance were not reported. Jervis Bardy et al.<sup>164</sup> reported on the microbiota of the middle ear in OME in an Australian Aboriginal cohort. They found that in 11 Aboriginal children, aged 3-9, that the most prevalent and abundant bacteria (>50% relative

abundance) were *A. otitidis* (6/11 samples, 55%), *H. influenzae* (3/11, 27%) and *Streptococcus spp.* (1/11, 9%). Other genera that were identified included *Moraxella*, *Corynebacteria*, *Staphylococcus* and *Turicella* (relative abundance not specified). These findings are consistent with the pre-existing literature, which has identified *H. influenzae* as the most common otopathogen found in OME<sup>37</sup>. In contrast, *A. otitidis*, due to its fastidious growth requirements, was not often reported from middle ear aspirates before widespread use of PCR. However, since the use of PCR, it has been shown to be the most abundant bacteria identified in OME (20-60%) (see table 1-1).

In contrast, *A. otitidis* is less commonly identified in AOM. In this setting, the classic otopathogens, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* predominate the microbiota of the middle ear. In a review of the literature, from 1970 to 2014, Ngo et al.<sup>37</sup> analysed the data from 66 articles reporting on the microbiology of the middle ear. Of the 66 articles, 38 studies involved sampling the middle ear in AOM. The authors reported that despite some geographical variation, *S. pneumoniae* (29/38 studies) was the most predominant bacterium, followed by *H. influenzae* (6/38). In addition, multispecies infection was common, with the frequency of polymicrobial detection high for *S. pneumoniae* (27.8%, range 9.9%-49.9%), *H. influenzae* (23.1%, range 5.0%-54.6%) and *M. catarrhalis* (7%, range 0.5%–27.8%). In contrast, in some regions (South America, Europe and Oceania) *H. influenzae* predominated in aspirates from recurrent AOM and those with AOM with treatment failure.

In cases of CSOM, *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) are considered the most common pathogens<sup>165</sup>. However, Neeff et al.<sup>166</sup> who conducted a 16S rRNA gene sequencing study to characterise the microbiota of 24 patients with CSOM and 22 control patients found that culture results under-represents the microbiota of CSOM. They reported that *P. aeruginosa* was only found in 9% of their cohort (4/46). *S. aureus* remained prevalent at 39% (18/46)

and they also found variable abundances of *Haemophilus*, *Staphylococcus*, *Alloiococcus*, and *Streptococcus* from these patients. There was a lower abundance of *Propionibacterium* in CSOM patients than controls, suggesting a potential protective role. Additionally, *Alloiococcus* and *Haemophilus* were detected commonly in CSOM patients with underlying cholesteatoma. Despite the detection of these extra bacteria, their role in CSOM remains undetermined.

### 1.2.3 *The nasopharynx as a reservoir for otitis media*

The nasopharynx is anatomically located at the confluence between the posterior nasal cavity, middle ear (via the ET) and the lower aerodigestive tract. As a result, it is an ecological niche for many commensal bacteria and potential respiratory or invasive pathogens. During times of health, there seems to be a complex symbiosis between the host, commensal bacteria and pathogenic bacteria, which maintains “health”. However, disturbance of a balanced nasopharyngeal (NP) microbiome might be involved in the onset of symptomatic infections with these pathogens. The exact mechanisms by which this occurs remain largely undetermined, although an imbalance in the composition of the microbiota caused by, for example, the arrival of new pathogens, viral co-infection or other factors, such as antibiotic use<sup>167</sup> and cigarette smoke exposure<sup>70</sup>, have been suggested.

The nasopharyngeal microbiota develops from the first day of life and evolves throughout the human lifespan. Bosch et al.<sup>168</sup> followed 102 children longitudinally from birth to six months and periodically swabbed the nasopharynx. They found that despite the significant difference between individuals on nasopharyngeal microbiota carriage during the study, that there was an initial *streptococcus viridans*-predomination across the population in the first week. Following that, infants were colonised with *S. aureus*, *Corynebacterium spp.*, *Dolosigranulum pigrum*, *M. catarrhalis*, *S. pneumoniae* and *H. influenzae*. Similarly, in a study of 96 healthy 18-month-old children, Bogaert et

al.<sup>169</sup> characterised the microbiota of the nasopharynx in healthy individuals using 16S rRNA gene sequencing. They described a core microbiota that was similar to Bosh et al, which consisted of: *Moraxella* (40% relative abundance), *Haemophilus* (20%) and *Streptococcus* (12%), *Dolosigranulum* (5%), and *Corynebacterium* (2%). In addition to the above, Bogaert et al.<sup>169</sup> also found a seasonal difference of the nasopharyngeal microbiota that was independent of antibiotic use or viral co-infection; with an increase in abundance of *Proteobacteria*, *Fusobacteria*, *Cyanobacteria* with less *Bacteroidetes* during winter months, which may reflect seasonal changes in OM.

Given the fact that classically associated otopathogens (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*) colonise the nasopharynx in high abundance, the nasopharynx is commonly considered as the reservoir for bacterial pathogens involved in middle ear infections<sup>170</sup>. In addition, colonisation of the nasopharynx with *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* at an early stage has been shown to predispose children to the development of rAOM.<sup>171,172</sup> The mechanism is via ascendance of nasopharyngeal contents into the middle ear via the Eustachian tube. This mechanism is illustrated by the fact that pepsin, an enzyme resident within the stomach, and its precursor pepsinogen have been demonstrated within middle ear fluid<sup>30,173</sup>.

Two studies, utilising culture-independent techniques have compared the nasopharyngeal microbiota in healthy children and those with AOM. In their study involving 163 infants (153 with AOM, 10 without) with sample collection over 6 years, Hilty et al.<sup>174</sup> found that control patients had a richer and more diverse microbiota, whereas patients with AOM showed a predominance of the three classic otopathogens in their nasopharynx. Specifically, control patients harboured both a higher prevalence and relative abundance of *Staphylococcaceae*, *Flavobacteriaceae*, *Carnobacteriaceae* and *Comamonadaceae*. Similarly, Pettigrew et al.<sup>159</sup>, found that colonisation of the nasopharynx by *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* was associated with lower

diversities of the microbiota. They found individuals with a high abundance of *Corynebacterium* and *Dolosigranulum* colonising the nasopharynx were less likely to have AOM. Additionally, patients treated with antibiotics in the preceding 6 months had a greater abundance of *Lactococcus* and *Propionibacterium* and were also less likely to have AOM. The authors suggest that these bacteria genera may thus play a probiotic or protective role in preventing pathogenic overgrowth in the nasopharynx.

#### 1.2.4 *The external ear canal as a potential reservoir for otitis media*

There are a number of bacterial species that are commonly aspirated from the middle ear effusions that are more often associated with colonising or causing infection in the external ear canal. These bacteria are *A. otitidis*, *Staphylococcus spp.* (including *S. aureus*), *P. aeruginosa*.

The predominant bacteria that colonize the external ear canal in healthy individuals have been reported by one culture independent study as: *A. otitidis* (57% relative abundance), *Corynebacterium otitidis* (20%) and *auris* (3%), *Staphylococcus auricularis* (10%) and *Propionibacterium acnes* (2%)<sup>175</sup>. Similarly, in a study combining PCR and culture, *De Baere et al.*<sup>176</sup> described the microbiota of the external ear canal to be predominated by *A. otitidis* (83%), *Corynebacteria spp. (otitidis* (55%) and *auris* (22%), but also a significant amount of *Staphylococcus spp. (auricularis, epidermidis, capitis, intermedius, 66% in total)*. Additionally, *P. aeruginosa* has also been suggested as a coloniser of the external canal<sup>177,178</sup>. In contrast, during episodes of AOM, there seems to be a shift in the microbiota that is identified from the external canal. *Heslop et al.*<sup>179</sup>, found that the predominant bacteria were instead: *P. aeruginosa* (36%), *S. pneumoniae* (27%), *S. aureus* (18%) and beta hemolytic group A *Streptococcus* (18%).

*P. aeruginosa* and *S. aureus* are considered the two most commonly identified bacterial causes of CSOM<sup>165</sup>. In contrast, they are seldomly found in middle ear aspirates in cases of AOM<sup>42</sup>, but are

reported in high prevalence in patients with otorrhea lasting up to 3 weeks<sup>180</sup>. These two characteristics suggest that in the presence of a tympanic perforation, whether from a spontaneous rupture or in the presence of a tympanostomy tube, that external ear canal bacteria can translocate into the middle ear to cause middle ear disease.

Another phenomenon that suggests that external canal bacteria can translocate into the middle ear and potentially cause disease lies in the fact that *A. otitidis* is commonly identified from middle ear aspirates in patients with OME<sup>181-183</sup>. *A. otitidis* is considered a commensal of the external ear canal and has in fact been shown to be the dominant organism from that biological niche<sup>175</sup>. Conflicting evidence exists regarding the presence of *A. otitidis* within the nasopharynx. Although *A. otitidis* has been identified from the nasopharynx from both healthy children<sup>184</sup> and children with OM<sup>38</sup>, several studies do not support this finding with *A. otitidis* being absent in both culture based studies<sup>169,185</sup><sup>132,186</sup> and 16S rRNA sequencing studies of the adenoid pad<sup>131,187</sup>.

Given the respective roles that *S. aureus*, *P. aeruginosa* and *A. otitidis* play in the external auditory canal, there remains the question as to whether these bacteria can translocate from the external ear canal to colonise the middle ear or whether they are merely contaminants as a result of sampling bacteria from the middle ear. There is clear evidence of *P. aeruginosa* and *S. aureus* infection of the external canal in the form of otitis externa, and also in CSOM<sup>165</sup>. In contrast, there remains no consensus on the role of *A. otitidis* in OM.

## 1.2.5 Bacteria associated with otitis media

### 1.2.5.1 *Alloiococcus otitidis*

*A. otitidis* is a strictly aerobic, gram-positive cocci first documented in MEF by Faden and Dryja in 1989<sup>188</sup>. It is weakly catalase positive and arranges in pairs, small clusters and tetrads. *A. otitidis* grows slowly with small alpha-haemolytic colonies when incubated on blood agar after 3-4 days of growth, which continue to remain small even after extended periods of growth. Due to the characteristic that *A. otitidis* is salt resistant, isolation of clinical strains is achieved when incubated on Brain Heart Infusion (BHI) supplemented with 6.5% sodium chloride<sup>189</sup>. However, despite this, *A. otitidis* is frequently not cultured from MEF samples and PCR has emerged as the gold standard for identification of *A. otitidis*<sup>190-194</sup>.

When using PCR for identification, *A. otitidis* is the most prevalent bacterial species found in patients with non-purulent OME (19 to 64%) (table 1-1) and has been identified from MEF samples as the single dominant bacterial species<sup>195</sup>. Moreover, it has been proposed that the presence of *A. otitidis* in the MEF correlates with a longer duration of OME<sup>181</sup>.

In contrast, in the setting of AOM, *A. otitidis* is less frequently identified from MEF. Kaur et al. found<sup>195</sup> that in 97 children with AOM, no children had *A. otitidis* within the middle ear effusions. However, Sillanpaa et al.<sup>205</sup> reported an incidence of 6.7% in 79 children. Furthermore, Leskinen et al.<sup>181</sup>, reported the presence of *A. otitidis* in up to 25% of children with AOM. However, spontaneous perforation occurs in 40% of AOM, which may have allowed *A. otitidis* to translocate prior to sampling. This can be illustrated by the fact that Marsh et al.<sup>183</sup> reported that 37% of Australian Aboriginal children with AOM with perforation were PCR positive for *A. otitidis*, suggesting the external ear canal bacteria has the opportunity to translocate into the middle ear in the presence of a tympanic membrane perforation.

**Table 1-1 Studies reporting the incidence of *A. otitidis* in otitis media with effusion (OME) using polymerase chain reaction**

<i>A. otitidis</i> identified by PCR in OME		
Year	Incidence (%)	Author ( <i>et al.</i> )
1997	5/25 (20)	Hendolin <sup>190</sup>
1999	6/12 (50)	Beswick <sup>196</sup>
1999	31/67 (46)	Hendolin <sup>191</sup>
2002	25/123 (20)	Leskinen <sup>197</sup>
2002	10/54 (19)	Kalcioglu <sup>192</sup>
2002	25/123 (20)	Leskinen <sup>198</sup>
2006	24/83 (29)	Harimaya <sup>199</sup>
2006	46/76 (61)	Harimaya <sup>194</sup>
2010	31/97 (32)	Kaur <sup>186</sup>
2012	15/65 (23)	Khoramrooz <sup>200</sup>
2012	12/34 (35)	Aydin <sup>185</sup>
2012	43/169 (25)	Holder <sup>201</sup>
2015	7/11 (64)	Jervis Bardy <sup>164</sup>
2015	63/245 (26)	Holder <sup>202</sup>
2015	18/55 (26)	Farajzadah <sup>203</sup>
2016	15/48 (31)	Slinger <sup>204</sup>

<i>A. otitidis</i> in AOM		
2004	30/118 (25)	Leskinen <sup>181</sup>
2006	20/40 (50)	Harimaya <sup>194</sup>
2012	7/38 (18)	Holder <sup>201</sup>
2015	7/31 (22)	Holder <sup>206</sup>
2016	6/90 (6.7)	Sillanpaa <sup>205</sup>
<i>A. otitidis</i> in AOM (with perforation)		
2009	5/5 (100)	De Baere <sup>207</sup>
2012	10/27 (37)	Marsh <sup>183</sup>

**Table 1-2 Studies reporting the incidence of *A. otitidis* in acute otitis media (AOM) using polymerase chain reaction**

As discussed above, *A. otitidis* has also been identified from the nasopharynx. In the literature it has been reported in four studies with incidence ranging for 0.5% to 11%, however an equal amount of studies show an absence from the nasopharynx (table 1-2). However, what is unclear is whether there is *A. otitidis* in the middle ear of those individuals and that in fact, the middle ear is the source of *A. otitidis*, which is travelling down the Eustachian tube to seed the nasopharynx.

<i>A. otitidis</i> identified on the Nasopharynx		
2002	4/50 (8)	Durmaz <sup>208</sup>
2003	6/56 (11)	Harimaya <sup>193</sup>
2009	0/70 (0)	Frank <sup>175</sup>
2011	2/386 (0.5)	Janapatla <sup>209</sup>
2012	0/34 (0)	Aydin <sup>185</sup>
2012	0/27 (0)	Marsh <sup>183</sup>
2015	0/11 (0)	Jervis Bardy <sup>164</sup>
2015	90/1021(8.8)	Teo <sup>210</sup>
<i>A. otitidis</i> in EAC		
2002	3/95 (3)	Durmaz <sup>208</sup>
2003	2/24 (8)	Frank <sup>175</sup>
2010	58/70 (83)	De Baere <sup>176</sup>

**Table 1-3 Studies reporting the incidence of *A. otitidis* from samples of the nasopharynx and external auditory canal using polymerase chain reaction**

*A. otitidis* is commonly considered a commensal of the external canal and has been identified in high incidence. In patients without OM, Frank et al. in their 16S rRNA gene sequencing study, reported that *A. otitidis* was an early coloniser of the EAC, with *A. otitidis* identified in 57% of their cohort of children 1-5 years of age<sup>175</sup>. Additionally, De Baere et al.<sup>176</sup> described the microbiota of the external canal to be predominated by *A. otitidis* (83%) in their study of 70 healthy young adults using PCR.

However, the potential for pathogenicity for *A. otitidis* has been documented; *A. otitidis* has been found to invade intracellularly<sup>188</sup>, to modulate immune responses *in vitro*<sup>211-216</sup> and has been implicated in cases of implant infection<sup>217,218</sup>, endocarditis<sup>219</sup>, and endophthalmitis<sup>217</sup>. In addition, when *A. otitidis* was introduced into the middle ear of rats, a serous OME developed, with the majority lasting longer than a week<sup>38</sup>. Despite these findings, consensus on the impact and role of *Alloiooccus* in the pathogenesis or development of OME remains elusive.

#### **1.2.5.1.1 Antibiotic susceptibility**

*A. otitidis* is  $\beta$ -lactamase negative bacteria<sup>189</sup>, and while being resistant to vancomycin, macrolides and trimethoprim<sup>189,220,221</sup>, it is known to be susceptible to penicillins, cephalosporins, tetracyclines<sup>189,220</sup>, and fluoroquinolones<sup>200</sup>. While little is known about resistant *A. otitidis* strains, *A. otitidis* is often identified within middle ear aspirates despite antibiotic treatment<sup>194</sup>.

#### **1.2.5.2 *Streptococcus pneumoniae***

*S. pneumoniae* is a Gram-positive, alpha haemolytic coccus that is often found to colonise the nasopharynx. It is a fastidious bacterium, growing best in 5 percent carbon dioxide, and requires a source of catalase (e.g., blood) to grow on agar plates. Virtually all strains of *S. pneumoniae* have a polysaccharide capsule, which is the basis for serotyping. There are at least 97 antigenically distinct serotypes of encapsulated *S. pneumoniae*<sup>222</sup>. Of these serotypes, 20 account for 80% of invasive pneumococcal disease<sup>223</sup>. The dominant serotypes associated with invasive pneumococcal disease worldwide include 1, 3, 4, 6A, 6B, 7F, 8, 9V, 14, 18C, 19F, and 23F. In young children, 6, 14, 18, 19, and 23F predominate. The serotypes most commonly associated with AOM are 3, 6A, 6B, 9V, 14, 19A, 19F, and 23F, with serotypes 1, 5, and 7F rarely associated<sup>224</sup>.

The pathogenesis of *S. pneumoniae* stems from its ability to colonise the nasopharynx. The rates of pneumococcal carriage have been reported to fluctuate over the course of the human life span;

peaking at 50-70% at the age of 2-3 years, before decreasing and stabilising to 5-10% carriage by the age of 10. The pneumococcal capsule not only plays a protective role against opsonisation and phagocytosis, but also promotes colonization of the nasopharynx by reducing mucosal clearance from the nasopharynx<sup>225-227</sup>. Once colonised within the nasopharynx, *S. pneumoniae* can migrate and cause a broad range of disease. The most common infections occur in the respiratory tract including: bronchitis, pneumonia and OM. *S. pneumoniae* can also invade and spread via the bloodstream, causing bacteraemia and meningitis<sup>228</sup>. Additionally, there is evidence that infection with influenza A virus induces commensal *S. pneumoniae* to disseminate beyond the nasopharynx and predisposes to *S. pneumoniae* AOM<sup>229</sup>. In AOM, *S. pneumoniae* has been identified in 30-40% of cases, with evidence that the same bacteria can be found to colonize the nasopharynx<sup>37,230</sup>. However, in contrast, *S. pneumoniae* has also been portrayed as a protective commensal bacterium. Once the nasopharynx is colonised *S. pneumoniae* inhibits colonization by other harmful potential pathogens, such as *Streptococcus pyogenes* and *Staphylococcus aureus* by “competitive exclusion”,<sup>231,232</sup>. However, more research is required to substantiate this aspect of *S. pneumoniae* colonisation.

#### 1.2.5.2.1 Antibiotic susceptibility

Penicillin resistant strains of *S. pneumoniae* were first identified in the 1970s<sup>223</sup> and since that time, the rates of resistance has rapidly increased. Patterns of drug resistance and the prevalence of specific clones are variable among different regions/countries and evolve over time and often reflect prescribing practices of that region. Six serotypes (i.e., 6A, 6B, 9V, 14, 19F, 23F) account for more than 80% of penicillin-resistant or macrolide-resistant *S. pneumoniae* worldwide. These strains are usually multi-drug resistant, while penicillins, cephalosporins and macrolides are ineffective. Penicillin resistance rates have been reported between <5-50% and macrolide resistance have been reported as high as 20% in some countries<sup>233</sup>.

Despite rising rates of resistance, there are a number of antibiotics that remain efficacious against *S. pneumoniae*. Pankuch et al.<sup>234</sup> found that vancomycin, teicoplanin, linezolid and quinupristin/dalfopristin were 100% effective in eradicating 346 *S. pneumoniae* strains tested. In addition, fluoroquinolones (levofloxacin, moxifloxacin, gatifloxacin) have also shown good microbiologic and clinical efficacy against penicillin-resistant pneumococci<sup>235</sup>. However, resistance to fluoroquinolone antibiotics has increased, again reflecting increased utilization of this class of antibiotics, but overall rates remain low (<2%) in most countries.

### 1.2.5.3 *Haemophilus influenzae*

*H. influenzae* is a gram positive, oxidase positive, facultatively anaerobic, and non-motile coccobacillus that is a commensal of the human pharynx. In clinical specimens obtained from patients who have received  $\beta$  lactam antibiotics, *H. influenzae* can instead appear as filamentous rods. By 18 months of age, one-third of children have had *H. influenzae* nasopharyngeal colonization with both typeable and non-typeable *H. influenzae* (NTHi)<sup>236,237</sup>. The name *Haemophilus* makes reference to the organisms' absolute nutritional requirement for haemin (factor X) and nicotinamide adenine dinucleotide (factor V) for growth. *H. influenzae* may be involved in a variety of invasive infections, mostly within the respiratory tract and is commonly associated with pneumonia, bronchitis, epiglottitis, sinusitis and otitis media. Outside of the respiratory tract, *H. influenzae* has been implicated in bacteraemia, cellulitis, septic arthritis and meningitis. As discussed, *H. influenzae* along with *S. pneumoniae* is one of the traditional pathogens in OM and is particularly associated with AOM in older children and with recurrent disease<sup>238</sup>. Historically, *H. influenzae* is second only to *S. pneumoniae* as the bacterial cause of AOM (23% vs 28% respectively). However compared to *S.*

*pneumoniae* AOM, *H. influenzae* AOM is considered less severe<sup>239</sup>. In contrast, *H. influenzae* is the most commonly cultured bacteria in cases of OME (11.6%)<sup>37</sup>.

*H. influenzae* is categorised into encapsulated or non-encapsulated (non-typeable) strains. For encapsulated (typeable) strains, there are six serologically distinct surface capsules that are designated serotypes a-f (based on agglutination of rabbit sera), whilst non-typeable strains do not agglutinate with a-f typing sera. The polysaccharide capsules of encapsulated *H. influenzae* are strongly negatively charged, which facilitates resistance of the bacteria to opsonisation and neutrophil phagocytosis<sup>240</sup>. Additionally, they are the most important virulence factors for invasive infections, for example, Hib strains are considered more pathogenic than the other five serotypes<sup>241</sup>, with 2-10% of *H. influenzae* infections attributed to this type. Fortunately, with the advent and widespread use of the Hib vaccine, these rates have dramatically dropped<sup>242</sup>.

Non-typeable *H. influenzae* (NTHi) on the other hand is non-encapsulated. Whilst the absence of a polysaccharide capsule contributes to the susceptibility of NTHi to complement-mediated bacteriolysis and phagocytosis, NTHi is responsible for the majority of *H. influenzae* associated OM. Differentiation of NTHi from capsule-producing *H. influenzae* depends on failure to detect a capsule. Traditionally, this is established by demonstrating agglutination of the bacteria in the presence of capsule specific types a-f antisera. However, the ability to detect the genes *bexA* and *bexB* in the capsule-region by PCR have been suggested to be more reliable, with false negative/positive rates of serotyping in the region of 40%<sup>243</sup>. With the advent of Hib vaccines and increasing survival of individuals with medical co-morbidities, invasive infections with NTHi are now more widespread among all age groups than those caused by encapsulated strains, with NTHi pharyngeal colonisation rates reported as high as 70%, with higher rates among younger children than older children and adults<sup>244</sup>.

Furthermore, the incidence of *H. influenzae* in AOM has increased since the widespread use of the pneumococcal vaccine. As previously discussed, the introduction and dissemination of the PCV7 and PCV 13 has resulted in reduction rates of *S. pneumoniae* pharyngeal carriage with a reciprocal rise in NTHi<sup>121,245</sup>.

#### 1.2.5.3.1 Antibiotic susceptibility

Although geographical variation in the resistance of *H. influenzae* to ampicillin exists, resistance rates have been consistently reported in the range of 20 to 30%. The major mechanism responsible for NTHi resistance is due to the production of plasmid mediated, A serine  $\beta$  lactamase; with 14-65% of NTHi reportedly having this trait<sup>246</sup>. This contributes to the majority of resistance to ampicillin and amoxicillin. In addition, *H. influenzae* has low susceptibility to macrolide antibiotics due to an membrane bound efflux pumps that are able to neutralise this class of antibiotics<sup>247</sup>. However of this class, azithromycin is more active in vitro against most strains of *H. influenzae* and has more rapid killing and a longer post-antibiotic effect than clarithromycin<sup>248</sup>.

Some *H. influenzae* strains that don't express  $\beta$ -lactamase are, nevertheless, resistant to ampicillin and amoxicillin. These strains are designated  $\beta$ -lactamase negative, ampicillin resistance (BLNAR) strains and gain their resistance due to characteristic *ftsI* gene mutations, which result in altered penicillin-binding protein 3 structure. BLNAR contribute up to 31% of resistant strains and are identified by having an MIC  $\geq$  4 mg/L to ampicillin and a negative test for  $\beta$ -lactamase<sup>249</sup>. In addition, there are *H. influenzae* strains that are known as  $\beta$ -lactamase positive, ampicillin and ampicillin/clavulanate resistant (BLPACR) strains. These strains both produce  $\beta$ -lactamase and possess an alteration in PBP3, which render them resistant to  $\beta$ -lactamase inhibitors. They have

ampicillin MICs  $\geq 4$  mg/L and co-amoxyclov MICs of  $\geq 8$  mg/L. BLPACR strains are less common and clinically are still susceptible to ampicillin/clavulanate<sup>250</sup>.

Despite the existence of BLNAR and BLPACR stains, generally speaking, drugs that achieve high rates of eradication of *H. influenzae* include drug combinations of penicillins with  $\beta$  lactamase inhibitors (co-amoxyclovanate) and extended-spectrum penicillin drugs (Piperacillin). Additionally, second and third generation cephalosporins and carbapenems are stable to  $\beta$  lactamase and are therefore effective. No extended spectrum  $\beta$  lactamase mediated resistance has yet been described in *H. influenzae*<sup>249</sup>.

#### 1.2.5.4 *Moraxella Catarrhalis*

*M. catarrhalis* is a large Gram-negative coccobacillus that is exclusively present in humans. *M. catarrhalis* has been reclassified in the last 40 years and has previously been called *Mima polymorpha*, *Neisseria catarrhalis* and *Branhamella catarrhalis*. Like *S. pneumoniae* and *H. influenzae*, *M. catarrhalis* had been considered a part of the normal flora of the respiratory tract, but is also associated with upper and lower respiratory tract infections, particularly in exacerbations of chronic obstructive pulmonary disease. *M. catarrhalis* is the third most identified in AOM with an average frequency of detection of 7.0% and less frequently in rAOM (4.1%)<sup>37</sup>. Less commonly, *M. catarrhalis* causes pneumonia, bacteraemia, periorbital cellulitis, neonatal meningitis, septic arthritis, and conjunctivitis.

Like *H. influenzae*, *M. catarrhalis* otitis media infections appear to be milder than pneumococcal otitis media<sup>239</sup>. In addition, compared with other bacterial pathogens, *M. catarrhalis* otitis media is

characterized by: a higher proportion of mixed bacterial infections, younger age at diagnosis, a lower proportion of spontaneous perforation of the tympanic membrane and no associated mastoiditis<sup>251</sup>.

#### 1.2.5.4.1 Antibiotic susceptibility

The majority of *M. catarrhalis* strains are considered resistant to penicillins, with 90-95 % of clinical isolates of *M. catarrhalis* found to express  $\beta$  lactamase.<sup>129,252</sup> However, there have been reports of successful treatment of  $\beta$  lactamase positive *M. catarrhalis* with penicillins<sup>253</sup>. The antibiotics of choice in the treatment of *M. catarrhalis* include: macrolides, combination penicillins with  $\beta$  lactamase inhibitors and fluoroquinolones<sup>254</sup>.

#### 1.2.5.5 *Staphylococcus aureus*

*S. aureus* is a Gram-positive coccus that is a commensal bacteria of the upper respiratory tract. It is found to colonize approximately one third of the population<sup>255,256</sup>. *S. aureus*, including methicillin-resistant strains (MRSA), are not commonly associated with AOM<sup>257</sup>, with only 1 case reported of MRSA OM in the literature for AOM<sup>179</sup>. In contrast, there are reports that methicillin-susceptible *S. aureus* (MSSA) AOM incidence ranges from 8 – 21%<sup>179</sup>.

There is evidence from observational studies that have shown that *S. aureus* prevalence within the nasopharynx is negatively associated with the presence of *S. pneumoniae*<sup>258</sup>. In fact, *S. pneumoniae* has been demonstrated to actively inhibit *S. aureus*, in addition to *H. influenzae* and *M. catarrhalis*, via a H<sub>2</sub>O<sub>2</sub> mediated mechanism. Moreover, there is a suggestion that with the introduction of the PCV7 vaccine, there was a replacement of vaccine serotypes with increased *S. aureus* colonization and a subsequent rise in *S. aureus* OM<sup>124</sup>. Additionally, *S. aureus* has been suggested to have an inverse relationship with *H. influenzae*<sup>259</sup>.

*S. aureus* is often identified in association with persistent otorrhea that follows insertion of tympanostomy tubes<sup>257</sup> and along with *P. aeruginosa*, *S. aureus* is the most commonly isolated aerobic bacteria (58.1%) in chronic suppurative otitis media (CSOM) and in mastoiditis<sup>163,165,260,261</sup>.

#### 1.2.5.5.1 Antibiotic susceptibility

MSSA strains are usually penicillin resistant but susceptible to flucloxacillin, macrolides and co-trimoxazole. However, MRSA is an increasing concern, especially in the hospital setting. In a review of 758 hospitalized patients, Davis et al.<sup>262</sup> found that at admission to hospital, 21% of patients were colonized with MSSA and 3.4 percent with MRSA. An MRSA strain is defined as having a minimum inhibitory concentrations (MIC) of  $\geq 4$  mcg/mL to oxacillin and is also resistant to all beta-lactam agents, including the majority of cephalosporins (except ceftobiprole and ceftaroline). Methicillin resistance is mediated by the *mecA* gene, which encodes for an abnormal low-affinity binding protein, PBP-2a. This protein allows MRSA strains to grow and divide in the presence of methicillin and other beta-lactam antibiotics. The *mecA* gene is located on a mobile genetic element called staphylococcal cassette chromosome and dissemination of resistance is possible via horizontal transfer of the *mecA* gene and related regulatory sequences<sup>263,264</sup>.

Oral antibiotics options in the treatment of MRSA include: clindamycin, trimethoprim/sulfamethoxazole and linezolid<sup>265</sup>. In the presence of a tympanic membrane perforation topical antibiotics containing gentamicin are also effective adjuvant treatments. Parenteral antimicrobials options include vancomycin, daptomycin, tigecycline, linezolid, and quinupristin/dalfopristin<sup>266</sup>.

#### 1.2.5.6 *Pseudomonas aeruginosa*

*P. aeruginosa* is a Gram negative bacillus and is a facultative anaerobe. It is considered an opportunistic bacterium in patients who are immunocompromised, but is also associated with disease for immunocompetent patients. *P. aeruginosa* is more commonly associated with colonizing the external ear canal<sup>38</sup> and the nasopharynx. It has also been found in biofilm form on the adenoids, tonsils and from the nasal cavity<sup>267,268</sup>.

*P. aeruginosa* is not commonly associated with AOM, but instead with CSOM and has been identified as the bacterial cause in 27.9% of cases<sup>165</sup>. Additionally, during episodes of chronic otitis media, *P. aeruginosa* can be found residing in the EAC, MEF and also in the nasopharynx<sup>131 207</sup>. Furthermore, *P. aeruginosa* is the most common bacterial pathogen in otitis externa<sup>269</sup>, in particular being isolated in 61% of cases of malignant otitis externa and also being the most common cause of petrositis<sup>270</sup>. These characteristics suggest that *P. aeruginosa* may primarily be a bacteria originating from the external auditory canal that can cause middle ear disease via a perforation in the tympanic membrane.

#### **1.2.5.6.1 Antibiotic susceptibility**

Fluoroquinolones are effective as first line topical treatment of suspected *P. aeruginosa* (or *Staphylococcus aureus*) otitis externa<sup>271</sup> with low levels of resistance. Ikeda et al., reported fluoroquinolone resistance in 12 to 28% of the 50 clinically isolated *P. aeruginosa* strains<sup>272</sup>, but suggested that 3<sup>rd</sup> and 4<sup>th</sup> generation fluoroquinolones remain effective, with sensitivity to garenoxacin, levofloxacin, ciprofloxacin and sitafloxacin. Additionally, Kim et al.<sup>165</sup> reported on 471 clinical *P. aeruginosa* strains isolated from patients with CSOM and found increasing rates of quinolone antibiotic resistance from 2001 to 2013 at their institution. However, they also reported that other antibiotics such as cefepime, ceftazidime and amikacin demonstrated good sensitivity profiles against *P. aeruginosa*

### 1.2.5.7 *Corynebacterium*

The genus *Corynebacterium* comprises a collection of irregular-formed, rod-shaped or coccoid bacteria. Although some members of the genus are major pathogens e.g. *Corynebacterium diphtheriae* (the etiological agent for diphtheria), the species frequently grown from MEE specimens are of low pathogenic potential. In fact, it has been suggested that some strains of *Corynebacterium* may have a protective role against pathogenic bacteria such as *S. aureus*. For example, *C. pseudodiphtheriticum* seems to be able to prevent colonization of the anterior nares by *S. aureus*<sup>273</sup>.

In the setting of OM, *Corynebacterium spp.* has been reported in 5.1%<sup>274</sup> and has been identified from patients with chronic suppurative OM<sup>275</sup>. However, *Corynebacterium spp.*, which are considered commensals of the external ear canal<sup>175</sup>, may well play a similar role in the middle ear<sup>274</sup>.

#### 1.2.5.7.1 *Antibiotic susceptibility*

To our knowledge, there is no literature addressing the treatment of *Corynebacterium spp.* in OM. However, based on EUCAST<sup>276</sup> guidelines, commonly used antibiotics in the treatment of this species includes penicillins, macrolides, fluoroquinolones, glycopeptides and tetracycline.

### ***1.2.6 The role of biofilm in otitis media***

As discussed above, bacteria are cultured from MEF in only 40% of case of OME, despite targeted PCR revealing the presence of bacterial DNA in 80% of cases. One hypothesis behind the low yield from culture is that the majority of the bacteria within the middle ear exist within a biofilm form. Since the demonstration of biofilm from the middle ear mucosa in children by Hall-Stoodley and colleagues<sup>162</sup>, OM has been considered a biofilm driven disease. The biofilm model of disease and the life-cycle of biofilms can be applied to explain the natural history of OM and also provides an explanation for its persistent and recurrent nature.

#### ***1.2.6.1 Background***

Bacteria are known to exist in one of two distinct growth phases: in a biofilm phase or a planktonic phase<sup>277</sup>. While these two phases represent distinctly different phenotypical modes of bacterial existence, they exist on a continuum in the life cycle of bacteria and are reflections of differential gene expression<sup>278</sup>. The biofilm phase is characterised by the formation of a multicellular community within a protective matrix that favours proliferation. In contrast, after being shed from the biofilm, the primary role of the planktonic bacteria is to facilitate dissemination and colonization of new environments, after which further biofilm can be formed.

In nature, bacteria predominantly exist in biofilm form, the formation of which, is in response to the proximity to a surface<sup>279</sup>. Biofilm is defined as “matrix-enclosed microbial populations adherent to each other and/or to surfaces or interfaces”<sup>277</sup>. It is a complex, three-dimensional structure made of an extracellular polysaccharide matrix and extracellular DNA produced by the bacteria. The matrix encloses the sessile cells and allows irreversible attachment to a surface<sup>280</sup>. The sessile cells within are subsequently able to exist in a coordinated, highly organised and stable physiological and functional population. This primitive physiological homeostasis provides bacteria with survival and

persistence advantage by allowing the sessile cells to express the most advantageous phenotype<sup>277</sup>.

The protective characteristics of biofilms are therefore a result of the protective physical barrier of the matrix and of the gene expression patterns of the sessile cells within.

The extracellular polysaccharide matrix protects bacteria within against not only environmental (e.g. fluctuations in temperature, oxygen tension, pH) and chemical (e.g. antibiotics) stressors but also against phagocytosis and humoral immunity<sup>280,281</sup>. Furthermore, there is evidence that bacterial species within polymicrobial biofilm can confer growth advantages and increased protection to each other, resulting in greater resistance than in single species biofilms<sup>282,283</sup>. The benefits of polymicrobial biofilm formation have been demonstrated *in vivo*<sup>282,283</sup> and in animal models<sup>283-286</sup>; however, this is not a universal finding<sup>246</sup>.

Biofilm formation is also known to provide increased antibiotic resistance. Bacteria in biofilm require antibiotic concentrations multitudes higher to eradicate than for the same bacteria in planktonic form<sup>280,287</sup>. Some of the recognised mechanisms behind increased resistance in biofilm include: the ability of the extracellular matrix to impede antibiotic penetration, decreased metabolic activity of the bacteria within the biofilm, increased adaptation via quorum sensing, increased levels of mutations in antibiotic target molecules and upregulated antibiotic resistance genes<sup>288</sup>. Furthermore, the transfer of antibiotic resistance genes between bacteria has been identified as another potential mechanism by which bacteria can gain antibiotic resistance and growth advantage<sup>289</sup>. Studies have shown that resistant *H. influenzae* strains can horizontally transfer *ftsI* genes (and thus resistance traits) to other *H. influenzae* strains<sup>290</sup>. Similarly, Armbruster and colleagues<sup>283</sup> demonstrated that polymicrobial biofilm formed by *H. influenzae* and *M. catarrhalis* conferred both  $\beta$  lactamase dependent and  $\beta$  lactamase-independent resistance to each other.

Additionally, infections caused by bacteria in planktonic and biofilm forms manifest with different disease characteristics. Planktonic bacteria are associated with acute infection, whereas biofilm driven disease, is more indolent. The presence of biofilm evokes a sustained inflammatory response from the host immune system, producing a persistent low-grade infection<sup>287</sup>. It is proposed that more than 65% of bacterial diseases are caused by bacteria existing in biofilm form<sup>291</sup>. Biofilm driven diseases are typified by a chronic illness of relapsing and remitting course, where culture of bacteria is variable, but also demonstrates a significant recalcitrance to antibiotic treatment. Among other things, cell detachment, endotoxin or exotoxin production, and resistance to host immune attack are all virulence mechanisms adopted by biofilm bacteria<sup>292</sup>.

#### ***1.2.6.2 Biofilm in Otitis media***

The life cycle of biofilms closely resembles the natural history of OM, which is characterised by frequent spontaneous resolution, but also by high recurrence rates and persistence of bacteria despite antibiotic treatment<sup>293</sup>. While in biofilm form, the bacteria may cause low-grade subacute inflammation and thus perpetuate middle ear effusion. However, through the planktonic shedding of the biofilm, an acute infection with an associated intense inflammatory response can result. As an example, patients with OME are 5 times more likely to develop AOM, than those without<sup>6</sup>.

In the setting of OM, the three most commonly recognised pathogenic agents, *S. pneumoniae*, nontypeable *H. influenzae* (NTHi) and *M. catarrhalis*, have been shown to form biofilms *in vivo*<sup>294</sup>. In addition, biofilms were first demonstrated in the middle ear in animal models of OM<sup>295,296</sup>. Post et al.<sup>295</sup> investigated the potential for *H. influenzae* to form biofilm in the middle ear of Chinchillas. They injected *H. influenzae* into the middle ear clefts and used scanning electron microscopy and confocal laser scanning microscopy to visualise the middle ear mucosa. They demonstrated that viable biofilm formed 48 hours after inoculation and persisted until 6 days. This study was followed

up by a similar experiment by Ehrlich et al.<sup>296</sup> with 48 adult chinchillas, in which they found middle ear mucosa biofilm formation within 24 hours of inoculation that persisted up to 21 days. Since these early experiments, biofilm has been demonstrated in these Chinchilla models from many bacterial strains including *S. pneumoniae*<sup>297</sup>, *M. catarrhalis*<sup>283</sup> and *P. aeruginosa*<sup>298</sup>.

The first study to demonstrate biofilm from the middle ear cleft in humans was by Hall-Stoodley et al.<sup>162</sup>. The study involved taking biopsies of the middle ear mucosa of 26 children with OME and 8 control patients. The samples were analysed with CSLM and FISH probes targeting *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. Not only was biofilm found in 46 out of 50 (92%) OME biopsies, but polymicrobial biofilms involving combinations of the three bacteria were visualised. Furthermore, biofilm was not identified from control patients. Similarly, Thornton et al.<sup>299</sup> demonstrated multispecies biofilm from mucosal samples of 20 children both with OME and rAOM (65% of samples). Additional to demonstrating biofilm, the authors also imaged intracellular bacteria resembling cocci within the mucosal epithelium using transmission electron microscopy. They note that, despite using targeted FISH probes for identification of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, that there remained species of bacteria within the samples that were unidentified. This finding suggests that previously unidentified bacteria exist in biofilm form within the middle ear of OME and rAOM.

In addition, middle ear biofilm has been shown in patients with chronic suppurative OM and cholesteatoma<sup>300</sup>. More recently, biofilm has been isolated on the surface of tympanostomy tubes<sup>301</sup> and also free floating within the middle aspirates of children with COME<sup>302</sup>.

### 1.3 Summary of literature review

Otitis media with effusion is a highly prevalent childhood disease that carries with it a significant morbidity to children across the globe. The most common impact of untreated OME is hearing loss to the child, with an associated delay in both social and learning development. These sequelae are especially pertinent in the Australian Aboriginal community, where there is a pre-existing gap in health outcomes, that is also reflected in the rates of OM.

While much is known about the factors contributing OME, there remain a number of key questions yet to be answered. Both host and environmental factors contribute to the development of OME. Host factors include age, sex, inherited predisposition to OME and anatomical factors. In addition, environmental factors such as pathogen exposure, tobacco smoke exposure, allergy, population density, seasonal variation and breastfeeding also play a role. Based on our current knowledge, these factors combine to contribute to Eustachian tube dysfunction. However, Eustachian tube dysfunction in itself is usually not sufficient to cause OME, but in the presence of a pathogen, whether through viral or bacterial infection, OM can develop, which subsequently results in OME in 40% of cases.

As a result, there is ample research on the role that viruses and bacteria play in the pathogenesis of OME. There are classically three bacteria that are identified as causes of OM. *S. pneumoniae* is the most common cause of AOM, whilst *H. influenzae* is more commonly found in the middle ear aspirates of patients with OME. *M. catarrhalis* is the third most commonly found bacteria and is associated with polymicrobial infection. However, with the evolution and advancement of culture-independent techniques, such as PCR and 16srRNA gene sequencing, previously unidentified bacteria have been demonstrated from the middle ear aspirates of OM sufferers. Of particular interest is the emergence of *A. otitidis* from patients with OME.

The nasopharynx is traditionally considered the reservoir for the middle ear as evidenced by the high incidence and abundance of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* described in the literature. In contrast, *A. otitidis* is often not found in the nasopharynx, even in children with concurrent otitis media. It is however commonly isolated from the external ear where, to date, its role is considered primarily commensal. There is evidence of external ear canal bacteria such as *S. aureus* and *P. aeruginosa* causing middle ear disease by translocating from the external ear canal into the middle ear following perforation of the tympanic membrane. Whether *A. otitidis* can translocate into the middle ear and play a pathogenic role is yet undetermined.

Regardless of the source of the bacteria, *A. otitidis* has been demonstrated in 20-60% of middle ear aspirates in OME, with evidence that it has pathogenic potential. *In vivo* studies have shown *A. otitidis* to have an immune-stimulatory potential on both myeloid and lymphoid cell types. Furthermore, *A. otitidis* has also been shown to induce otitis media with effusion in a rat model. Additionally, *A. otitidis* has been implicated in cases of implant and chronic infection, which are typically associated with biofilm processes. This is relevant because OM is thought to be a biofilm driven disease, with the persistent and recurrent nature of OM being typical of biofilm infection. Furthermore, biofilm has been directly visualised on the middle ear mucosa in patients with OME. Of particular interest is a comment by Thornton et al.<sup>299</sup>, who identified *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in biofilm from middle ear mucosa samples using targeted FISH probes. They reported that when visualising the biofilm, that there remained a proportion of the biofilm with unidentified bacteria. Whilst, the ability of *A. otitidis* has not been investigated, given that *A. otitidis* is found in high abundance in middle ear aspirates in OME, it seems a potential candidate to contribute to biofilm formation in OME.

## 1.4 Aims

1. Characterise the resident microbiota of the middle ear in children with OME using culture-independent 16S rRNA gene sequencing techniques
2. Identify the bacterial reservoirs for the middle ear by comparing the microbiota of the middle ear with that of the adenoid pad and external auditory canal
3. Analyse the microbial communities within the middle ear to further understand the complex microbial relationships within the middle ear and to explore ways to manipulate this microbiome
4. Determine the potential pathogenic role of *A. otitidis* in otitis media by elucidating its growth characteristics and whether it can form biofilm
5. Examine the possible relationships between *A. otitidis* and common otitis media pathogens

## *Chapter 2: The microbiome of otitis media with effusion*

---

Conducted in the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, Australia and the Department of Otolaryngology Head and Neck Surgery, Women's and Children's Hospital, Adelaide, Australia

Financial assistance provided by the University of Adelaide Postgraduate Award Scholarship

## STATEMENT OF AUTHORSHIP

### **Chun Ling Chan**

Project design, patient recruitment, tissue collection, analysis of data, manuscript preparation

### **David Wabnitz**

Project design, tissue collection, manuscript editing

### **Jake Jervis Bardy**

Data Analysis, manuscript preparation and editing

### **Ahmed Bassiouni**

Data Analysis, manuscript preparation and editing

### **Peter-John Wormald**

Principal project supervision, manuscript editing

### **Sarah Vreugde**

Project co-supervisor, manuscript editing

### **Alkis James Psaltis**

Project co-supervisor, manuscript editing

**Accepted for publication:**

**The microbiome of otitis media with effusion**

**Chan C, Wabnitz, D, Jarvis-Bardy J, Bassiouni A, Vreugde, S, Wormald PJ, Psaltis AJ**

The Laryngoscope, published online, 23/06/16

# Statement of Authorship

Title of Paper	The microbiome of otitis media with effusion		
Publication Status	<input checked="" type="checkbox"/> Published		
Publication Details	Published online in "The Laryngoscope", 23/06/16		

## Principal Author

Name of Principal Author (Candidate)	Chun Chan		
Contribution to the Paper	Project design, patient recruitment, tissue collection, analysis of data, manuscript preparation		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	31/10/16

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David Wabnitz		
Contribution to the Paper	Project design, tissue collection, manuscript editing		
Signature		Date	12 October 2016

Name of Co-Author	Jake Jarvis Bardy		
Contribution to the Paper	Data Analysis, manuscript preparation and editing		
Signature		Date	12 October 2016

Name of Co-Author	Ahmed Bassiouni		
Contribution to the Paper	Data Analysis, manuscript preparation and editing		
Signature		Date	12 October 2016

Name of Co-Author	Peter John Wormald		
Contribution to the Paper	Principal project supervision, manuscript editing		
Signature		Date	11 October 2016

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature		Date	11 October 2016

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature		Date	11 October 2016

## 2.1 Abstract

**Objective:** The adenoid pad has been considered a reservoir for bacteria in the pathogenesis of otitis media with effusion. This study aims to characterize the middle ear microbiota in children with otitis media with effusion and establish whether a correlation exists between the middle ear and adenoid microbiota.

**Study design:** Prospective, controlled study

**Methods:** Middle ear aspirates adenoid pad swabs were collected from twenty-three children undergoing ventilation tube insertion. Adenoid swabs from patients without ear disease were controls. Samples were analysed using 16S rRNA sequencing on the Illumina MiSeq platform.

**Results:** Thirty-five middle ear samples were collected. The middle ear effusion microbiota was dominated by *Alloiococcus (otitidis)* (23% mean relative abundance), *Haemophilus* (22%), *Moraxella* (5%), and *Streptococcus* (5%). *Alloiococcus* shared an inverse correlation with *Haemophilus* ( $p = 0.049$ ) and was found in greater relative abundance in unilateral effusion ( $p = 0.004$ ). The microbiota of bilateral effusions from the same patient were similar ( $p < 0.001$ ). However, the OME microbiota was found to be dissimilar to that of the adenoid ( $p = 0.01$ ), whilst the adenoid microbiota of OME and control patients were similar ( $p > 0.05$ ) (PERMANOVA).

**Conclusions:** Dissimilarities between the local microbiota of the adenoid and the middle ear question the theory that the adenoid pad is a significant reservoir to the middle ear in children with otitis media with effusion. *A. otitidis* had the greatest cumulative relative abundance, particularly in unilateral effusions and shares an inverse correlation with the relative abundance of *Haemophilus*.

## 2.2 Introduction

Otitis media with effusion (OME) is the most common cause of hearing loss in childhood<sup>303</sup>. It has been estimated that the point prevalence of OME at age two is one in five children, with four in every 5 children affected at least temporarily by the age of 10 years<sup>303</sup>.

The pathogenesis of OME remains unclear, although the Eustachian tube (ET) is thought to play a central role<sup>303</sup>. It has been postulated that the ET's short length, horizontal position, and reduced rigidity in the paediatric population may permit the reflux of naso- and oro-pharyngeal microbes in to the middle ear cavity, explaining the higher incidence of OME in children compared to adults<sup>303</sup>.

Traditional culture based techniques have isolated *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* as the most common pathogens in OME. With the advancement of culture independent techniques such as 16S rRNA pyrosequencing, diverse and previously unknown bacterial communities have now been demonstrated within the middle ear<sup>132,304</sup>. One such emerging potential pathogen is *A. otitidis*, which has been isolated from middle ear aspirates with increasing frequency in the last decade<sup>182,194,305,306</sup>.

The aim of this study was to use culture independent techniques to compare the local microbial population of the adenoids in patients with and without OME and test the hypothesis that this region acts as a bacterial reservoir, predisposing to OME.

## ***2.3 Materials and methods***

Ethics approval was obtained from the institutional research boards of the Women and Children's Hospital and the University of Adelaide (approval HREC/14/WCHN/42).

### ***2.3.1 Selection Criteria***

Children wait-listed for ventilation tube (grommet) insertion with adenoidectomy for chronic OME (>3 months) were enrolled. Control patients were approached if they had no history of otitis media in the last 12 months and were undergoing other forms of surgery requiring general anaesthetic and endotracheal intubation that was anatomically distant from the nasopharynx. Written parent/guardian consent was obtained prior to enrolment. Exclusion criteria included previous adenoidectomy, acute otitis media, resolved effusions or upper respiratory tract infection at time of surgery, and antibiotic or steroid use in the four weeks preceding surgery. Demographic data including age, gender and parental smoking status as well as clinical data including: presenting symptoms, previous tympanometry, medication history, and previous medical and surgical history were collected.

### ***2.3.2 Sample collection***

Samples were collected whilst the patient was under general anaesthetic, prior to commencement of the planned procedure. All specimens were placed on ice immediately after collection and transferred for storage in a -80° freezer.

#### ***2.3.2.1 Middle ear effusion collection***

Following aural toilet of the external canal, all instruments were replaced with new sterile equipment. Myringotomy was performed and the effusion aspirated with microsuction attached to an Argyle™ specimen trap (Covidien, Mansfield, Massachusetts, USA). The circuit was then flushed

with 2 microliters of sterile normal saline. Caution was exerted to ensure that no instrument touched the canal wall skin. If this occurred the instruments were exchanged for sterile ones.

#### **2.3.2.2 *Adenoid swab:***

To avoid contamination all swabs were taken transorally under direct vision. A sterile flocked swab (Copan Italia S.p.A., Brescia, Italy) was guided trans-orally avoiding contact with the oropharyngeal mucosa. The swab was then brushed over the surface of the adenoid pad five times and transferred immediately into a cryotube.

#### **2.3.3 *DNA extraction***

DNA extraction was carried out using PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories, Salona Beach, CA). Swab heads and tissue were thawed on ice and placed directly on to the beads for homogenisation. Total DNA was extracted from all clinical samples and two DNA extraction negative controls containing extraction reagents only. The remainder of the extraction protocol was performed as per the manufacturer's protocol. Extracted DNA was stored at -80°C until sequencing.

#### **2.3.4 *PCR amplification of the 16S rRNA gene and sequencing***

PCR amplification and sequencing was performed by the Australian Genome Research Facility (AGRF). Libraries were generated by amplifying the V3–V4 (341F – 806R) hypervariable region of the 16S rRNA gene. PCR amplicons were generated using the primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia) following local protocol. The resulting amplicons were measured by Fluorometry (Invitrogen Picogreen) and normalised. The equimolar pool was then quantified by qPCR (KAPA) and setup for sequencing on the Illumina MiSeq with Paired End Chemistry. Reads from Illumina sequencing were used as raw data for bioinformatic analyses.

### *2.3.5 Bioinformatics pipeline*

The Paired-End reAd mergeR (PEAR)<sup>307</sup> v.0.9.5 was used to pair forward and reverse reads in each sample, and quality filter the paired reads. Open-reference OTU picking strategy with pre filter threshold of 0.80 was used to cluster OTUs<sup>308</sup>. Within the open-reference method, UCLUST<sup>309</sup> v.1.2.22 was used to cluster OTUs at 97% similarity. SILVA (release 111)<sup>310</sup> was used for the reference-based step of the OTU picking strategy and for taxonomic assignment at 95% similarity.

### *2.3.6 Biostatistical analysis*

Prior to downstream analysis, artefactual OTUs arising from possible reagent contamination were identified by analysing the OTU distribution relative to amplicon concentration obtained after library preparation of each sample. OTUs with cumulative relative abundance ranked in the top ten of all OTUs in the dataset were filtered from the data if the relative abundance distribution was inversely correlated with amplicon concentration<sup>311</sup>.

Pursuant to rarefaction, all samples were subsampled to 2000 reads. Diversity estimates were performed on subsampled data. Simpson's index of diversity (1-D) was used to estimate diversity. The Simpson's index of diversity takes into account the number of species present, and their relative abundance, providing a value between 0 and 1, with higher numbers representing a more diverse bacterial community. A Mann Whitney U-test was used to determine significance of variation in diversity across sample types. GraphPad v6 (GraphPad Software Inc, California, USA) was used to generate box plots and calculate the statistical tests used.

Mean relative abundance of top genera were calculated for the middle ear fluid (MEF), adenoid and control groups. The mean of top genera in the MEF, adenoid and control groups were compared using a Wilcoxon Signed Ranks Test, Two Samples Students T-Test and independent samples T-Test as applicable. Bray-Curtis (BC) similarity matrices were generated, providing a value from 0 to 1

estimating the similarity between samples (with higher numbers representing greater similarity). The BC matrix was used to create a hierarchical group-average cluster analysis in PRIMER v6 (PRIMER-E Ltd, Plymouth, UK)<sup>312</sup>. The BC matrix was also used to summarise the distance between MEF samples from the same patient (within patient), and the MEF samples from all patients (between patient) in QIIME. A two-sided Student's two-sample t-test was used to test significance between the within patient and between patient summaries. A permutational MANOVA (PERMANOVA) test was also performed in PRIMER to test whether there was a statistically significant difference between the bacterial communities in the MEF and the adenoids samples (as a fixed factor), with samples from the same patients grouped in the analysis (as a random factor). Microsoft Excel v 14.4.5 (Microsoft Corporation, Washington, USA) was used to generate bar plots of community composition. Cytoscape v2.8.2<sup>313</sup> was used to create a co-occurrence model.

Species level presumptive identification was performed on OTUs classified as *Alloiooccus*, using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database<sup>314</sup>. Taxonomic assignment for all other OTUs remained at genus or family (if genera not possible)-level during taxonomic assignment.

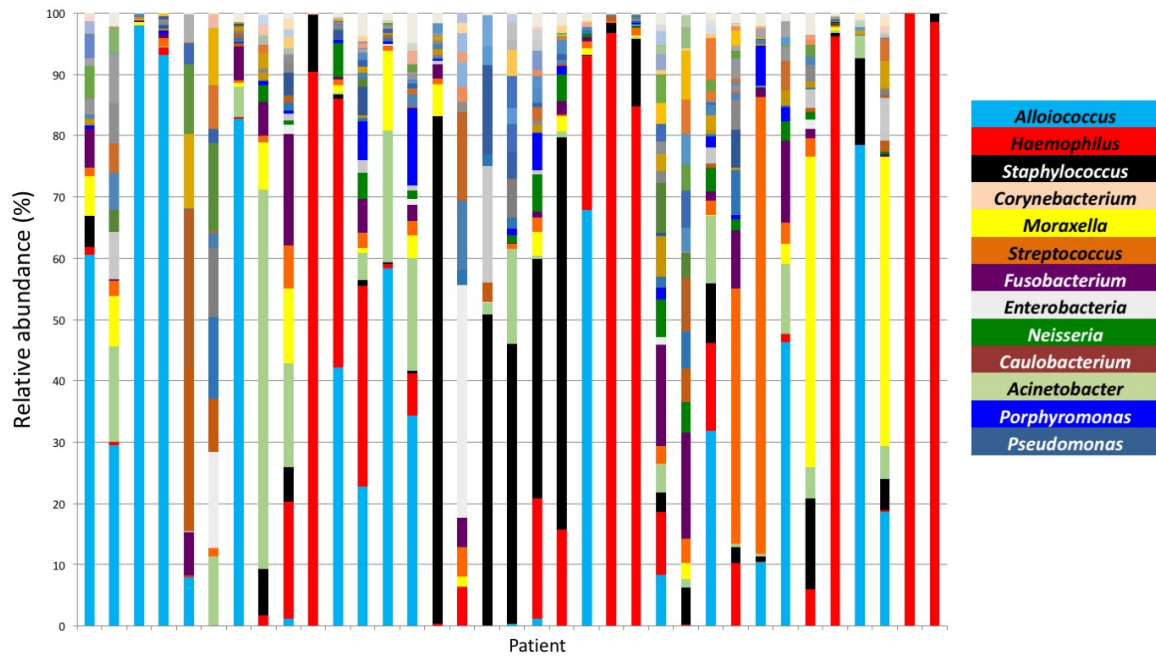
## 2.4 Results

### 2.4.1 Demographic data:

Twenty-three patients (14 male) with OME, aged 1-8 years (mean = 40 months, SD = 21), and 10 control patients aged 1-12 years (73 months, 56), were recruited. Thirty-five middle ear effusion samples were collected, with 12 (60%) OME patients presenting with bilateral middle ear effusions. The control group included 7 patients undergoing laryngobronchoscopy for investigation of laryngomalacia and/or aspiration, with the remaining 3 undergoing general anaesthetic thyroidectomy, pre-auricular sinus repair and airway foreign body retrieval respectively. There was no significant difference between the groups with univariate analysis of age, sex and exposure to a member of the same household smoking.

### 2.4.2 Middle ear microbiome

The bacterial composition of MEF was predominated by aerobic bacteria, but relative abundances were highly variable between patients (fig 2-1). The genera with the highest cumulative relative abundance was *Alloiococcus* (presumptive identification of OTUs classified to genus level as *Alloiococcus* using BLAST, confirmed >99% similarity to *A. otitidis*), with otopathogenic genera (*Moraxella*, *Haemophilus* and *Streptococcus*) also well represented (table 1). In addition, seven MEF samples demonstrated a dominant genus (relative abundance > 90%, *Alloiococcus*, 2/7 and *Haemophilus*, 5/7).



**Figure 2-1 The microbiome of otitis media with effusion.** Microbiome of MEF, represented in relative abundance. Data are presented for genera that were present at  $\geq 1\%$  relative abundance in at least 1 sample. The thirteen most abundant genera are identified (see key).

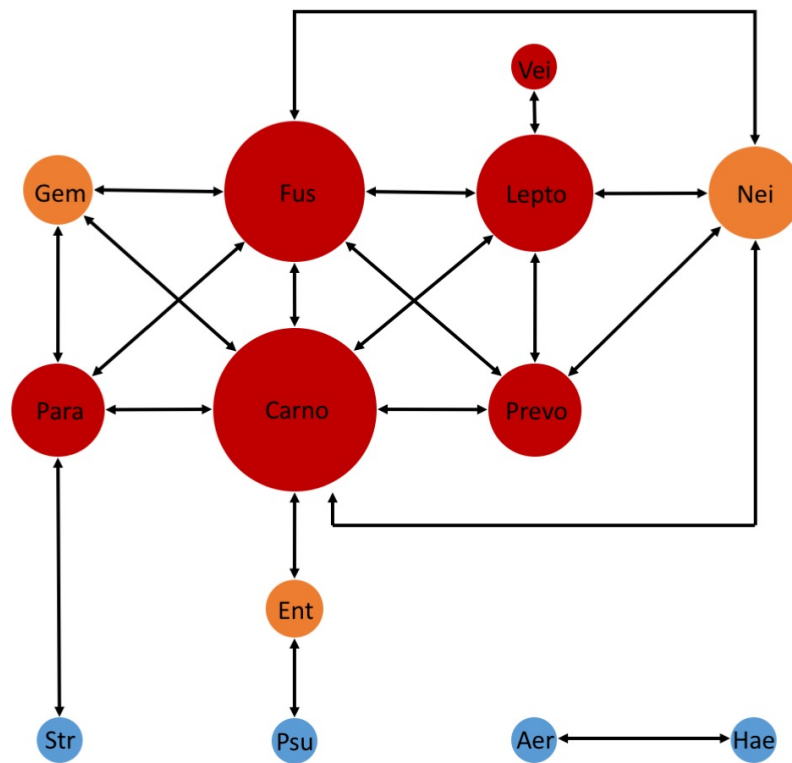
Inverse correlation was demonstrated between relative abundances of *Alloiococcus* and *Haemophilus* ( $p = 0.049$ ). In addition, subgroup analysis demonstrated that *Alloiococcus* was more abundant in unilateral effusions than in bilateral effusions ( $p = 0.004$ , Mann Whitney U-Test)

Correlation was observed between the relative abundance of different bacteria within the middle ear microbiome (Figure 2-2, table 2-1). In summary, there was a core of anaerobic and facultative anaerobic bacterial genera that were consistently abundant across all MEF samples.

Bacterial genera	Prevalence		Relative abundance		
	MEF (%)	Adenoids	MEF (%)	Adenoids (%)	p value
<b>Aerobes:</b>					
<i>Alloiococcus</i>	69	78	23	< 1	<b>&lt; 0·001</b>
<i>Haemophilus</i>	100	100	22	25	0·271
<i>Staphylococcus</i>	66	87	11	< 1	<b>0·004</b>
<i>Corynebacteria</i>	66	70	6	< 1	<b>0·008</b>
<i>Streptococcus</i>	97	96	5	13	<b>0·028</b>
<i>Moraxella</i>	89	96	5	14	0·271
<i>Neisseria</i>	80	100	1	7	<b>0·001</b>
<i>Pseudomonas</i>	68	not found	1	not found	<b>0·017</b>
<b>Anaerobes:</b>					
<i>Fusobacteria</i>	100	100	4	11	<b>0·002</b>
<i>Porphyromonas</i>	74	100	1	5	<b>0·031</b>
<i>Prevotella</i>	69	100	< 1	3	<b>0·005</b>
<i>Enterobacteria</i>	66	not found	2	not found	0·149
<i>Gemella</i>	63	100	< 1	3	<b>&lt; 0·001</b>
<i>Paraprevotella</i>	46	100	< 1	2	<b>0·001</b>
<i>Veillonella</i>	43	100	< 1	1	<b>0·02</b>
<i>Caulobacteria</i>	29	not found	1	not found	0·096

**Table 2-1 The bacteria found on the adenoid pad and from middle ear effusion.** The prevalence and relative abundance of bacterial genera from adenoid swabs and MEF of patients with otitis media with effusion.

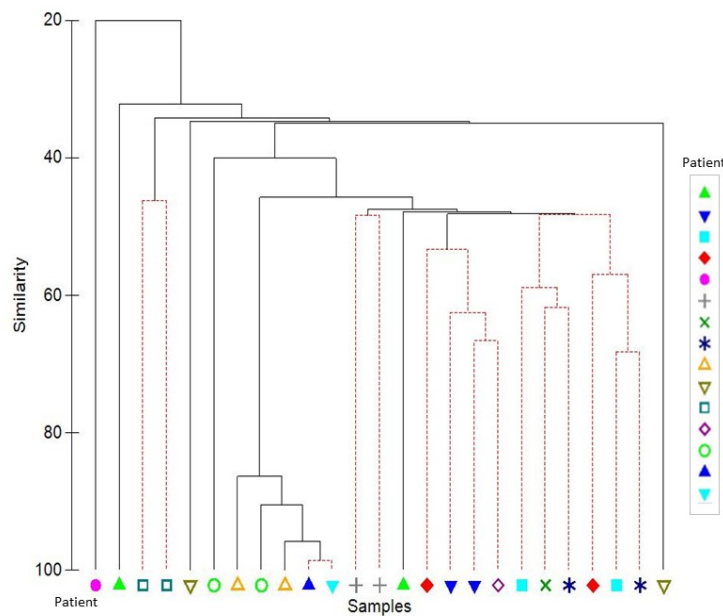
**Bacterial correlation ( $p < 0.05$ ) - MEF**



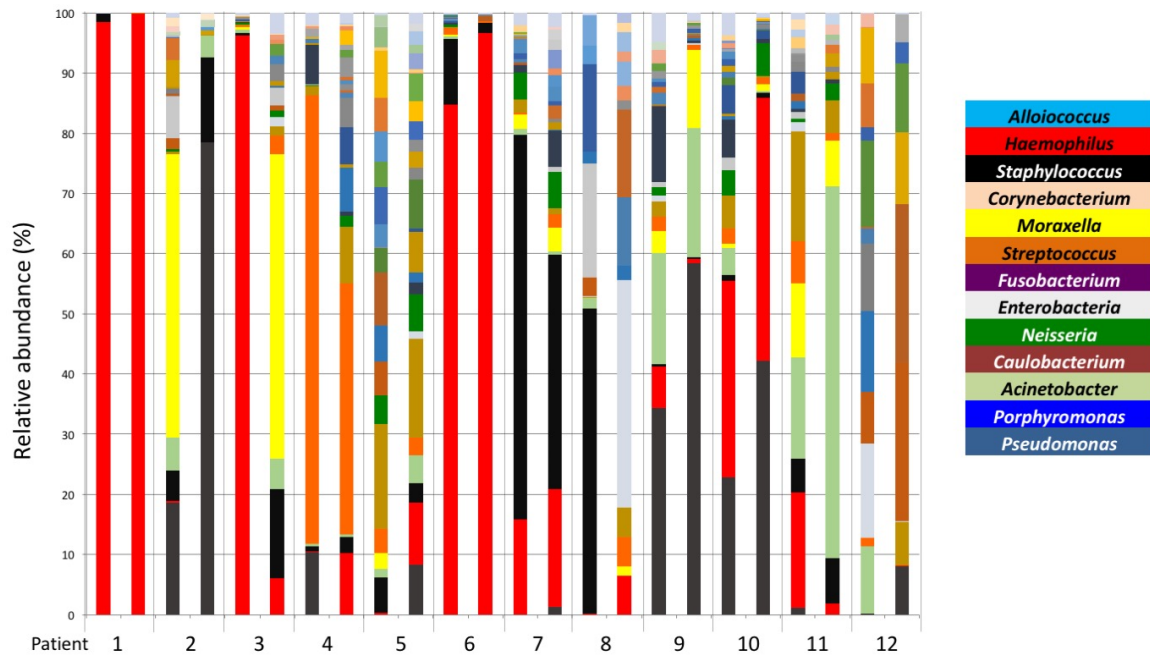
**Figure 2-2 Bacterial correlations within middle ear fluid:** Graphic representation of the correlation between bacterial genera (family if unspecified) within the MEF ( $p < 0.05$ , Two Samples T-test). Red: anaerobic bacteria, orange: facultative anaerobic bacteria, blue: aerobic bacteria. The size of the circle is relative to the number of correlations with other bacteria.

Legend: Allo – *Alloiococcus*, Hae – *Haemophilus*, Psu – *Pseudomonas*, Str – *Streptococcus*, Ent – *Enterobacteriaceae* (family), Neiss – *Neisseria*, Gem – *Gemellaceae* (family), Fuso – *Fusobacteria*, Lepto – *Leptotrichiaceae* (family), Vei – *Veillonella*, Para – *Paraprevotella*, Carno – *Carnobacteria*, Prevo - *Prevotella*

Bilateral effusions were similar within the same patient (Two tailed Students T-tests,  $p < 0.05$ ) (fig 2-3 & fig 2-4), as well as between the effusions of different patients with bilateral OME (PERMANOVA analysis,  $p < 0.001$ ). Specifically, there were strong correlations in relative abundance between sides with common otopathogenic genera: *Haemophilus* ( $p = 0.007$ ), *Streptococcus* ( $p < 0.001$ ), *Staphylococcus* ( $p = 0.025$ ), and *Corynebacteria* ( $p = 0.006$ ) using paired T-tests.



**Figure 2-3 Cluster analysis of the similarities between MEF samples:** Hierarchical Group Average Cluster Analysis (HCAN) based upon Bray-Curtis similarity of genus level data comparing left and right ear effusions from the same patient. Percent similarity is indicated on the Y-axis.

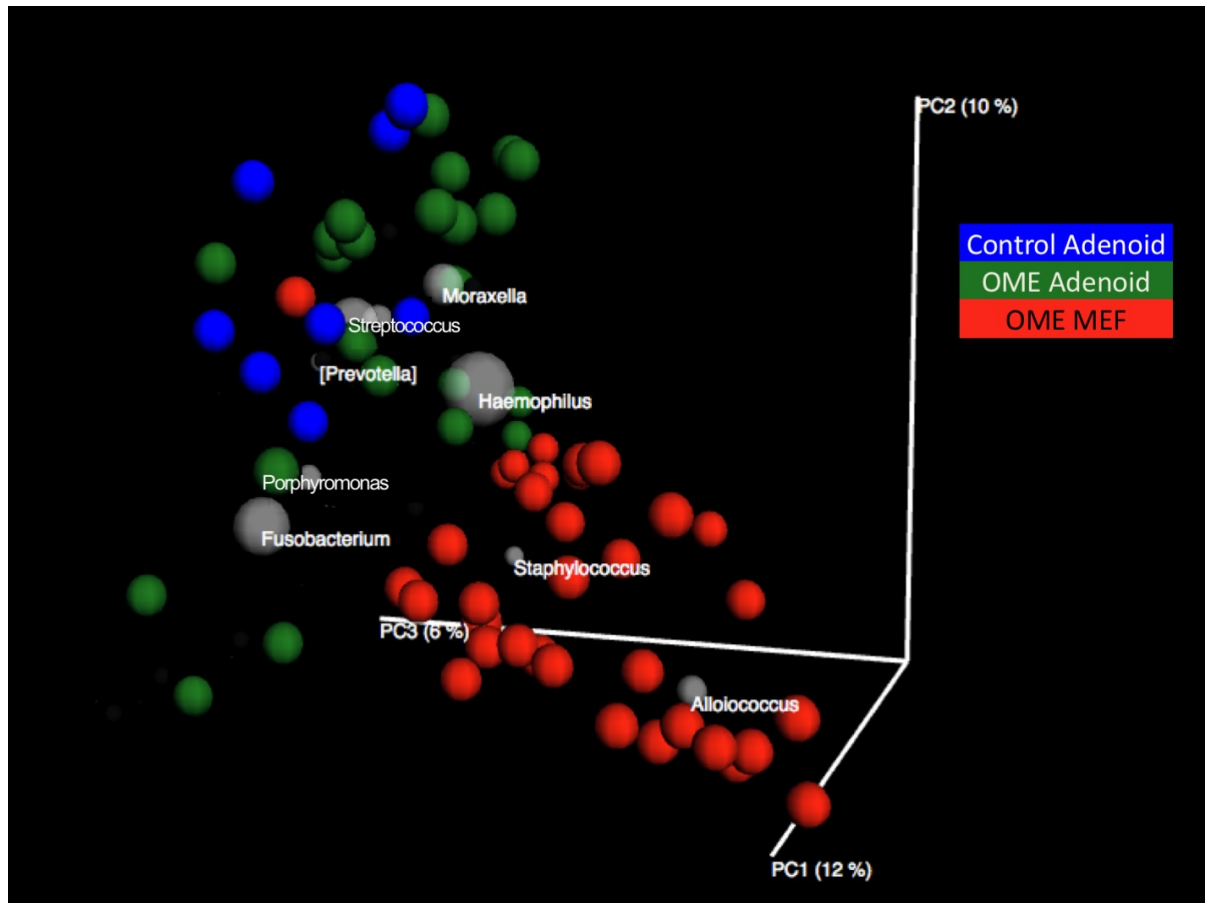


**Figure 2- 4 Microbiome of bilateral MEF.** The microbiome of bilateral MEF, represented in relative abundance. Data are presented for genera that were present at  $\geq 1\%$  relative abundance in at least 1 sample. The thirteen most abundant genera are identified (see key).

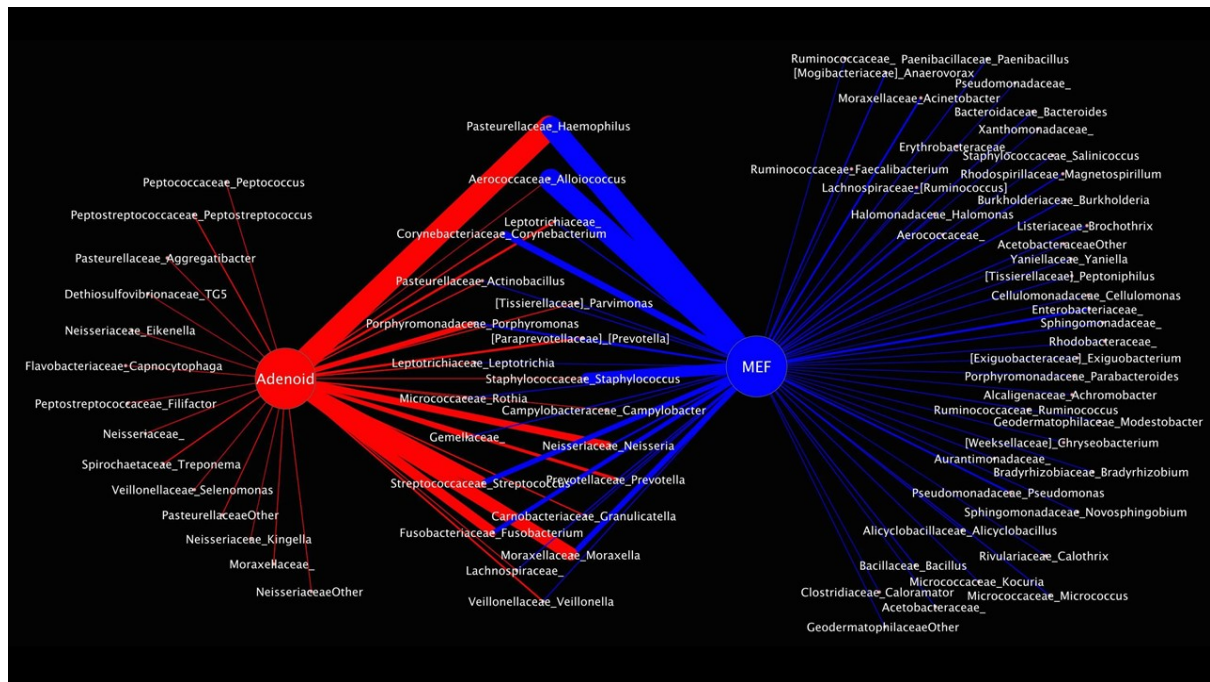
### 2.4.3 Adenoid microbiome versus middle ear microbiome

The microbiome of the middle ear cavity and the adenoid were dissimilar (fig 2-5, table 2-2). Alpha diversity between adenoid swabs from patients with OME and from control adenoid swabs were similar. Thirteen of the seventeen most abundant bacterial genera demonstrated a significant difference in relative abundance between the different sites, with three bacterial families (*Pseudomonas*, *Enterobacteria* and *Caulobacteria*) only demonstrated in MEF (table 2-1). This was confirmed with PERMANOVA multivariate analysis (controlled for paired design) testing beta diversity ( $p = 0.001$ ) and was modelled with co-occurrence (fig 2-6).

Adenoid tissue showed an increase in the abundance of anaerobes, accounting for five out of the ten most abundant genera found in the adenoid, representing almost half of the total abundance of genera from adenoid samples (48% versus 6%,  $p < 0.001$ ).



**Figure 2-5 Principal Co-ordinates Analysis of the microbiota of the adenoid and MEF:** Principal Co-ordinates Analysis (PCoA) biplot based on Bray-Curtis similarity of genus level data. Colored spheres indicate individual samples. Top 10 genera are displayed in grey spheres with volume indicating relative abundance (larger volumes indicate increased relative abundance).



**Figure 2-6 Co-occurrence comparisons of the adenoid pad and MEF:** Co-occurrence Model showing average proportional relative abundance of genus level data by sample type. Genera with  $\geq 1\%$  relative abundance in at least 1 sample are included.

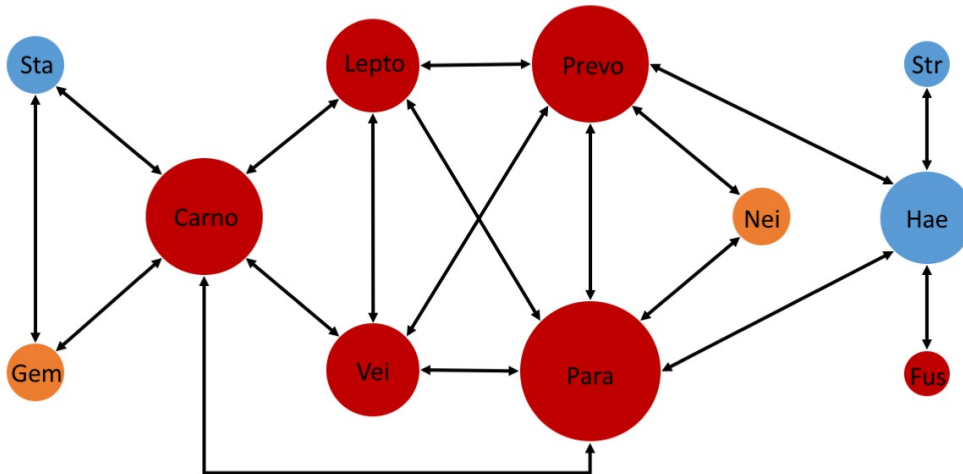
#### 2.4.4 MEF adenoid microbiome versus control

Adenoid samples from OME patients and control patients were similar in regards to diversity (Mann Whitney U Test,  $p = 0.1$ ) and relative abundance (Independent Samples T test,  $P > 0.05$  throughout) (Table 3). PERMANOVA again confirmed this, with similarity demonstrated ( $p = 0.4$ ). Notably, there was a greater (non-significant) abundance of genera associated with middle ear disease (*Haemophilus*, *Streptococcus* and *Moraxella*) observed in the OME adenoids. In addition, the same anaerobic and facultative anaerobic bacteria that were highly correlated in abundance in MEF were found to have similar relationships on the adenoid (Supplement 1/Figure 2-7).

<b>Correlating bacteria</b>		<b>p value</b>
<i>Fusobacterium</i>	<i>Neisseria</i>	0.012
	<i>Gemellaceae (family)</i>	0.02
	<i>Prevotella</i>	0.002
	<i>Leptotrichiaceae (family)</i>	0.001
	<i>Paraprevotella</i>	0.021
	<i>Carnobacterium</i>	0.007
<i>Carnobacterium</i>	<i>Neisseria</i>	0.031
	<i>Gemellaceae (family)</i>	0.013
	<i>Prevotella</i>	<0.001
	<i>Leptotrichiaceae (family)</i>	<0.001
	<i>Paraprevotella</i>	<0.001
<i>Leptotrichiaceae (family)</i>	<i>Veillonella</i>	0.001
	<i>Prevotella</i>	<0.001
	<i>Neisseria</i>	0.004
<i>Paraprevotella</i>	<i>Streptococcus</i>	0.042
	<i>Gemella</i>	<0.001
<i>Alloiococcus</i>	<i>Haemophilus</i>	0.049
<i>Pseudomonas</i>	<i>Enterobacteriaceae (family)</i>	0.023
<i>Neisseria</i>	<i>Prevotella</i>	0.002

**Table 2-2 Bacterial correlations in MEF:** Correlations between bacterial genera (family if unspecified) found in MEF, all with p value < 0.05

**Bacterial correlation ( $p < 0.05$ ) - Adenoid**



**Figure 2-7 Bacterial correlations on the adenoid pad.** Graphic representation of the correlation between bacterial genera (family if unspecified) within the adenoid. Red: anaerobic bacteria, orange: facultative anaerobic bacteria, blue: aerobic bacteria. The size of the circle is relative to the number of correlations with other bacteria.

Legend: Allo – *Alloiococcus*, Hae – *Haemophilus*, Psu – *Pseudomonas*, Str – *Streptococcus*, Ent – *Enterobacteriaceae* (family), Neiss – *Neisseria*, Gem – *Gemellaceae* (family), Fuso – *Fusobacteria*, Lepto – *Leptotrichiaecae* (family), Vei – *Veillonella*, Para – *Paraprevotella*, Carno – *Carnobacteria*, Prevo - *Prevotella*

## 2.5 Discussion

This study is the largest published study to date utilising culture-independent, 16S rRNA sequencing techniques to characterize the middle ear and adenoid microbiome in patients with OME. It provides further evidence that OME is not a sterile condition and importantly casts doubt on the commonly held belief that the adenoid pad serves as a bacterial reservoir for OME.

We found that the microbiome of the middle ear consisted of six main genera of bacteria: (in descending order) *Alloiococcus*, *Haemophilus*, *Staphylococcus*, *Corynebacteria*, *Streptococcus* and *Moraxella*. These findings are consistent with previously published works using both traditional culture, culture independent PCR<sup>182,198,315</sup> and a 16S pyrosequencing study by Jervis-Bardy and colleagues<sup>132</sup>.

Analysis of our data shows distinctly different microbiota composition between middle ear effusions and the adenoid pad, with thirteen of the seventeen most abundant genera showing a statistically significant difference in relative abundance. The most striking of which was the difference in relative abundance demonstrated for *Alloiococcus* with <1% in the adenoid sample versus 23% (most abundant) in the MEF. *A. otitidis* is commonly considered a commensal of the external canal<sup>38,175,315</sup> and has been reported as the most prevalent bacterial species in patients with non-purulent OME (20 to 40%)<sup>182,197,315</sup>. Our findings are consistent with the literature, with *A. otitidis* having a prevalence of 69% and representing the greatest mean relative abundance across our cohort, particularly in patients with unilateral effusions.

In addition, *Alloiococcus*, along with *Haemophilus* species, were the only two bacterial genera shown to be the dominant bacteria (>90% relative abundance) within the MEF, with an inverse correlation

observed between the two. Although this is the first study to report possible bacterial interference between *A. otitidis* and *Haemophilus*, numerous studies have shown *H. influenzae* to have bacterial interference with *S. aureus* and *S. pneumonia* within the respiratory tract<sup>316-318</sup>.

Interestingly, the microbiota of bilateral effusions within patients were similar, despite a significant difference with that of the adenoid tissue of the same patient. This suggests that the microenvironment of the middle ear itself may play a greater role in influencing the constitution of the microbial communities within them, rather than bacteria from the nasopharynx. This finding is supported by the wider microbiome literature, which reports distinctly different microbiomes within specific body sites<sup>153,155</sup> and that the composition at each site remains relatively stable over time<sup>153,155,156</sup>. These findings imply that a core microbiota exist within body niches and that they are somewhat resistant to external factors. Whilst our study design precluded longitudinal analysis (only five patients have required repeat tympanostomy tube insertion to date), we believe that this is likely true of the middle ear as well.

In our patients, we found that core bacteriology of OME involves complex polymicrobial communities rather than merely the presence of dominant bacteria. Only a minority of MEF samples displayed a dominant species and even in these cases there were traces of other bacteria. Moreover, we observed a correlation in relative abundance between multiple genera, particularly anaerobic and facultative anaerobic bacteria. Interestingly, a similar correlation was also observed in the adenoid tissue. One possible explanation for this is the high prevalence of bacterial biofilms demonstrated in both middle ear effusions and adenoid tissue<sup>162</sup>. These three dimensional structures are known to create microenvironments of low oxygen tension which may favour anaerobic growth<sup>319</sup>.

Based on the findings of this study, we propose a microbiome model of the pathogenesis of otitis media. We postulate that the microbiome of the healthy middle ear is likely distinctly different to that in the setting of OME and also to that of acute otitis media. We believe that adenoidal hypertrophy and Eustachian tube dysfunction predispose to OME resulting in the formation of a stable OME microbiome (and likely biofilm), similar to that described in this paper. However, subsequent changes in the local environment within the middle ear, whether via direct inflammation (for example, secondary to viral infection<sup>320</sup>) or host immunomodulation, creates a disequilibrium of the local microbiota, thus resulting in the emergence of a dominant species and potentially acute disease. For example, when the micro-environment favours the classic otopathogens (*H. influenzae*, *M. Catarrhalis*, *S. Pneumonia*) acute otitis media may be favoured, yet when a less virulent bacterium, such as *A. otitidis* is dominant, then perhaps serous effusion persists. This is reflected in the fact that effusions with a high abundance of *Haemophilus* were noted as being more viscous and consistent with glue ear than effusions with high abundance of *Alloiococcus*.

Finally, the similarity of the microbiota of the MEF and external ear canal; in particular high prevalence of *Alloiococcus*, *Staphylococcus* and *Corynebacterium* is reported in the literature<sup>175</sup>. This does raise the question of whether the external ear itself may act as a reservoir for the middle ear. Although, bacterial translocation through an intact healthy tympanic membrane would seem unlikely, contamination through macro- or micro-perforations cannot be excluded. Furthermore, changes in the barrier function and permeability of the tympanic membrane during an acute infection remain unknown and so are the current subject of an in vitro study in our department. The possibility of contamination during sampling may also explain our finding, although the methodology employed for this study aimed to minimize the likelihood of this.



## 2.6 Conclusion

The microbiome of the middle ear in OME is diverse with both aerobic and anaerobic bacteria represented. *A. otitidis* is found in high abundance and may play a major role in the pathogenesis of OME, especially unilateral effusions and displays an inverse relationship with *Haemophilus*. Furthermore, dissimilarities between the local microbiota of the adenoid and the middle ear were demonstrated, suggesting that the microenvironment of the middle ear plays a greater role in the composition of the microbiota than the potential bacterial seeding from the adenoid pad to the middle ear in children with otitis media with effusion.

### Acknowledgements:

Many thanks to the theatre and administration staff and at the Women's and Children's hospital for their support of this research

## *Chapter 3: The external ear canal as a reservoir for the middle ear: a 16S phylogenetic analysis*

---

Conducted in the Department of Otolaryngology Head and Neck Surgery, University of Adelaide, Adelaide, Australia and the Department of Otolaryngology Head and Neck Surgery, Women's and Children's Hospital, Adelaide, Australia

Financial assistance provided by the University of Adelaide Postgraduate Award Scholarship.

## STATEMENT OF AUTHORSHIP

### **Chun Ling Chan**

Project design, patient recruitment, tissue collection, analysis of data, manuscript preparation

### **David Wabnitz**

Project design, tissue collection, manuscript editing

### **Ahmed Bassiouni**

Data Analysis, manuscript preparation and editing

### **Peter-John Wormald**

Principal project supervision, manuscript editing

### **Sarah Vreugde**

Project co-supervisor, manuscript editing

### **Alkis James Psaltis**

Project co-supervisor, manuscript editing

Accepted for publication:

**The external ear canal as a reservoir for the middle ear: a 16S analysis**

**Chan C**, Bassiouni A, Vreugde, S, Wormald PJ, Psaltis AJ

JAMA Otolaryngology, Head and Neck Surgery, accepted for publication 16/08/16 (in press)

# Statement of Authorship

Title of Paper	The external ear canal as a reservoir for the middle ear: a 16S phylogenetic analysis
Publication Status	<input checked="" type="checkbox"/> Accepted for Publication
Publication Details	Accepted for publication in "JAMA otolaryngology – Head and Neck Surgery". Proposed publication date 03/11/16

## Principal Author

Name of Principal Author (Candidate)	Chun Chan		
Contribution to the Paper	Project design, patient recruitment, tissue collection, analysis of data, manuscript preparation		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature	<table border="1"> <tr> <td>Date</td> <td>31/10/16</td> </tr> </table>	Date	31/10/16
Date	31/10/16		

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	David Wabnitz		
Contribution to the Paper	Project design, tissue collection, manuscript editing		
Signature	<table border="1"> <tr> <td>Date</td> <td>12 October 2016</td> </tr> </table>	Date	12 October 2016
Date	12 October 2016		

Name of Co-Author	Ahmed Bassiouni		
Contribution to the Paper	Data Analysis, manuscript preparation and editing		
Signature	<table border="1"> <tr> <td>Date</td> <td>11 October 2016</td> </tr> </table>	Date	11 October 2016
Date	11 October 2016		

Name of Co-Author	Peter John Wormald		
Contribution to the Paper	Principal project supervision, manuscript editing		
Signature		Date	11 October 2016

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature		Date	12 October 2016

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature		Date	11 October 2016

### 3.1 Abstract

**Objective:** The adenoid pad has long been considered a reservoir for bacteria in the pathogenesis of otitis media with effusion. However, bacteria more reminiscent of external ear canal commensals are often demonstrated within middle ear aspirates. This study aimed to compare the microbiota of the external ear canal, middle ear in otitis media with effusion and adenoid pad to further clarify the true source of middle ear bacteria.

**Methods:** Middle ear aspirates and external canal lavages were collected from children with otitis media with effusion undergoing ventilation tube insertion in two consecutive years (2014-2015). Samples were analysed using 16S rRNA sequencing on the Illumina MiSeq platform. Previously collected microbiota data from the adenoid pad was collated for analysis.

**Results:** 18 paediatric patients with chronic otitis media with effusion were recruited prospectively, with 34 paired middle ear and external ear canal samples. The microbiota consisted of *Alloiococcus (otitidis)* (38.5% mean relative abundance), *Haemophilus* (14.4%), *Moraxella* (10%), *Staphylococcus* (8.2%) and *Streptococcus* (3.8%). The microbiota of the external ear canal demonstrated a sparsity of classic otopathogens (*Haemophilus*, *Moraxella*, *Streptococcus*), but had high abundance of *Alloiococcus* (58%), *Staphylococcus* (20.8%) and *Pseudomonas* (3.2%). In contrast, based on previous collected data, the microbiota of the adenoid pad showed high abundance of the classic otopathogens with a sparsity of external canal genera. The microbiota of the middle ear collected over two consecutive years were similar.

**Conclusion:** Both the external auditory canal and the nasopharynx could serve as reservoirs for the middle ear. Furthermore, the microbiota of the middle ear with effusion appears to be relatively stable overtime and between populations with otitis media with effusion.

### 3.2 Introduction

The typical bacteria responsible for otitis media are *S. pneumoniae*, *M. catarrhalis* and *Haemophilus influenzae*. These bacteria are commensals within the oro- and naso-pharynx and it is commonly believed that these pathogens ascend from the aerodigestive tract into the middle ear via the Eustachian tube<sup>303</sup>.

In recent times, with the increasing interest in and access to culture independent techniques (polymerase chain reaction, fluorescence in situ hybridisation and most recently 16S rRNA sequencing) *A. otitidis* has emerged as a bacterial species that has been reported in high abundance from the middle ear of patients<sup>220,293,321</sup>. However, *A. otitidis*, whilst being commonly considered as a commensal of the external canal, is often not found in the nasopharynx.

We recently characterised the microbiome of the middle ear<sup>322</sup> and found that *A. otitidis* was the most abundant bacteria found within middle ear effusions of children (23% cumulative relative abundance). Furthermore, external canal commensals such as *Pseudomonas*, *Staphylococcus* and *Corynebacterium* species were also demonstrated in high abundance from middle ear samples. We therefore, designed a follow up study to investigate whether the external canal could be a potential reservoir for the middle ear.

### 3.3 *Methods*

#### 3.3.1 *Study group*

This study was conducted following ethics approval from the institutional research boards of the Women and Children's Hospital and the University of Adelaide (approval HREC/14/WCHN/42). Collection of tissue was carried out over the Australian winter period (June – September) 2015. All patients were aged between 1-16 years of age, with written parental/guardian consent obtained prior to enrolment. Children wait-listed for ventilation tube (grommet) insertion +/- adenoidectomy for OME were enrolled. Exclusion criteria included: effusions that had resolved by the time of surgery, upper respiratory tract infection on the day of surgery and antibiotic or steroid use in the four weeks preceding surgery. Demographic data including age, gender and parental smoking status as well as clinical data including presenting symptoms, previous tympanometry, medication history, and previous medical and surgical history were collected.

#### 3.3.2 *Sample collection*

Swabs and aspirates were obtained under general anaesthetic. All samples were immediately placed on ice and transferred to a negative - 80°C freezer for storage.

##### 3.3.2.1 *External canal aspirate:*

The ears were examined using an operating microscope. The external canal was lavaged with 1mL of sterile normal saline. This was then aspirated into a specimen trap (Argyle, Covidien, Mansfield, Massachusetts, USA).

##### 3.3.2.2 *Middle ear effusion collection:*

Middle ear effusions were sampled using a standardized protocol previously described<sup>322</sup>. In brief, a myringotomy, using sterile instruments, was performed and the middle ear effusion was aspirated into a specimen trap through the incision site. The utmost care was taken to avoid direct contact

with the external ear canal or other previously used instruments. If contact with the external canal inadvertently occurred, the equipment was exchanged for a new sterile set. Following collection of the aspirate, 2 microliters of sterile normal saline was used to flush the circuit.

### ***3.3.3 DNA extraction***

DNA extraction was carried out using PowerLyzer® PowerSoil® DNA Isolation Kit (MoBio Laboratories, Salona Beach, CA). Samples were thawed on ice and placed directly on to the beads for homogenisation. Total DNA was extracted from all clinical samples and two DNA extraction negative controls containing extraction reagents only. The remainder of the extraction protocol was performed as per the manufacturer's protocol. Extracted DNA was stored at -80°C until sequencing.

### ***3.3.4 PCR amplification of the 16S rRNA gene and sequencing***

PCR amplification and sequencing was performed by the Australian Genome Research Facility (AGRF). Libraries were generated by amplifying the V3–V4 (341F – 806R) hypervariable region of the 16S rRNA gene. PCR amplicons were generated using the primers CCTAYGGGRBGCASCAG in the forward sequence and GGACTACNNGGGTATCTAAT in the reverse sequence, using AmpliTaq Gold 360 mastermix (Life Technologies, Australia) following local protocol. The resulting amplicons were measured by Fluorometry (Picogreen, Thermo Fisher Scientific, Eugene, OR) and normalised. The equimolar pool was then quantified by qPCR (KAPA) and setup for sequencing on the Illumina MiSeq with Paired End Chemistry. Reads from Illumina sequencing were used as raw data for bioinformatic analysis.

### ***3.3.5 Bioinformatics pipeline***

The Paired-End reAd mergeR (PEAR)<sup>323</sup> v.0.9.5 was used to pair forward and reverse reads in each sample, and quality filter the paired reads. Open-reference operational taxonomic unit (OTU) picking strategy in QIIME v.1.8 was used to cluster OTUs<sup>324</sup>. Within the open-reference method, UCLUST<sup>10</sup> v.1.2.22 was used to cluster OTUs at 97% similarity. The Greengenes 16S database was used for reference-based OTU picking and for taxonomic assignment. Taxonomic assignment for all other OTUs remained at genus-level during taxonomic assignment. All samples were subsampled to 950 reads (rarefaction). Diversity estimates were performed on subsampled data.

### 3.3.6 Biostatistical analysis

Shannon's index of diversity was used for alpha diversity. A Friedman rank sum test was used to determine whether a significant difference of alpha diversity between sample types (while controlling for the repeated measure of "side in patient").

Distance matrices for beta diversity metrics (Bray-Curtis, unweighted UniFrac, and weighted UniFrac) were generated using QIIME. Distances in these matrices were used to calculate mean distances within- and between- sample type groups. Mean "within-sample type" and "between-sample type" distances were then compared using non-parametric Mann-Whitney U-tests. A permutational MANOVA (PERMANOVA) test was also performed to test whether there was a statistically significant difference between the bacterial communities in the MEF and the canal samples, with samples from the same side (of the same patient) grouped in the analysis (as a random factor).

Species level presumptive identification was performed on OTUs classified as *A. otitidis*, using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database<sup>15</sup>. Taxonomic assignment for all other OTUs remained at genus or family (if genera not possible)-level during taxonomic assignment.

Pearson's GraphPad v6 (GraphPad Software Inc, California, USA) was used to analyse the data, generate box plots and calculate the statistical tests used. Mean relative abundance of top genera were calculated for the middle ear fluid (MEF), external ear canal (EAC) groups. The mean of top genera from these two sites were compared using a paired T-test. Correlation between genera was performed using Pearson's coefficient ( $r$ ), which measures the linear correlation between two variables.

### 3.4 Results

18 patients with OME, aged 1 – 14 years old (mean 48 months, SD =36) were recruited. 66% (12/18) of the patients were male. 3 patients (17%) had undergone previous ventilation tube insertion. 16 patients had bilateral effusions (total number of MEF samples = 34). No patients were primary smokers, although one patient co-habitated with at least one carer who smoked.

#### 3.4.1 Microbiome of middle ear with effusion

The genera of bacteria that occurred at greater than or equal to 1% mean (cumulative) relative abundance were: *Alloiococcus* (38.5%), *Haemophilus* (14.4%), *Moraxella* (10.0%), *Staphylococcus* (8.2%), *Streptococcus* (3.8%), *Corynebacteria* (3.1%) and *Pseudomonas* (3.1%) (Table 3-1, figure 3-1). Genera occurring at less than 1% were reported as absent, due to the potential for background noise confounding these findings. Furthermore, *Alloiococcus* shared an inverse correlation relationship with *Staphylococcus* (Pearson correlation co-efficient ( $r$ ) = -0.433,  $p$  = 0.011)(figure 3-2).

#### 3.4.2 Microbiome of the external ear canal

The genera of bacteria that occurred at greater than 1% mean (cumulative) relative abundance were: *Alloiococcus* (58%), *Staphylococcus* (20.8%), *Pseudomonas* (3.2%), *Corynebacteria* (2.8%) and *Enterobacteria* (1.4%). *Haemophilus* (0.3%), *Moraxella* (0.3%), *Streptococcus* (0.2%) were less abundant, despite prevalence ranging from 17-31% (Table 3-1, figure 3-1).

There were inverse correlation relationships between the relative abundance of bacterial genera (fig 2): *Alloiococcus* with *Staphylococcus* ( $r$  = -0.787,  $p$  < 0.001), *Haemophilus* ( $r$  = -0.356,  $p$  = 0.039), *Moraxella* ( $r$  = -0.456,  $p$  = 0.007) and *Streptococcus* ( $r$  = -0.419,  $p$  = 0.014). *Haemophilus* shared correlation with *Moraxella* ( $r$  = 0.794,  $p$  < 0.001) and *Corynebacteria* ( $r$  = 0.632,  $p$  < 0.001), whilst *Moraxella* also had a positive correlation with *Streptococcus* ( $r$  = 0.736,  $p$  < 0.001).

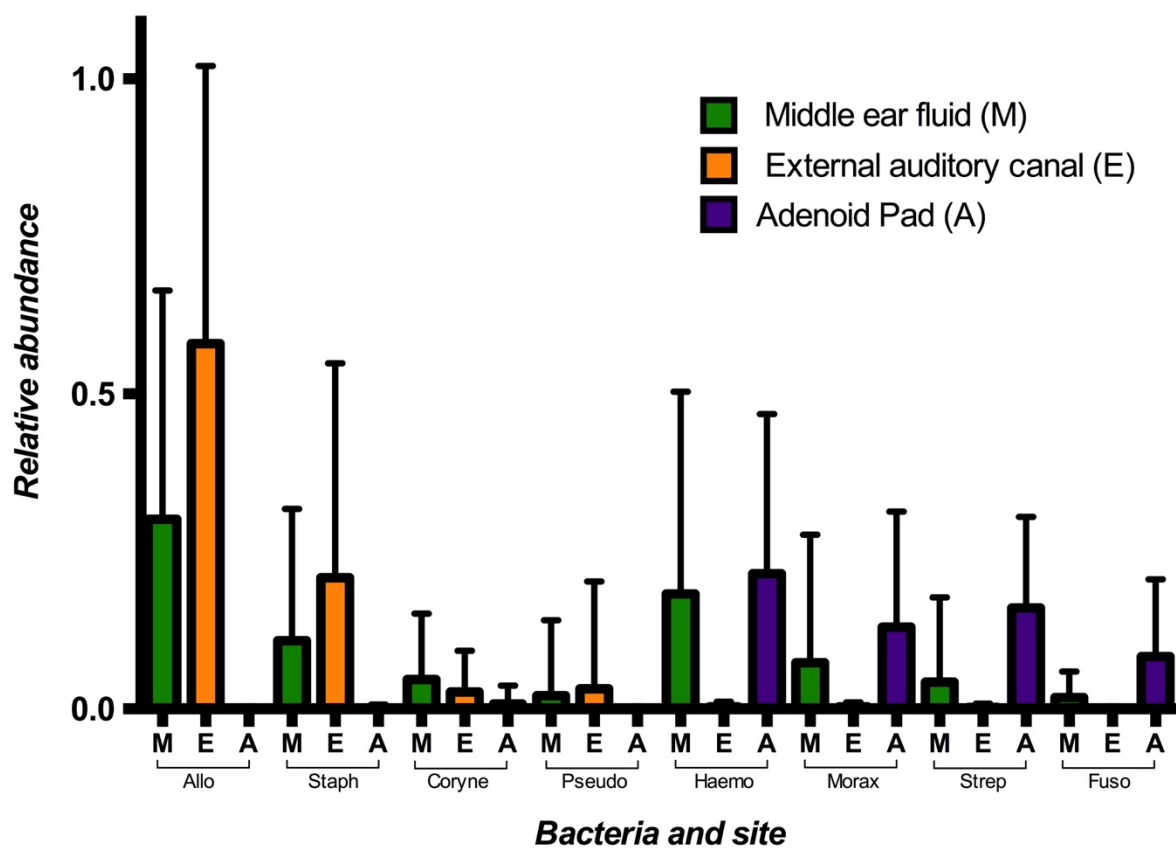


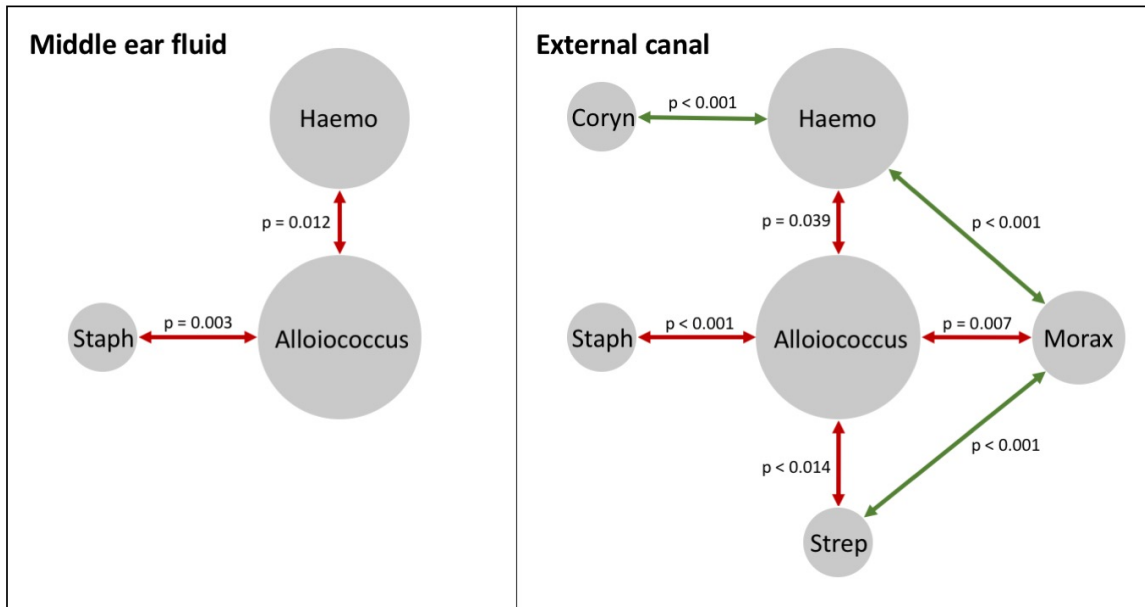
Figure 3-1 Comparing the microbiome of the adenoid, middle ear and external ear canal. Graphical representation of the relative abundance of microbiota found at three different sites from collated data over the two consecutive years: middle ear fluid (green), external auditory canal (orange) and adenoid pad (purple)

Legend: Allo – *Alloiococcus*, Staph – *Staphylococcus*, Coryne – *Corynebacterium*, Pseudo – *Pseudomonas*, Haemo – *Haemophilus*, Morax – *Moraxella*, Strep – *Streptococcus*, Fuso – *Fusobacteria*

	Prevalence (%)			Mean relative abundance (%)		
	EAC	MEF	Adenoids	EAC	MEF	Adenoids
<i>Haemophilus</i>	14.3	31.4	100.0	0.3	14.4	25.0
<i>Moraxella</i>	17.1	20.0	96.0	0.3	10.0	14.0
<i>Streptococcus</i>	11.4	17.1	96.0	0.2	3.8	13.0
<i>Alloiococcus</i>	74.3	65.7	78.0	58.0	38.6	0.1
<i>Staphylococcus</i>	51.4	37.1	87.0	21.8	8.1	0.2
<i>Corynebacterium</i>	28.6	51.4	70.0	2.7	3.1	0.8
<i>Pseudomonas</i>	14.3	6.7	not found	3.2	3.1	not found
<i>Enterobacteria</i>	14.3	2.9	not found	1.4	0.7	not found

**Table 3-1 Distribution of bacteria from the adenoid pad, external ear canal and middle ear fluid:**

The prevalence and mean relative abundance in percentage of bacterial genera from the external auditory canal (EAC), middle ear fluid (MEF) and Adenoid pad (from previously published data).



**Figure 3-2 Bacterial correlations within the middle ear and external canal:** Graphic representation of the correlation between bacterial genera within: left pane – within middle ear fluid, right pane – within external ear canal. Green arrows represent positive correlation; red arrows represent inverse correlation.

Legend: Staph – *Staphylococcus*, Coryn – *Corynebacterium*, Haemo – *Haemophilus*, Morax – *Moraxella*, Strep – *Streptococcus*.

### 3.4.3 Comparing the microbiome of the middle ear to the external canal

The microbiome of the two sites were dissimilar (figure 3-1). The microbiome of the middle ear aspirate was more diverse (Simpson's Diversity Index); EAC was 1.169 compared with that of the MEF, which was 1.702 (PERMANOVA,  $p < 0.001$ ). Analysis of beta diversity also showed a difference between MEF and EAC microbiota based on the beta diversity (mean of distances within samples vs mean of distances between samples = 0.654 vs 0.667, Mann Whitney U test  $p = 0.004$ , PERMANOVA

$p < 0.001$ ). However, despite overall dissimilarities between the two areas, there was positive correlation between bacterial genera commonly associated with external canal commensals: *Alloiococcus* (Pearson  $r = 0.680$ ,  $p < 0.001$ ), *Staphylococcus* ( $0.868$ ,  $p < 0.001$ ) and *Corynebacteria* ( $0.963$ ,  $p < 0.001$ ). Patients with previous myringotomy were found to have a high abundance of *Alloiococcus* (73-94%) or *Staphylococcus* (69%) (when *Aerococcus* was found at a relative abundance of 5%), however, due to the small sample size, no formal statistical analysis was possible.

#### 3.4.4 Bilateral effusions

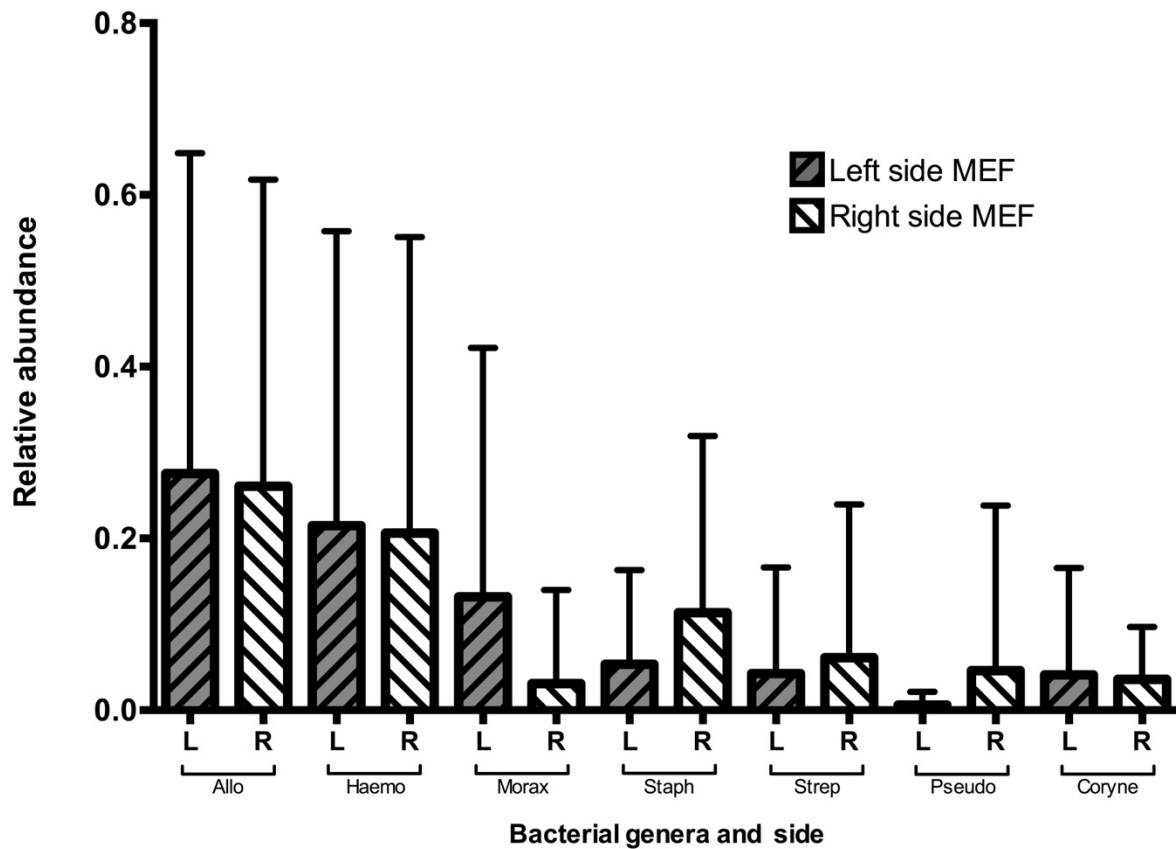
Bilateral effusions were similar in composition (One-way ANOVA,  $p < 0.001$ ) and there were no significant differences between matched groups for individual genera (Sidaks multiple comparison's test) (fig 3).

#### 3.4.5 Combined with previous data set

When combined with our previously published data<sup>322</sup>, there were a total of 69 MEF samples, with 24 paired, matched adenoid samples and 34 paired, matched EAC samples (figure 3-1). The two data sets had comparable distribution of age (mean age 40 (SD = 21) vs 48 (SD = 36)  $p = 0.39$ ) and gender (male 74% vs 66%  $p = 0.84$ ). The microbiome of the middle ears was similar when comparing the 2014 to the 2015 cohort (One-way ANOVA  $p < 0.001$ ) (fig 3-4), however on further analysis there was a greater relative abundance of *Alloiococcus* in the second year (23% vs 38.5% Sidaks multiple comparisons test,  $p < 0.05$ ).

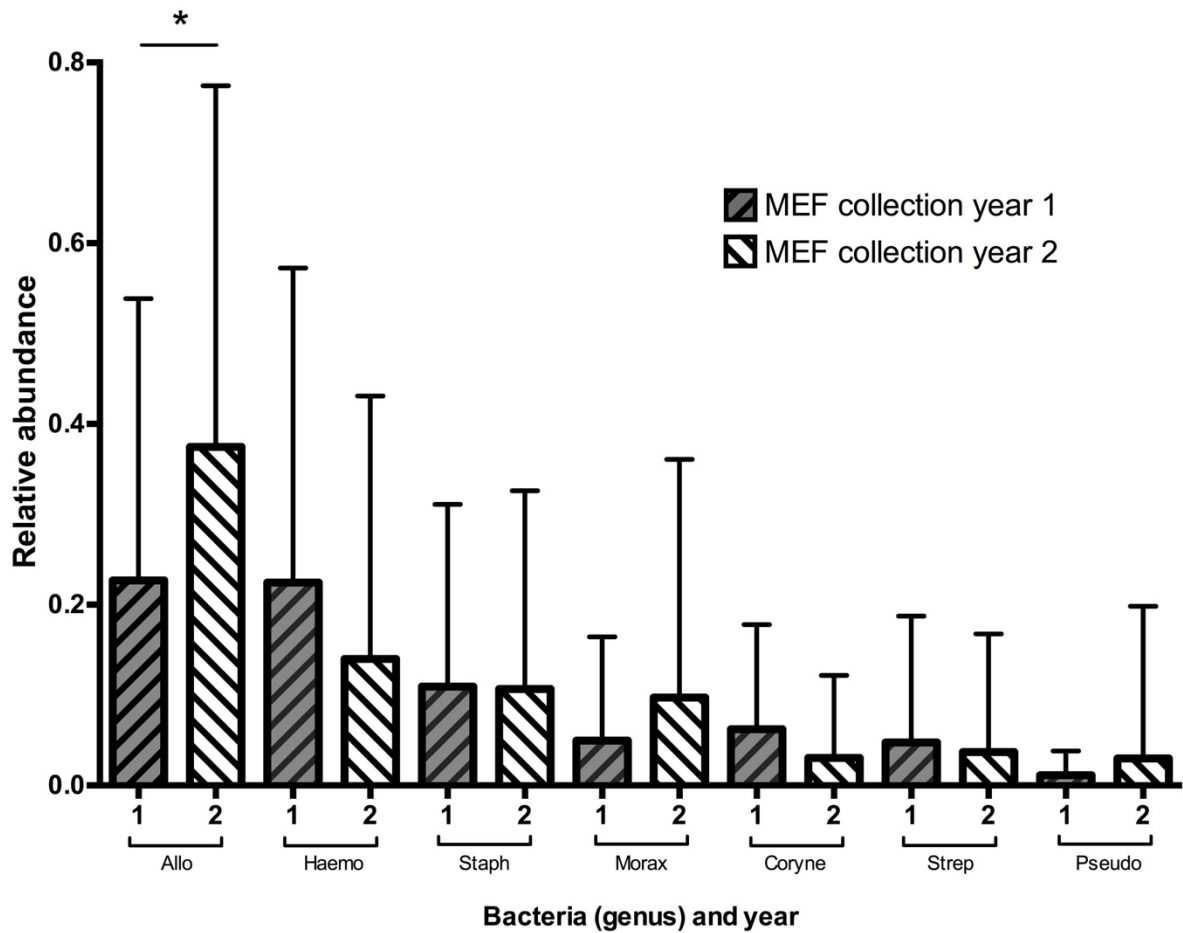
With the combined data set for MEF, analysis again revealed inverse correlation between *Alloiococcus* and *Staphylococcus* ( $r = 0.356$ ,  $p = 0.003$ ). In addition, a further inverse correlation was demonstrated between *Alloiococcus* with *Haemophilus* ( $r = -0.300$ ,  $p = 0.012$ ).

When comparing the microbiome of the adenoid with the external ear canal (Mann Whitney U-test), there were significant differences in relative abundance for *Alloiococcus*, *Haemophilus*, *Streptococcus*, *Moraxella* and *Staphylococcus* ( $p < 0.05$ ).



**Figure 3-3 Microbiome of bilateral ear effusions.** Graphical representation of the mean relative abundance of microbiota when comparing between left and right ears for patients with bilateral effusions

Legend: Allo – *Alloiococcus*, Haemo – *Haemophilus*, Morax – *Moraxella*, Staph – *Staphylococcus*, Strep – *Streptococcus*, Pseudo – *Pseudomonas*, Coryne – *Corynebacterium*



**Figure 3-4 Microbiome of the middle ear over time.** Graphical representation of the cumulative relative abundance of microbiota found in middle ear effusions when comparing two cohorts of patients over consecutive years with otitis media with effusion

Legend: Allo – *Alloiococcus*, Haemo – *Haemophilus*, Staph – *Staphylococcus*, Morax – *Moraxella*, Coryne – *Corynebacterium*, Strep – *Streptococcus*, Pseudo – *Pseudomonas*

### 3.5 Discussion

This is a follow up study to our previously published study<sup>322</sup>, which was the first to describe the microbiome of the middle ear in children with otitis media with effusion in a non-indigenous Australian population using 16S rRNA sequencing. The microbiota of the middle ears from this and the previous study were similar between the two cohorts. *Alloiococcus* was again demonstrated to be the most abundant middle ear bacterial genera found within the middle ear, with the classic ear pathogens (*Moraxella*, *Haemophilus* and *Streptococcus*) also present in abundance. The only significant difference was that there was an increase in the mean relative abundance of *Alloiococcus* in the second year. The reason for this difference is unclear, but may reflect a natural variation in commensal carriage of the external canal/middle ear cleft between populations. Alternatively, as discussed above, there was a greater number of patients with previous myringotomy in our second cohort, leading to a greater opportunity for the external auditory canal bacteria, including *Alloiococcus*, to translocate into the middle ear. However, the ecological niche subsequently created by the development of an effusion within the middle ear cavity is such that *Alloiococcus* growth is favoured over that of other external canal bacteria (i.e. *Staphylococcus* which *Pseudomonas*), which consequently do not exhibit a proportional rise in abundance. Despite this variation in relative abundance, we believe that bacteria genera identified from the middle ears of our cohort reflect a 'core' microbiome of the middle ear, which remains relatively stable over time and between different populations, a characteristic which has been reported at other body sites<sup>325,326</sup>.

Our findings support those of a previous culture-independent phylogenetic study by Frank et al.<sup>327</sup>. Despite differences in sampling techniques, geographical location and seasonal/climatic factors, Frank et al. also demonstrated a major abundance of *Alloiococcus* (57%). Their study also showed abundance of *Corynebacterium* (24%) and *Staphylococcus* (10%), but only minimal abundance of *Pseudomonas* (<1%).

Interestingly, we found that the microbiome of the middle ear in OME appear to reflect both the microbiome of the adenoid pad and the external canal microbiomes (fig 1, table 1). *Haemophilus*, *Streptococcus* and *Moraxella* were in high abundance in both the adenoids and the middle ear but absent (<1%) in the external canal. Conversely, *Alloiococcus*, *Staphylococcus* and *Pseudomonas* were found in high relative abundance in the external canal, but absent in the adenoids. We therefore propose it is not only the adenoid that seeds the middle ear as previously described<sup>303</sup>, but that the external ear canal may also serve as a bacterial reservoir for middle ear infections.

Although bacterial translocation across intact mucosal surfaces has been previously documented in gut mucosa in certain pathological states<sup>328,329</sup>, translocation across an intact tympanic membrane has not been demonstrated. In health, the tympanic membrane has been shown to only allow oxygen and carbon dioxide to diffuse through it<sup>330</sup>. This is largely due to its multi-layered, multicellular structure, consisting of: epidermis (external layer), a radiating layer of collagen fibres, a circular layer of collagen fibres and layer of simple cuboidal epithelium (middle ear mucosa). Furthermore during acute infections the thickness of the tympanic membrane has been found to increase in thickness further, due to oedema and inflammation<sup>331</sup>.

Given the above, it would seem that the presence of a tympanic perforation would be needed to facilitate entry of bacteria from the external canal into the middle ear. Epidemiological studies suggest that almost 80% of children will suffer an episode of acute otitis media by age three<sup>6</sup>, with perforations reported in up to 30% of such cases<sup>3</sup>. It is not unforeseeable then, that such an episode could be responsible for the translocation of external ear canal bacteria observed in our study. Unfortunately, a past history of perforation was not obtained from our patient cohort, therefore this cannot be commented on directly. However, as mentioned above, we observed a statistically significant increase in the mean relative abundance of *Alloiococcus* in our current patient cohort

compared with the first. This finding may potentially reflect the greater number of patients with previous ventilation tube insertion [6% (1/17 patients) in the first study vs 17% (3/18) in the second], thus allowing for a greater chance of external canal bacteria translocating in to the middle ear. In fact, examples of external ear canal commensals causing middle ear disease via perforation is evident in the frequent bacterial culture of *P. aeruginosa* and *S. aureus* in cases of chronic suppurative otitis media<sup>332,333</sup>.

Yet, unlike *P. aeruginosa* and *S. aureus*, the role of *A. otitidis* in otitis media remains unclear. *A. otitidis* is an aerobic, gram-positive cocci first documented in MEF by Faden and Dryja in 1989<sup>188</sup>. It is commonly considered a commensal of the external canal and is not commonly identified with standard techniques<sup>38,182,194,198</sup>. However, potential for pathogenicity has been documented: *A. otitidis* has been found to invade intracellularly<sup>188</sup>, to modulate immune responses *in vitro*<sup>211,334</sup> and has been implicated in cases of endocarditis<sup>219</sup>, and endophthalmitis<sup>217</sup>. In addition, *A. otitidis* is the most prevalent bacterial species found in patients with non-purulent OME (20 to 40%)<sup>181-183</sup> and has been reported to persist within middle ear aspirates despite antibiotic treatment<sup>194</sup>. However, the impact and role of *Alloiococcus* in the pathogenesis or development of OME remains elusive. Yet, we believe that further investigation to elucidate the role of *Alloiococcus* in OME is warranted.

However, given the similarity between the microbiome of the external ear canal and middle ear, the possibility of contamination must be considered. We believe this is less likely for the following reasons. Firstly, to reduce contamination prior to myringotomy and aspiration, the external canal is first toileted and washed. The aspiration technique itself then avoids any external canal contact, with minimal contact with the tympanic membrane itself (video 1). Although there is a theoretical risk of contamination with the brief contact with the external surface of the tympanic membrane,

the comparatively large volume of middle ear aspirate relative to this brief contact, would unlikely yield such high relative abundances seen in our study. Therefore, we believe that our results accurately represent the microbiota within the middle ear with effusion.

### **3.6** *Conclusion*

We have demonstrated that the microbiome of the middle ear with effusion is grossly stable over time and across populations. Furthermore, due to the distribution of the microbiome characterised, we postulate that both the nasopharynx and external ear canal contribute the microbiota within the middle ear.

*Chapter 4: Indirect pathogenicity of Alloiococcus  
otitidis in multispecies biofilm with Haemophilus  
influenzae: effects on antibiotic sensitivity and  
persistence*

---

Conducted in the Department of Otolaryngology Head and Neck Surgery, University of Adelaide,  
Adelaide, Australia

Financial assistance provided by the University of Adelaide Postgraduate Award Scholarship

STATEMENT OF AUTHORSHIP

**Chun Ling Chan**

Project design, analysis of data, manuscript preparation

**Katharina Richter**

Analysis of data, SEM preparation, manuscript editing

**Peter-John Wormald**

Principal project supervision, manuscript editing

**Alkis James Psaltis**

Project co-supervisor, manuscript editing

**Sarah Vreugde**

Principal project supervisor, project design, manuscript editing

**Submitted for publication**

**Indirect pathogenicity of *Alloiococcus otitidis* in multispecies biofilm with *Haemophilus influenzae*:  
effects on antibiotic sensitivity and persistence**

**Chan C, Richter K, Wormald PJ, Psaltis AJ, Vreugde, S,**

Submitted to PLOS Pathogens 28/10/16 (in review)

# Statement of Authorship

Title of Paper	Alloiococcus forms multispecies biofilm with Haemophilus influenzae: effects on antibiotic sensitivity and persistence
Publication Status	<input checked="" type="checkbox"/> Submitted for Publication
Publication Details	Submitted to PLOS Pathogens 28/10/16

## Principal Author

Name of Principal Author (Candidate)	Chun Chan			
Contribution to the Paper	Project design, <i>in vitro</i> experimentation, CSLM preparation and imaging, analysis of data, manuscript preparation			
Overall percentage (%)	90%			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.			
Signature	<table border="1"> <tr> <td></td> <td>Date</td> <td>31/10/16</td> </tr> </table>		Date	31/10/16
	Date	31/10/16		

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Katharina Richter			
Contribution to the Paper	Analysis of data, SEM preparation, manuscript editing			
Signature	<table border="1"> <tr> <td></td> <td>Date</td> <td>28/10/2016</td> </tr> </table>		Date	28/10/2016
	Date	28/10/2016		

Name of Co-Author	Peter John Wormald			
Contribution to the Paper	Principal project supervision, manuscript editing			
Signature	<table border="1"> <tr> <td></td> <td>Date</td> <td>11 October 2016</td> </tr> </table>		Date	11 October 2016
	Date	11 October 2016		

Name of Co-Author	Sarah Vreugde		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature		Date	11 October 2016

Name of Co-Author	Alkis James Psaltis		
Contribution to the Paper	Project co-supervisor, manuscript editing		
Signature	↵	Date	12 October 2016

## 4.1 Abstract

**Background:** *A. otitidis* is one of the most commonly found bacteria within middle ear aspirates of children with otitis media with effusion. However, its phenotype and role in the pathogenesis of this condition remains unclear. Recent studies have observed an inverse relationship between the relative abundance of *A. otitidis* and *H. influenzae* within middle ear aspirates. The aim of this study was to characterize *A. otitidis* and investigate the relationship between these two bacteria *in vitro*.

**Methods:** The ability of *A. otitidis* to form both single-species and polymicrobial biofilms with *H. influenzae* reference strains and clinical isolates was explored. Biofilm was imaged using confocal scanning laser microscopy and scanning electron microscopy. Quantification of biofilm biomass and bacterial volume was assessed using a crystal violet assay and viable cell counting. Antimicrobial sensitivity was assessed using a microdilution method and E-test for amoxicillin and ciprofloxacin.

**Results:** *A. otitidis* was able to form both single-species and polymicrobial biofilms with *H. influenzae*. *A. otitidis* promoted *H. influenzae* persistence and survival by increasing biofilm production in adverse growth conditions and by augmenting antimicrobial resistance in both planktonic and polymicrobial biofilm.

**Conclusion:** *A. otitidis* may play an indirect pathogenic role in otitis media by altering *H. influenzae* antibiotic sensitivity and persistence.

## 4.2 Background

*Alloiococcus otitidis* (*A. otitidis*) is a commonly identified bacteria within middle ear aspirates of children with chronic otitis media with effusion (COME). In the past, *A. otitidis* had been considered to be commensal. Recent studies, however, suggest that *A. otitidis* may not be an innocent bystander. *In vivo* studies have shown *A. otitidis* to have an immune-stimulatory potential on both myeloid<sup>334</sup> and lymphoid<sup>216</sup> cell types. Furthermore, *A. otitidis* has also been shown to induce otitis media with effusion in a rat model<sup>306</sup>.

Recently, using culture-independent 16S rRNA sequencing, we confirmed that *A. otitidis* is indeed the most abundant bacteria in middle ear aspirates from two cohorts of children with COME<sup>335,336</sup>. Additionally, we found an inverse correlation between the relative abundance of non-typeable *Haemophilus influenzae* (NTHi) and *A. otitidis*. This finding suggests a possible interaction between these two genera of bacteria that may be crucial in the pathogenesis of OME. NTHi is a coloniser of the human pharynx and is associated with a range of local mucosal and invasive infections<sup>337</sup>. Since the wide-spread introduction of pneumococcal vaccination, NTHi has emerged as the most common cause of acute otitis media<sup>245,338,339</sup> and has been implicated in 55-95% of cases of all otitis media (OM)<sup>337</sup>. Also, NTHi is also frequently demonstrated in middle ear aspirates of patients with COME<sup>132,340-342</sup> and has been shown to form biofilms on the middle-ear mucosa<sup>343,344</sup>.

The identification of biofilm within the middle ear of children with COME has led to the concept that OM could be a biofilm driven disease<sup>162,299</sup>. Biofilms are complex three dimensional structures made of extracellular DNA, bacterial cells and the extracellular polysaccharide (EPS) matrix that they produce. The EPS matrix encloses the bacterial cells and allows irreversible attachment to a substratum, an interface or to each other<sup>280</sup>. The formation of biofilm is significant, as bacteria within the biofilm are conferred protection from environmental, host and chemical stressors, and

have an increased antibiotic resistance<sup>280,281</sup>. Moreover, bacteria in polymicrobial biofilms have been shown to further reinforce this protection through quorum sensing and the transfer of antibiotic resistance genes between strains<sup>283-286</sup>.

The aim of this study was to further elucidate the role of *A. otitidis* in the pathogenesis of otitis media and to explore the potential interactions between *H. influenzae* and *A. otitidis*, in regards to biofilm formation and antibiotic susceptibility.

## 4.3 Methods

### 4.3.1 Bacterial strains and handling

*A. otitidis* strain 51267 was purchased from the American Type Culture Collection (ATCC). This strain was subcultured from freezer stocks onto Brain Heart Infusion agar supplemented with 5% defibrinated sheep blood (BHlb) and incubated for 4 days at 37°C at normal atmospheric conditions. All subsequent cultures were derived from colonies isolated from these plates and were incubated in either normal atmosphere or 5% CO<sub>2</sub>.

The biofilm forming *H. influenzae* ATCC 33391 was purchased from ATCC. Also, two clinical NTHi strains designated NT176 and NT1159, both from middle ear isolates, were obtained from the Department of Molecular and Cellular Biology at the University of Adelaide. For each experiment, strains were subcultured from freezer stocks onto BHI supplemented with 10µg/mL hemin (factor X) and 0.2µg/mL of β-nicotinamide-adenine-dinucleotide (factor V) (BHIs) and incubated overnight at 37 °C in 5% CO<sub>2</sub>. All subsequent cultures were derived from colonies isolated from these plates and were incubated in 5% CO<sub>2</sub>. All experiments described below were replicated at least in triplicate.

### 4.3.2 Planktonic cell assays

#### 4.3.2.1 Single Colony Forming Unit (SCFU) assay

Tenfold dilutions of bacterial solutions were made and plated onto BHlb and BHIs for enumeration. The requirement of factor V and X for *H. influenzae* growth is well documented<sup>345</sup>; therefore BHlb was used as a selective medium for *A. otitidis*. Therefore, for *A. otitidis* enumeration, single colonies were counted after incubating for 3 days in normal atmosphere. *H. influenzae* was enumerated on BHIs agar plates after 24 hours in 5% CO<sub>2</sub> after initial testing showed no growth of *A. otitidis* after this time.

#### 4.3.2.2 *Planktonic co-culture*

Bacterial solutions of *H. influenzae* and *A. otitidis* were made up in 0.45% normal saline and added to BHIs. Specifically, 1mL of 1.0 McFarlane units of the candidate strain of *H. influenzae* and 1mL of 3.0 McFarlane units of *A. otitidis* were added to 8mL of BHIs and co-incubated for 24 hours in 5% CO<sub>2</sub>. This was equivalent to a starting suspension of  $2.0 \times 10^7$  single colony forming units per mL (SCFU/mL) of *A. otitidis*,  $1.4 \times 10^8$  SCFU/mL of ATCC 33391,  $4.2 \times 10^7$  SCFU/mL of NT 176 and  $4.8 \times 10^7$  SCFU/mL of NT 1159. Serial dilutions ( $10^0 - 10^{-10}$ ) of the resulting culture were plated onto selective growth agar (as above) to determine cell viability at 2, 6, 8, 10, 12 and 24 hours following incubation. The above co-culture method was also carried out with incubation at 30°C.

#### 4.3.2.3 *Growth of Haemophilus influenzae in depleted media*

In addition, the co-culture assay was repeated in BHI only, BHI with factor X only and BHI with factor V only. Viable bacterial enumeration was carried out as above.

### 4.3.3 *Biofilm assays*

#### 4.3.3.1 *A. otitidis biofilm*

Bacterial colonies were collected from subcultured bacteria (as described above) with a sterile swab and added to 2mL of 0.45% sodium chloride. This mixture was adjusted to achieve a bacterial suspension with a McFarland unit of 3.0 and combined with BHIs or BHIb to a ratio of 1: 14 (bacterial solution: media). 180 µL of this mixture was transferred into each of the wells of a 96 well plate. The 96 well plate was then incubated for the desired time (24 to 168 hours) at 37°C on a gyro-shaker at 70 rpm.

#### 4.3.3.2 *H. influenzae biofilm*

Bacterial colonies were suspended in 2 mL of 0.45% sodium chloride (as above). A bacterial suspension with a McFarland unit of 1.0 was prepared and added to BHIs to a ratio of 1:14. 180 µL of

this mixture was transferred into each of the wells of a 96 well plate. The 96 well plate was then incubated for the desired time (24 to 168 hours) at 37°C in 5% CO<sub>2</sub> (2 litre Gas jar) on a gyro-shaker at 70 rpm.

#### 4.3.3.3 *Co-culture of biofilm*

Bacterial suspensions for biofilm formation were prepared in BHIs as described above. 90 µL of each bacterial suspension was added to the 96 well plate and mixed by pipetting up and down. The plates were incubated in 5% CO<sub>2</sub> for 24 and 48 hours. Control wells contained 90 µL *A. otitidis* with 90 µL of BHIs, 90 µL *H. influenzae* with 90 µL of BHIs or BHIs alone.

In addition, as described above, the co-culture biofilm assay was repeated with both bacteria suspended in either BHI only, BHI with factor X only and BHI with factor V only. Furthermore, a culture at 30°C with BHIs was also performed. Viable bacterial enumeration was carried out with SCFU as above.

#### 4.3.4 *Crystal Violet Assay*

Biofilms were washed twice in Phosphate buffered saline (PBS) to remove non-adherent cells. 200 µL of 100% methanol was added to each well and incubated at room temperature for 15 minutes. The methanol was removed, and the wells were allowed to air dry. The wells were then stained with 200 µL of crystal violet 10%w/w and allowed to incubate at room temperature for 15 mins. For quantification of biofilm formation, 210 µL of 95% ethanol (w/w) was added to the wells and incubated at 37°C for 1 hour to extract the crystal violet. Optical density was read at 595 nm using a plate reader instrument (BMG Labtech, Fluostar Optima, Offenburg, Germany).

#### 4.3.4.1 *Colony forming unit assay*

Biofilms were washed twice in PBS to remove non-adherent cells. Cells were re-suspended in 100  $\mu$ L of PBS by sonicating for 10 minutes, followed by pipetting up and down. Bacteria were enumerated on selective plates as described above.

#### 4.3.4.2 *Fluorescence microscopy*

Biofilms were formed in 8 chamber glass slides as reported above. After the final PBS wash, biofilms were prepared for confocal laser scanning microscopy as previously described<sup>346</sup>. Briefly, biofilms were fixed for 15 minutes in 2.5% glutaraldehyde at room temperature, followed by fluorescent staining for 15 minutes at room temperature with SYTO 9 (1  $\mu$ L/mL) and propidium iodide (1  $\mu$ L/mL) in PBS (Live/Dead® *Ba*clight™ bacterial viability kit, Thermo Fischer Scientific, Inc, Waltham, MA, USA). The slides were examined on a Zeiss LSM700 Confocal Laser Scanning Microscope (CLSM) (Carl Zeiss, Jena, Germany).

#### 4.3.4.3 *Scanning electron microscopy*

Multi-species biofilms were grown for 168 hours on pegs in the Calgary Biofilm Device under the conditions mentioned above. Biofilms on pegs were washed with PBS, fixed with 2.5% glutaraldehyde and exposed to 1% osmiumtetroxide, followed by a dehydration series ranging from 50% to 100% ethanol. The final dehydration step was repeated twice before addition of hexamethyldisilazane to preserve the biofilm structure. Biofilms on pegs were then sputter-coated with 5 nm gold and visualised by scanning electron microscopy (Zeiss Gemini 2, Carl Zeiss, Jena, Germany).

#### 4.3.5 *Antibiotic susceptibility testing*

Breakpoints were determined as outlined by the EUCAST 2016 guidelines<sup>276</sup> and were classified as sensitive (S) or resistant (R) as follows for *H. influenzae*: amoxicillin S  $\leq$  2  $\mu$ g/mL, R > 2  $\mu$ g/mL, ciprofloxacin S  $\leq$  0.5  $\mu$ g/mL, R > 0.5  $\mu$ g/mL. Antibiotic minimum inhibitory concentration (MIC)

breakpoints have not been defined for *A. otitidis*. Therefore, guidelines for *S. pneumoniae* were used to interpret the results as previously reported<sup>347</sup>: amoxicillin S ≤ 0.06 µg/mL, R ≥ 2 µg/mL, ciprofloxacin S ≤ 0.125 µg/mL, R ≥ 2 µg/mL.

#### 4.3.5.1 Planktonic assays

Modified minimum inhibitory concentration assays as previously described<sup>348</sup> were carried out. 50µL of *H. influenzae* alone, *A. otitidis* alone, or *H. influenzae* and *A. otitidis* together were added to 96-well polypropylene plates in BHIs and BH1b. Bacterial suspensions were mixed in the wells by pipetting up and down. Serial dilutions of amoxicillin and ciprofloxacin were made up in BHIs, and 50µL was added to the wells. The plates were incubated at 37°C in an orbital shaker at 70 rpm for 24 h, and bacterial viability was monitored by the SCFU assay.

Further, antibiotic testing was carried out by E-Test<sup>®</sup> (bioMérieux, SA, Marcy l'Etoile, France) following the EUCAST guidelines<sup>276</sup>. Again combinations of *H. influenzae* and *A. otitidis* were investigated as above. These techniques are not calibrated for *A. otitidis*; therefore modifications were made to obtain sufficient growth of this species. BH1b agar was utilized for testing of *A. otitidis* alone, and BHIs was used for testing of *H. influenzae* and when *H. influenzae* was in combination with *A. otitidis*. 1mL of bacterial suspension in 0.45% normal saline equivalent of no. 3 turbidity McFarland standard was added to 30mL of agar. For *H. influenzae* species, no. 1 McFarland standard solution was made up. Plates were grown for 96 hours in both atmospheric and 5%CO<sub>2</sub>. The E-test<sup>®</sup> was used to assess sensitivity to ciprofloxacin and amoxicillin.

#### 4.3.5.2 Biofilm assays

Biofilms were formed as described above. After a 24-h incubation at 37°C, plates were washed three times with PBS to remove nonadherent cells. Antimicrobials (100 µl) in BHIs were added to the

washed biofilm in serial dilutions (as above). The plates were incubated for an additional 24 h, and bacterial viability was monitored by the SCFU assay to establish minimal bactericidal concentrations (MBC).

#### 4.3.6 Statistical Analysis

Pearson's GraphPad v6 (GraphPad Software Inc, California, USA) was used to analyze the data, generate box plots and calculate the statistical tests used. The student *t*-test (two-tailed, unequal variance) and Mann-Whitney U test were used to analyze the significance of differences between two experimental groups where appropriate. Data with a *p*-value of 0.05 or less were considered to be significant.

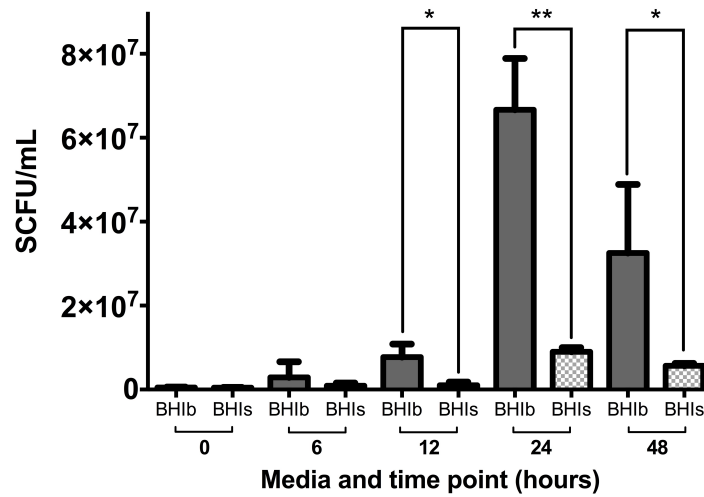
### 4.4 Results

#### 4.4.1 *A. otitidis* planktonic growth and biofilm formation

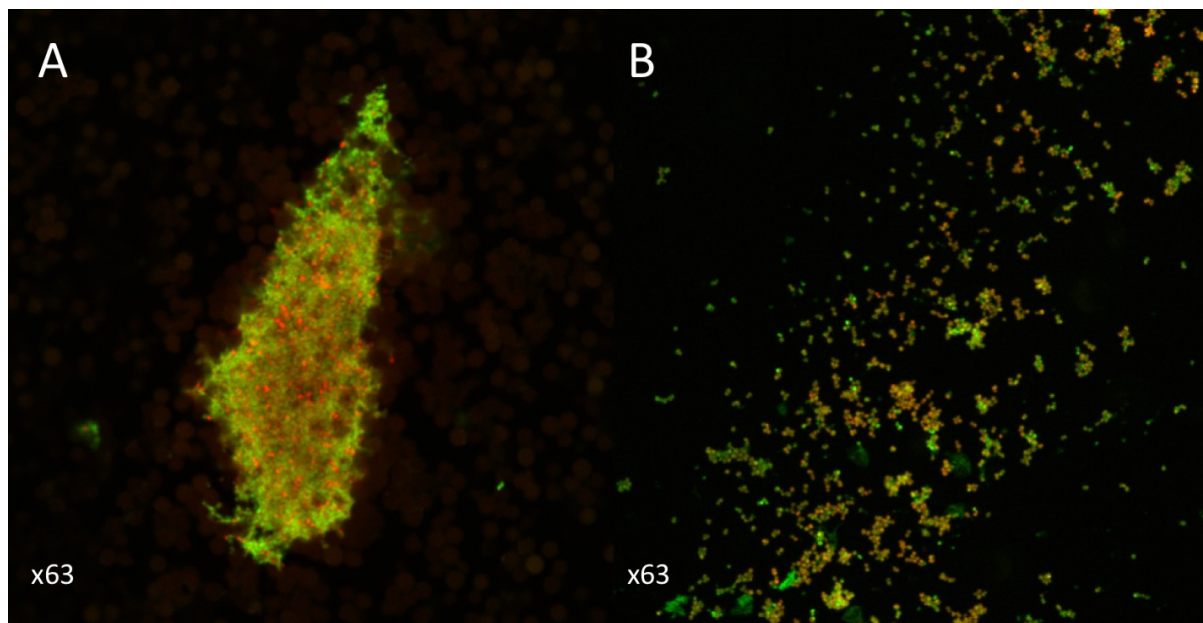
*A. otitidis* grew both in BHlb and BHls. Single colonies achieved a stable size by day 4 in both media but were larger when grown in BHlb. Peak growth of bacteria in broth was at 24 hours post incubation for both media. There were a greater number of viable cells in BHlb than in BHls at 12 hours (BHlb, Mean [SD] =  $7.7 \times 10^6$ , [ $3.1 \times 10^6$ ] vs BHls,  $1.0 \times 10^6$  [ $7.5 \times 10^5$ ],  $p = 0.02$ ) 24 hours (BHlb,  $6.7 \times 10^7$  [ $1.2 \times 10^7$ ] vs BHls,  $9.0 \times 10^6$  [ $1.0 \times 10^6$ ],  $p = 0.001$ ) and 48 hours (BHlb,  $3.2 \times 10^7$  [ $1.6 \times 10^7$ ] vs BHls,  $5.6 \times 10^6$  [ $5.7 \times 10^5$ ],  $p = 0.046$ )(Figure 4-1). There was no difference in growth when incubated at normal atmosphere or in 5% CO<sub>2</sub>.

CLSM with LIVE/DEAD Baclight Bacterial Viability stains indicated that when grown in BHlb, *A. otitidis* formed dense, satellite-aggregations of cells with extracellular matrix, consistent with biofilm (Figure 4-2A). When grown in BHls, cells appeared more dispersed, and aggregations were smaller in size

(Figure 4-2B). Peak viability of cells and biofilm biomass assessed with SCFU and crystal violet assay occurred at 48 hours of incubation (data not shown).



**Figure 4-1 A. *otitidis* growth in BHIs and BHib.** Single colony forming units per mL (SCFU/mL) of *A. otitidis* when incubated in Brain Heart Infusion with 5% sheep blood (BHib) (Solid, dark grey columns) and Brain Heart Infusion supplemented with 10µg/mL hemin (0.1% (w/v) hemin (factor X), 0.1% (w/v) l-histidine) (BHIs) (Checked column) when incubated over a 48-hour period. \*, p < 0.05, \*\*, p < 0.01.



**Figure 4-2 Confocal laser scanning microscopy image of *A. otitidis* biofilm.** Confocal scanning laser microscopy (CSLM) of dense satellite-aggregations of *A. otitidis* in BHIb (A) and sparser aggregations in BHIs (B) at X63 magnification. Live/Dead Baclight Bacterial Viability staining was used: live cells (with intact cell membranes) stain green and dead or dying cells (with compromised cell membranes) stain red.

#### 4.4.2 Co-culture of *H. influenzae* and *A. otitidis* and formation of polymicrobial biofilm

As expected, *H. influenzae* required factors V and X to grow<sup>349</sup> and did not grow in BHIb. Therefore, to facilitate co-culture experimentation, experiments were carried out in BHIs. In planktonic co-culture, there was no significant change in the number of viable cells or optical density of the broth, of co-cultured *H. influenzae* and *A. otitidis* compared to single-species controls in the first 48 hours of growth (data not shown).

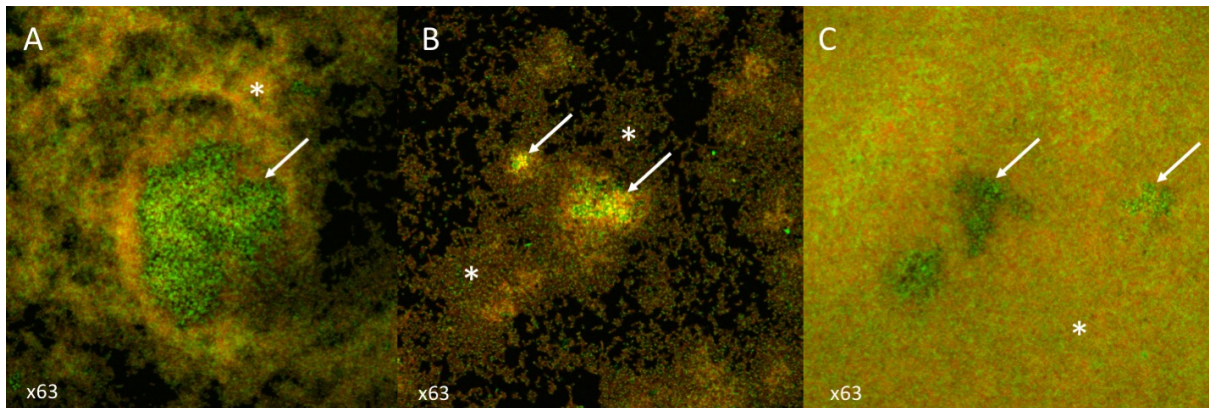
All three strains of *H. influenzae* formed biofilm in BHIs. When co-cultured with *A. otitidis* in BHIs, polymicrobial biofilm was observed with all three strains. In these polymicrobial biofilms, *A. otitidis* appeared to form larger and denser aggregations of cells compared to biofilm formed with single

species *A. otitidis* in BHIs. The aggregates of *A. otitidis* biofilm formed in polymicrobial biofilm were reminiscent of those demonstrated when *A. otitidis* was cultured alone in BH1b (Figure 4-3A-C). *A. otitidis* biofilm (Figure 4-4A) and polymicrobial biofilms (Figure 4-4B) were furthermore visualised by scanning electron microscopy, where clusters of *A. otitidis* were observed next to *H. influenzae* biofilm.

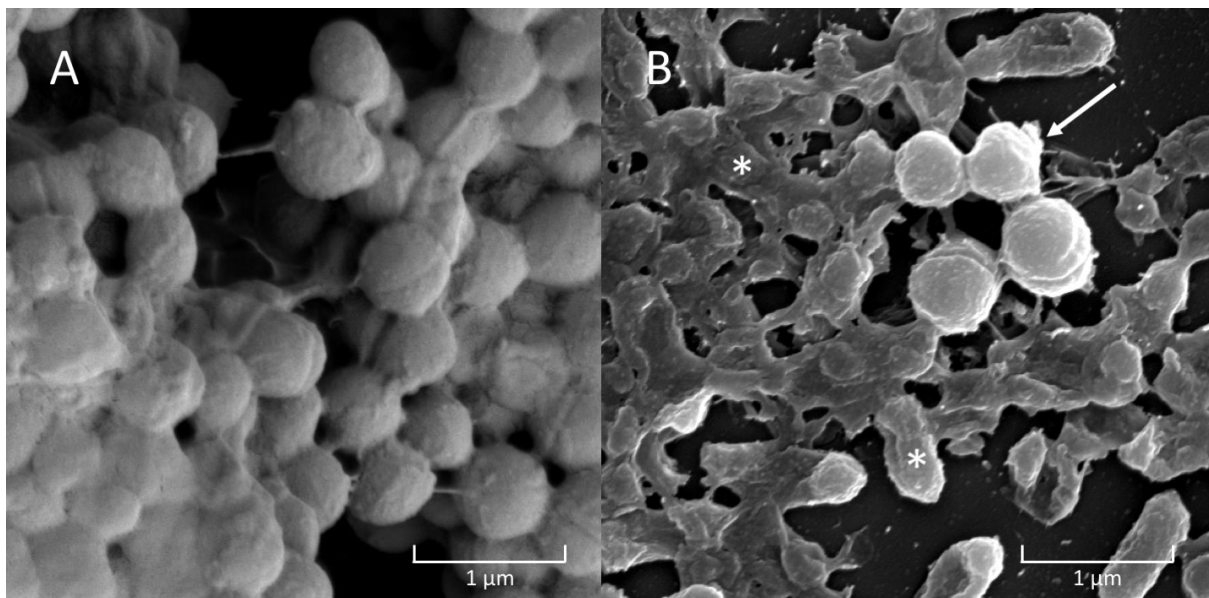
The number of viable *H. influenzae* cells via SCFU counting was similar from mono- or co-cultured biofilm. Additionally, total biofilm biomass, as reflected in the results of the crystal violet assays and SCFU counts, was similar for co-culture with all three *H. influenzae* strains (Figure 4-5).

In contrast, when *H. influenzae* was cultured in suboptimal conditions (i.e. BHI in the absence of factor V or X or growth at 30°C), the addition of *A. otitidis* increased the total number of viable *H. influenzae* cells and the total biofilm biomass. No biofilm was demonstrated at 24 hours in BHI only, for either *H. influenzae* only, *H. influenzae* with *A. otitidis* or *A. otitidis* only (results not shown). However, when *H. influenzae* ATCC 33391 was grown in BHI with Factor V, there was a significant increase in absorbance for co-cultured biofilm (-0.01 SD 0.01 vs 0.08 SD 0.01, 95% CI 0.05 to 0.13,  $p < 0.001$ )(Figure 4-6A) and absolute cell count ( $1.4 \times 10^4$  SD  $4.4 \times 10^3$  vs  $1.365 \times 10^7$  SD  $5.0 \times 10^6$ ,  $p = 0.002$ )(Figure 4-6B). Likewise, when *H. influenzae* ATCC 33391 was grown in BHI with Factor X, the presence of *A. otitidis* increased the absorbance (0.22 SD 0.14 vs 0.38 SD 0.12, 95% CI 0.06 to 0.18  $p < 0.001$ ) (Figure 4-6C) and absolute number of viable cells ( $1.5 \times 10^6$  SD  $6.9 \times 10^5$  vs  $5.1 \times 10^6$  SD  $8.8 \times 10^5$ ,  $p = 0.009$ ) (Figure 4-6D).

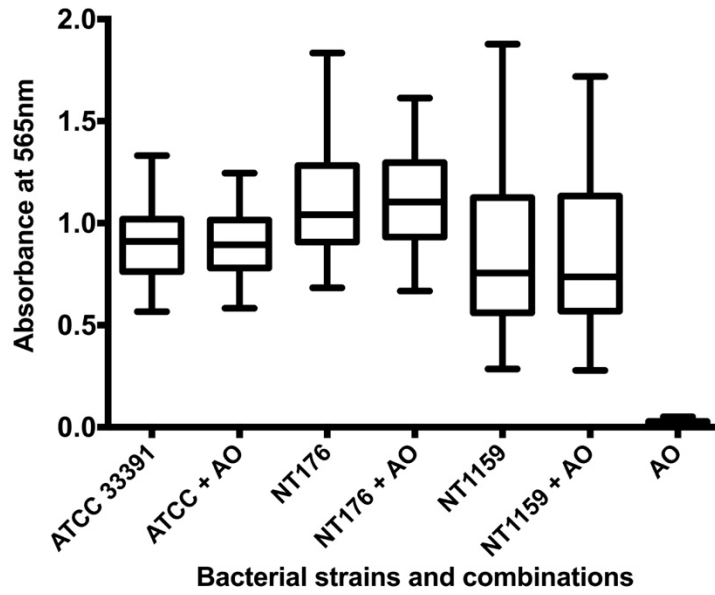
For culture at 30°C, there was a significant increase in biofilm mass demonstrated using the crystal violet assay for the non-typeable *H. influenzae* strains NT 176 and NT 1159 (0.34 SD 0.05 vs 0.39 SD 0.07, 95% CI 0.03 to 0.08,  $p < 0.001$  and 0.09 SD 0.06 vs 0.17 SD 0.06 CI 0.05 to 0.11,  $p < 0.001$ ) when grown in co-culture with *A. otitidis* (Figure 4-7B and 4-7C).



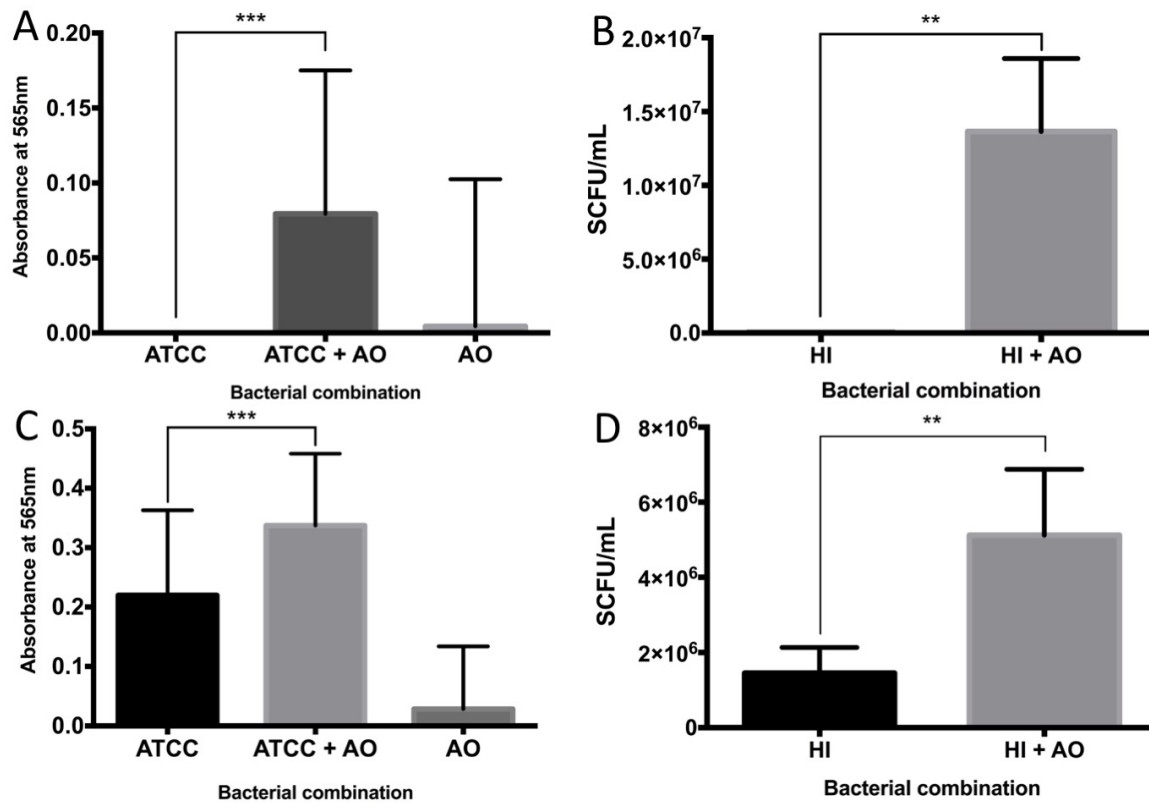
**Figure 4-3 Confocal scanning laser microscopy images of *A. otitidis* and *H. influenzae* polymicrobial biofilm.** CSLM of multispecies biofilm in BHIs at 63X magnification, after LIVE/DEAD *Ba*clight Bacterial Viability staining consisting of *A. otitidis* (Green cocci, arrows) and *H. influenzae* (mixed, smaller, orange-green coccobacilli, asterisks). The *H. influenzae* strains shown are NT 1159 (A), NT 176 (B) and ATCC 33391 strain (C).



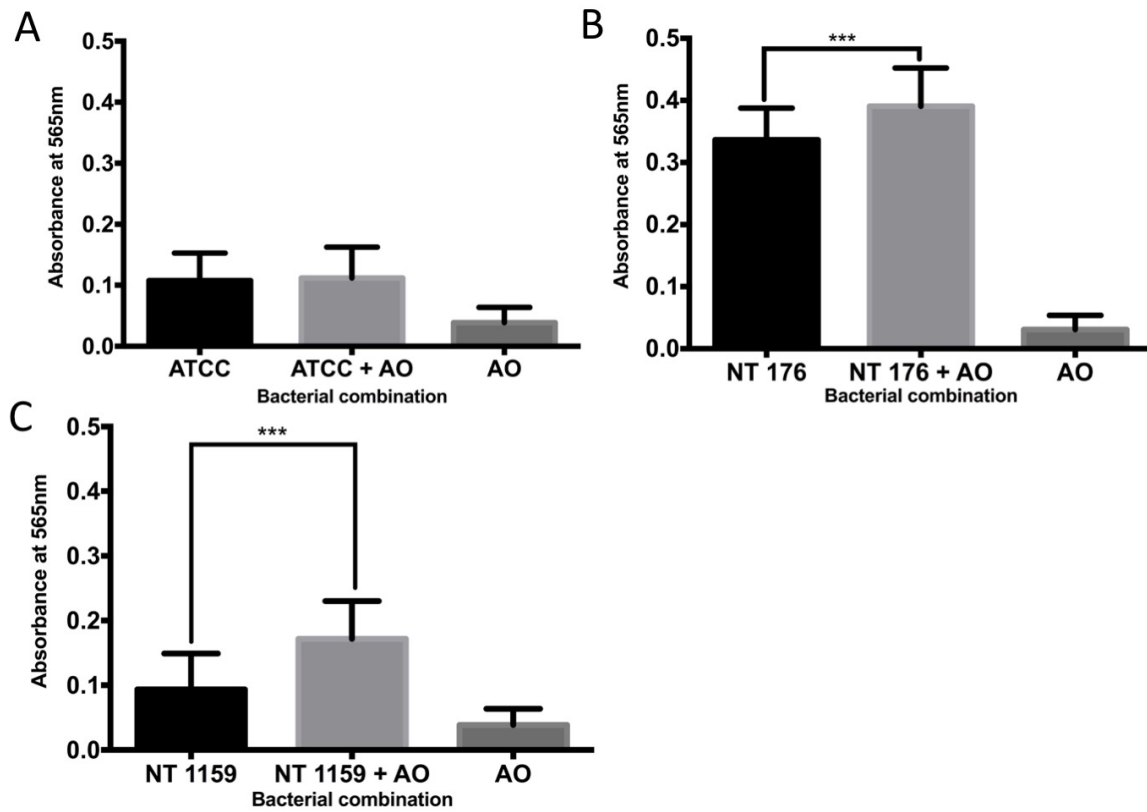
**Figure 4-4: SEM of multispecies biofilm.** *A. otitidis* single species biofilm in BHIb (A) and multispecies biofilm in BHIs of *A. otitidis* (cocci, arrows) and *H. influenzae* (rods, asterisks).



**Figure 4-5 Crystal violet assay of mono and polymicrobial biofilm.** Biofilm biomass of mono- and poly-species biofilm with 3 different *H. influenzae* strains (ATCC 33391, NT176 and NT1159) alone or in combination with *A. otitidis* (AO), as assessed using crystal violet staining and absorbance reading at 565nm, after 24 hours of incubation at 5% CO<sub>2</sub> in BHIs.



**Figure 4-6 *H. influenzae* growth alone and in combination with *A. otitidis* in reduced media.** Quantification of *H. influenzae* ATCC 33391 (ATCC) biofilm alone and in the presence of *A. otitidis* (AO) in reduced brain heart infusion media; with added Factor V (A-B) or Factor X (C-D). Panels represent crystal violet staining and absorbance reading at 565nm (A and C) with corresponding SCFU counts (B and D respectively). Data are presented as means  $\pm$  SD for n=4. \*\*, p < 0.01, \*\*\*, p < 0.001.



**Figure 4-7** *H. influenzae* growth alone and in combination with *A. otitidis* at 30°C. Quantification of *H. influenzae* ATCC 33391 (ATCC) (A), NT 176 (B) and NT 1159 (C) biofilm biomass in the presence or absence of *A. otitidis* biofilm grown for 24 hours at 30° C in BHIs. Biofilm biomass was measured using crystal violet staining and absorbance reading at 565nm. Data are presented as means  $\pm$  SD for n = 4. \*\*\*, p < 0.001.

### 4.4.3 Antibiotic susceptibility

#### 4.4.3.1 *A. otitidis*

In planktonic form, *A. otitidis* was found to be sensitive to both amoxicillin and ciprofloxacin when compared with clinical breakpoint guidelines<sup>276</sup>, both alone and in combination with the *H. influenzae* strains (clinical breakpoints MIC  $\leq$ 0.06  $\mu\text{g}/\text{mL}$  and S  $\leq$  0.125  $\mu\text{g}/\text{mL}$  respectively) (Table 1).

Interestingly, when *A. otitidis* was co-cultured with the *H. influenzae* strains, there was a change in the MIC for some combinations. For amoxicillin, the MIC for *A. otitidis* alone was 0.016  $\mu\text{g}/\text{mL}$ . However, when in combination with all 3 strains of *H. influenzae*, there was a 2.9-fold decrease in sensitivity with a MIC of 0.047  $\mu\text{g}/\text{mL}$  ( $p < 0.05$ ). For ciprofloxacin, the MIC of *A. otitidis* alone was 0.016  $\mu\text{g}/\text{mL}$ . In combination with *H. influenzae* strain NT176, the MIC fell to 0.012  $\mu\text{g}/\text{mL}$  ( $p < 0.05$ ). *H. influenzae* strains NT 1159 and ATCC 33391 did not alter the MIC of *A. otitidis*.

In biofilm form, the MBC of *A. otitidis* for amoxicillin and ciprofloxacin was 0.125  $\mu\text{g}/\text{mL}$  and 0.25  $\mu\text{g}/\text{mL}$  respectively. With the addition of *H. influenzae* strain NT 176, the MBC significantly decreased for both amoxicillin and ciprofloxacin. Again, *H. influenzae* strains NT 1159 and ATCC did not have an effect on MBC.

**Table 4-1: Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentration (MBC) for *Alloiococcus otitidis* (AO) planktonic and biofilm antibiotic susceptibility.**

Bacterial combinations	MIC (µg/mL) (Planktonic)	MBC (µg/mL) (Biofilm)
<b>Amoxicillin:</b>		
<i>A. otitidis</i> (AO)	0.016 (S)	0.125
AO + ATCC	0.047 (S)*	0.125
AO + NT 176	0.047 (S)*	0.06 *
AO + NT 1159	0.047 (S)*	0.125
<b>Ciprofloxacin:</b>		
AO	0.016 (S)	0.25
AO + ATCC	0.016 (S)	0.25
AO + NT 176	0.012 (S)*	0.125 *
AO + NT 1159	0.016 (S)	0.25

Minimum inhibitory concentrations (MIC) and Minimum Bactericidal Concentration (MBC) for *A. otitidis* (AO) planktonic and biofilm antibiotic susceptibility respectively. MIC was assessed by *E*-test with BHib agar, of *A. otitidis* monospecies or in co-culture with *H. influenzae* (ATCC 33391, NT176 and NT1159 strains). All strains were sensitive (S) to amoxicillin and ciprofloxacin in planktonic form (MIC ≤ 0.06 µg/mL and S ≤ 0.125 µg/ml respectively). MBC of *A. otitidis* monospecies or in co-culture with *H. influenzae* was determined by SCFU counting. \*, p < 0.05

#### 4.4.3.2 *H. influenzae*

In planktonic form, *H. influenzae* was sensitive to both amoxicillin and ciprofloxacin when compared with clinical breakpoints guidelines<sup>276</sup>, both alone and in combination with the *H. influenzae* strains (clinical breakpoints MIC ≤ 2 µg/mL and ≤ 0.5 µg/mL respectively) (Table 2).

Co-culture with *A. otitidis* altered the sensitivity in some combinations. For amoxicillin, the MICs for the *H. influenzae* strains were: ATCC 33391 = 1.0 µg/mL, NT176 = 1.5 µg/mL and NT 1159 = 1.0

$\mu\text{g/mL}$ . In co-culture with *A. otitidis*, there was a relative decrease in the sensitivity of NT 176 to amoxicillin (MIC = 2.0  $\mu\text{g/mL}$ ). There was no change to the MICs of strains ATCC 33391 or NT 1159. For ciprofloxacin, the MICs for the *H. influenzae* strains were: ATCC 33391 = 0.008  $\mu\text{g/mL}$ , NT176 = 0.012  $\mu\text{g/mL}$  and NT 1159 = 0.008  $\mu\text{g/mL}$ . In co-culture with *A. otitidis*, there was a relative decrease in sensitivity for strains ATCC 33391 (MIC = 0.012  $\mu\text{g/mL}$ ) and NT 1159 (MIC = 0.012  $\mu\text{g/mL}$ ). There was no change in MIC for strain NT 176.

In biofilm form, for amoxicillin, the MBC of all three *H. influenzae* strains were: ATCC = 32  $\mu\text{g/mL}$ , NT176 = 32  $\mu\text{g/mL}$  and NT 1159 = 32  $\mu\text{g/mL}$ . In combination with *A. otitidis*, there was a relative decrease in sensitivity for *H. influenzae strain NT 176* with a MBC of 64  $\mu\text{g/mL}$ . For ciprofloxacin, the MBC of the *H. influenzae* strains were: ATCC 33391 = 0.1  $\mu\text{g/mL}$ , NT176 = 0.05  $\mu\text{g/mL}$  and NT 1159 = 0.023  $\mu\text{g/mL}$ . Again, in combination with *A. otitidis*, there was a relative decrease in sensitivity for *H. influenzae strain NT 176* with a MBC of 0.1  $\mu\text{g/mL}$ . There was no change in MBC for strains NT1159 or ATCC 33391 when in co-culture with *A. otitidis* for either amoxicillin or ciprofloxacin.

**Table 4-2: Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentration (MBC) for *Haemophilus influenzae* (HI) (strains ATCC, NT 176 and NT 1159) in planktonic and biofilm antibiotic susceptibility.**

<i>H. influenzae</i> strain	MIC HI alone	MIC with <i>A. otitidis</i> in co-culture (µg/mL)	MBC HI alone	MBC with <i>A. otitidis</i> in co-culture (µg/mL)
Amoxicillin:				
ATCC	1.0 (S)	1.0 (S)	32	32
NT 176	1.5 (S)	2.0 (S)*	32	64*
NT 1159	1.0 (S)	1.0 (S)	32	32
Ciprofloxacin:				
ATCC	0.008 (S)	0.012 (S)*	0.1	0.1
NT 176	0.012 (S)	0.012 (S)	0.05	0.1*
NT 1159	0.008 (S)	0.012 (S)*	0.023	0.023

Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentration (MBC) for *H. influenzae* (HI) (strains ATCC 33391, NT 176 and NT 1159) in planktonic and biofilm antibiotic susceptibility respectively. MIC was assessed by *E-test* method with BHIs agar of *H. influenzae* monospecies or in co-culture with *A. otitidis*. All strains were sensitive (S) to amoxicillin and ciprofloxacin in planktonic form (MIC  $\leq$  2 µg/ml and  $\leq$  0.5 µg/ml respectively). MBC of *H. influenzae* monospecies or in co-culture with *A. otitidis* was determined by SCFU counting \*,  $p < 0.05$

## 4.5 Discussion

This paper is the first study to our knowledge to establish that *A. otitidis* forms biofilm *in vitro*. Additionally, we demonstrated that *A. otitidis* and *H. influenzae* form polymicrobial biofilm and that once in this form, these bacteria develop traits that promote persistence and recalcitrance to treatment.

*A. otitidis* is the most prevalent bacterial species found in patients with non-purulent OME (20 to 40%)<sup>181-183</sup>. Our department recently confirmed this using culture-independent 16S rRNA sequencing in two cohorts of children with COME<sup>336,342</sup>. In these studies, we also presented evidence that the external ear canal is likely the reservoir by which *A. otitidis* is seeded into the middle ear (via tympanic membrane perforation). In the past, *A. otitidis* has been considered as merely a commensal of the external ear canal<sup>38,175</sup>. However, consensus on the role of *A. otitidis* remains divided, as *A. otitidis* has been shown to have pathogenic traits including eliciting immune responses *in vitro*<sup>211-216</sup>, invading intracellularly<sup>188</sup> and having been identified as a pathogen in device related<sup>217</sup> and chronic infection<sup>219</sup>. Furthermore, during phylogenetic analysis of our data we found an inverse relationship in relative abundance between *A. otitidis* and *H. influenzae*<sup>335</sup>, suggesting the potential for bacterial interaction in these patients.

In showing that *A. otitidis* forms biofilm, we have added to the evidence that this species of bacteria has pathogenic potential. OM appears to be a biofilm driven disease and the ability of *A. otitidis* to form biofilm is consistent with this perception<sup>162</sup>. The life cycle of biofilms closely resembles the natural history of OM, which is characterized by frequent spontaneous resolution, but also by high recurrence rates and persistence of bacteria despite antibiotic treatment. While in biofilm form, the bacteria may cause low grade subacute inflammation and thus perpetuate middle ear effusion<sup>287</sup>. However, through planktonic shedding of the biofilm an acute infection with an associated intense

inflammatory response can result. Additionally, the formation of biofilm provides bacteria a survival and persistence advantage due to the protection conferred by the extracellular polysaccharide matrix. This matrix protects bacteria within against not only environmental (e.g. fluctuations in temperature, oxygen tension, pH) and chemical (e.g. antibiotics) stressors but also against phagocytosis and humoral immunity<sup>280,281</sup>.

In the setting of OM, biofilms were first demonstrated in the middle ear in an animal model of OM<sup>295,296</sup>. Since that time, middle ear biofilm has been shown in patients with chronic suppurative OM and cholesteatoma<sup>300</sup>. More recently, biofilm has been isolated on the surface of tympanostomy tubes<sup>301</sup> and also free floating within the middle ear aspirates of children with COME<sup>302</sup>. A further two studies have confirmed the presence of biofilm on middle ear mucosa (MEM) from children with COME. Using fluorescence in situ hybridisation (FISH) and SEM Hall-Stoodley et al<sup>162</sup> demonstrated that 92% of the children in their study with COME and recurrent AOM had polymicrobial MEM biofilms, 70% of which, were identified as comprising *H. influenzae*. Similarly, Thornton et al<sup>299</sup> found 64% of their cohort had demonstrable MEM biofilm, with 45% positive for *H. influenzae*. Interestingly, Thornton et al. noted that "a significant proportion" of MEM biofilm samples had bacteria not identifiable by the FISH probes they used. Given our findings that *A. otitidis* forms biofilm and the fact that it exists in high relative abundance within the middle ear cleft of COME, a portion of these unidentifiable bacteria may, in fact, be *A. otitidis*. Further experiments using *A. otitidis* specific probes will be needed to confirm this hypothesis.

Another significant finding of this study was to demonstrate that *A. otitidis* and *H. influenzae* formed polymicrobial biofilm. As discussed above, multispecies biofilm is prevalent within the middle ear cavities of children with COME. There is evidence that bacterial species within polymicrobial biofilm can confer growth advantages and increased protection to each other, resulting in greater resistance

than in single species biofilms<sup>282,283</sup>. Consistent with this we found that the presence of *A. otitidis* increased production of *H. influenzae* biofilm extracellular matrix in low temperatures and increased the number of viable cells despite an absence of critical nutrients (factors X and V). The implication of this finding is that in the setting of OM with effusion, polymicrobial biofilm formation with *A. otitidis* may result in the persistence of *H. influenzae* in biofilm during times of adverse growth conditions (e.g., Limited nutrients, low temperature, extremes of pH, low oxygen tension). When conditions are more conducive to growth, *H. influenzae* may then re-emerge in planktonic form and potentially cause infection.

Similarly, we showed that in the absence of sheep blood, *A. otitidis* formed dense biofilm only when co-cultured with *H. influenzae* biofilm. This indicates that the presence of *H. influenzae* allows *A. otitidis* cells to overcome the growth restrictions it incurs when grown in BHIs media (in the absence of sheep blood). It will be interesting to unravel the mechanism of this observation and to identify putative factors produced by *H. influenzae* that may promote *A. otitidis* biofilm formation. Alternatively, these findings could indicate that, for *A. otitidis* to optimally form biofilm, it requires a lattice from which to form on.

In addition, not only did polymicrobial biofilm support *A. otitidis* and *H. influenzae* growth, it was also associated with an increase in antibiotic resistance for both *A. otitidis* and *H. influenzae*. Biofilm formation is regarded as providing increased antibiotic resistance, with bacteria in biofilm requiring antibiotic concentrations multitudes higher to eradicate than for the same bacteria in planktonic form<sup>280,287</sup>. There is evidence in animal models to suggest that bacteria within a polymicrobial biofilm can confer even greater protection from host defences and antimicrobials than that of single-species biofilms<sup>283-286</sup>; however, this is not a universal finding<sup>246</sup>. Some of the recognised mechanisms behind increased resistance in biofilm include: the ability of the extracellular matrix to

impede antibiotic penetration, decreased metabolic activity of the bacteria within the biofilm, increased adaptation via quorum sensing, increased levels of mutations in antibiotic target molecules and upregulated antibiotic resistance genes<sup>288</sup>.

*A. otitidis* is a  $\beta$ -lactamase negative bacteria<sup>189</sup>, and while being resistant to vancomycin, macrolides and trimethoprim<sup>189,220,221</sup>, it is known to be susceptible to penicillins, cephalosporins, tetracyclines<sup>189,220</sup>, and fluoroquinolones<sup>200</sup>. While little is known about resistant *A. otitidis* strains, *A. otitidis* is often identified within middle ear aspirates despite antibiotic treatment<sup>194</sup>. In contrast, 14-65% of NTHi produce  $\beta$  lactamase<sup>246</sup>, with ampicillin resistance thought to be greater than 30%<sup>350</sup>. In addition, emerging strains of NTHi also possess a non  $\beta$  lactamase mediated ampicillin resistance, a trait originating from *ftsI* gene mutations<sup>250</sup>, which alter the structure of penicillin binding protein 3. Antibiotic resistance gene mutations and the transfer of these genes between bacterial strains has been identified as a cornerstone of bacteria evolving antibiotic resistance<sup>289</sup>. As an example, studies have shown *H. influenzae* can horizontally transfer *ftsI* genes (and thus resistance traits) between bacterial strains<sup>290</sup>. Similarly, Armbruster and colleagues<sup>283</sup> demonstrated that polymicrobial biofilm formed by *H. influenzae* and *Moraxella catarrhalis* conferred both  $\beta$  lactamase dependent and  $\beta$  lactamase-independent resistance to each other.

In our study, we found that polymicrobial biofilm had a protective effect against antibiotics for one species and a deleterious effect on the other. When *A. otitidis* and *H. influenzae* strain NT176 formed polymicrobial biofilm, a decrease in sensitivity of *H. influenzae* to both antibiotics was seen. In contrast, however, there was an increase in sensitivity for *A. otitidis*. Given that *A. otitidis* is not known to produce  $\beta$  lactamase, *A. otitidis* seems to confer a further  $\beta$  lactamase-independent mode of resistance to *H. influenzae* NT176. It is unclear why *A. otitidis* should be more sensitive to antibiotics when in polymicrobial biofilm than single-species biofilm, however, bacterial interference

between *H. influenzae* and other respiratory tract bacteria has been identified in the past<sup>351</sup> and seems to be a strain specific interaction.

While *A. otitidis* was more sensitive to ciprofloxacin in the presence of *H. influenzae* strain NT176, *A. otitidis* demonstrated decreased sensitivity to amoxicillin when any of the three *H. influenzae* strains was added to planktonic co-culture. Likewise, planktonic cells of *H. influenzae* strains NT1159 and ATCC 33391 showed decreased sensitivity to ciprofloxacin in the presence of *A. otitidis*. The significance of these findings is not clear and warrants further investigation. Whilst our findings were reproducible in at least 3 independent experiments, the E-test used was not specifically designed and validated for polymicrobial sensitivity. The act of suspending *A. otitidis* or *H. influenzae* in agar for the E-test may indeed alter the phenotype of these bacteria, thereby altering their sensitivity to antibiotics. In vivo studies will likely better replicate physiological conditions and allow for further elucidation of these relationships.

A limitation of this study is the small number of bacterial strains investigated. However, both commercially available type strains and clinical strains were investigated, and we believe that these results highlight an area for potential research. We have added to our current understanding of the role of *A. otitidis* in OM and reinforced the value of biofilm, in particular polymicrobial biofilm, as a mechanism for bacteria to alter their sensitivity to antibiotics. Further studies, both *in vitro* and *in vivo*, exploring the ability for the formation of polymicrobial biofilm with clinical isolates of *A. otitidis* and their interactions with *H. influenzae* and other classically accepted otopathogens, such as *Moraxella catarrhalis* and *Streptococcus pneumoniae*, will likely provide further insight into the role of *A. otitidis* in the pathogenesis of OM.

## 4.6 Conclusion

In this study, we have described another potential role that *A. otitidis* plays in the pathogenesis of OM. We demonstrated that *A. otitidis* is capable of forming both single species and also polymicrobial biofilm with *H. influenzae*. In addition, when in polymicrobial biofilm, *A. otitidis* promotes *H. influenzae* persistence and survival by increasing biofilm production in adverse growth conditions and by augmenting antimicrobial resistance.

## *Chapter 5: Final discussion*

---

## 5.1 Thesis Synopsis

This thesis has characterised the microbiome within the middle ear of children with otitis media with effusion and further defined a potential pathogenic role of *A. otitidis* in OME. OME is a multifactorial disease that affects 1 in 5 children at any one time. It is the most common cause of hearing loss and balance disorder in childhood. While there are many factors identified that contribute to the aetiology of OME, bacteria seem to play a prominent role.

In the past, identification of these bacteria was limited to their ability to be cultured in media. However, these techniques had an inherent bias based on the fact that bacteria often have a specific growth requirement and that fastidious strains were unable to be identified. With the introduction of culture-independent techniques such as; FISH, PCR, mass spectrometry and DNA microarray, many of the restrictions of culture techniques were overcome, allowing increasing identification and characterization of bacteria. Despite this, a selection bias remained, in that all the aforementioned techniques remained targeted. Given that 16S rRNA is uniformly present in all bacteria, the advent of 16S rRNA gene sequencing, allowed researchers to identify all of the microbes within a sample non-selectively. This technology provided researchers with a greater insight into the pathogenesis of many diseases by completely characterising the flora from a sample, allowing for the study of the complex inter-relationships and symbiotic associations within.

Using 16S rRNA gene sequencing we characterised the microbiota of the middle ear in OME in two cohorts of children over two years. Prior to our study, there were only two much smaller studies that had used 16S rRNA to identify the microbiota of the middle ear in OME. Liu et al.<sup>131</sup>, described the microbial population within the middle ear of one child with COME and Jervis Bardy et al.<sup>132</sup> characterised those of a cohort of 11 Australian Aboriginal children in Central Australia which was

published during the course of this project. Our studies were the first and largest to our knowledge to sample a population of urban children presenting to a tertiary referral hospital.

The first of the studies involved the recruitment of 30 children (23 with OME and 7 control patients), during the Australian winter season from the Women's and Children's Hospital in Adelaide, Australia in 2014. 35 middle ear fluid samples and 23 adenoid swabs were collected from patients with OME and 7 adenoid swab samples from controls. We found that there was considerable variation in the microbiota of the OME between patients. However, *Aerococcus* was the most abundant genera (mean relative abundance 23%), followed by *Haemophilus* (22%), *Staphylococcus* (11%), *Corynebacterium* (6%), *Streptococcus* (5%) and *Moraxella* (5%). These findings were consistent with the findings previously published by Jervis Bardy et al.<sup>164</sup> and suggest that the microbiota of the middle ear in OME is consistent between different demographic and geographical groups.

Additionally, we found that the microbiota resident on the adenoid pads of control patients and those with OME were similar, which was contrary to expectation. The microbiotas were both dominated by the classic otopathogens (*Haemophilus*, *Streptococcus* and *Moraxella*), with high prevalence of anaerobes (predominantly *Fusobacteria*). These findings are consistent with the current understanding of the commensals of the nasopharynx, but also suggest that the bacteria colonising the adenoid pad do not change in the presence of OME and therefore may not have a significant influence on the microbiota of the middle ear in this setting. This concept was further supported by the observation that the microbiota of the middle ear in OME was significantly dissimilar to that of the adenoid pad. While the two sites shared similar abundances of *Haemophilus*, *Streptococcus* and *Moraxella*, the adenoid pad showed a far greater number of anaerobes and a reduction of *Staphylococcus*, *Corynebacterium* and *Aerococcus*.

*Staphylococcus*, *Corynebacterium* and *Aerococcus* are commonly considered commensals of the EAC. The results from our first study thus prompted us to consider whether the EAC could act as a potential reservoir for middle ear bacteria. We therefore designed a follow-up study to compare the microbiota of the external ear canal to that of the middle ear in OME. This study involved recruiting 18 patients from the same hospital in the winter period of 2015 (one year after the first study). We sampled 35 middle ear effusions and 35 paired external ear canal lavages from these patients. We found that despite an overall dissimilarity between the microbiota of the middle ear and EAC, there was a strong correlation between *Aerococcus* (*A. otitidis*), *Staphylococcus*, *Pseudomonas* and *Corynebacteria*. This finding suggests that the external ear canal was possibly serving as a reservoir for the middle ear, with bacteria likely translocating through perforations of the tympanic membrane, a precedence for which is seen in chronic suppurative otitis media being caused by primarily external ear canal bacteria.

When the data was collated from both studies, we found that the microbiota from the two cohorts were similar. This finding intimates that there exists a core microbiota within the middle ears of children with OME, consisting of *Aerococcus*, *Haemophilus*, *Corynebacteria*, *Staphylococcus*, *Streptococcus* and, *Moraxella*, which appears to be stable over time and between populations. Moreover, the results suggested that both adenoid pad and external auditory canals act as reservoirs for the middle ear; the adenoid pad seeds the middle ear with *Haemophilus*, *Streptococcus* and *Moraxella*, while the EAC seeds the middle ear with *Aerococcus*, *Corynebacteria* and *Staphylococcus*. Additionally, when analysing the relative abundances of bacteria within the middle ear fluid from our two studies, we found that there was a negative correlation between *A. otitidis* and *H. influenzae*.

We set about exploring the growth characteristics of *A. otitidis*, specifically whether *A. otitidis* is a biofilm forming bacteria. OME is a biofilm driven disease, as illustrated by the demonstration of polymicrobial biofilm on the middle ear mucosa of children with OME<sup>162,299</sup>. Therefore, we hypothesised that should *A. otitidis* play a role in the pathogenesis of OME, it is likely in biofilm form. In our *in vivo* experiments, using confocal laser scanning microscopy and scanning electron microscopy, we confirmed that *A. otitidis* formed biofilm. Additionally, when investigating the bacterial interactions between *H. influenzae* and *A. otitidis*, we found that *A. otitidis* formed polymicrobial biofilm with *H. influenzae*. Additionally, whilst in co-culture, *A. otitidis* added to the potential virulence of *H influenzae* by promoting its persistence in adverse growth conditions and by altering the antibiotic resistance. However, this effect was strain and antibiotic dependent.

## ***5.2 Future Directions***

We have described the bacterial communities that reside within the middle ear in children with middle ear effusion with 16S rRNA gene sequencing. However, next generation gene sequencing technology continues to evolve. Whilst this technology is considered novel in the field of otolaryngology, 16S sequencing is now considered mainstream in medical research and science. Work is being carried out by researchers to not only identify the bacteria within the samples but also the entire genomes unique to those bacteria. This is known as the study of metagenomics, which allows researchers to investigate the functional characteristics of the microbiome. Additionally, advanced techniques have also delved into characterising protein expression (metaproteomics), metabolites (metabolomics) and mRNA products (metatranscriptomics). When these technologies become more accessible to the general research community, they could be applied to further elucidating the microbiome of the middle ear.

An example of the need for further clarifying the relationships within the microbiome is with the interpretation of our 16S rRNA gene sequencing findings. We found an inverse correlation between *A. otitidis* and *H. influenzae*. Initially, we had hypothesised that this indicated a potential for direct bacterial interference between the two bacteria. However, we now believe that the inverse correlation found was likely a reflection of fact that *A. otitidis* and *H. influenzae* require different growth conditions. We hypothesise that the environment within the middle ear cleft in the presence of a stable, non-purulent effusion, is more conducive to the promotion of *A. otitidis* growth and persistence. During this time, *H. influenzae*, and potentially other otopathogens are promoted to persist in biofilm form in the presence of a high abundance of *A. otitidis*. However, subsequent changes in the local environment within the middle ear, whether via direct inflammation (for example, secondary to viral infection) or host immunomodulation, lead to an environment more advantageous to *H. influenzae* proliferation. The result is the emergence of *H. influenzae* as the more dominant species, thus potentially resulting in acute disease. As discussed, there is an established chinchilla model of otitis media and biofilm<sup>295,296</sup> that may be suitable to explore this phenomenon further. Unfortunately, chinchillas are not readily available in Australia, yet, rat models of otitis media exist that may be suitable substitute biofilm model.

Another limitation to our *in vitro* work lies in the limited number of strains that were tested. Unfortunately, we were unable to obtain clinical isolates of *A. otitidis*. However, we believe that applying clinical isolates of *A. otitidis* and greater numbers of *H. influenzae* to our experimental model will further elucidate the role that this bacterium plays in OME. Furthermore, the inverse correlation between *A. otitidis* and *Staphylococcus* species was not explored and may again shed light on the potential role of *A. otitidis*.

### *5.3 Conclusion*

This thesis has made a significant contribution to our understanding of the complex role that bacteria play in otitis media with effusion using novel molecular sequencing techniques. In addition, we have added to the growing evidence that *A. otitidis*, whether directly or indirectly, plays a pathogenic role in OME.

## Bibliography

- 1 Hoffman H, Hudgins PA. Head and Skull Base Features of Nine Egyptian Mummies: Evaluation with High-Resolution CT and Reformation Techniques. *American Journal of Roentgenology* 2002; **178**: 1367–76.
- 2 Kong K, Coates HLC. Natural history, definitions, risk factors and burden of otitis media. *The Medical journal of Australia* 2009; **191**: S39–43.
- 3 Berger G. Nature of spontaneous tympanic membrane perforation in acute otitis media in children. *J Laryngol Otol* 1989; **103**: 1150–3.
- 4 Kubba H, Pearson JP, Birchall JP. The aetiology of otitis media with effusion: a review. *Clinical otolaryngology and allied sciences* 2000; **25**: 181–94.
- 5 Casselbrant ML, Villardo RJM, Mandel EM. Balance and otitis media with effusion. *International journal of audiology* 2008; **47**: 584–9.
- 6 Teele DW, Klein JO, Rosner B. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. *J Infect Dis* 1989; **160**: 83–94.
- 7 Zielhuis GA, Rach GH, Van Den Broek P. Screening for otitis media with effusion in preschool children. *Lancet* 1989; **1**: 311–4.
- 8 Mandel EM, Doyle WJ, Winther B, Alper CM. The incidence, prevalence and burden of OM in unselected children aged 1-8 years followed by weekly otoscopy through the 'common cold' season. *Int J Pediatr Otorhinolaryngol* 2008; **72**: 491–9.
- 9 Engel J, Anteunis L, Volovics A, Hendriks J, Marres E. Prevalence rates of otitis media with effusion from 0 to 2 years of age: healthy-born versus high-risk-born infants. *Int J Pediatr Otorhinolaryngol* 1999; **47**: 243–51.
- 10 Kvaerner KJ, Tambs K, Harris JR, Magnus P. Distribution and heritability of recurrent ear infections. *The Annals of otology, rhinology, and laryngology* 1997; **106**: 624–32.
- 11 Kalm O, Johnson U, Prellner K. HLA frequency in patients with chronic secretory otitis media. *Int J Pediatr Otorhinolaryngol* 1994; **30**: 151–7.
- 12 Carrie S, Hutton DA, Birchall JP, Green GG, Pearson JP. Otitis media with effusion: components which contribute to the viscous properties. *Acta Otolaryngol* 1992; **112**: 504–11.
- 13 Pedersen M, Mygind N. Rhinitis, sinusitis and otitis media in Kartagener's syndrome (primary ciliary dyskinesia). *Clinical otolaryngology and allied sciences* 1982; **7**: 373–80.
- 14 el-Sayed Y, al-Sarhani A, al-Essa AR. Otological manifestations of primary ciliary dyskinesia. *Clinical otolaryngology and allied sciences* 1997; **22**: 266–70.

- 15 Quaranta N, Iannuzzi L, Gelardi M. Does the type of rhinitis influence development of otitis media with effusion in children? *Current allergy and asthma reports* 2014; **14**: 472.
- 16 Minovi A, Dazert S. Diseases of the middle ear in childhood. *GMS current topics in otorhinolaryngology, head and neck surgery* 2014; **13**: Doc11.
- 17 Coticchia JM, Chen M, Sachdeva L, Mutchnick S. New paradigms in the pathogenesis of otitis media in children. *Frontiers in pediatrics* 2013; **1**: 52.
- 18 Sadé J, Weissman Z. Middle ear mucosa and secretory otitis media. *Arch Otorhinolaryngol* 1977; **215**: 195–205.
- 19 Takahashi H, Hayashi M, Sato H, Honjo I. Primary deficits in eustachian tube function in patients with otitis media with effusion. *Arch Otolaryngol Head Neck Surg* 1989; **115**: 581–4.
- 20 Iwano T, Kinoshita T, Hamada E, Doi T, Ushiro K, Kumazawa T. Otitis media with effusion and eustachian tube dysfunction in adults and children. *Acta Otolaryngol Suppl* 1993; **500**: 66–9.
- 21 Ovesen T, Børglum JD. New aspects of secretory otitis media, eustachian tube function and middle ear gas. *Ear Nose Throat J* 1998; **77**: 770–7.
- 22 Hoa M, Syamal M, Schaeffer MA, Sachdeva L, Berk R, Coticchia J. Biofilms and chronic otitis media: an initial exploration into the role of biofilms in the pathogenesis of chronic otitis media. *American journal of otolaryngology* 2010; **31**: 241–5.
- 23 Saafan ME, Ibrahim WS, Tomoum MO. Role of adenoid biofilm in chronic otitis media with effusion in children. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies* 2013; **270**: 2417–25.
- 24 Saylam G, Tatar EC, Tatar I, Ozdek A, Korkmaz H. Association of adenoid surface biofilm formation and chronic otitis media with effusion. *Arch Otolaryngol Head Neck Surg* 2010; **136**: 550–5.
- 25 Dhillon RS. The middle ear in cleft palate children pre and post palatal closure. *J R Soc Med* 1988; **81**: 710–3.
- 26 Robinson PJ, Lodge S, Jones BM, Walker CC, Grant HR. The effect of palate repair on otitis media with effusion. *Plast Reconstr Surg* 1992; **89**: 640–5.
- 27 Huang MH, Lee ST, Rajendran K. A fresh cadaveric study of the paratubal muscles: implications for eustachian tube function in cleft palate. *Plast Reconstr Surg* 1997; **100**: 833–42.
- 28 Maxwell KS, Fitzgerald JE, Burleson JA, Leonard G, Carpenter R, Kreutzer DL. Interleukin-8 expression in otitis media. *The Laryngoscope* 1994; **104**: 989–95.
- 29 Jousimies-Somer H, Grénman R, Rintala A. Bacteriological investigation of secretory

- otitis media in children with cleft palate. *Scand J Plast Reconstr Surg* 1986; **20**: 297–302.
- 30 Tasker A, Dettmar PW, Panetti M, Koufman JA, P Birchall J, Pearson JP. Is gastric reflux a cause of otitis media with effusion in children? *The Laryngoscope* 2002; **112**: 1930–4.
- 31 Boronat-Echeverría N, Aguirre-Mariscal H, Carmolinga-Ponce M, *et al.* Helicobacter pylori detection and clinical symptomatology of gastroesophageal reflux disease in pediatric patients with otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2016; **87**: 126–9.
- 32 Kariya S, Okano M, Nishizaki K. An association between Helicobacter pylori and upper respiratory tract disease: fact or fiction? *World J Gastroenterol* 2014; **20**: 1470–84.
- 33 Holla-Bhar R, Iellamo A, Gupta A, Smith JP, Dadhich JP. Investing in breastfeeding - the world breastfeeding costing initiative. *Int Breastfeed J* 2015; **10**: 8.
- 34 Li L, Borland R, Yong H-H, *et al.* The association between exposure to point-of-sale anti-smoking warnings and smokers' interest in quitting and quit attempts: findings from the International Tobacco Control Four Country Survey. *Addiction* 2012; **107**: 425–33.
- 35 Gould GS, Watt K, Stevenson L, McEwen A, Cadet-James Y, Clough AR. Developing anti-tobacco messages for Australian Aboriginal and Torres Strait Islander peoples: evidence from a national cross-sectional survey. *BMC Public Health* 2014; **14**: 250.
- 36 Zielhuis GA, Heuvelmans-Heinen EW, Rach GH, Van Den Broek P. Environmental risk factors for otitis media with effusion in preschool children. *Scand J Prim Health Care* 1989; **7**: 33–8.
- 37 Ngo CC, Massa HM, Thornton RB, Cripps AW. Predominant Bacteria Detected from the Middle Ear Fluid of Children Experiencing Otitis Media: A Systematic Review. *PLoS ONE* 2016; **11**: e0150949.
- 38 Tano K, Essen von R, Eriksson PO, Sjostedt A. Alloiococcus otitidis--otitis media pathogen or normal bacterial flora? *APMIS* 2008; **116**: 785–90.
- 39 Henderson FW, Collier AM, Sanyal MA, *et al.* A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. *N Engl J Med* 1982; **306**: 1377–83.
- 40 Winther B, Alper CM, Mandel EM, Doyle WJ, Hendley JO. Temporal relationships between colds, upper respiratory viruses detected by polymerase chain reaction, and otitis media in young children followed through a typical cold season. *Pediatrics* 2007; **119**: 1069–75.
- 41 Chonmaitree T, Heikkinen T. Viruses and acute otitis media. *The Pediatric infectious disease journal* 2000; **19**: 1005–7.
- 42 Ruohola A, Meurman O, Nikkari S, *et al.* Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. *Clinical infectious*

diseases: an official publication of the Infectious Diseases Society of America 2006; **43**: 1417–22.

- 43 Wren JT, Blevins LK, Pang B, *et al.* Influenza A virus alters pneumococcal nasal colonization and middle ear infection independently of phase variation. *Infection and immunity* 2014; **82**: 4802–12.
- 44 Allen EK, Koepfel AF, Hendley JO, Turner SD, Winther B, Sale MM. Characterization of the nasopharyngeal microbiota in health and during rhinovirus challenge. *Microbiome* 2014; **2**: 22.
- 45 Shaw CB, Obermyer N, Wetmore SJ, Spirou GA, Farr RW. Incidence of adenovirus and respiratory syncytial virus in chronic otitis media with effusion using the polymerase chain reaction. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1995; **113**: 234–41.
- 46 Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. *N Engl J Med* 1999; **340**: 260–4.
- 47 Pitkäranta A, Rihkanen H, Carpén O, Vaheri A. Rhinovirus RNA in children with longstanding otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002; **66**: 247–50.
- 48 Heikkinen T. The role of respiratory viruses in otitis media. *Vaccine* 2000; **19 Suppl 1**: S51–5.
- 49 Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM. Otitis media. *Lancet* 2004; **363**: 465–73.
- 50 Stenström C, Ingvarsson L. General illness and need of medical care in otitis prone children. *Int J Pediatr Otorhinolaryngol* 1994; **29**: 23–32.
- 51 Chantzi FM, Kafetzis DA, Bairamis T, *et al.* IgE sensitization, respiratory allergy symptoms, and heritability independently increase the risk of otitis media with effusion. *Allergy* 2006; **61**: 332–6.
- 52 Kreiner-Møller E, Chawes BLK, Caye-Thomasen P, Bønnelykke K, Bisgaard H. Allergic rhinitis is associated with otitis media with effusion: a birth cohort study. *Clin Exp Allergy* 2012; **42**: 1615–20.
- 53 Kwon C, Lee HY, Kim MG, Boo SH, Yeo SG. Allergic diseases in children with otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2013; **77**: 158–61.
- 54 Tomonaga K, Kurono Y, Mogi G. The role of nasal allergy in otitis media with effusion. A clinical study. *Acta Otolaryngol Suppl* 1988; **458**: 41–7.
- 55 Lack G, Caulfield H, Penagos M. The link between otitis media with effusion and allergy: a potential role for intranasal corticosteroids. *Pediatr Allergy Immunol* 2011; **22**: 258–66.

- 56 Alles R, Parikh A, Hawk L, Darby Y, Romero JN, Scadding G. The prevalence of atopic disorders in children with chronic otitis media with effusion. *Pediatr Allergy Immunol* 2001; **12**: 102–6.
- 57 Sobol SE, Taha R, Schloss MD, *et al.* T(H)2 cytokine expression in atopic children with otitis media with effusion. *J Allergy Clin Immunol* 2002; **110**: 125–30.
- 58 Collins MP, Church MK, Bakhshi KN, Osborne J. Adenoid histamine and its possible relationship to secretory otitis media. *J Laryngol Otol* 1985; **99**: 685–91.
- 59 Ulualp SO, Sahin D, Yilmaz N, Anadol V, Peker O, Gursan O. Increased adenoid mast cells in patients with otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 1999; **49**: 107–14.
- 60 Quaranta N, Milella C, Iannuzzi L, Gelardi M. A study of the role of different forms of chronic rhinitis in the development of otitis media with effusion in children affected by adenoid hypertrophy. *Int J Pediatr Otorhinolaryngol* 2013; **77**: 1980–3.
- 61 Owen MJ, Baldwin CD, Swank PR, Pannu AK, Johnson DL, Howie VM. Relation of infant feeding practices, cigarette smoke exposure, and group child care to the onset and duration of otitis media with effusion in the first two years of life. *J Pediatr* 1993; **123**: 702–11.
- 62 Rasmussen F. Protracted secretory otitis media. The impact of familial factors and day-care center attendance. *Int J Pediatr Otorhinolaryngol* 1993; **26**: 29–37.
- 63 Wald ER, Dashefsky B, Byers C, Guerra N, Taylor F. Frequency and severity of infections in day care. *J Pediatr* 1988; **112**: 540–6.
- 64 Pukander J, Luotonen J, Timonen M, Karma P. Risk factors affecting the occurrence of acute otitis media among 2-3-year-old urban children. *Acta Otolaryngol* 1985; **100**: 260–5.
- 65 Jacoby PA, Coates HL, Arumugaswamy A, *et al.* 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. *The Medical journal of Australia* 2008; **188**: 599–603.
- 66 Agius AM, Wake M, Pahor AL, Smallman LA. Smoking and middle ear ciliary beat frequency in otitis media with effusion. *Acta Otolaryngol* 1995; **115**: 44–9.
- 67 Agius AM, Wake M, Pahor AL, Smallman A. The effects of in vitro cotinine on nasal ciliary beat frequency. *Clinical otolaryngology and allied sciences* 1995; **20**: 465–9.
- 68 Etzel RA, Pattishall EN, Haley NJ, Fletcher RH, Henderson FW. Passive smoking and middle ear effusion among children in day care. *Pediatrics* 1992; **90**: 228–32.
- 69 Ahmer El OR, Essery SD, Saadi AT, *et al.* The effect of cigarette smoke on adherence of respiratory pathogens to buccal epithelial cells. *FEMS Immunol Med Microbiol* 1999; **23**: 27–36.

- 70 Brook I. Effects of exposure to smoking on the microbial flora of children and their parents. *Int J Pediatr Otorhinolaryngol* 2010; **74**: 447–50.
- 71 Antonelli PJ, Daly KA, Juhn SK, Veum EJ, Adams GL, Giebink GS. Tobacco smoke and otitis media in the chinchilla model. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1994; **111**: 513–8.
- 72 Coggins CR, Lovejoy HM, McGuirt WF, Sagartz JW, Hayes AW, Ayres PH. Relevant exposure to environmental tobacco smoke surrogate does not produce or modify secretory otitis media in the rat. *Toxicol Pathol* 1997; **25**: 395–7.
- 73 Kitchens GG. Relationship of environmental tobacco smoke to otitis media in young children. *The Laryngoscope* 1995; **105**: 1–13.
- 74 Ladomenou F, Kafatos A, Tselentis Y, Galanakis E. Predisposing factors for acute otitis media in infancy. *J Infect* 2010; **61**: 49–53.
- 75 Uhari M, Mäntysaari K, Niemelä M. A meta-analytic review of the risk factors for acute otitis media. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 1996; **22**: 1079–83.
- 76 Ladomenou F, Moschandreas J, Kafatos A, Tselentis Y, Galanakis E. Protective effect of exclusive breastfeeding against infections during infancy: a prospective study. *Arch Dis Child* 2010; **95**: 1004–8.
- 77 Paradise JL, Elster BA, Tan L. Evidence in infants with cleft palate that breast milk protects against otitis media. *Pediatrics* 1994; **94**: 853–60.
- 78 Jakaitis BM, Denning PW. Human breast milk and the gastrointestinal innate immune system. *Clin Perinatol* 2014; **41**: 423–35.
- 79 Bode L. The functional biology of human milk oligosaccharides. *Early Hum Dev* 2015; **91**: 619–22.
- 80 Hunt CE, Lesko SM, Vezina RM, *et al*. Infant sleep position and associated health outcomes. *Arch Pediatr Adolesc Med* 2003; **157**: 469–74.
- 81 Harrison LM, Morris JA, Telford DR, Brown SM, Jones K. The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol Med Microbiol* 1999; **25**: 19–28.
- 82 Morris PS, Leach AJ, Silberberg P, *et al*. Otitis media in young Aboriginal children from remote communities in Northern and Central Australia: a cross-sectional survey. *BMC Pediatr* 2005; **5**: 27.
- 83 Leach AJ, Boswell JB, Asche V, Nienhuys TG, Mathews JD. Bacterial colonization of the nasopharynx predicts very early onset and persistence of otitis media in Australian aboriginal infants. *The Pediatric infectious disease journal* 1994; **13**: 983–9.

- 84 Currie BJ, Brewster DR. Childhood infections in the tropical north of Australia. *J Paediatr Child Health* 2001; **37**: 326–30.
- 85 Moran DJ, Waterford JE, Hollows F, Jones DL. Ear disease in rural Australia. *The Medical journal of Australia* 1979; **2**: 210–2.
- 86 Morris PS. A systematic review of clinical research addressing the prevalence, aetiology, diagnosis, prognosis and therapy of otitis media in Australian Aboriginal children. *J Paediatr Child Health* 1998; **34**: 487–97.
- 87 Sun W, Jacoby P, Riley TV, *et al.* Association between early bacterial carriage and otitis media in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia: a cohort study. *BMC infectious diseases* 2012; **12**: 366.
- 88 Watson K, Carville K, Bowman J, *et al.* Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. *The Pediatric infectious disease journal* 2006; **25**: 782–90.
- 89 McDonald E, Bailie R, Brewster D, Morris P. Are hygiene and public health interventions likely to improve outcomes for Australian Aboriginal children living in remote communities? A systematic review of the literature. *BMC Public Health* 2008; **8**: 153.
- 90 Jeffries-Stokes C, Lehmann D, Johnston J, *et al.* Aboriginal perspective on middle ear disease in the arid zone of Western Australia. *J Paediatr Child Health* 2004; **40**: 258–64.
- 91 Robb PJ. Childhood otitis media with effusion. *Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery* 2006; **31**: 535–7.
- 92 Rosenfeld RM, Shin JJ, Schwartz SR, *et al.* Clinical Practice Guideline: Otitis Media with Effusion Executive Summary (Update). *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery*. 2016; **154**: 201–14.
- 93 Golz A, Angel-Yeger B, Parush S. Evaluation of balance disturbances in children with middle ear effusion. *Int J Pediatr Otorhinolaryngol* 1998; **43**: 21–6.
- 94 Shekelle P, Takata G, Chan LS, *et al.* Diagnosis, natural history, and late effects of otitis media with effusion. *Evid Rep Technol Assess (Summ)* 2002; : 1–5.
- 95 Sabo DL, Paradise JL, Kurs-Lasky M, Smith CG. Hearing levels in infants and young children in relation to testing technique, age group, and the presence or absence of middle-ear effusion. *Ear Hear* 2003; **24**: 38–47.
- 96 Fria TJ, Cantekin EI, Eichler JA. Hearing acuity of children with otitis media with effusion. *Archives of otolaryngology* 1985; **111**: 10–6.
- 97 Hunter LL, Margolis RH, Giebink GS. Identification of hearing loss in children with otitis media. *The Annals of otology, rhinology & laryngology Supplement* 1994; **163**: 59–61.

- 98 Brody R, Rosenfeld RM, Goldsmith AJ, Madell JR. Parents cannot detect mild hearing loss in children. First place--Resident Clinical Science Award 1998. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1999; **121**: 681–6.
- 99 Stewart MG, Ohlms LA, Friedman EM, *et al.* Is parental perception an accurate predictor of childhood hearing loss? A prospective study. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 1999; **120**: 340–4.
- 100 Widen JE, Folsom RC, Cone-Wesson B, *et al.* Identification of neonatal hearing impairment: hearing status at 8 to 12 months corrected age using a visual reinforcement audiometry protocol. *Ear Hear* 2000; **21**: 471–87.
- 101 Harlor ADB, Bower C, Committee on Practice and Ambulatory Medicine, Section on Otolaryngology-Head and Neck Surgery. Hearing assessment in infants and children: recommendations beyond neonatal screening. *Pediatrics*. 2009; **124**: 1252–63.
- 102 Rosenfeld RM, Kay D. Natural history of untreated otitis media. *The Laryngoscope* 2003; **113**: 1645–57.
- 103 Venekamp RP, Burton MJ, van Dongen TMA, van der Heijden GJ, van Zon A, Schilder AGM. Antibiotics for otitis media with effusion in children. *Cochrane Database Syst Rev* 2016; : CD009163.
- 104 Berkman ND, Wallace IF, Steiner MJ, *et al.* Otitis Media With Effusion: Comparative Effectiveness of Treatments. 2013; published online May.
- 105 Bhargava R, Chakravarti A. A double-blind randomized placebo-controlled trial of topical intranasal mometasone furoate nasal spray in children of adenoidal hypertrophy with otitis media with effusion. *American journal of otolaryngology* 2014; **35**: 766–70.
- 106 Cengel S, Akyol MU. The role of topical nasal steroids in the treatment of children with otitis media with effusion and/or adenoid hypertrophy. *Int J Pediatr Otorhinolaryngol* 2006; **70**: 639–45.
- 107 Browning GG, Rovers MM, Williamson I, Lous J, Burton MJ. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. *Cochrane Database Syst Rev* 2010; : CD001801.
- 108 Gates GA, Avery CA, Prihoda TJ, Cooper JC. Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. *N Engl J Med* 1987; **317**: 1444–51.
- 109 Maw AR, Bawden R. The long term outcome of secretory otitis media in children and the effects of surgical treatment: a ten year study. *Acta Otorhinolaryngol Belg* 1994; **48**: 317–24.
- 110 Kuo C-L, Tsao Y-H, Cheng H-M, *et al.* Grommets for otitis media with effusion in

- children with cleft palate: a systematic review. *Pediatrics* 2014; **134**: 983–94.
- 111 Kay DJ, Nelson M, Rosenfeld RM. Meta-analysis of tympanostomy tube sequelae. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2001; **124**: 374–80.
- 112 Rosenfeld RM, Schwartz SR, Pynnonen MA, *et al*. Clinical practice guideline: Tympanostomy tubes in children. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery*. 2013; **149**: S1–35.
- 113 Paradise JL, Bluestone CD, Colborn DK, *et al*. Adenoidectomy and adenotonsillectomy for recurrent acute otitis media: parallel randomized clinical trials in children not previously treated with tympanostomy tubes. *JAMA* 1999; **282**: 945–53.
- 114 Nguyen LHP, Manoukian JJ, Yoskovitch A, Al-Sebeih KH. Adenoidectomy: selection criteria for surgical cases of otitis media. *The Laryngoscope* 2004; **114**: 863–6.
- 115 Boonacker CWB, Rovers MM, Browning GG, Hoes AW, Schilder AGM, Burton MJ. Adenoidectomy with or without grommets for children with otitis media: an individual patient data meta-analysis. *Health technology assessment* 2014; **18**: 1–118.
- 116 Mikals SJ, Brigger MT. Adenoidectomy as an adjuvant to primary tympanostomy tube placement: a systematic review and meta-analysis. *JAMA otolaryngology-- head & neck surgery* 2014; **140**: 95–101.
- 117 Health AGDO. Immunise - Children birth - 4 years.  
<http://www.immunise.health.gov.au/internet/immunise/publishing.nsf/Content/parents-of-children>.
- 118 Hausdorff WP, Hoet B, Adegbola RA. Predicting the impact of new pneumococcal conjugate vaccines: serotype composition is not enough. *Expert Review of Vaccines* 2014; **14**: 413–28.
- 119 Fortanier AC, Venekamp RP, Boonacker CWB, *et al*. Pneumococcal conjugate vaccines for preventing otitis media. *Cochrane Database Syst Rev* 2014; : CD001480.
- 120 Revai K, McCormick DP, Patel J, Grady JJ, Saeed K, Chonmaitree T. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. *Pediatrics* 2006; **117**: 1823–9.
- 121 Taylor S, Marchisio P, Vergison A, Harriague J, Hausdorff WP, Haggard M. Impact of pneumococcal conjugate vaccination on otitis media: a systematic review. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2012; **54**: 1765–73.
- 122 Eskola J, Kilpi T, Palmu A, *et al*. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med* 2001; **344**: 403–9.
- 123 Sarasoja I, Jokinen J, Lahdenkari M, Kilpi T, Palmu AA. Long-term effect of

- pneumococcal conjugate vaccines on tympanostomy tube placements. *The Pediatric infectious disease journal* 2013; **32**: 517–20.
- 124 Veenhoven R, Bogaert D, Uiterwaal C, *et al.* Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* 2003; **361**: 2189–95.
- 125 Williams SR, Mernagh PJ, Lee MHT, Tan JT. Changing epidemiology of invasive pneumococcal disease in Australian children after introduction of a 7-valent pneumococcal conjugate vaccine. *The Medical journal of Australia* 2011; **194**: 116–20.
- 126 Leach AJ, Wigger C, Beissbarth J, *et al.* General health, otitis media, nasopharyngeal carriage and middle ear microbiology in Northern Territory Aboriginal children vaccinated during consecutive periods of 10-valent or 13-valent pneumococcal conjugate vaccines. *Int J Pediatr Otorhinolaryngol* 2016; **86**: 224–32.
- 127 Murphy TF. Vaccines for Nontypeable Haemophilus influenzae: the Future Is Now. *Clinical and vaccine immunology : CVI* 2015; **22**: 459–66.
- 128 Khan MN, Ren D, Kaur R, Basha S, Zagursky R, Pichichero ME. Developing a vaccine to prevent otitis media caused by nontypeable Haemophilus influenzae. *Expert Review of Vaccines* 2016; **15**: 863–78.
- 129 Hassan F. Molecular mechanisms of moraxella catarrhalis-induced otitis media. *Current allergy and asthma reports* 2013; **13**: 512–7.
- 130 Cox MJ, Cookson WOCM, Moffatt MF. Sequencing the human microbiome in health and disease. *Human molecular genetics* 2013; **22**: R88–94.
- 131 Liu CM, Cosetti MK, Aziz M, *et al.* The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. *Arch Otolaryngol Head Neck Surg* 2011; **137**: 664–8.
- 132 Jervis-Bardy J, Rogers GB, Morris PS, *et al.* The microbiome of otitis media with effusion in Indigenous Australian children. *Int J Pediatr Otorhinolaryngol* 2015; published online July 17. DOI:10.1016/j.ijporl.2015.07.013.
- 133 Ishmael FT, Stellato C. Principles and applications of polymerase chain reaction: basic science for the practicing physician. *Annals of allergy* 2008.
- 134 Chial H. DNA sequencing technologies key to the Human Genome Project. *Nature Education* 2008.
- 135 Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA* 1998; **279**: 296–9.
- 136 Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen K-Y. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clinical microbiology and infection : the official publication*

- of the European Society of Clinical Microbiology and Infectious Diseases 2008; **14**: 908–34.
- 137 Van de Peer Y, Chapelle S, De Wachter R. A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Research* 1996; **24**: 3381–91.
- 138 Chakravorty S, Helb D, Burday M, Connell N, Alland D. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J Microbiol Methods* 2007; **69**: 330–9.
- 139 McDonald D, Price MN, Goodrich J, *et al.* An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* 2012; **6**: 610–8.
- 140 Quast C, Pruesse E, Yilmaz P, *et al.* The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 2013; **41**: D590–6.
- 141 Cole JR, Wang Q, Cardenas E, *et al.* The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Research* 2009; **37**: D141–5.
- 142 Ashelford KE, Chuzhanova NA, Fry JC, Jones AJ, Weightman AJ. At least 1 in 20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl Environ Microbiol* 2005; **71**: 7724–36.
- 143 Meyer F, Paarmann D, D'Souza M, *et al.* The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 2008; **9**: 386.
- 144 Caporaso JG, Kuczynski J, Stombaugh J, *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010; **7**: 335–6.
- 145 Schloss PD, Westcott SL, Ryabin T, *et al.* Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009; **75**: 7537–41.
- 146 Hill MO. Diversity and Evenness: A Unifying Notation and Its Consequences. *Ecology* 1973; **54**: 427–32.
- 147 Bray JR, Curtis JT. An ordination of the upland forest communities of southern Wisconsin. *Ecological monographs* 1957.
- 148 Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 2005.
- 149 Rooks MG, Veiga P, Wardwell-Scott LH, *et al.* Gut microbiome composition and function in experimental colitis during active disease and treatment-induced remission. *ISME J* 2014; **8**: 1403–17.
- 150 Abt MC, Artis D. The intestinal microbiota in health and disease: the influence of

- microbial products on immune cell homeostasis. *Curr Opin Gastroenterol* 2009; **25**: 496–502.
- 151 Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. *Science* 2001; **292**: 1115–8.
- 152 Vollaard EJ, Clasener HA. Colonization resistance. *Antimicrobial agents and chemotherapy* 1994; **38**: 409–14.
- 153 Zhou Y, Gao H, Mihindukulasuriya KA, *et al.* Biogeography of the ecosystems of the healthy human body. *Genome Biol* 2013; **14**: R1.
- 154 Li K, Bihan M, Methe BA. Analyses of the stability and core taxonomic memberships of the human microbiome. *PLoS ONE* 2013; **8**: e63139.
- 155 Li K, Bihan M, Methe BA. Analyses of the stability and core taxonomic memberships of the human microbiome. *PLoS ONE* 2013; **8**: e63139.
- 156 Microbial co-occurrence relationships in the human microbiome. *PLoS Comput Biol* 2012; **8**: e1002606.
- 157 Segata N, Haake SK, Mannon P, *et al.* Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. *Genome Biol* 2012; **13**: R42.
- 158 Bardy JJ, Psaltis AJ. Next Generation Sequencing and the Microbiome of Chronic Rhinosinusitis A Primer for Clinicians and Review of Current Research, Its Limitations, and Future Directions. *The Annals of otology, rhinology, and laryngology* 2016; **125**: 0003489416641429–621.
- 159 Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl Environ Microbiol* 2012; **78**: 6262–70.
- 160 Khoruts A, Sadowsky MJ. Understanding the mechanisms of faecal microbiota transplantation. *Nature Reviews Gastroenterology & ...* 2016.
- 161 Young VB. Therapeutic Manipulation of the Microbiota: Past, Present and Considerations for the Future. *Clinical Microbiology and Infection* 2016; published online Sept. DOI:10.1016/j.cmi.2016.09.001.
- 162 Hall-Stoodley L, Hu FZ, Gieseke A, *et al.* Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 2006; **296**: 202–11.
- 163 Neeff M, Biswas K, Hoggard M, Taylor MW, Douglas R. Molecular microbiological profile of chronic suppurative otitis media. *J Clin Microbiol* 2016; : JCM.01068–16.
- 164 Jarvis-Bardy J, Rogers GB, Morris PS, *et al.* The microbiome of otitis media with effusion in Indigenous Australian children. *Int J Pediatr Otorhinolaryngol* 2015; **79**: 1548–55.

- 165 Kim SH, Kim MG, Kim SS, Cha SH, Yeo SG. Change in Detection Rate of Methicillin-Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* and Their Antibiotic Sensitivities in Patients with Chronic Suppurative Otitis Media. *J Int Adv Otol* 2015; **11**: 151–6.
- 166 Neeff M, Biswas K, Hoggard M, Taylor MW, Douglas R. Molecular Microbiological Profile of Chronic Suppurative Otitis Media. *J Clin Microbiol* 2016; **54**: 2538–46.
- 167 Sevillano D, Aguilar L, Alou L, *et al.* Beta-lactam effects on mixed cultures of common respiratory isolates as an approach to treatment effects on nasopharyngeal bacterial population dynamics. *PLoS ONE* 2008; **3**: e3846.
- 168 Bosch AATM, Levin E, van Houten MA, *et al.* Development of Upper Respiratory Tract Microbiota in Infancy is Affected by Mode of Delivery. *EBioMedicine* 2016; **9**: 336–45.
- 169 Bogaert D, Keijsers B, Huse S, *et al.* Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS ONE* 2011; **6**: e17035.
- 170 Marchisio P, Claut L, Rognoni A, *et al.* Differences in nasopharyngeal bacterial flora in children with nonsevere recurrent acute otitis media and chronic otitis media with effusion: implications for management. *The Pediatric infectious disease journal* 2003; **22**: 262–8.
- 171 Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis* 1997; **175**: 1440–5.
- 172 Stol K, Verhaegh SJC, Graamans K, *et al.* Microbial profiling does not differentiate between childhood recurrent acute otitis media and chronic otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2013; **77**: 488–93.
- 173 Al-Saab F, Manoukian JJ, Al-Sabah B, *et al.* Linking laryngopharyngeal reflux to otitis media with effusion: pepsinogen study of adenoid tissue and middle ear fluid. *J Otolaryngol Head Neck Surg* 2008; **37**: 565–71.
- 174 Hilty M, Qi W, Brugger SD, *et al.* Nasopharyngeal microbiota in infants with acute otitis media. *J Infect Dis* 2012; **205**: 1048–55.
- 175 Frank DN, Spiegelman GB, Davis W, Wagner E, Lyons E, Pace NR. Culture-independent molecular analysis of microbial constituents of the healthy human outer ear. *J Clin Microbiol* 2003; **41**: 295–303.
- 176 De Baere T, Vanechoutte M, Deschaght P, Huyghe J, Dhooge I. The prevalence of middle ear pathogens in the outer ear canal and the nasopharyngeal cavity of healthy young adults. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2010; **16**: 1031–5.
- 177 Campos A, Arias A, Betancor L, *et al.* Study of common aerobic flora of human cerumen. *J Laryngol Otol* 1998; **112**: 613–6.

- 178 Brook I. Microbiological studies of the bacterial flora of the external auditory canal in children. *Acta Otolaryngol* 1981; **91**: 285–7.
- 179 Heslop A, Ovesen T. Severe acute middle ear infections: microbiology and treatment. *Int J Pediatr Otorhinolaryngol* 2006; **70**: 1811–6.
- 180 Roland PS, Parry DA, Stroman DW. Microbiology of acute otitis media with tympanostomy tubes. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2005; **133**: 585–95.
- 181 Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. *Alloiococcus* otitidis in acute otitis media. *Int J Pediatr Otorhinolaryngol* 2004; **68**: 51–6.
- 182 Guvenc MG, Midilli K, Inci E, *et al.* Lack of *Chlamydomphila pneumoniae* and predominance of *Alloiococcus* otitidis in middle ear fluids of children with otitis media with effusion. *Auris Nasus Larynx* 2010; **37**: 269–73.
- 183 Marsh RL, Binks MJ, Beissbarth J, *et al.* Quantitative PCR of ear discharge from Indigenous Australian children with acute otitis media with perforation supports a role for *Alloiococcus* otitidis as a secondary pathogen. *BMC Ear Nose Throat Disord* 2012; **12**: 11.
- 184 Teo SM, Mok D, Pham K, *et al.* The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell host & microbe* 2015; **17**: 704–15.
- 185 Aydin E, Tastan E, Yucel M, *et al.* Concurrent assay for four bacterial species including *alloiococcus* otitidis in middle ear, nasopharynx and tonsils of children with otitis media with effusion: a preliminary report. *Clinical and experimental otorhinolaryngology* 2012; **5**: 81–5.
- 186 Kaur R, Adlowitz DG, Casey JR, Zeng M, Pichichero ME. Simultaneous assay for four bacterial species including *Alloiococcus* otitidis using multiplex-PCR in children with culture negative acute otitis media. *The Pediatric infectious disease journal* 2010; **29**: 741–5.
- 187 Ren T, Glatt DU, Nguyen TN, *et al.* 16S rRNA survey revealed complex bacterial communities and evidence of bacterial interference on human adenoids. *Environmental microbiology* 2013; **15**: 535–47.
- 188 Faden H, Dryja D. Recovery of a unique bacterial organism in human middle ear fluid and its possible role in chronic otitis media. *J Clin Microbiol* 1989; **27**: 2488–91.
- 189 Bosley GS, Whitney AM, Pruckler JM, *et al.* Characterization of ear fluid isolates of *Alloiococcus* otitidis from patients with recurrent otitis media. *J Clin Microbiol* 1995; **33**: 2876–80.
- 190 Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. *J Clin Microbiol* 1997; **35**: 2854–8.

- 191 Hendolin PH, Kärkkäinen U, Himi T, Markkanen A, Ylikoski J. High incidence of *Alloiococcus* otitis in otitis media with effusion. *The Pediatric infectious disease journal* 1999; **18**: 860–5.
- 192 Kalcioglu MT, Oncel S, Durmaz R, Otlu B, Miman MC, Ozturan O. Bacterial etiology of otitis media with effusion; focusing on the high positivity of *Alloiococcus* otitidis. *The new microbiologica* 2002; **25**: 31–5.
- 193 Harimaya A, Takada R, Somekawa Y, Fujii N, Himi T. High frequency of *Alloiococcus* otitidis in the nasopharynx and in the middle ear cavity of otitis-prone children. *Int J Pediatr Otorhinolaryngol* 2006; **70**: 1009–14.
- 194 Harimaya A, Takada R, Hendolin PH, Fujii N, Ylikoski J, Himi T. High incidence of *Alloiococcus* otitidis in children with otitis media, despite treatment with antibiotics. *J Clin Microbiol* 2006; **44**: 946–9.
- 195 Kaur R, Czap K, Casey JR, Pichichero ME. Correlation of nasopharyngeal cultures prior to and at onset of acute otitis media with middle ear fluid cultures. *BMC infectious diseases* 2014; **14**: 640.
- 196 Beswick AJ, Lawley B, Fraise AP, Pahor AL, Brown NL. Detection of *Alloiococcus* otitis in mixed bacterial populations from middle-ear effusions of patients with otitis media. *Lancet* 1999; **354**: 386–9.
- 197 Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. The clinical role of *Alloiococcus* otitidis in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002; **66**: 41–8.
- 198 Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. The clinical role of *Alloiococcus* otitidis in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002; **66**: 41–8.
- 199 Harimaya A, Takada R, Somekawa Y, Fujii N, Himi T. High frequency of *Alloiococcus* otitidis in the nasopharynx and in the middle ear cavity of otitis-prone children. *Int J Pediatr Otorhinolaryngol* 2006; **70**: 1009–14.
- 200 Khoramrooz SS, Mirsalehian A, Imaneini H, *et al.* Characterization of *Alloiococcus* otitidis strains isolated from children with otitis media with effusion by Pulsed-Field Gel Electrophoresis. *Int J Pediatr Otorhinolaryngol* 2012; **76**: 1658–60.
- 201 Holder RC, Kirse DJ, Evans AK, *et al.* One third of middle ear effusions from children undergoing tympanostomy tube placement had multiple bacterial pathogens. *BMC Pediatr* 2012; **12**: 87.
- 202 Holder RC, Kirse DJ, Evans AK, *et al.* Otopathogens Detected in Middle Ear Fluid Obtained during Tympanostomy Tube Insertion: Contrasting Purulent and Non-Purulent Effusions. *PLoS ONE* 2015; **10**: e0128606.
- 203 Farajzadah Sheikh A, Saki N, Roointan M, *et al.* Identification of *Alloiococcus* otitidis, *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae* in

- Children With Otitis Media With Effusion. *Jundishapur J Microbiol* 2015; **8**: e17985.
- 204 Slinger R, Duval M, Langill J, *et al.* Direct molecular detection of a broad range of bacterial and viral organisms and Streptococcus pneumoniae vaccine serotypes in children with otitis media with effusion. *BMC Res Notes* 2016; **9**: 247.
- 205 Sillanpää S, Oikarinen S, Sipilä M, *et al.* Moraxella catarrhalis Might Be More Common than Expected in Acute Otitis Media in Young Finnish Children. *J Clin Microbiol* 2016; **54**: 2373–9.
- 206 Holder RC, Kirse DJ, Evans AK, *et al.* Otopathogens Detected in Middle Ear Fluid Obtained during Tympanostomy Tube Insertion: Contrasting Purulent and Non-Purulent Effusions. *PLoS ONE* 2015; **10**: e0128606.
- 207 De Baere T, Hollants J, Waeytens A, *et al.* Otitis media microbes: culture, PCR, and confocal laser scanning microscopy. *B-Ent* 2009; **5**: 65–72.
- 208 Durmaz R, Ozerol IH, Kalcioğlu MT, *et al.* Detection of Alloiococcus otitidis in the nasopharynx and in the outer ear canal. *The new microbiologica* 2002; **25**: 265–8.
- 209 Janapatla R-P, Chang H-J, Hsu M-H, Hsieh Y-C, Lin T-Y, Chiu C-H. Nasopharyngeal carriage of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and Alloiococcus otitidis in young children in the era of pneumococcal immunization, Taiwan. *Scand J Infect Dis* 2011; **43**: 937–42.
- 210 Teo SM, Mok D, Pham K, *et al.* The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell host & microbe* 2015; **17**: 704–15.
- 211 Harimaya A, Takada R, Himi T, Yokota S, Fujii N. Evidence of local antibody response against Alloiococcus otitidis in the middle ear cavity of children with otitis media. *FEMS Immunol Med Microbiol* 2007; **49**: 41–5.
- 212 Himi T, Kita H, Mitsuzawa H, *et al.* Effect of Alloiococcus otitidis and three pathogens of otitis media in production of interleukin-12 by human monocyte cell line. *FEMS Immunol Med Microbiol* 2000; **29**: 101–6.
- 213 Harimaya A, Himi T, Fujii N, *et al.* Induction of CD69 expression and Th1 cytokines release from human peripheral blood lymphocytes after in vitro stimulation with Alloiococcus otitidis and three middle ear pathogens. *FEMS Immunol Med Microbiol* 2005; **43**: 385–92.
- 214 Harimaya A, Koizumi J, Fujii N, Himi T. Interleukin-8 induction via NF-kappaB, p38 mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 pathways in human peripheral blood mononuclear cells by Alloiococcus otitidis. *Int J Pediatr Otorhinolaryngol* 2007; **71**: 1465–70.
- 215 Harimaya A, Fujii N, Himi T. Preliminary study of proinflammatory cytokines and chemokines in the middle ear of acute otitis media due to Alloiococcus otitidis. *Int J Pediatr Otorhinolaryngol* 2009; **73**: 677–80.

- 216 Tarkkanen J, Himi T, Harimaya A, Carlson P, Ylikoski J, Mattila PS. Stimulation of adenoidal lymphocytes by *Alloiococcus otitidis*. *The Annals of otology, rhinology, and laryngology* 2000; **109**: 958–64.
- 217 Marchino T, Vela JI, Bassaganyas F, Sanchez S, Buil JA. Acute-Onset Endophthalmitis Caused by *Alloiococcus otitidis* following a Dexamethasone Intravitreal Implant. *Case Rep Ophthalmol* 2013; **4**: 37–41.
- 218 Guler A, Sahin MA, Gurkan Yesil F, Yildizoglu U, Demirkol S, Arslan M. Chronic Otitis Media Resulting in Aortic Valve Replacement: A Case Report. *J Tehran Heart Cent* 2015; **10**: 98–100.
- 219 Cakar M, Demirbas S, Yildizoglu U, *et al.* First report of endocarditis by *Alloiococcus otitidis* spp. in a patient with a history of chronic otitis. *J Infect Public Health* 2013; **6**: 494–5.
- 220 Ashhurst-Smith C, Hall ST, Stuart J, *et al.* *Alloiococcus otitidis*: an emerging pathogen in otitis media. *J Infect* 2012; **64**: 233–5.
- 221 Stroman DW, Roland PS, Dohar J, Burt W. Microbiology of normal external auditory canal. *The Laryngoscope* 2001; **111**: 2054–9.
- 222 Geno KA, Gilbert GL, Song JY, *et al.* Pneumococcal Capsules and Their Types: Past, Present, and Future. *Clinical Microbiology Reviews* 2015; **28**: 871–99.
- 223 Lynch JP, Zhanel GG. *Streptococcus pneumoniae*: does antimicrobial resistance matter? *Semin Respir Crit Care Med* 2009; **30**: 210–38.
- 224 Rodgers GL, Arguedas A, Cohen R, Dagan R. Global serotype distribution among *Streptococcus pneumoniae* isolates causing otitis media in children: potential implications for pneumococcal conjugate vaccines. *Vaccine* 2009; **27**: 3802–10.
- 225 Nelson AL, Roche AM, Gould JM, Chim K, Ratner AJ, Weiser JN. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infection and immunity* 2007; **75**: 83–90.
- 226 Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* 2008; **6**: 288–301.
- 227 Magee AD, Yother J. Requirement for capsule in colonization by *Streptococcus pneumoniae*. *Infection and immunity* 2001; **69**: 3755–61.
- 228 Shak JR, Vidal JE, Klugman KP. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends Microbiol* 2013; **21**: 129–35.
- 229 Short KR, Habets MN, Hermans PWM, Diavatopoulos DA. Interactions between *Streptococcus pneumoniae* and influenza virus: a mutually beneficial relationship? *Future Microbiol* 2012; **7**: 609–24.

- 230 Tonnaer EL, Rijkers GT, Meis JF, *et al.* Genetic relatedness between pneumococcal populations originating from the nasopharynx, adenoid, and tympanic cavity of children with otitis media. *J Clin Microbiol* 2005; **43**: 3140–4.
- 231 Lewnard JA, Givon Lavi N, Huppert A, *et al.* Epidemiological Markers for Interactions Among *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* in Upper Respiratory Tract Carriage. *J Infect Dis* 2016; **213**: 1596–605.
- 232 Biesbroek G, Wang X, Keijser BJ, *et al.* Seven-valent pneumococcal conjugate vaccine and nasopharyngeal microbiota in healthy children. *Emerging infectious diseases* 2014; **20**: 201–10.
- 233 Lynch JP, Zhanel GG. *Streptococcus pneumoniae*: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. *Curr Opin Pulm Med* 2010; **16**: 217–25.
- 234 Pankuch GA, Jacobs MR, Appelbaum PC. Bactericidal activity of daptomycin against *Streptococcus pneumoniae* compared with eight other antimicrobials. *J Antimicrob Chemother* 2003; **51**: 443–6.
- 235 Paradisi F, Corti G, Cinelli R. *Streptococcus pneumoniae* as an agent of nosocomial infection: treatment in the era of penicillin-resistant strains. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2001; **7 Suppl 4**: 34–42.
- 236 Rao VK, Krasan GP, Hendrixson DR, Dawid S, St Geme JW. Molecular determinants of the pathogenesis of disease due to non-typable *Haemophilus influenzae*. *FEMS Microbiology Reviews* 1999; **23**: 99–129.
- 237 Aniansson G, Alm B, Andersson B, *et al.* Nasopharyngeal colonization during the first year of life. *J Infect Dis* 1992; **165 Suppl 1**: S38–42.
- 238 Leibovitz E, Jacobs MR, Dagan R. *Haemophilus influenzae*: a significant pathogen in acute otitis media. *The Pediatric infectious disease journal* 2004; **23**: 1142–52.
- 239 Rodriguez WJ, Schwartz RH. *Streptococcus pneumoniae* causes otitis media with higher fever and more redness of tympanic membranes than *haemophilus influenzae* or *moraxella catarrhalis*. *The Pediatric infectious disease journal* 1999; **18**: 942–4.
- 240 Inzana TJ. Capsules and virulence in the HAP group of bacteria. *Can J Vet Res* 1990; **54 Suppl**: S22–7.
- 241 MacNeil JR, Cohn AC, Farley M, *et al.* Current epidemiology and trends in invasive *Haemophilus influenzae* disease--United States, 1989-2008. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2011; **53**: 1230–6.
- 242 Martin NG, Sadarangani M, Pollard AJ, Goldacre MJ. Hospital admission rates for meningitis and septicaemia caused by *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* in children in England over five decades: a population-based observational study. *Lancet Infect Dis* 2014; **14**: 397–405.

- 243 LaClaire LL, Tondella MLC, Beall DS, *et al.* Identification of Haemophilus influenzae serotypes by standard slide agglutination serotyping and PCR-based capsule typing. *J Clin Microbiol* 2003; **41**: 393–6.
- 244 Kuklinska D, Kilian M. Relative proportions of Haemophilus species in the throat of healthy children and adults. *Eur J Clin Microbiol* 1984; **3**: 249–52.
- 245 Casey JR, Adlowitz DG, Pichichero ME. New Patterns in the Otopathogens Causing Acute Otitis Media Six to Eight Years After Introduction of Pneumococcal Conjugate Vaccine. *The Pediatric infectious disease journal* 2009; : 1.
- 246 Westman E, Lundin S, Hermansson A, Melhus A. Beta-lactamase-producing nontypeable Haemophilus influenzae fails to protect Streptococcus pneumoniae from amoxicillin during experimental acute otitis media. *Antimicrobial agents and chemotherapy* 2004; **48**: 3536–42.
- 247 Peric M, Bozdogan B, Jacobs MR, Appelbaum PC. Effects of an efflux mechanism and ribosomal mutations on macrolide susceptibility of Haemophilus influenzae clinical isolates. *Antimicrobial agents and chemotherapy* 2003; **47**: 1017–22.
- 248 Zuckerman JM, Qamar F, Bono BR. Macrolides, ketolides, and glycylicyclines: azithromycin, clarithromycin, telithromycin, tigecycline. *Infect Dis Clin North Am* 2009; **23**: 997–1026–ix–x.
- 249 Witherden EA, Tristram SG. Prevalence and mechanisms of  $\beta$ -lactam resistance in Haemophilus haemolyticus. *J Antimicrob Chemother* 2013; **68**: 1049–53.
- 250 Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial Resistance in Haemophilus influenzae. *Clinical Microbiology Reviews* 2007; **20**: 368–89.
- 251 Broides A, Dagan R, Greenberg D, Givon Lavi N, Leibovitz E. Acute otitis media caused by Moraxella catarrhalis: epidemiologic and clinical characteristics. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2009; **49**: 1641–7.
- 252 Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. Moraxella catarrhalis: from emerging to established pathogen. *Clinical Microbiology Reviews* 2002; **15**: 125–44.
- 253 Doern GV, Brueggemann AB, Pierce G, Hogan T, Holley HP, Rauch A. Prevalence of antimicrobial resistance among 723 outpatient clinical isolates of Moraxella catarrhalis in the United States in 1994 and 1995: results of a 30-center national surveillance study. *Antimicrobial agents and chemotherapy* 1996; **40**: 2884–6.
- 254 Leibovitz E. The challenge of recalcitrant acute otitis media: pathogens, resistance, and treatment strategy. *The Pediatric infectious disease journal* 2007; **26**: S8–S11.
- 255 Gorwitz RJ, Kruszon-Moran D, McAllister SK, *et al.* Changes in the prevalence of nasal colonization with Staphylococcus aureus in the United States, 2001–2004. *J Infect Dis* 2008; **197**: 1226–34.

- 256 van Belkum A, Verkaik NJ, de Vogel CP, *et al.* Reclassification of *Staphylococcus aureus* nasal carriage types. *J Infect Dis* 2009; **199**: 1820–6.
- 257 Hartnick CJ, Shott S, Willging JP, Myer CM. Methicillin-resistant *Staphylococcus aureus* otorrhea after tympanostomy tube placement: an emerging concern. *Arch Otolaryngol Head Neck Surg* 2000; **126**: 1440–3.
- 258 van den Bergh MR, Biesbroek G, Rossen JWA, *et al.* Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS ONE* 2012; **7**: e47711.
- 259 Reiss-Mandel A, Regev-Yochay G. *Staphylococcus aureus* and *Streptococcus pneumoniae* interaction and response to pneumococcal vaccination: Myth or reality? *Hum Vaccin Immunother* 2016; **12**: 351–7.
- 260 Verhoeff M, van der Veen EL, Rovers MM, Sanders EAM, Schilder AGM. Chronic suppurative otitis media: a review. *Int J Pediatr Otorhinolaryngol* 2006; **70**: 1–12.
- 261 Yeo SG, Park DC, Hong SM, Cha CI, Kim MG. Bacteriology of chronic suppurative otitis media--a multicenter study. *Acta Otolaryngol* 2007; **127**: 1062–7.
- 262 Davis KA, Stewart JJ, Crouch HK, Florez CE, Hospenthal DR. Methicillin-resistant *Staphylococcus aureus* (MRSA) nares colonization at hospital admission and its effect on subsequent MRSA infection. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2004; **39**: 776–82.
- 263 Herold BC, Immergluck LC, Maranan MC, *et al.* Community-acquired methicillin-resistant *Staphylococcus aureus* in children with no identified predisposing risk. *JAMA* 1998; **279**: 593–8.
- 264 Crisóstomo MI, Westh H, Tomasz A, Chung M, Oliveira DC, de Lencastre H. The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones. *Proc Natl Acad Sci USA* 2001; **98**: 9865–70.
- 265 Brook I. Recovery of methicillin resistant *Staphylococcus aureus* from a child with acute otitis media. *The Pediatric infectious disease journal* 2008; **27**: 372–3.
- 266 Al-Shawwa BA, Wegner D. Trimethoprim-sulfamethoxazole plus topical antibiotics as therapy for acute otitis media with otorrhea caused by community-acquired methicillin-resistant *Staphylococcus aureus* in children. *Arch Otolaryngol Head Neck Surg* 2005; **131**: 782–4.
- 267 Liu CM, Kohanski MA, Mendiola M, *et al.* Impact of saline irrigation and topical corticosteroids on the postsurgical sinonasal microbiota. *Int Forum Allergy Rhinol* 2015; **5**: 185–90.
- 268 Swidsinski A, Goktas O, Bessler C, *et al.* Spatial organisation of microbiota in quiescent adenoiditis and tonsillitis. *Journal of clinical pathology* 2007; **60**: 253–60.

- 269 Roland PS, Stroman DW. Microbiology of acute otitis externa. *The Laryngoscope* 2002; **112**: 1166–77.
- 270 Stern Shavit S, Soudry E, Hamzany Y, Nageris B. Malignant external otitis: Factors predicting patient outcomes. *American journal of otolaryngology* 2016; **37**: 425–30.
- 271 Rosenfeld RM, Schwartz SR, Cannon CR, *et al*. Clinical practice guideline: acute otitis externa. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2014; **150**: S1–S24.
- 272 Ikeda K, Misawa S, Kusunoki T. Comparative bactericidal activity of four fluoroquinolones against *Pseudomonas aeruginosa* isolated from chronic suppurative otitis media. *BMC Ear Nose Throat Disord* 2015; **15**: 5.
- 273 Uehara Y, Nakama H, Agematsu K, *et al*. Bacterial interference among nasal inhabitants: eradication of *Staphylococcus aureus* from nasal cavities by artificial implantation of *Corynebacterium* sp. *The Journal of hospital infection* 2000; **44**: 127–33.
- 274 Holzmann D, Funke G, Linder T, Nadal D. *Turicella* otitidis and *Corynebacterium auris* do not cause otitis media with effusion in children. *The Pediatric infectious disease journal* 2002; **21**: 1124–6.
- 275 Jonsson L, Schwan A, Thomander L, Fabian P. Aerobic and anaerobic bacteria in chronic suppurative otitis media. A quantitative study. *Acta Otolaryngol* 1986; **102**: 410–4.
- 276 EUCAST. EUCAST 2016 clinical breakpoints.  
[http://www.eucast.org/clinical\\_breakpoints](http://www.eucast.org/clinical_breakpoints).  
[http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/) (accessed Aug 5, 2016).
- 277 Costerton JW, Lewandowski Z. Microbial biofilms. *Annual Reviews in ...* 1995.
- 278 Berlanga M, Guerrero R. Living together in biofilms: the microbial cell factory and its biotechnological implications. *Microb Cell Fact* 2016; **15**: 165.
- 279 Fletcher M. The physiological activity of bacteria attached to solid surfaces. *Advances in microbial physiology* 1991.
- 280 Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews* 2002; **15**: 167–93.
- 281 Fergie N, Bayston R, Pearson JP, Birchall JP. Is otitis media with effusion a biofilm infection? *Clinical otolaryngology and allied sciences* 2004; **29**: 38–46.
- 282 Weimer KED, Armbruster CE, Juneau RA, Hong W, Pang B, Swords WE. Coinfection with *Haemophilus influenzae* Promotes Pneumococcal Biofilm Formation during Experimental Otitis Media and Impedes the Progression of Pneumococcal Disease. *J Infect Dis* 2010; **202**: 1068–75.
- 283 Armbruster CE, Hong W, Pang B, *et al*. Indirect pathogenicity of *Haemophilus*

influenzae and *Moraxella catarrhalis* in polymicrobial otitis media occurs via interspecies quorum signaling. *MBio* 2010; **1**: e00102–10–e00102–19.

- 284 Brook I, Gilmore JD. Evaluation of bacterial interference and beta-lactamase production in management of experimental infection with group A beta-hemolytic streptococci. *Antimicrobial agents and chemotherapy* 1993; **37**: 1452–5.
- 285 Brook I. In vitro susceptibility vs. in vivo efficacy of various antimicrobial agents against the *Bacteroides fragilis* group. *Rev Infect Dis* 1991; **13**: 1170–80.
- 286 Hackman AS, Wilkins TD. In vivo protection of *Fusobacterium necrophorum* from penicillin by *Bacteroides fragilis*. *Antimicrobial agents and chemotherapy* 1975; **7**: 698–703.
- 287 Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; **358**: 135–8.
- 288 Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 2010; **35**: 322–32.
- 289 Davies J. Inactivation of antibiotics and the dissemination of resistance genes. *Science* 1994; **264**: 375–82.
- 290 Takahata S, Ida T, Senju N, *et al.* Horizontal gene transfer of *ftsI*, encoding penicillin-binding protein 3, in *Haemophilus influenzae*. *Antimicrobial agents and chemotherapy* 2007; **51**: 1589–95.
- 291 Potera C. Forging a link between biofilms and disease. *Science* 1999.
- 292 Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999.
- 293 Harimaya A, Takada R, Hendolin PH, Fujii N, Ylikoski J, Himi T. High incidence of *Alloiococcus otitidis* in children with otitis media, despite treatment with antibiotics. *J Clin Microbiol* 2006; **44**: 946–9.
- 294 Bakaletz LO. Bacterial biofilms in the upper airway - evidence for role in pathology and implications for treatment of otitis media. *Paediatr Respir Rev* 2012; **13**: 154–9.
- 295 Post JC. Direct evidence of bacterial biofilms in otitis media. *The Laryngoscope* 2001; **111**: 2083–94.
- 296 Ehrlich GD, Veeh R, Wang X, *et al.* Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* 2002; **287**: 1710–5.
- 297 Murrah KA, Pang B, Richardson S, *et al.* Nonencapsulated *Streptococcus pneumoniae* causes otitis media during single-species infection and during polymicrobial infection with nontypeable *Haemophilus influenzae*. *Pathogens and disease* 2015; **73**: ftu011–1.
- 298 Byrd MS, Pang B, Hong W, *et al.* Direct evaluation of *Pseudomonas aeruginosa* biofilm

- mediators in a chronic infection model. *Infection and immunity* 2011; **79**: 3087–95.
- 299 Thornton RB, Rigby PJ, Wiertsema SP, *et al.* Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr* 2011; **11**: 94.
- 300 Park YH, Kim EH, Seo ST, *et al.* Formation of Biofilm in Patients with Chronic Otitis Media and Cholesteatoma. *Korean Journal of Otolaryngology-Head and Neck Surgery* 2009; **52**: 124.
- 301 Wang JC, Hamood AN, Saadeh C, Cunningham MJ, Yim MT, Cordero J. Strategies to prevent biofilm-based tympanostomy tube infections. *Int J Pediatr Otorhinolaryngol* 2014; **78**: 1433–8.
- 302 Van Hoecke H, De Paepe A-S, Lambert E, *et al.* Haemophilus influenzae biofilm formation in chronic otitis media with effusion. *European archives of oto-rhino-laryngology : official journal of the European Federation of Oto-Rhino-Laryngological Societies* 2016; : 1–8.
- 303 Minovi A, Dazert S. Diseases of the middle ear in childhood. 2014; **13**: Doc11.
- 304 Liu CM, Cosetti MK, Aziz M, *et al.* The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. *Arch Otolaryngol Head Neck Surg* 2011; **137**: 664–8.
- 305 Leskinen K, Hendolin P, Virolainen-Julkunen A, Ylikoski J, Jero J. The clinical role of *Alloiooccus otitidis* in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2002; **66**: 41–8.
- 306 Tano K, Essen von R, Eriksson PO, Sjöstedt A. *Alloiooccus otitidis*--otitis media pathogen or normal bacterial flora? *APMIS* 2008; **116**: 785–90.
- 307 Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 2014; **30**: 614–20.
- 308 Rideout JR, He Y, Navas-Molina JA, *et al.* Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* 2014; **2**: e545.
- 309 Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010; **26**: 2460–1.
- 310 Pruesse E, Quast C, Knittel K, *et al.* SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research* 2007; **35**: 7188–96.
- 311 Jervis-Bardy J, Leong LEX, Marri S, *et al.* Deriving accurate microbiota profiles from human samples with low bacterial content through post-sequencing processing of Illumina MiSeq data. *Microbiome* 2015; **3**: 1193.
- 312 CLARKE KR. Non-parametric multivariate analyses of changes in community structure.

*Austral Ecology* 1993; **18**: 117–43.

- 313 Shannon P, Markiel A, Ozier O, *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* 2003; **13**: 2498–504.
- 314 Wheeler DL, Church DM, Lash AE, *et al.* Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research* 2001; **29**: 11–6.
- 315 Marsh RL, Binks MJ, Beissbarth J, *et al.* Quantitative PCR of ear discharge from Indigenous Australian children with acute otitis media with perforation supports a role for *Alloiococcus otitidis* as a secondary pathogen. *BMC Ear Nose Throat Disord* 2012; **12**: 11.
- 316 The bacterial interactions in the nasopharynx of children receiving adenoidectomy. *BioMedicine* 2015; **5**: 6.
- 317 Pettigrew MM, Gent JF, Revai K, Patel JA, Chonmaitree T. Microbial Interactions during Upper Respiratory Tract Infections. *Emerging infectious diseases* 2008; **14**: 1584–91.
- 318 Korona-Glowniak I, Niedzielski A, Kosikowska U, Grzegorzczak A, Malm A. Nasopharyngeal vs. adenoid cultures in children undergoing adenoidectomy: prevalence of bacterial pathogens, their interactions and risk factors. *Epidemiology and infection* 2015; **143**: 821–30.
- 319 Aruni AW, Dou Y, Mishra A, Fletcher HM. The Biofilm Community: Rebels with a Cause. *Current oral health reports* 2015; **2**: 48–56.
- 320 The virome in host health and disease. *Immunity* 2015; **42**: 805–13.
- 321 Bosley GS, Whitney AM, Pruckler JM, *et al.* Characterization of ear fluid isolates of *Alloiococcus otitidis* from patients with recurrent otitis media. *J Clin Microbiol* 1995; **33**: 2876–80.
- 322 Yi HJ, Guo W, Wu N, *et al.* The Microbiome of Otitis Media with Effusion. *Acta Otolaryngol* 2013; **134**: 23–33.
- 323 Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* 2014; **30**: 614–20.
- 324 Rideout JR, He Y, Navas-Molina JA, *et al.* Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ* 2014; **2**: e545.
- 325 Zhou Y, Gao H, Mihindukulasuriya KA, *et al.* Biogeography of the ecosystems of the healthy human body. *Genome Biol* 2013; **14**: R1.
- 326 Li K, Bihan M, Methé BA. Analyses of the Stability and Core Taxonomic Memberships of the Human Microbiome. *PLoS ONE* 2013; **8**: e63139.
- 327 Frank DN, Spiegelman GB, Davis W, Wagner E, Lyons E, Pace NR. Culture-independent

- molecular analysis of microbial constituents of the healthy human outer ear. *J Clin Microbiol* 2003; **41**: 295–303.
- 328 Márquez M, Fernández Gutiérrez Del Álamo C, Girón-González JA. Gut epithelial barrier dysfunction in human immunodeficiency virus-hepatitis C virus coinfecting patients: Influence on innate and acquired immunity. *World J Gastroenterol* 2016; **22**: 1433–48.
- 329 Faderl M, Noti M, Corazza N, Mueller C. Keeping bugs in check: The mucus layer as a critical component in maintaining intestinal homeostasis. *IUBMB Life* 2015; **67**: 275–85.
- 330 Yuksel S, Swartz JD, Banks J, Seroky JT, Doyle WJ. In vivo measurement of O<sub>2</sub> and CO<sub>2</sub> gas exchange across the human tympanic membrane. *Acta Otolaryngol* 2009; **129**: 716–25.
- 331 Guan X, Jiang S, Seale TW, Hitt BM, Gan RZ. Morphological changes in the tympanic membrane associated with *Haemophilus influenzae*-induced acute otitis media in the chinchilla. *Int J Pediatr Otorhinolaryngol* 2015; **79**: 1462–71.
- 332 Mittal R, Lisi CV, Gerring R, *et al.* Current concepts in the pathogenesis and treatment of chronic suppurative otitis media. *J Med Microbiol* 2015; **64**: 1103–16.
- 333 Liu CM, Cosetti MK, Aziz M, *et al.* The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16S rRNA gene-based pyrosequencing. *Arch Otolaryngol Head Neck Surg* 2011; **137**: 664–8.
- 334 Himi T, Kita H, Mitsuzawa H, *et al.* Effect of *Alloiooccus* otitidis and three pathogens of otitis media in production of interleukin-12 by human monocyte cell line. *FEMS Immunol Med Microbiol* 2000; **29**: 101–6.
- 335 Chan CL, Wabnitz D, Bardy JJ, *et al.* The microbiome of otitis media with effusion. *The Laryngoscope* 2016; published online June 23. DOI:10.1002/lary.26128.
- 336 Chan CL, Richter K, Wormald P-J, Vreugde S, Psaltis AJ. A phylogenetic analysis to identify the bacterial reservoirs for the middle ear in otitis media. *JAMA otolaryngology-- head & neck surgery* 2016; published online Nov 3.
- 337 Van Eldere J, Slack MPE, Ladhani S, Cripps AW. Non-typeable *Haemophilus influenzae*, an under-recognised pathogen. *Lancet Infect Dis* 2014; **14**: 1281–92.
- 338 Revai K, McCormick DP, Patel J, Grady JJ, Saeed K, Chonmaitree T. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. *Pediatrics* 2006; **117**: 1823–9.
- 339 Coker TR, Chan LS, Newberry SJ, *et al.* Diagnosis, microbial epidemiology, and antibiotic treatment of acute otitis media in children: a systematic review. *JAMA* 2010; **304**: 2161–9.
- 340 Post JC. Molecular analysis of bacterial pathogens in otitis media with effusion. *JAMA: The Journal of the American Medical Association* 1995; **273**: 1598–604.

- 341 Daniel M, Imtiaz-Umer S, Fergie N, Birchall JP, Bayston R. Bacterial involvement in otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2012; **76**: 1416–22.
- 342 Chan CL, Wabnitz D, Bardy JJ, *et al.* The microbiome of otitis media with effusion. *The Laryngoscope* 2016; published online June 23. DOI:10.1002/lary.26128.
- 343 Hall-Stoodley L, Hu FZ, Gieseke A, *et al.* Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 2006; **296**: 202–11.
- 344 Ehrlich GD, Veeh R, Wang X, *et al.* Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* 2002; **287**: 1710–5.
- 345 Baron S, Musher DM. *Haemophilus Species*. 1996.
- 346 Richter K, Ramezanpour M, Thomas N, Prestidge CA, Wormald P-J, Vreugde S. Mind ‘De GaPP’: in vitro efficacy of deferiprone and gallium-protoporphyrin against *Staphylococcus aureus* biofilms. *Int Forum Allergy Rhinol* 2016; **6**: 737–43.
- 347 Ashhurst-Smith C, Hall ST, Walker P, Stuart J, Hansbro PM, Blackwell CC. Isolation of *Alloiococcus otitidis* from Indigenous and non-Indigenous Australian children with chronic otitis media with effusion. *FEMS Immunol Med Microbiol* 2007; **51**: 163–70.
- 348 Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols* 2008; **3**: 163–75.
- 349 Evans NM, Smith DD, Wicken AJ. Haemin and Nicotinamide Adenine Dinucleotide Requirements of *Haemophilus Influenzae* and *Haemophilus Parainfluenzae*. *J Med Microbiol* 1974; **7**: 359–65.
- 350 Rennie RP, Ibrahim KH. Antimicrobial resistance in *Haemophilus influenzae*: how can we prevent the inevitable? Commentary on antimicrobial resistance in *H. influenzae* based on data from the TARGETed surveillance program. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2005; **41 Suppl 4**: S234–8.
- 351 Tano K, Håkansson EG, Holm SE, Hellström S. Bacterial interference between pathogens in otitis media and alpha-haemolytic *Streptococci* analysed in an in vitro model. *Acta Otolaryngol* 2002; **122**: 78–85.