THE ASSOCIATION OF OUTER EAR COMMENSAL BACTERIA WITH OTITIS MEDIA IN CHILDREN

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science in Biological Sciences

At

The University of Waikato

By

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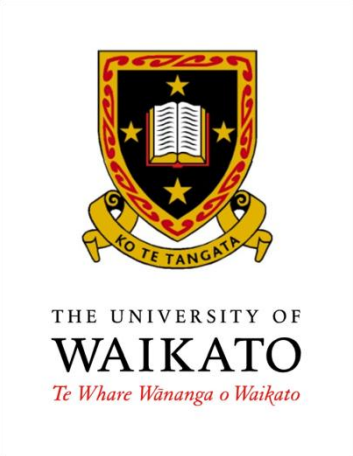

The University of Waikato

2012

Abstract

Background

Middle ear infections (otitis media) are a significant burden on children's health. They are one of the more common childhood ailments and are responsible for about a third of children's general practitioner visits. Three bacteria of the nasopharynx have been well documented as true pathogens of otitis media. These are *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*. A newer bacterium, *Alloiococcus otitidis*, which is more difficult to culture than the initial three pathogens, was discovered in 1989 by Faden & Dryja.

The pathogenicity of *A. otitidis* in otitis media is controversial, as is the association of two other bacteria, *Turicella otitidis* and *Corynebacterium auris*. These three bacteria are regarded as commensals of the outer ear canal, and how they move into the middle ear is subject to much debate. My hypothesis is that during a primary middle ear infection, commensal bacteria of the external ear canal may be able to move through the inflamed tympanic membrane and into the middle ear where they act as opportunistic pathogens. This may create a more persistent bacterial infection, with greater resistance to antibiotic therapies.

Methods

DNA was extracted directly from swabs of the outer ear canal and nasopharynx of children with otitis media and otitis-free controls, and from the middle ear exudates of the otitis media study group. This DNA was then amplified using primers specific for each of the organisms of interest. The presence of a specific PCR product, confirmed by DNA sequencing, indicated a positive result.

Swabs from each of the sites of interest were also grown on 10% Columbia blood agar, 10% Columbia chocolate agar and in BHI broth supplemented with foetal calf serum. These results gave further information on bacterial presence in New Zealand children beyond the six studied.

Sau-PCR microbial community profiling was used to demonstrate the diversity of species present in the polymicrobial samples. DNA was restriction digested and the resulting banding patterns were compared between study sites, and study groups.

Results and Conclusions

Moraxella species colonised the outer ear canal of control participants more regularly than the outer ear canal of otitis media patients (58% vs. 33%). This difference was found to be statistically significant (p= 0.0023). Differences between the colonisation of the outer ear canal and nasopharyngeal body sites were found at a statistically significant level in control participants with *A. otitidis* (p=0.0433), *C. auris* (p=0.0000), *T. otitidis* (p=0.0050) and *Moraxella* spp. (p=0.0005). Of the same body sites in otitis media patients, only the colonisation of *C. auris* (p=0.0002) and *T. otitidis* (p=0.0004) were found to be statistically significant. This shows that four of the six studied bacteria colonise both anatomical sites in otitis media patients in a similar manner (without statistical significance), as opposed to only two in the control participants.

Of the six bacterial species analysed using non-culture techniques, *S. pneumoniae*, *H. influenzae* and *C. auris* failed to be cultured. This may show that storage prior to analysis made samples less suitable for culture based research. A number of other species were cultured, with subsequent sequencing identifying them. This included species thought to be associated with a decreased risk of developing otitis media, found in both of the control participant nasopharynxes that were studied, and in only one nasopharynx from the otitis media group. *A. otitidis* was cultured regularly in outer ear samples, and one middle ear effusion.

Sau-PCR microbial profiling was an effective but crude indication of microbial diversity. Otitis media patient nasopharyngeal profiles resembled each other, with resemblance extending to the middle ear effusion profile. Control participant banding patterns within sites were similar, but this resemblance did not extend to the otitis media group profiles. Comparisons between the banding patterns from known bacterial species to those of unknown polymicrobial species were useful if the identifying species had a unique banding pattern, as with *A. otitidis*. Sau-PCR therefore proved useful in analysing the complexities that are polymicrobial samples, however identifications of these species by cloning proved unsuccessful.

Further research using all the techniques above with increased numbers of participants is necessary to substantiate results of this study. Information on antibiotic resistance, viral presence, and biofilm status could prove useful information in helping treat infection.

Acknowledgements

To begin with, I would like to thank my supervisors Ray Cursons and Tony Cecire, without whom I would not have been able to complete this research. I thank you both for your guidance and assistance throughout these past two years. I have learnt a lot and it has definitely been an experience!

Other contributors I would like to thank include Judi McWhirti, for putting me on the right track in the statistical analysis of my results; Jan Bird of Pathlab Hamilton, for supplying the pure cultures of three of the studied organisms; and Kim Hunter for her help with vaccination status and recruitment of control children.

I must also thank my wonderful lab mates Claire Mulholland, Tania Law, Nadine Huitema, and Jo Duyvestyn. I couldn't have picked a better bunch of girls to go on this roller coaster ride that is a Masters' thesis. Thanks for laughing with me during the good times, and for your support through the tough ones.

Further, I would like to thank my parents and my grandparents for their continued support and advice. I cannot begin to tell you how grateful I am to have you in my life. Thank you for a wonderful upbringing and for so constantly telling me how proud you were of me. I am truly blessed to have such a supportive bunch of people behind me all the way!

A big thank you goes to the Todd Foundation, the Waikato Graduate Women Charitable Trust, the Bing's Scholarship and the University of Waikato for providing financial stability during my thesis year. This was thoroughly appreciated and well used.

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List of Abbreviations

AO – *Alloiococcus otitidis* primer

CA – *Corynebacterium auris* primer

HI – *Haemophilus influenzae* primer

MS – *Moraxella* species primer

TO – *Turicella otitidis* primer

SP – *Streptococcus pneumoniae* primer

OM – Otitis media

AOM – Acute otitis media

rAOM – Recurrent acute otitis media

CSOM – Chronic suppurative otitis media

OME – Otitis media with effusion

PCR – Polymerase chain reaction

DNA – Deoxyribose nucleic acid

MEE – Middle ear effusion

LM – Left middle ear cavity

RM – Right middle ear cavity

OE – Outer ear canal

LE – Left outer ear canal

RE – Right outer ear canal

NP – Nasopharynx or nasopharyngeal

mRNA – messenger ribose nucleic acid

HMP - Human Microbiome Project

URTI – Upper respiratory tract infection

URT – Upper respiratory tract

OTU– Operational Taxonomic Unit

Chapter 1: Introduction and Literature Review

1.1 Introduction: Middle ear disease

Middle ear infection (Otitis media (OM)) is the most common childhood illness throughout the world and represents the leading reason for prescription of antibiotic agents (O'Brien et al. 2009). Unfortunately, antimicrobial therapy is sometimes ineffective against these polymicrobial infections, leading to the necessity for surgical intervention (Brogden & Guthmiller 2002). Tympanostomy-tube insertions, a procedure specifically for the treatment of OM, are at present the most frequently performed surgical procedure on children in the United States (O'Brien et al. 2009).

Acute otitis media (AOM) is the initial form of infection. Symptoms include pain, caused by an inflamed tympanic membrane (ear drum), and fluid in the middle ear (Barnett 2007). AOM can be referred to as recurrent acute otitis media (rAOM) after 3 or 4 cases over a 6 month or 12 month period, respectively. Chronic forms of infection include otitis media with effusion (OME), in which a middle ear effusion (MEE) fills the middle ear cavity. This MEE is a fluidic substance that can decrease hearing capabilities, but is often painless and if left untreated, can lead to mastoiditis (inflammation of the mastoid bone) or even deafness (Hoberman et al. 2011).

The nasopharynx (NP), of the upper respiratory system, is thought to play a role in episodes of OM. Also involved are two areas of the auricular system, the middle ear, and the outer ear canal (OE) (see Figure 1.1). Extensive bacterial colonisation is proven in the NP (Brook 2011) and the OE (De Baere et al. 2009). There is little evidence of bacterial colonisation of the healthy middle ear (prior to an episode of OM), therefore it is currently perceived to be sterile (Murphy et al. 2009).

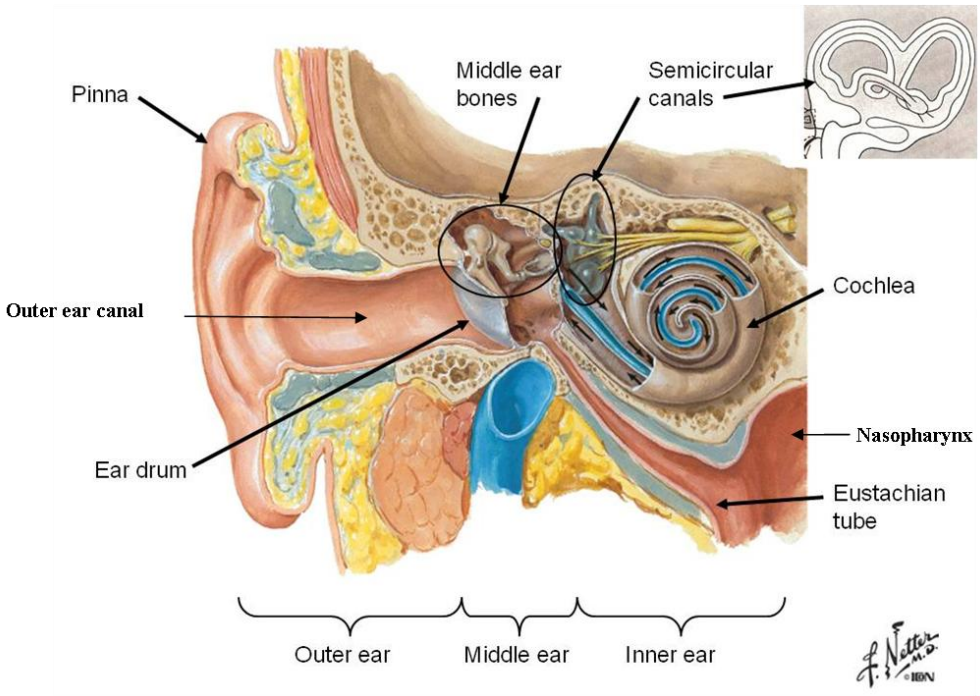


Figure 1.1 Schematic of the auricular system, including the nasopharynx. Key areas to note are the middle ear, the outer ear canal, and the nasopharynx (ME220: Introduction to Sensors 2008).

Three commensal bacteria of the NP (Figure 1.1) are known and proven pathogens of OM. These are *Streptococcus pneumoniae*, non-typable *Haemophilus influenzae*, and *Moraxella catarrhalis* (De Baere et al. 2009). These asymptomatically frequent the NP of many children. Pathogenicity is selected for during times of immune impairment, such as those caused by contraction of a viral agent. An episode of OM is thought to be provoked by the impaired functioning of the immune response in the Eustachian tubes, which would allow bacteria from the NP to ascend into the middle ear cavity (Figure 1.1).

Three other opportunistic bacteria that have a potential role in OM are *Alloiococcus otitidis*, *Corynebacterium auris*, and *Turicella otitidis*. These are all commensal bacteria of the OE with unknown and/or controversial roles in OM (Holzmann et al. 2002; Tano et al. 2008). Potentially, an initial episode of OM may lead to the weakening of the ear drum, allowing bacteria to invade the middle ear cavity from the OE. No research into the subject has currently been completed to neither confirm nor deny this possibility.

If this were to occur, the potential for exacerbation of infection by addition of further biofilm contributors or antibiotic resistant species could lead to more

chronic forms of infection. All six of the aforementioned bacteria have been found in the MEE of OM patients, leading to their association with the disease.

1.2 Common flora of the auricular system

The nasopharynx, oropharynx, oral cavity, and auricular system represent a number of ecological niches. This allows for a vast array of both eukaryotic, but predominantly prokaryotic colonisation. Greater than 800 bacteria are attributed to these areas, but of this array of species, only a small subset are thought to cause clinical infections (Bogaert et al. 2011).

Polymerase chain reaction (PCR) and DNA based analysis have made the detection of bacterial or viral presence possible without initial culture. Viable organisms are often not culturable (Morris et al. 2009). This could be due to dormancy (common in a biofilm environment), death from previous antibiotic treatment, or because specific nutritional requirements are not present in conventional microbiological media. However, using these DNA based methods, bacterial presence can be registered without initial culture. This has allowed for a more complete picture of the microbiota associated with the NP and auricular system.

The Human Microbiome Project (HMP) involves a number of organisations and institutions which collectively are attempting to isolate and sequence all the bacteria associated with the human body (NIH Human Microbiome Project 2011). The HMP catalogue holds current data describing the different bacteria isolated, and has the option of filtering by body site.

As of the 23rd of January 2012, 3 bacteria of the nose and 388 of the oral cavity had been identified, and were in the process of being analysed by the HMP. Although the species present in the OE and NP are yet to be analysed extensively by this organisation, papers that look into the microbiome of these areas have been published. Both culture and non-culture based techniques have been used to analyse these areas.

1.2.1 Outer ear bacteria

Extensive diversity of bacterial species has been noted in the OE. Evidence of this is shown in a study by Frank et al (2003) who cloned and sequenced the 16S gene of OE organisms from 24 individuals, avoiding culture bias by extracting DNA

directly from samples (see Table 1.1) (Frank et al. 2003). Eukaryotes, such as the fungus *Tilletiaria anomala*, and the mite, *Trhypochthonius tectorum* were present amongst the predominantly prokaryotic microbiota.

Using these culture-independent techniques, *A. otitidis* (Table 1.1 refers to this species as *A. otitis*) was seen to be the most prevalent bacteria of this area (Frank et al. 2003). *A. otitidis* is slow growing and requires a particular media of 5% blood agar, therefore complicating identification by culture (Faden & Dryja 1989). *T. otitidis* (in Table 1.1 referred to as *C. otitidis*, the name was later revised) was the next most prevalent species, with *C. auris* a distant fourth. These three organisms are of great importance to this research, and will be discussed in further detail in coming sections.

Stroman et al (2001) also looked at the microbiology of the OE, but used culture techniques instead (Stroman et al. 2001). Of 310 canal samples, 7 were positive for *C. auris*, 36 for *T. otitidis* and 20 for *A. otitidis*. In 314 cerumen samples, 8 were positive for *C. auris*, 38 for *T. otitidis*, and 28 for *A. otitidis*. The most prevalent species was *Staphylococcus auricularis* (63 and 66 respectively), which was the third most prevalent in the culture-independent study by Frank et al (2003). This demonstrates how different colonisation can appear when using culture as opposed to molecular genetic techniques. *S. auricularis* has not been linked to OM as frequently as the other species and therefore is not included to a great extent in this study.

T. otitidis and *C. auris* share many characteristics. This makes them difficult to tell apart using standard clinical tests. The API Coryne system is a rapid but quite accurate method for the identification of Corynebacterium species (Soto et al. 1994). However, this system cannot tell *T. otitidis* and *C. auris* apart, with the API Coryne system numerical profile for both species very often 210004 (Gomez-Garces et al. 2004). Despite this, relatively simple morphological and metabolic characteristics allow their differentiation (Gomez-Garces et al. 2004).

Table 1.1 Diversity of microbiota associated with human ear canals, identified using 16S rDNA culture-independent techniques (Frank, et al., 2003).

Nearest relative[a]	Clade[b]	% ID[c]	No. of clones[d]	% of total
A. otitis	Low G + C	96-98	1,221	56.71
C. otitidis	Actinobacteria	96-99	440	20.44
S. auricularis	Low G + C	98-99	212	9.85
C. auris	Actinobacteria	98	67	3.11
Tilletiaria anomala	Eucaryote	94-95	65	3.02
P. acnes	Actinobacteria	99	35	1.63
Corynebacterium sp. strain 61720	Actinobacteria	97	17	0.79
M. obscurus fungus	Eucaryote	94-97	10	0.46
Stephanoascus ciferrii	Eucaryote	98	8	0.37
Trhypochthonius tectorum	Eucaryote	91	8	0.37
S. epidermidis	Low G - C	95	6	0.28
Fusobacterium periodonticum	Fusobacteria	98	4	0.19
Homo sapiens (mitochondrion)	Plastid	97	4	0.19
Pseudomonas lanceolata	γProteobacteria	99	4	0.19
Streptococcus gordonii	Low G + C	99	4	0.19
Aspergillus fumigatus	Eucaryote	96	3	0.14
P. pubis fungus	Eucaryote	99	3	0.14
Sclerotium sp. strain BSC-97	Eucaryote	96	3	0.14
Enterobacter sp. strain B509	γProteobacteria	99	3	0.14
Staphylococcus hominis	Low G - C	90	3	0.14
Kocuria erythromyxa	Actinobacteria	94	2	0.09
Uncultured bacterium strain BPC009	Actinobacteria	95-98	2	0.09
Enterobacter agglomerans	γProteobacteria	99	2	0.09
Bacillus licheniformis	Low G - C	98	2	0.09
Streptococcus sp. oral strain B5SC	Low G - C	98	2	0.09
Alpha proteobacterium strain VUN10077	αProteobacteria	99	1	0.05
Uncultured bacterium 16S rRNA	ß Proteobacteria	98	1	0.05
Acinetobacter sp.	γProteobacteria	98	1	0.05
Haemophilus paraphrophilus	γProteobacteria	96	1	0.05
Brevibacterium avium	Actinobacteria	98	1	0.05
Brevibacterium sp.	Actinobacteria	98	1	0.05
Corynebacterium sp. strain X82493	Actinobacteria	95	1	0.05
Dietzia maris	Actinobacteria	95	1	0.05
Gram-positive bacterium strain Wuba45	Actinobacteria	97	1	0.05
Human oral bacterium strain AC1	Actinobacteria	98	1	0.05
Synechococcus sp. strain ATCC 700246	Cyanobacteria	95	1	0.05
Tetranychus urticae	Eucaryote	95	1	0.05
Abiotrophia defectiva	Low G + C	96	1	0.05
Carnobacterium sp. strain LV62LW1	Low G + C	92	1	0.05
Staphylococcus piscifermentans	Low G + C	96	1	0.05
Streptococcus mitis	Low G + C	99	1	0.05
Streptococcus sp. oral clone BW009	Low G + C	99	1	0.05
Veillonella sp. oral clone X042	Low G + C	98	1	0.05
Uncultured bacterium strain WCHA2-01	OP11	100	1	0.05
Uncultured bacterium 16S rRNA, strain BD7-4	OP11	87	1	0.05
Total			2,150	100.00

[a] Based on BlastN search.
[b] Kingdom (eucaryote)- or division (bacteria)-level phylogenetic affiliation.
[c] BlastN score. ID, identity.
[d] Total number of clones screened in all libraries.

1.2.2 Nasopharyngeal bacteria

The bacteria of the NP consist of benign commensals and asymptomatic pathogens (Harrison et al. 1999). It is known to be a reservoir for potentially pathogenic species of the upper respiratory tract (URT), and has therefore been more extensively studied than the OE.

A recent metagenomic analysis was carried out on the bacterial DNA from the NP of 96 healthy 18 month olds. A metagenomic analysis allows the identification of the species present in a polymicrobial population through the use of 454 pyrosequencing, without initial isolation of the species. The top 30 operational taxonomic units (OTU), representing 30 different DNA sequences and therefore bacterial species, are presented in Table 1.2. It is important to note that the top three most common OTUs at the genus level are of the *Moraxella* genus, *Streptococcus* genus, and *H. influenzae* (identified to the species level).

The carriage rates and dominance of species in the NP is determined by a number of factors. The presence of other bacteria, exposure to cigarette smoke, age, presence of respiratory illnesses, season, use of day care facilities, sibling number, diet and sleeping position are all noted factors.

Interactions between colonising bacteria have been shown to influence the NP carriage of potentially pathogenic bacteria. Non-pathogenic bacteria, such as α-haemolytic *streptococci*, *Peptostreptococcus anaerobius* and *Prevotella melaninogenica*, are known to interfere with the ability of potential pathogens, such as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*, to colonise this area (Brook & Gober 1998). This could demonstrate the importance of 'interfering organisms' in inhibiting pathogen colonisation. These interactions are important to understand in order to treat OM appropriately.

Smoking has been shown to have a marked effect on the colonising organisms of the respiratory tract (Brook 2011). Affecting both the smoker and those exposed second hand, smoke has been shown to decrease the number of organisms with interfering capacities, therefore increasing the number of nasopharyngeal colonising pathogens with the potential to cause OM. *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*, are seen to colonise in increased numbers in children who are prone to OM (Brook & Gober 1998).

Table 1.2 Thirty most common OTUs ('species-level' phylotypes), ranked by absolute presence among the ~1100000 reads(Bogaert et al. 2011).

	Phylum	Class	Order	Family	Genus	Species	OTU level classified	Overall presence (% of reads)	Relative presence (n =)	Relative presence (>0.1% of reads) (n =)	Core microbiome
1	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Moraxella	NA	genus	38.11	95	91	All
2	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	influenzae	species	19.16	84	61	All
3	Fimicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	genus	12.98	96	88	All
4	Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	Flavobacterium	NA	genus	10.07	80	47	Spring
5	Fimicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Dolosigranulum	NA	genus	4.80	86	75	All
6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Moraxella	NA	genus	2.22	39	28	
7	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	propinquum	species	1.65	80	57	All
8	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria	meningitidis	species	1.19	62	22	
9	Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium	necrophorum	species	0.96	8	4	
10	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	influenzae	species	0.77	16	10	
11	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Neisseria	polysaccharea	species	0.65	16	7	
12	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Helcococcus	NA	genus	0.57	31	23	
13	Firmicutes	NA	NA	NA	NA	NA	phylum	0.57	49	23	
14	Actinobacteria	Actinobacteria	Actinomycetales	Dermabacteraceae	Brachybacterium	NA	genus	0.56	16	11	
15	Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA	genus	0.40	21	2	
16	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	NA	genus	0.37	95	73	All
17	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Haemophilus	NA	genus	0.34	14	8	
18	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	catoniae	species	0.27	29	9	
19	Fimicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	genus	0.24	17	9	
20	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Porphyromonas	catoniae	species	0.21	23	9	
21	Fimicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Parvimonas	NA	genus	0.19	8	5	
22	Cyanobacteria	NA	NA	NA	NA	NA	phylum	0.18	83	46	Fall/Winter
23	Fimicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	genus	0.17	25	5	
24	Fimicutes	Bacilli	Bacillales	Paenibacillaceae	Brevibacillus	brevis	species	0.16	43	34	Spring
25	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella	shahii	species	0.15	1	1	
26	Fimicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	NA	genus	0.14	42	33	Spring
27	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	NA	genus	0.13	90	43	Fall/Winter
28	Fimicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	NA	genus	0.12	80	31	
29	Fimicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA	family	0.12	15	7	
30	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	NA	genus	0.11	86	31	

Nr. of samples (of total of 96 samples) containing each OTU in >0% or >0.1% of the reads is stated. Core microbiome: OTUs found in >50% of the samples in >0.1% of reads per sample (All: OTU found in >50% of samples; Spring and Fall/Winter: OTU found in >50% of samples obtained in spring or fall/winter, respectively). NA: not assigned.
doi:10.1371/journal.pone.0017035.t001

Using culture techniques, Harrison et al (1999) showed how the bacterial species of the NP changed as infants grew, and tested for variations in bacterial colonisation between periods of upper respiratory tract infections (URTI) (Harrison et al. 1999). Statistically significant differences in bacterial carriage were found when comparing NP populations in children <3 months of age to those >3 months of age, indicating age has an effect on the carriage of many bacterial species (Harrison et al. 1999).

When comparing infants with URTI to age and gender matched URTI-free controls, it was shown that URTI had little effect on bacterial carriage in the NP (Harrison et al. 1999). Statistically significant differences were found only in *Staphylococcus epidermidis* (a benign commensal), *M. catarrhalis* (referred to in this paper under its previous name, *Branhamella catarrhalis*) and α- or non-haemolytic *streptococci*. The use of culture techniques may limit these results; though diverse growth conditions were used to minimise potential bias (Harrison et al. 1999).

Historically, NP cultures were used in an attempt to predict the pathogens present in the middle ear (Gehanno et al. 1996). However, evaluation of this technique found that pathogens in the NP did not directly correspond to their involvement in OM. It was found however, that a negative culture for any NP pathogens had a 96% predictor value in determining the lack of pathogens in the MEE (Gehanno et al. 1996). This means, that if no pathogens were cultured from the NP, then the prediction that there were no pathogens in the middle ear was accurate 96% of the time.

1.3 Pathogens of middle ear disease

1.3.1 Koch's postulates

Historically, Koch's postulates determined whether or not a microorganism was causative of a disease (Fredricks & Relman 1996). Briefly, Koch's postulates state that for an organism to be a pathogen; the parasite occurs in every case of the disease; and exists in no other disease by chance or asymptomatically; and that it can be isolated in pure culture, re-inciting disease state upon introduction to a new susceptible individual (Fredricks & Relman 1996).

These postulates were published in the late 18th century. Advancements in technology that have allowed detection of organisms without first isolating in pure culture, and the fact that viruses cannot be grown in pure cell-free culture, have led to their re-evaluation (Fredricks & Relman 1996). Using OM as an example, known pathogens can colonize the NP asymptomatically, and there are instances of bacteria-free infections, facts which go against Koch's postulates (Vergison 2008).

1.3.2 Nasopharyngeal pathogens and viruses in otitis media

Early in the 20th century, group A *streptococcus* were the leading cause of OM in children (Vergison 2008). Good oral hygiene and the use of antibiotics have led to a shift in the etiology of the disease. Further shifts have been caused by the introduction of vaccines, such as the 7-valent pneumococcal conjugate vaccine (PCV7) effective against targeted serotypes of *S. pneumoniae*. Predominantly, the NP pathogens *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* make up the triad currently causing OM. Despite vaccine availability differing between countries, and causing differences in the epidemiological landscape, this triad are still the leading OM pathogens globally (Vergison 2008). Table 1.3 shows the frequency in which these three organisms are cultured in MEEs.

S. pneumoniae can invade the URT in both normal and immune impaired individuals (Murphy et al. 2009). The large carriage rate (see section 1.3.4 of this literature review for examples) in healthy individuals has a function in facilitating its transmission between individuals, and could cause problems in those with less immunity against them. This species has a polysaccharide capsule, with a function in increasing pathogenicity (Murphy et al. 2009). 91 different specific polysaccharide capsules, or 'serotypes', of *S. pneumoniae* exist. The capsule has a function in protecting the organism from host immune responses, which can increase pathogenesis in cases of AOM and other *Streptococcus* related ailments (Murphy et al. 2009).

Between 1999 and 2002, it was found that cases of AOM caused by non-PCV7 targeted serotypes of *S. pneumoniae* increased from 12% to 32% (Vergison 2008). Further, AOM caused by non-typeable *H. influenzae* increased in incidence. Non-typeable *H. influenzae* are the non-encapsulated serotype of this species, and are thought to cause mucosal infections, such as OM (Murphy et al. 2009). Although

they can colonise the NP asymptomatically, the observation has been made that colonisation in the first few months of life is associated with recurrent OM.

For decades, *M. catarrhalis* was thought to have very little or no pathogenic abilities. This was concluded despite its detection more often than the other two pathogens in sputum samples from sufferers of the URTI chronic obstructive pulmonary disease (COPD) (Murphy et al. 2009). It was later discovered to be an important pathogen in disease exacerbation. Further, *M. catarrhalis* has been found in the middle ear fluid of OM sufferers. With a potential function in increasing the biofilm, and by possibly providing passive antibiotic resistance to other species, *M. catarrhalis* is now seen as an important contributor to OM infections (Armbruster et al. 2010).

Table 1.3 Pathogens cultured from middle ear effusions (Poetker et al., 2005).

Pathogen	**Total effusions (*n* = 233)**	**Total isolates (*n* = 148)**	**Right effusions (*n* = 118)**	**Right isolates (*n* = 65)**	**Left effusions (*n* = 115)**	**Left isolates (*n* = 83)**
No growth	99 (42.5)		61 (51.7)		38 (33)	
Staphylococcus (NOS)	38 (16.3)	38 (25.7)	14 (11.9)	14 (21.5)	24 (20.9)	24 (28.9)
Haemophilus influenzae	24 (10.3)	24 (16.2)	12 (10.2)	12 (18.5)	12 (10.4)	12 (14.5)
Moraxella catarrhalis	15 (6.4)	15 (10.1)	12 (10.2)	12 (18.5)	3 (2.6)	3 (3.6)
Neisseria meningitidis	11 (4.7)	11 (7.4)	5 (4.2)	5 (7.7)	6 (5.2)	6 (7.2)
Staphylococcus aureus	11 (4.7)	11 (7.4)	3 (2.5)	3 (4.6)	8 (7)	8 (9.7)
Corynebacterium	11 (4.7)	11 (7.4)	4 (3.4)	4 (6.2)	7 (6.1)	7 (8.4)
Staphylococcus epidermidis	9 (3.7)	9 (6.1)	2 (1.7)	2 (3.1)	7 (6.1)	7 (8.4)
Streptococcus pneumoniae	6 (2.6)	6 (4.1)	3 (2.5)	3 (4.6)	3 (2.6)	3 (3.6)
Streptococcus mitis	3 (1.3)	3 (2)	1 (0.8)	1 (1.5)	2 (1.7)	2 (2.4)
Staphylococcus warneri	3 (1.3)	3 (2)	2 (1.7)	2 (3.1)	1 (0.9)	1 (1.2)
Staphylococcus capitis	2 (0.8)	2 (1.4)	1 (0.8)	1 (1.5)	1 (0.9)	1 (1.2)
Streptococcus salivarius	2 (0.8)	2 (1.4)	1 (0.8)	1 (1.5)	1 (0.9)	1 (1.2)
Rare Coagulase Negative Staphylococcus	2 (0.8)	2 (1.4)	1 (0.8)	1 (1.5)	1 (0.9)	1 (1.2)
Candida species	2 (0.8)	2 (1.4)	0	0	2 (1.7)	2 (2.4)
Leuconostoc	2 (0.8)	2 (1.4)	0	0	2 (1.7)	2 (2.4)
Escherichia coli	1 (0.4)	1 (0.7)	1 (0.8)	1 (1.5)	0	0
Bacillus species	1 (0.4)	1 (0.7)	1 (0.8)	1 (1.5)	0	0
Staphylococcus saprophyticus	1 (0.4)	1 (0.7)	1 (0.8)	1 (1.5)	0	0
Streptococcus pyogenes	1 (0.4)	1 (0.7)	1 (0.8)	1 (1.5)	0	0
Branhamella caterrhalis	1 (0.4)	1 (0.7)	0	0	1 (0.9)	1 (1.2)
Aerococcus viridans	1 (0.4)	1 (0.7)	0	0	1 (0.9)	1 (1.2)
Lactis cremoris	1 (0.4)	1 (0.7)	0	0	1 (0.9)	1 (1.2)

Many upper respiratory viruses are commonly associated with OM. 20% of AOM cases are thought to be caused by viruses alone, while another 65% of infections involve co-infection with a virus and bacteria species (Vergison 2008). Viruses with associations to OM include Human rhinovirus, respiratory syncytial virus (RSV), influenza viruses A and B, and parainfluenza 1, 2 and 3. These are thought to play a role in facilitating bacterial invasion of the middle ear cavity (Binks et al. 2011). It is environmental factors, including the presence of a virus, which can cause commensal bacteria of the NP to become primary pathogens of OM (Bakaletz 2009).

Studies have shown that certain strains of *M. catarrhalis* are able to adhere to epithelial cells much more effectively when an RSV infection is also present (Binks et al. 2011). Cilial beating and the human immune system normally keeps potential pathogens of OM in the NP. However, it is thought that viral infection can contribute to inflammation in the Eustachian tube, decreasing cilial beat frequency and the ability of the epithelial cells to resist bacterial invasion. This may lead to Eustachian tube ascension by opportunistic pathogens and therefore OM (Binks et al. 2011).

Using animal models, it has been shown that co infection with a virus, such as influenza or parainfluenza, and a bacterial pathogen, such as *S. pneumoniae*, left animals more susceptible to developing OM than those with only the bacteria present (in the NP) (Murphy et al. 2009). These two viruses possess neuraminidase activity which removes the sialic acid from host cell receptors. This may provide an avenue for *S. pneumoniae* attachment to the cell surface and subsequent spread of the bacterium. If this activity was used on the mucosa of the Eustachian tube, this would impair functioning and increase the likelihood of its ascension and subsequent OM (Bakaletz 2009).

1.3.3 Outer ear bacteria and otitis media

It has been postulated that the conditions of the middle ear can determine whether bacteria becomes a coloniser or a pathogen (Gomez-Garces et al. 2004). For example, the inflammation cause by a primary middle ear infection, and/or the antibiotics prescribed to combat it, may lead to an imbalance of the microbial flora. This imbalance may cause a change in the previously harmless bacteria,

causing them to overgrow and extend into a pathogenic role (Gomez-Garces et al. 2004).

The possibility of OE commensal bacteria crossing through the inflamed tympanic membrane into the middle ear cavity during an initial infection and subsequently complicating treatment is a leading point in this research. Ruptures of the ear drum either from previous grommets or from spontaneous rupturing during infection, is another possible avenue (Frank et al. 2003). How regularly these spontaneous ruptures occur during AOM is unknown.

As previously mentioned, the three OE bacteria with controversial associations to OM are *A. otitidis*, *C. auris*, and *T. otitidis* (Holzmann et al. 2002; Gomez-Garces et al. 2004; Ashhurst-Smith et al. 2007; Harimaya et al. 2007). All have been cultured from the MEEs of patients with OM. There is a possibility that the OE bacteria present in samples obtained from the middle ear cavity may be contaminates from the OE cerumen (Frank et al. 2003). Though, the occasional intracellular location of *A. otitidis* could be used as evidence to prove that *A. otitidis* at least enters the middle ear in one way or another during OM (Faden & Dryja 1989).

To test the theory of *A. otitidis* as a true OM pathogen, an enzyme-linked immunosorbent assay (ELISA) was used to measure the level of proinflammatory cytokines and chemokines released when it was the sole pathogen. Results were then compared with the levels present when the known pathogen *S. pneumoniae* caused the infection (Harimaya et al. 2009). These proinflammatory molecules increased in a similar manner between both *A. otitidis* and *S. pneumoniae*, suggesting *A. otitidis* is a true pathogen of OM (Harimaya et al. 2009).

A study that looked into the prevalence of *A. otitidis* in MEE during cases of AOM and OME found that it occurred more regularly in OME cases (Leskinen et al. 2002). They postulated that this correlation to OME could mean *A. otitidis* was a factor in increasing chronicity and have consequences in prolongation of inflammation. Further, *A. otitidis* has been found in the NP and MEE of otitis prone children, more often than in non-otitis prone children, providing evidence towards a role in chronicity (Harimaya et al. 2006) The increased colonisation of the NP by this organism may also have relevance to OM episodes, or could simply be the opportunistic colonisation of the area when it becomes accessible.

If *A. otitidis* is found to be a true pathogen of OM, or a secondary infectious agent, this could have a significant impact on how we treat OM. This is because *A. otitidis* displays resistance to amoxicillin/clavulanate, the sulfa drugs, and erthyromycin, all prescribed for the treatment of OM (Coates 2007).

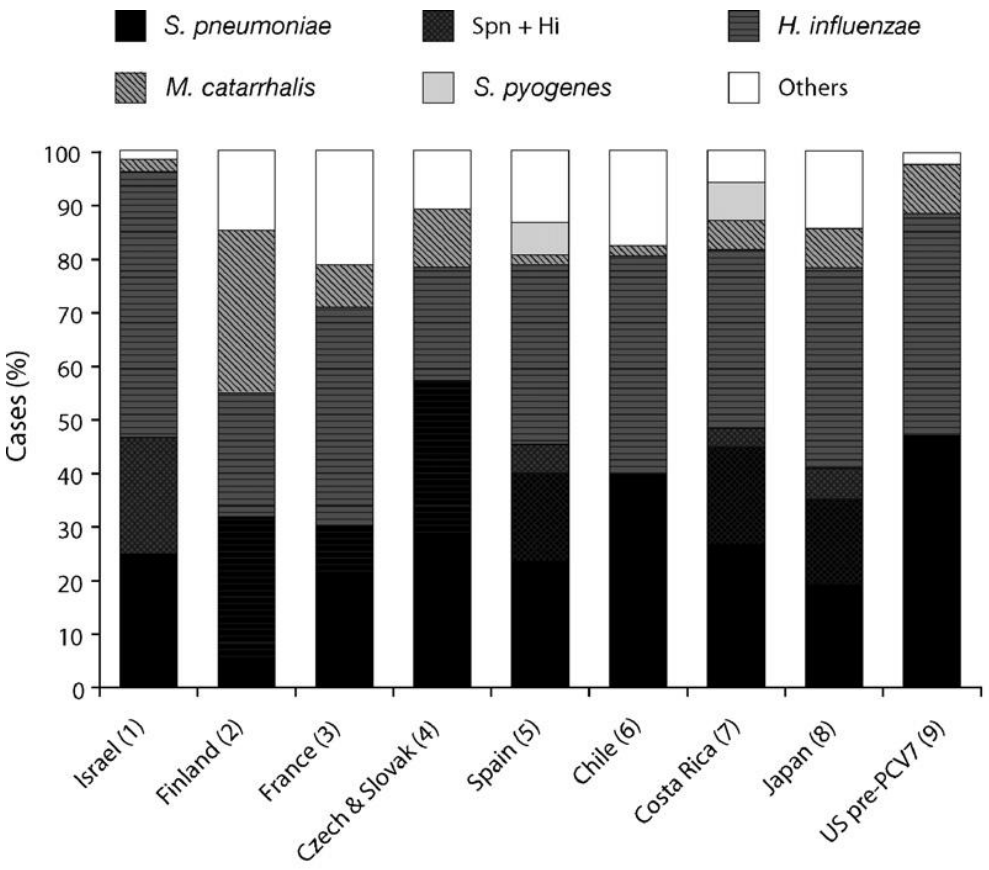
C. auris is frequently penicillin resistant, with a MIC of >1 mg/L (Gomez-Garces et al. 2004). If this bacterium played a role in OM, this could complicate penicillin treatment. *C. auris'* association with OM is also unknown and controversial, with many studies concluding it is part of the normal OE flora (Holzmann et al. 2002). This despite *Corynebacterium* spp. and *C. auris* itself being cultured from the MEE of patients with OM (see Table 1.3) (Stuart et al. 2003; Poetker et al. 2005).

T. otitidis is thought to be found exclusively in the OE, and whether it is a true pathogen of OM is a controversial possibility (Gomez-Garces et al. 2004). It was originally isolated from patients suffering from OM under the genus *Corynebacterium*. The classification of *T. otitidis* under a different genus (*Turicella*) is due to the chemotaxonomic and phylogenetic distinctiveness of this bacterium from its coryneform counterparts (Funke et al. 1994).

1.3.4 Global differences

Worldwide, the distribution of OM pathogens from the NP has been seen to differ (Vergison 2008). A comparison of a number of aetiology results from around the world by Vergison et al (2008) indicated that *H. influenzae* and *S. pneumoniae* dominate the microbial flora in patients with OM, with *M. catarrhalis* coming third. Group A *streptococcus* were the most geographically distinct species, with a range of 1% to 5% of cases containing this species worldwide (see Figure 1.2).

Carriage rates of the three major NP pathogens have been shown to be highly diverse depending on geographic location, age group and socioeconomic status (Garcia-Rodriguez & Martinez 2002). Using *S. pneumoniae* as an example, the lowest carriage rate was a group of 261 Swedish adults, at only 0.8%. A Swedish group of 620 healthy children, in contrast, had a carriage rate of 52%. This suggests the possibility of an immune response in the former. The highest carriage rate was seen in 102 Gambian children, of 87.2%, demonstrating the effect of socioeconomic status on colonisation. (Garcia-Rodriguez & Martinez 2002).



M. catarrhalis, Moraxella catarrhalis; PCV7, seven-valent pneumococcal conjugate vaccine; *S. pneumoniae, Streptococcus pneumoniae*; Spn + Hi, *S. pneumoniae* plus *Haemophilus influenzae*; *S. pyogenes, Streptococcus pyogenes.*

(1) Leibovitz *et al.*, 2007 [25]; (2) Eskola *et al.*, 2001 [20];
(3) Gehanno *et al.*, 2001 [21]; (4) Prymula *et al.*, 2006 [26];
(5) Del Castillo *et al.*, 1996 [19]; (6) Rosenblüt *et al.*, 2001 [27];
(7) Guevara *et al.*, 2008 [22]; (8) Suzuki *et al.*, 2005 [28];
(9) Block *et al.*, 2004 [18].

Figure 1.2 Distribution of key OM pathogens worldwide (Vergison., 2008). Results compiled from a number of references, stated above.

Vaccination availability differs globally and can influence the etiology of the disease. PCV7 is effective against 7 of the 91 serotypes of *S. pneumoniae*, and is used in the fight against pneumococcal species colonisation. This vaccine was licensed for use in 2000, and has been widely used since then (National Network for Immunization Information 2010).

PCV7 became available to Taiwanese children in October of 2005 (Kuo et al. 2011). As vaccination rates increased through the Taiwanese population, a study looked at the presence of *S. pneumoniae* in 6057 children. No significant decrease in the carriage rate of *S. pneumoniae* in the NP was found between July 2005 and July 2008, possibly indicating the replacement of vaccine-serotypes with non-vaccine serotypes.

A Lebanese study on OM pathogens demonstrates the importance of moderation of antibiotic treatment (Nasser et al. 2011). In Lebanon, antibiotics are available without a prescription, leading to the potential for overuse. This study found that about half of the *H. influenzae* cultured from patients with OME were beta-lactamase-negative, ampicillin resistant strains. It is thought this resistance comes from altered penicillin binding proteins, which lowers the affinity for β-lactams (Nasser et al. 2011). More alarming is the pattern of increased antibiotic resistance of pathogens from children that are regularly exposed to cigarette smoking, a habit ubiquitous in many societies.

Looking specifically at New Zealand children, the microbiology of the NP and OE has not been studied extensively. However, two studies have looked at bacteria present in MEEs. One study on Auckland children from 1996 used culture techniques to determine the pathogens present in MEEs (Watson et al. 1996). Of 105 ears, 67 were culture negative. The majority of culture positive samples were of the three known pathogens, indicating New Zealand's is also caused by this triad.

A more recent study looked at the colonisation of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *A. otitidis* in the MEEs of Hamilton children in New Zealand (Cecire et al. 2009). 98 specimens were collected, and using non-culture techniques, 160 positives were found across these four bacteria. This demonstrates the polymicrobial nature of infection, and further shows that New Zealand children have a high carriage rate of NP pathogens. For example, of 98 specimens, 35% were positive for *S. pneumoniae*. This is a similarly high carriage rate as seen in global studies (Garcia-Rodriguez & Martinez 2002)

1.3.5 Biofilms and Antibiotic Resistance

Biofilms exist in nature as complex communities of bacteria encased in an exopolymeric matrix (Hall-Stoodley et al. 2006). They are often closely associated to a surface, such as a grommet, but can also form on mucosal surfaces, such as those present in the Eustachian tube or middle ear (Murphy et al. 2009). These polymicrobial communities have been shown to have a different transcriptome, a reduced growth rate and an increased frequency of genetic material exchange by horizontal gene transfer, when compared to free living organisms. This allows them to optimise their existence to account for growth

limiting environments that occur *in situ*. Sometimes antibiotic resistance genes are shared, potentially complicating treatment (Murphy et al. 2009).

MEEs from patients with chronic OM, such as OME and rOAM, have previously been visualised using a confocal scanning laser microscope. These images supported the hypothesis that these chronic forms of middle ear infection are biofilm-related (Hall-Stoodley et al. 2006). As well as providing a physical impediment against antibiotic attack, enzymes such as β-lactamase have been recognised within some biofilms (Fergie et al. 2004). β-lactamase targets the β-lactam ring present in antibiotics such as ampicillin, inhibiting its antibacterial properties. Antibiotic susceptible bacteria may therefore be protected from destruction due to the presence of resistance enzymes produced by other species in the biofilm extracellularly.

A possible example of this has been observed between *H. influenzae* and *M. catarrhalis in vitro* (Armbruster et al. 2010). As observed using scanning electron microscopy, when infected with both species a biofilm is formed which is more substantial than when formed by the species individually. As well as a physical barrier to immune response, *H. influenzae* is further provided with passive resistance to antibiotics such as β-lactam, as *M. catarrhalis* produces the enzyme β-lactamase directly into the biofilm (Armbruster et al. 2010).

Further support for the biofilm model comes from experiments looking at the effects of co infection of a viral pathogen and *S. pneumoniae* on chinchilla NP and ME biofilm status (Hoa et al. 2009). Using scanning electron microscopy, the middle ears of control chinchillas and infected chinchillas were compared. Of those infected chinchillas observed to have developed OM, NP biofilms were found in 83% and ME biofilms were found in 67%. All animals with ME biofilms also had NP biofilms (Hoa et al. 2009). When compared to controls, Chinchillas with AOM demonstrated a higher proportion of NP biofilms. This demonstrates the influence the NP flora and biofilm status has on AOM development.

Bacteria in biofilms tend to have a slow growth rate and low metabolic activity, which could explain the large proportion of culture negative but PCR positive samples (Rayner et al. 1998). It could also show the ability of biofilms to retard the degradation of DNA and mRNA, in which case DNA based technologies may identify bacteria long dead from antibiotic treatments, as its genomic remains were still present in the biofilm (Fergie et al. 2004). However, Rayner et al (1998)

provided solid evidence for the presence of metabolically active *H. influenzae* in a culture negative samples by detecting unstable mRNA molecules which would not have been present if the organism had been long dead (Rayner et al. 1998).

1.4 Treatment of otitis media

1.4.1 Current treatments

1.4.1.1 **Vaccines**

As previously mentioned, a 7-valent pneumococcal conjugate vaccine (PCV7) is currently in use (Morris et al. 2009). This targets *S. pneumoniae*, an important pathogen of OM. The use of this vaccine has been shown to decrease the colonisation of the serotypes of *S. pneumoniae* that it targets, but has led to an increase in the non-targeted serotypes, potentially counteracting the decrease in other serotypes (Vergison 2008). Despite this, it has been shown that vaccinations can decrease the incidence of OM by about 6%, and markedly decrease the number of ventilation tube insertions by about 24% (Fireman et al. 2003).

An unfortunately side effect of vaccination and antibiotic treatment has been the increase in prevalence and the spread of a penicillin resistant *S. pneumoniae* strain. This has complicated treatment and modified the epidemiological landscape of OM (Vergison 2008) A newly developed 13-valent pneumococcal conjugate vaccine (Prevnar-13), which targets 13 pneumococcal serotypes, has recently been approved for routine vaccination (Sucher et al. 2011). As this vaccine targets six more serotypes than PCV7, it is hoped that its introduction will decrease both the serotype replacement effect seen when using PCV7 and the spread of resistant strains.

Vaccines directly for the prevention of OM are not currently in use, as the causative agents can differ quite drastically between cases (Arar et al. 2008). Since the introduction of the aforementioned vaccine, and other polyvalent vaccines, the most commonly isolated pathogen of AOM has become *H. influenzae* (Coates 2007).

1.4.1.2 **Antibiotics and surgical interventions**

Antibiotic resistance is a major problem in the fight against OM. For non-severe infections, the use of antibiotics is restricted. Watchful waiting remains a treatment for OM, as often these infections can resolve themselves (Morris et al.

2009). However, a blind trial comparing the use of amoxicillin-clavulanate to a placebo showed that the children given the antimicrobial therapy had better resolution of the disease (Tahtinen et al. 2011). Amoxicillin-clavulanate is a drug that demonstrates activity against β-lactamase-producers as well as penicillin resistant *S. pneumoniae* strains (Hoberman et al. 2002). A similar study by Hoberman et al. (2011) compared the efficiency of amoxicillin-clavulanate to the use of a placebo on AOM (Hoberman et al. 2011). 16% (amoxicillin-clavulanate) versus 51% (placebo) of children persisted to have signs of AOM after 10 – 12 days.

These studies may have been skewed to favour antibiotic treatment by their stringent selection criteria resulting in the inclusion of children with more advanced OM. Therefore, their conclusion may indicate that children with more severe OM should be treated with antibiotics, and watchful waiting continue to be used in monitoring less severe cases for spontaneous resolution or increasing severity.

In the more severe cases of rAOM, the Centre for Disease Control and Prevention (CDC) recommends treatment with three sequential courses of antibiotics. These are amoxicillin/clavulanate (as discussed earlier), cefuroxime and ceftriaxone. This is to treat against bacterial species that may be resistant to one or two of these, but not all (Coates 2007).

Antibiotic treatment should be prescribed carefully, as it can increase the reservoir potential of the NP by facilitating the survival of antibiotic resistant organisms. For example, if broad spectrum antibiotics are used, numbers of competing or 'interfering' organisms could be decreased (Hoa et al. 2009). This could decrease the resistance capabilities of the respiratory system against pathogen colonisation. Interfering commensal organisms are needed to compete against pathogenic bacteria for establishment in the child's respiratory system (Brook & Gober 1998).

Using culture techniques, Brook & Gober (1998) determined the efficiency of two regularly used antibiotics against OM (amoxicillin/clavulanic acid, and cefprozil) for efficacy in killing OM pathogens while leaving non-pathogenic bacteria unscathed (Brook & Gober 1998). It was shown that, while both antibiotics attacked and killed non-pathogenic interfering bacteria, cefprozil was more specific to the pathogenic bacteria. However, amoxicillin/clavulanic acid remains a staple in treatment of OM. Samples of the NP were not taken after an extended

period of time to determine the re-establishment of pathogenic bacteria after antibiotic treatment, nor were future bouts of ear infection reported, limiting results.

Surgical interventions are widely used on infections that fail to respond effectively to antibiotics. Insertion of a ventilation tube (or grommet) is common practice in cases of rAOM and OME. This allows the release of the pressure built up behind the ear drum, due to the blocking of the Eustachian tube, and also may have a function to dry out the biofilm (Fergie et al. 2004).

1.4.2 Future treatments

OM caused by *H. influenzae* may prove to be vaccine preventable (Morris et al. 2009). A pneumococcal- *H. influenzae* protein D conjugate vaccine that could potentially vaccinate against 11 serotypes of *S. pneumoniae* and non-typeable *H. influenzae*, is currently being assessed for clinical use (Haggard 2008). These bacteria are both important pathogens of OM, and therefore this could potentially be the first vaccine against OM itself. Trials on this vaccine have already been associated with a reduction in the risk of developing OM by 34% (Jansen et al. 2009). This is much more substantial than the modest 6-7% reduction in OM episodes reported from the use of PCV7 (Binks et al. 2011).

A potential target for a vaccine against *A. otitidis* has been identified in the form of an acidic capsule polysaccharide. This capsule may provide a highly charged cell surface, and some form of antibiotic resistance potentially important in the pathogenicity of the organism (Arar et al. 2008). If vaccines against the primary pathogens of OM were to become more frequently administered, *A. otitidis* could fill the gap this creates and itself become a primary infectious organism. Therefore, it is important to assess possible targets of these other organisms pre-emptively, as it may become necessary to administer both vaccines for effective OM protection.

1.5 Research outline

Using both molecular genetic techniques adapted from those previously described (Hendolin et al. 1997), and culture techniques, this research looks to determine the presence of six bacteria (*H. influenzae, S. pneumoniae, T. otitidis, C. auris, A. otitidis* and *M. catarrhalis*) linked to OM in the NP, MEE and OE. Emphasis will be on non-culture based techniques as to avoid culture bias and potential bias

caused by incomplete antibiotic profiles of the children involved. Comparisons will be made between the presence/absence of these six bacteria between children with OM and OM-free control patients, and between studied body sites. Polymicrobial status between the groups and studied sites will also be analysed.

Culture techniques will be used on a subset of samples to determine species prevalence beyond that of the six species of interest. This will provide information on the species present in the NP, OE and MEE that are culture-viable.

To further determine microbial diversity of these areas, a Sau-PCR microbial profiling technique will be used. This uses a restriction enzyme to cut at specific sites to create a banding pattern of which comparisons can be made between the differences/similarities of the microbes present between sampling variables. Cloning of the brightest bands (and therefore the most prevalent DNA sequences) will be undertaken in an attempt to identify the more dominant species.

The vaccination status of all participating children will be compared with the prevalence of *S. pneumoniae* in their studied sites in an attempt to determine vaccination efficiency in firstly preventing *S. pneumoniae* colonisation, and secondly preventing OM.

1.6 Techniques and Data analysis

1.6.1 Previously used techniques

Culture techniques are often used when determining the microbial composition of those areas involved in OM. Though this provides a definitive answer as to the culturable organisms present in the sample, organisms that are viable but non-culturable could be falsely negative (Rayner et al. 1998).

Multiplex PCR is used when studying specific bacteria involved in OM (Hendolin et al. 1997). A multiplex PCR utilizes a number of forward primers and one universal reverse primer to identify the presence of bacterial DNA. The size of the PCR products indicates which species were positively identified (Hendolin et al. 1997).

Microarrays are another valid option for identifying bacterial species present in polymicrobial samples. These microarrays work by the attachment of an oligonucleotide probe to a surface, such as glass, followed by the hybridization of complimentary DNA from targeted species in a mixed population. An example of

use can be seen in the paper by Cannon et al (2010) who used a low density oligonucleotide microarray to detect with high sensitivity and specificity, the presence of viral and bacterial DNA in a mixed population (Cannon et al. 2010).

1.6.2 Techniques of this study

To identify bacteria present in specimens taken from the NP, OE and the MEE, both culture techniques and non-culture based direct DNA extraction will be used:

10% Columbia blood agar plates, 10% Columbia chocolate agar plates and brain heart infusion with foetal calf serum (BHI+FCS) broth will be used for culturing organisms. DNA will then be extracted from isolated cultures and subsequent sequencing of the 16S gene or DNA *gyrase* subunit B (*gyr*B) will allow for their identification. Alternatively, a Sau-PCR DNA will be used with a Sau-PCR microbial profiling technique for microbial community assessment.

A Proteinase K enzyme lysis method will be utilized to extract genomic DNA directly from samples. This will be followed by a nested PCR for presence/absence analysis of six species of interest (*A. otitidis*, *C. auris*, *T. otitidis*, *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*). Nested PCR step 1 amplifies bases 63 to 941 of the 16S gene using universal primers. Then a second set of species specific primers, which anneal within the initial PCR product, will be used in a second cycle of amplification. Sequencing of PCR products obtained from a subset of the second round products will be completed to ensure accuracy of positive results.

Sau-PCR will be used to further assess the microbiota in the sampled sites (Corich et al. 2005). This is in an attempt to further determine the community profile differences between the sites being analysed, and study groups (control and OM patients). Differences in banding patterns between culture and non-culture methods of analysis will also be assessed.

1.6.3 Data Analysis

A chi-squared test will be applied to determine the statistical significance of differences in colonisation of the six bacteria of interest between controls and patients in the same body site.

Due to the lack of independence between left ear canal and right ear canal of each subject, as well as the lack of independence between the middle ears of the

patients, a chi-squared test cannot be used to find statistical significance between the NP and OE colonisation of species. A McNemar's test will therefore be used to determine if there is a statistical significant difference in colonisation between the NP and OE. The different study groups will be considered separately.

1.7 Study objectives

The main objectives of this study are as follows;

1) To determine whether there is a correlation between the presence of otitis media pathogens/potential pathogens and the presence of OM.
2) To determine whether the nasopharynx has any significant colonisation by *A. otitidis, C. auris*, or *T. otitidis* in either controls or OM patients.
3) To determine colonisation differences between the sample sites.
4) Using culture techniques, to determine the bacteria present in New Zealand children further than the six of interest.
5) Using Sau-PCR, to compare the bacterial diversity present between the sites, the study populations, and the DNA isolation techniques (culture vs. non-culture).

1.8 Hypothesis

1) I hypothesise that samples from children with otitis media will have more *M. catarrhalis*, *H. influenzae*, and *S. pneumoniae* present in their NP than children without OM.
2) I hypothesise that *A. otitidis*, *T. otitidis* and *C. auris* will be present in the nasopharynx and outer ears of OM children to a more significant level than OM-free control children.
3) I hypothesise that there will be a difference in bacterial colonisation between the NP and OE in both OM patients and OM-free children.

1.9 Null hypothesis

1) There is no difference in colonisation of the six bacterium of interest when comparing otitis free controls and otitis media patients.
2) There is no difference in colonisation of these six bacteria between the outer ear canal and nasopharynx.

Chapter 2: Materials and Methods

2.1 Sample collection

Ethical approval for this study was received from the University of Waikato Ethics Committee, and the Northern Y Regional Ethics Committee, Ministry of Health (Reference Number: NTY/11/03/029). Ethical approval information can be found in Appendix I.

Prior to their procedure, parents of patients were approached either in the outpatient setting or in the admission unit for surgical procedures of the participating hospital by staff of the otolaryngology department. Information sheets and consent forms (Appendix I) were provided and any questions answered. In the case of controls, parents of patients were sent information sheets and consent forms by mail and then were contacted by telephone prior to the procedures to clarify queries. Some personal and medical details were obtained for help in analysis including: age, sex, ethnicity, operation undertaken, history of OM including number of ventilation tube insertions, right or middle ear fluid presence, and professional medical diagnosis. This information can be found in Appendix II. Participating health centres include Waikato Hospital, Hamilton, Southern Cross Hospital, Hamilton, Thames Hospital, Thames, and Anglesea Procedure Centre, Hamilton.

Samples of the NP flora were taken via the nasal cavity using urethral swabs (Copan Italia). These were taken through a speculum inserted into the nose in an attempt to bypass the nasal cavity flora. Paediatric swabs (Copan Italia) were used for OE sampling of patients undergoing ventilation tube insertion. Flocked swabs, a newly developed swab for more sensitive microbial sampling, were not used in this research as they were not the standard of use (Goldfarb et al. 2009).

MEEs were collected in patients that had a visible discharge using a shortened 2mL syringe and a blunt 18G aspirating needle connected to wall suction. The sides of the outer ear canal were cleaned of cerumen beforehand, and avoided as best as possible during collection. The needle was discarded before transfer of the effusion into a sterile 1.7mL tube (Axygen).

Control samples were collected at the same hospitals as above and in a similar manner, but from patients undergoing non-OM related procedures.

All swab samples were stored in Liquid Amies Medium, while MEEs were stored either in sterile saline solution or with no additives. All samples were labelled using a numbering system, and were stored in a -80°C freezer until required.

2.2 Cultured isolates of six species of interest

Live and growing cultures of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* were obtained from Pathlab Hamilton, while *T. otitidis*, *C. auris* and *A. otitidis* were sourced from the New Zealand Reference Culture Collection (NZRM) as freeze dried specimens. These freeze dried samples were streaked onto 10% blood agar, and grown at 37°C for 48 hours.

DNA from all reference bacteria was extracted using the procedure detailed in section 2.3.2 for DNA isolation from cultured species. These isolated specimens were used to determine efficiency of DNA extraction protocols, specificity of primers, and the correct use of culture media.

2.3 Culture analysis

2.3.1 Culturing of organisms

Swabs were streaked onto 10% Columbia blood agar plates and 10% Columbia chocolate agar plates. Some were also placed into 2mL brain heart infusion + foetal calf serum (BHI+FCS) broth. Alternatively, 100µL of MEE was added to the plates. These were incubated at 37°C (in a Precision incubator) for 7 days.

10% Columbia blood agar plates were made as follows: In a 500mL SCHOTT bottle, 22g of Columbia blood agar (Difco^{TM}) was added to 450mL of double distilled water (Barnstead). After a thorough mixing, the bottle was pressure cooked (duo) for twenty minutes, then put into a water bath (Julabo 12B) set at 50°C until a temperature equilibrium was reached. In this time, a 50mL sample of sheep blood (Invitrogen – BL204BSL) was placed into a 37°C incubator (Precision).

Once the temperature of the bottle and contents had reached approximately 50°C, the sheep blood was added. Approximately 30mL of the blood agar was then aseptically poured into standard 8cm petri dishes.

10% Columbia chocolate agar was made using a similar procedure to the blood agar, only the blood was added when the agar was at approximately 80°C. After

blood addition, the bottle was returned to the water bath for 5 minutes with periodic swirling to ensure complete blood cell lysis.

For BHI+FCS broth: 37 grams of BHI broth (Bacto^{TM}) was added to 1L of distilled water in a 1L SCHOTT bottle. This mix was pressure cooked (duo) for twenty minutes, and then allowed to cool in a water bath to approximately 50°C. 5.5mL of FCS was added to this sterilized BHI broth before this solution was filtered through a 0.2µm filter (Whatman). Aliquots of approximately 2mL were aseptically transferred into 5mL screw capped sterile tubes (SARSTEDT) using a sterile 3mL pipette. Unused broth was stored at 4°C until required.

2.3.2 DNA extraction from isolated species in culture

Species that grew on plates were isolated in pure culture for separate DNA analysis. Species that grew in FCS+BHI broth were first isolated from the broth by spinning for 30 seconds at 16.1rcf. The supernatant was completely removed before extraction commenced.

DNA was extracted from cultured organisms using a lysozyme step followed by an SDS lysis protocol as follows:

To a 1.7mL microcentrifuge tube, 90µL of TE and 10µL of 100mg/µL Lysozyme solution (100mg/mL) were added, followed by bacterial colonies using a disposable loop until the solution was cloudy. Vortexing was followed by incubation in a thermomixer at 37°C for 15 minutes at 800rpm. 350µL of SDS lysis solution was added, before thermomixing at 95°C for 10 minutes (800rpm). 500µL of 5M Guanidinium thiocyanate (GITC), 55µL of sodium acetate (3M NaAc pH 5.2) and approximately 0.5mL of chloroform were added, mixed, and then put on a rotating wheel for 10 minutes. The DNA was precipitated using isopropanol and washed using 70% ethanol. 50µL of 1X TE was used for resuspension. DNA was quantified using an ND-1000 spectrophotometer (Nanodrop).

2.4 DNA Quantification and Quality assessment

DNA quantification results were obtained using an ND-1000 spectrophotometer (Nanodrop). Samples that were above 100ng/µL were diluted to this concentration in 1X TE. Purity of the DNA sample was assessed using the ratio between the 260nm reading and the 280nm reading. A 260/280 ratio of 1.8 – 2 was considered ideal, however often this was unobtainable. The CTAB clean-up step was added

to remove as much of this contamination as possible, and all samples were analysed despite 260/280 readings.

2.5 Identification of cultured organisms

2.5.1.1 **Gram stain**

Gram staining allows for the differentiation of gram positive and gram negative species, as well as the determination of morphology. In this study, gram staining was used to correctly identify two samples whose 16S sequence aligned with *M. catarrhalis* and *M. nonliquefaciens*. The former is morphologically a coccus, and the latter a rod. All reagents were from Ajax Finechem Pty Ltd, and were mixed locally. Staining procedure follows.

Cells were heat fixed onto a microscope slide before a drop of crystal violet solution (2g Crystal violet (certified 90% dye content), 20 mL 95% ethanol (vol/vol), 0.8g ammonium oxalate, distilled water to 100mL) was added and left for one minute. Rinsing in tap water was followed by the addition of Gram's iodine (1.0 g iodine, 2.0g potassium iodide, 300mL of distilled water). This was left for two minutes before it was rinsed off and the cells decolourised using 95% ethanol (vol/vol). The final counterstain used was dilute carbol fuchsin. Carbol fuchsin is made using: 1g basic fuchsin, 5g phenol, 10mL absolute ethanol, and 100mL water; this is then diluted 1:10 with distilled water to make dilute carbol fuchsin. This was applied to the cells for one minute before it was rinsed off, and the slide left in the 80°C incubator until dry.

Visualisation was achieved using a Zeiss Axiostar plus microscope, at 1000x magnification under oil immersion.

2.5.1.2 **16S Sequencing**

The 16S gene was amplified using either the 63f and COM2R or 63f and DG74 primer pairs (Table 2.1). Alternatively, the functional gene for DNA gyrase subunit B (gyrB) was amplified using specific gyrB forward and reverse primers. Reactions were completed in 0.2mL thin walled PCR tubes (Axygen). The reaction mixture used included 1X Buffer B (Solis Biodyne), Hot FIREPol DNA Polymerase (Solis Biodyne) at a concentration of 0.5U per 20µL reaction, 2mM $MgCl_2$, and 250µM of each dNTP (A, G, C and T, Solis Biodyne). Primers were added at a concentration of 0.4µM. Parameters for the 63f/COM2R were as

follows: 95℃ for 15 minutes, followed by 39 cycles of; 95℃ for 20 seconds, 60℃ for 20 seconds, then 72℃ for 1 minute. Reaction ended with a 5 minute extension step (72℃).

Table 2.1 Forward and reverse primer sequences used throughout this research. They consist of both universal 16S or *gyr*B primers, and species specific forward primers. Primer names are abbreviations of species names as follows; *A. otitidis* primer: AO, *C. auris* primer: CA, *H. influenzae* primer: HI, *Moraxella* species primer: MS, *S. pneumoniae* primer: SP, *T. otitidis* primer: TO.

Species	Primer Sequence
AO	5'-GGG GAA GAA CAC GGA TAG GA-3'
CA	5' - TGG ATA GGA CTG CTG GTT - 3'
HI	5'-CGT ATT ATC GGA AGA TGA AAG TGC-3'
MC	5'-CCC ATA AGC CCT GAC GTT AC-3'
SP	5'-AAG GTG CAC TTG CAT CAC TAC C-3'
TO	5' - AAC TGG GTC TAA TTC CCG - 3'
702R	5'-CTA CGC ATT TCA CCG CTA CAC-3'
COM2R	5' - CCG TCA ATT CCT TTG AGT TT - 3'
63f	5' - CAG GCC TAA CAC ATG CAA GTC - 3'
DG74	5'-AGG AGG TGA TCC AAC CGC A-3'
gyrB F	5'-CGI CCI GGK ATG TAY ATH GG-3'
gyrB R	5'-RMI CCW ACI CCR TGT AGI CCI CC-3'
1195R	5' GAC GTC RTC CNC DCC TTC CTC - 3'

When using the primer pair 63f and DG74, the reaction mix required treatment with Shrimp DNAse recombinant (usb) for 16S contamination removal. 0.5U/20µL of Shrimp DNAse recombinant was added before incubation at 37℃ for 15 minutes, then 80℃ for 15 minutes. DNA was then added and the following PCR parameters used; 95℃ for 15 minutes, followed by 10 cycles of: 95℃ for 20 seconds, 65℃ for 20 seconds (decreasing by 1℃ per cycle), then 72℃ for 1 minute and 30 seconds. This is followed by 25 cycles of: 95℃ for 20 seconds, 55℃ for 20 seconds, then 72℃ for 1 minute 30 seconds. Cycling is completed on a 72℃ extension step for 5 minutes.

PCR products were visualized on a 1% (w/v) TAE gel stained with ethidium bromide, as detailed in section 2.6.

PCR products being sequenced were isolated using either the PEG purification method, or the rAPid Alkaline Phosphatase purification method. Sequencing used the 16S universal primers 702R, COM2R or 1195R (Table 2.1).

2.6 Agarose gel electrophoresis – visualisation of PCR products

An Owl gel electrophoresis system was used to visualise PCR products. For both the nested PCR and cultured products, a 1% (w/v) agarose gel (usb) was cast using 1X Tris-Acetate-EDTA (TAE) buffer (40mM Tris acetate, 1mM EDTA) stained with ethidium bromide (1µg/mL). 10µL of amplified product was then added to the gel wells before being electrophoresed for approximately 30 minutes at 300A and either 100V (30mL gel) or 110V (100mL gel). A 100 base pair DNA ladder (Invitrogen) was used as a size reference and migrated with the DNA products. Visualisation occurred under UV light (TFX-35M, Life Technologies).

If the DNA was to be cloned, as is the case with Sau-PCR products, a 2% TAE gel stained with RedSafe (5µL/100mL of 20,000X, Invitrogen) was used. The DNA was then visualised on a Safe Imager (Invitrogen).

2.7 PCR product isolation for sequencing

Two methods were used to isolate PCR products for sequencing. The first was a Polyethylene glycol (PEG) DNA isolation method, the second used a rAPid Alkaline Phosphatase protocol.

The PEG PCR product isolation method follows:

PCR product was added to a 1.7mL microcentrifuge tube. PEG solution (10.0g Polyethylene glycol 800, 7.3g NaCl), in a 1:1 ratio with the PCR product (generally 40µL), was further added. Solution was mixed and left at room temperature for 30 minutes, before centrifugation at 16.1 rcf for 10 minutes. The supernatant was removed before the addition of 1mL of 100% ethanol. Centrifugation at 16.1 rcf for 5 minutes followed. The precipitate was subsequently washed with 70% ethanol and resuspended in 10µLs of 1X TE. DNA was quantified using a Nanodrop (as detailed in section 2.4).

The rAPid Alkaline Phosphatase protocol was as follows:

To 10µL of PCR reaction components (including PCR product of interest), 0.5µL of Exonuclease I (10U) was added, along with 0.5µL of FastAP Thermosensitive Alkaline Phosphatase (1U). This was then heated at 37°C for 30 minutes, followed by 15 minutes at 85°C. The concentration of the resulting purified PCR

product was estimated using a crude gel quantification method (detailed in section 2.7.1).

2.7.1 Gel DNA quantification method after rAPid Alkaline Phosphatase PCR product purification

This method relies on the comparison between the brightness of a known concentration of DNA in the DNA ladder, and that of the unknown product.

5µL of the recently purified PCR product was loaded onto a 1% (w/v) TAE agarose gel with the same volume of a 100bp DNA ladder (Invitrogen or Solis Biodyne) in an adjacent lane. The gel was then run on the Owl system at 100V and 300A for approximately 30 minutes. Comparisons were then made between the brightness of the bands to determine an approximate measure of DNA concentration.

2.8 Isolation of genomic DNA directly from samples

Genomic DNA for this study was isolated directly from the samples using an adapted proteinase K enzyme lysis method, followed by a DNA clean up using a Cetrimonium bromide (CTAB) protocol.

Proteinase K enzyme lysis method:

Swabs were either cut (urethral swabs), or snapped (paediatric swabs) into a 1.7mL microcentrifuge tube (Axygen). In the case of a mucosal MEE, 100µL was added to the tube. 350µL of sodium dodecyl sulphate (SDS) lysis solution (1M tris pH 9, 50mM EDTA, 1% SDS), followed by 10µLs of Proteinase K solution was added to the swabs/effusion. The Proteinase K solution contained 0.06g of powder Proteinase K (final conc. 60µg/µL), 50mL glycerol, 0.01M Tris pH 8, 0.2M calcium chloride (Ajax Finechem Pty Ltd) and was made up to 100mL using MQ-H_20 (Barnstead). This was incubated in a thermomixer (Eppendorf) at 56°C for 2 hours, shaking at 800rpm.

A 0.6mL microcentrifuge tube, (Axygen) that has previously had a hole burnt into the bottom, was put inside a 1.7mL microcentrifuge tube, before the swabs were added to the smaller tube. Centrifugation (Biofuge pico Heraeus) at 16.1 rcf for 10 seconds followed. The freed liquid was collected and returned to its respective tube, while swabs were appropriately discarded.

To this solution, 350µL of 5M lithium chloride (Ajax Finechem Pty Ltd) was added, and then an equal volume (approximately 750µL) of chloroform (Ajax Finechem Pty Ltd). After a vigorous mixing, the samples were put onto a rotating wheel (Labinco) for 15 minutes then centrifuged at 16.1rcf for 10 minutes. DNA was precipitated overnight using an equal volume of isopropanol (Ajax Finechem Pty Ltd) (approx. 750µL) and subsequently washed with 70% ethanol.

500µL of 1X tris (hydroxymethyl) aminomethane (tris) ethylenediaminetetra-acetic acid (EDTA) (1X TE: 10mM tris, 1mM EDTA pH 8) buffer was added in preparation for the following step in DNA purification. MEE samples did not require this clean up method, therefore only 20µL of 1X TE was added to these.

CTAB purification step:

30µL of 10% SDS (Ajax Finechem Pty Ltd) and 100µL of 5M sodium chloride (Ajax Finechem Pty Ltd) was added to the samples needing further purification. 80µLs of CTAB/NaCl that had been in an 80°C incubator for 10 minutes was further added (10% CTAB in 0.7M NaCl). Incubation in a thermomixer at 65°C for 10 minutes followed. An equal volume of chloroform (Ajax Finechem Pty Ltd) was then added, mixed by inversion, and put on a rotating wheel for 10 minutes. Centrifugation followed at 16.1 rcf for 10 minutes. DNA was precipitated overnight using an equal volume of isopropanol before being washed with 70% ethanol. After resuspension in 20µL of 1X TE, DNA was quantified using a Nanodrop.

Efficiency of this extraction method on the species of interest was assessed by running a swab over a mixed culture of all six species, storing in liquid Amies medium in a -80°C freezer, and then extracting the DNA the next day via the above method.

2.9 Molecular genetic techniques (non-culture based)

Both multiplex PCR and a nested PCR were trialled for efficiency in analysing the DNA from direct extraction. The primer pair 63f and COM2R were used in the first round of the nested PCR to amplify universal 16S DNA. Previously described primers specific for the 16S gene of *A. otitidis*, *Moraxella* species, *H. influenzae*, and *S. pneumoniae* were used, with the universal 16S 702R reverse primer, in both the multiplex PCR and the second round of the nested PCR (Hendolin et al. 1997).

C. auris and *T. otitidis* specific primers were designed using Primrose primer design technology, in conjunction with the rdp database and NCBI database for specificity assessment (Ashelford et al. 2002). These two primers had amine groups attached the 5' end, as they were initially designed to act as probes for a failed in-house microarray protocol. See Table 2.1 for primer sequences.

2.9.1 *Moraxella catarrhalis* vs. *Moraxella nonliquefaciens*

The *Moraxella* species primer was initially thought to be specific to *M. catarrhalis* (and was named MC, not MS). However, it was later discovered that *M. catarrhalis* and *M. nonliquefaciens* have near identical 16S sequence identity (sequence alignment demonstrated in Appendix III). Therefore, the primer described in Hendolin et al (1997) and used in this research has 100% identity not only with *M. catarrhalis*, but also with *M. nonliquefaciens*. This led to the reclassification of the *M. catarrhalis* results to that of *Moraxella* species in this current research.

M. nonliquefaciens is present in the nasal cavity, and therefore NP samples may be contaminated with this species during sampling (Wilson 2005). However, use of a speculum to bypass the flora of the nasal cavity in order to sample only the flora of the NP decreases this likelihood. Culture results discussed in the coming chapter indicate the species initially positive for *M. nonliquefaciens* were identified instead as *M. catarrhalis*. This increases the likelihood that the majority of *Moraxella* spp. positives are for *M. catarrhalis*, as opposed to *M. nonliquefaciens*.

2.9.2 16S rRNA vs. DNA gyrase B

The use of DNA gyrase subunit B (gyrB) as the discriminating gene between the six species of interest was investigated (Watanabe et al. 2001). Protein coding genes can have higher sequence variation when compared to the 16S gene, and therefore allow further species distinction. Limited horizontal transfer of this gene between species makes it an ideal candidate for phylogenetic analysis and species identifier. Unfortunately, the gyrB gene had only been sequenced for the three known pathogens, and not the three OE bacteria. Species specific primers therefore could not be designed for this gene, and therefore was not suitable for use as a species identifier.

2.10 Primer binding sites

2.10.1 16S universal primers:

2.10.1.1 63f binding position in the 16S sequence of the studied species (listed as 3' – 5' of genomic sequence):

H. influenzae; 44 – 64 (Genbank accession no. AF076035).

M. catarrhalis; 39 – 59 with one mismatch at base 6 (Genbank accession no. AF192341).

S. pneumoniae; 19 – 35 (only partial alignment with the 16S sequence (Genbank accession number - GU326247).

A. otitidis; 21 – 38 with a mismatch in base 7 of the primer (Genbank accession number - AY957475). Note that the BLAST database lists *A. otitidis* as *A. otitis*.

C. auris; 15 bases of the 21 base primer bind between 9 – 23 at 100% identity (Genbank accession number - NR_026211).

T. otitidis; 20 – 34 (15 bases of the 21 base pair primer matches the *T. otitidis* 16S gene). (Genbank accession number - NR_026120).

2.10.1.2 COM2R binding position in the 16S sequence of studied species (listed as 3' to 5' of genomic sequence):

H. influenzae; 885–866 (one mismatch; Genbank accession number - HQ846514).

M. catarrhalis; 881–862 (one mismatch at 871; Genbank accession number- AF005185).

S. pneumoniae; 904 – 885 (Genbank accession number - GU326247)

A. otitidis; 909 – 890 (Genbank accession number - AY957475)

T. otitidis; 902 – 883 (Genbank accession number - NR_026120)

C. auris; 867 – 848 (Genbank accession number - NR_026211)

2.10.2 Second round primers

2.10.2.1 702R binding positions

702R binds in the following positions of the six bacteria of interest:

676 to 696 in *A. otitidis* sequence (EMBL accession no. 59765), 679 to 699 in *H. influenzae* sequence (GenBank accession no. M35019), positions 630 to 650 in

the *M. catarrhalis* sequence (GenBank accession no. L13736), and positions 567 to 587 in the *S. pneumoniae* sequence (GenBank accession no. X58312) (Hendolin et al. 1997), 702 and 682 *Corynebacterium* spp. (Genbank accession no. AY259129.1), and 696 and 679 in an uncultured *Turicella* spp. (with one mismatch at 689; Genbank accession no. AY494657.1).

2.10.2.2 **Second round species specific forward primers**

Binding positions of the species specific forward primers (accession numbers given for the primers designed by this study):

AO primer 437 to 456; CA primer 133 – 150 (NCBI ref seq: NR_026211.1); TO primer 138 – 155 (NCBI ref seq: NR_026120.1); HI primer - 177 to 200; MS primer - 416 to 435; SP primer - 106 to 127.

Specific primers give the following product sizes when used with the 702R reverse primer:

HI;525, SP;484, AO;264, MS;237, CA;569, TO;564.

2.11 Multiplex PCR

In an attempt to optimize a multiplex reaction, the primer concentrations stated in Hendolin et al (1997) were used with the four specific forward primers, as well as the 702R reverse primer (Hendolin et al. 1997). Concentrations were as follows: AO- 1.6µM, HI- 1.4µM, MS- 0.2µM, and SP- 0.04µM. 702R reverse primer was added at a concentration of 0.4µM. Reactions were completed in 0.2mL thin walled PCR tubes (Axygen) using Genscript PCR premix 2X (in a 1:1 ratio of PCR grade water) which contained all reagents for the reaction, including "Hot Start" Script DNA polymerase which requires a 15 minute 95°C activation step.

PCR parameters were as follows: 95°C for 15 minutes, Followed by 38 cycles of: 95°C for 30 seconds, 66°C for 45 seconds and 72°C for 1 minute. A final extension step of 72°C for 5 minutes ended the reaction.

Products were identified and visualised using agarose gel electrophoresis as described in section 2.6. In confirming the multiplex PCR results, PCR products were isolated for sequencing. This used a Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research) to isolate the differently sized bands separately. PEG and rAPid Alkaline Phosphatase protocols could not be used with the multiplex protocol, as more than one PCR product was present in the reaction vessel.

2.12 Nested PCR optimization

This nested PCR protocol involves the amplification of universal 16S DNA in an initial round of cycling, followed by species specific DNA amplification in a second amplification cycle.

2.12.1 First round primer concentration optimisation

Variations of first round primer concentrations were tested to determine which yielded optimum results. Final concentrations chosen were 0.125µM of COM2R and 0.05µM of 63f.

2.12.2 Gradient PCR

PCR parameters for both the gradients described in the following sections were as follows: 95°C for 3 minutes, followed by 30 cycles of; 95°C for 20 seconds, either 50°C to 60°C or 54°C to 66°C for 20 seconds, then 72°C for 30 seconds. Reaction ended on a 72°C extension step for 5 minutes.

2.12.2.1 **First round annealing temperature optimization**

A gradient PCR was used to determine the best annealing temperature for the first round primers 63f and COM2R. This utilised *Staphylococcus aureus* DNA and used a Peltier thermal cycler (PTC-200). Temperatures 50°C to 60°C were tested. This gradient also determined amplification efficiency at two different DNA concentrations (600ng/20µL and 50ng/20µL).

The reaction mixture used included 1X Buffer B (Solis Biodyne), 0.5U of FIREpol DNA Polymerase (Solis Biodyne) per 20µL reaction, 2mM $MgCl_2$, and 250µM of each dNTP (A, G, C and T, Solis Biodyne). Note that this is not the reaction mix subsequently chosen for first round use.

55°C was the annealing temperature chosen for use in the first round.

2.12.2.2 **Second round annealing temperature optimisation**

Hendolin et al (1997) used the annealing temperature of 66°C for their four specific primers AO, SP, HI, and MS (Hendolin et al. 1997). TO was observed to anneal at this temperature, however CA did not. Using 702R as the reverse primer, a gradient of annealing temperatures between 54°C and 66°C was run on a Peltier thermal cycler (PTC-200). Reaction mix was the same as used in the first gradient.

This led to the use of 61°C as the second round annealing temperature.

2.12.3 First round reagents and PCR parameters:

Both first and second round amplification cycles were completed on a Bioer LifePro Thermal cycler (Model number: TC-96/G/H(b)A) using SnapStrip* 8 strip 0.2mL PCR tubes (Scientific Specialties Inc.).

After initially using the same reaction mix as the second round of amplification, the first round reaction mix was changed to: 1:1 ratio of 2X GoTaq Hot Start Green Master Mix (Promega) to MQ-H_2O (Barnstead), 0.1µM of COM2R and 0.05µM of 63f.

First round parameters were as follows: 3 minutes at 95℃, 25 cycles of; 20 seconds at 95℃, 20 seconds at 55℃, and 45 seconds at 72℃. Then a final extension step of 72℃ for 5 minutes.

2.12.4 Second round reagents and PCR parameters;

1µL of the first round products (taken straight from the PCR vessel) were added to the following reagents;

1X Buffer B (Solis Biodyne), 0.5U per 20µL reaction of Hot FIREpol (Solis Biodyne), 2mM $MgCl_2$, and 250µM of each dNTP (A, G, C and T, Solis Biodyne). Respective forward primers and the reverse primer were used at a concentration of 0.4µM.

A touchdown PCR was utilised for increased specificity in the second round of PCR. Parameters were as follows:

15 minutes at 95℃ followed by;

8 cycles of; 20 seconds at 95℃, 20 seconds at 68℃ to 61℃ (with temperature decreasing 1℃ per cycle), 30 seconds at 72℃.

Then 22 cycles of; 20 seconds at 95℃, 20 seconds at 61℃, 30 seconds at 72℃. Cycling ends with a 5 minute extension step (72℃).

To ensure amplification accuracy, a subset of second round products were isolated for sequencing and sent for sequencing. These products were isolated using the rAPid Alkaline Phosphatase purification method, followed by gel quantification.

2.13 Statistical analysis

A chi-squared test was used to determine the statistical significance of in colonisation between OM study group and the OM-free control group.

The lack of independence between the left ear canal and the right ear canal complicated statistical analysis. Each patient has one NP and two OEs. A simple chi-squared test could not be used to compare the OEs with the colonisation results in the NP as these three orifices are not completely independent of each other. Therefore, a McNemar's test which takes into account the lack of independence was used to determine statistical significance in colonisation between the OE and NP.

2.14 Sau-PCR: genetic fingerprinting of polymicrobial populations.

Sau-PCR creates a distinct banding pattern or 'fingerprint' which differs between isolated organisms and can be used to compare between mixed populations. This can be used as a crude method to determine species diversity in an unknown bacterial population or could be useful in comparing a known species banding pattern with those of unknown species/mixed population, in an attempt to identify whether that bacteria is present in the sample.

This fingerprint is created by the digestion of genomic DNA using the Sau3AI restriction enzyme, which cuts DNA at specific sites leaving sticky ends (Corich et al. 2005). Specific primers based on the restriction enzymes recognition sequence then amplify the fragmented DNA, creating a fingerprint based on the genomic sequence. This fingerprint will differ depending on the number of recognition sites in an isolated species, or in a mixed population of species (Corich et al. 2005).

The Sau-PCR procedure is as follows:

Digestion:

10µL of DNA solution (200ng total) was digested at 37°C overnight with 10 units of Sau3AI or MboI (2µL). The reaction mix contained the appropriate restriction buffer (2µL of 10X NE Buffer 1 + bovine serum albumin (BSA) or NE Buffer 4) in a final volume of 20µL (made up with 6µL MilliQ H_2O). After incubation was

complete, the temperature was raised to 65°C for 20 minutes, in order to deactivate the restriction enzyme.

Amplification cycles:

The Sau-PCR was completed in a reaction volume of 25µL in 0.2mL microcentrifuge tubes (eppendorf) using a Peltier Thermal Cycler. Reagent concentrations were as follows; 2µL of Sau3AI or MboI-digested DNA (10 ng/µL), 200µM deoxynucleoside triphosphates, 10mM Tris-HCl (pH 9.0), 50mM KCl, 1.5mM $MgCl_2$, 0.5 U Taq polymerase (Hot FIREpol, Solis Biodyne), 2µM primer (Corich et al. 2005). Primers used are listed in Table 2.2 (Corich et al., 2005). SauC was the most regularly used.

Table 2.2 Length, sequence and annealing temperature of Sau-PCR primers. (Corich et al, 2005).

Primer	Length (nt)	Sequence 5' to 3'	Annealing T (°C)
SauA	12	CCGCCGCGATCA	44
SauC	12	CCGCCGCGATCC	46
SauG	12	CCGCCGCGATCG	46
SauT	12	CCGCCGCGATCT	44
SAG	13	CCGCCGCGATCAG	48
SCA	13	CCGCCGCGATCCA	48
STG	13	CCGCCGCGATCTG	48
SGAG	14	CCGCCGCGATCGAG	52

Sau-PCR parameters were adapted from the paper by Corich et al (2005) with one major difference in the initial activation step, as we used Hot FIREpol DNA polymerase (Corich et al. 2005). PCR parameters are as follows:

95°C for 15 minutes;

DNA was then added before;

25°C for 0:05 seconds;

Increase in temperature by 0.1°C/s until 60°C is reached;

60°C for 0:30 seconds.

The following is cycled through twice:

95°C for 1 minute;

50°C for 0:15 seconds;

Decrease in temperature by 0.1°C/s to 25°C;

Increase in temperature by 0.1°C/s to 50°C;

50°C for 0:30 seconds.

The following is cycled through 39 times;

95°C for 0:15 seconds, 48°C for 1:00, 65°C for 2:00 minutes.

Reaction is ended with a 65°C extension step for 5 minutes.

Cloning of the Sau-PCR products was attempted using DNA acquired from band stabbing 2% agarose gels stained with RedSafe.

2.15 Sequencing reactions

Samples of interest were sequenced at the Waikato DNA Sequencing Facility. DNA sequences were resolved using a 3130xl Genetic Analyzer System fitted with 50 cm capillary arrays (Applied Biosystems) loaded with POP-7 polyacrylamide matrix (Applied Biosystems). DNA templates were prepared using Big Dye v3.1 terminator chemistry (Applied Biosystems).

Chapter 3: Results

3.1 Study participants

Full patient information can be found in Appendix II. Basic statistics on the age, gender, ethnicity, and PCV7 vaccine dosage are listed in Table 3.1 (control group) and Table 3.2 (OM patient group). One control participant's bacterial results were excluded from the study due to a history of OM (Patient #1C).

Controls were on average 2 month older than OM patients. A total of 54 males and 24 females participated in the study. The control group demonstrated more variation in ethnicity than the OM group, however Maori and New Zealand Europeans dominated both. Overall, 23 Maori, 46 New Zealand Europeans, and 9 other ethnicities participated in the study. The variation in bacterial prevalence between the ethnicities was not determined.

Table 3.1 Basic information of the control study group involved in this study.

Control patients	
Average age:	3yrs 7mnths
Gender: M	27
F:	9
Ethnicities:	
Maori	11
NZ European	16
European	3
Maori-Polynesian	1
Other European	1
Polynesian	1
Indian	1
Cook Island Maori	1
Somalian	1
PCV7 dose number:	
1	0
2	0
3	2
4	14
0	20

Table 3.2 Basic information on the otitis media patient group involved in this study.

OM patients	
Average age:	3yrs 5mnths
Gender: M	27
F:	15
Ethnicities:	
Maori	12
NZ European	30
OM Diagnosis:	
Unknown	4
OME	24
RAOM	13
AOM	1
PCV7 dose number:	
1	1
2	0
3	1
4	13
0	27

3.2 Culture

3.2.1 Culture analysis

Culture results are a snap shot of the bacteria that were present, and culture viable using particular conditions, during sample collection. In this research, culture was used to determine the bacterial prevalence in New Zealand children beyond the six bacteria of interest.

Direct DNA extraction from swabs, as well as culture analysis was unable to be undertaken on the same samples. This is because the streaking of the swab onto culture plates removes many microorganisms that would subsequently be lysed in the direct DNA isolating technique, decreasing the amount of DNA obtained for non-culture analysis. MEE samples can however be used in more than one type of analysis, as only 100µL is used in direct DNA extraction. Therefore 5 MEEs were analysed by both direct extraction and culture.

Further problems with culture analysis include:

- A lack of antibiotic profiles. As such, the use of unknown antibiotics could introduce bias into culture analysis.

- Selective media is not available that grows all six bacteria of interest equally.

Therefore, culture analysis was only completed on a small subset of samples.

DNA from mixed culture was not used with the nested PCR protocol. The reason behind this can be seen when looking at the results using DNA extracted from the non-selective media, BHI+FCS broth (Figure 3.1). Increased background and non-specific activity made positive and negative results unclear. Due to this difficulty in interpretation, sequencing of the 16S or *gyr*B gene was used to identify more exactly the isolated species from culture samples, beyond those of the species of interest.

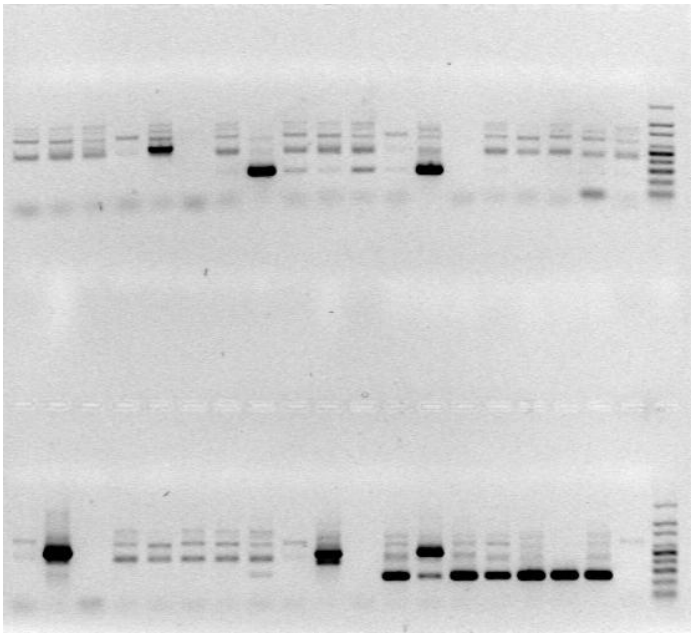


Figure 3.1 The results using the nested PCR protocol on DNA isolated from BHI+FCS non-selective broth. Though clear positives are present, a number of non-specific bands are visible, complicating results interpretation.

Contamination issues with the 16S universal primers required the introduction of the Shrimp DNAse recombinant step before amplification. Further, the use of *gyr*B primers was abandoned early due to the lack of sequencing information on this gene in BLAST for sequence comparisons.

3.2.2 Culture results

Of 42 OM patients; 11 OE, 7 NP, and 8 MEE samples were analysed using culture. Further, of the 36 control patients 4 OE and 2 NP samples were analysed. 10% blood agar and 10% chocolate agar were used as the culture medium.

Species cultured in BHI+FCS were not identified (as detailed in section 3.2.1), however some of these samples were used with the Sau-PCR protocol (see section 3.12). Extended BLAST results and a subset of electropherogram data from the sequenced organisms can be found in Appendix V, Section 1.

Basic information on the cultured organisms can be found in Table 3.3. Species noted on this table are not a 100% confirmation of their presence, as many 16S sequences of the same genus are very similar. For example, sample 25CNP-C was initially identified as *M. nonliquefaciens*. However, the same degree of alignment can be found with *M. catarrhalis*. Electropherogram results account for a number of the discrepancies seen in the sequence comparisons, as well as the low e score of a small number of samples (see Appendix V).

Not all of the organisms that were cultured were also sequenced. There are many reasons behind this, which include; early cultured organisms were used in an attempt to optimise protocols that were eventually abandoned, the DNA was unable to be successfully extracted (or amplified in some cases), and in some cases only the most dominant species on the plate were isolated. Further, a number of samples were culture negative. For example, of the eight cultured MEE samples, three yielded no bacterial growth after an extended period of incubation.

The inability to align the sequence of 26CNP-B further than its genus to sequences in the BLAST database demonstrates the complexity of the microbiome of this site. Reanalysis of this DNA sequence in years, or even months to come, may lead to a species level identification. A similar problem was found in the use of the gyrB sequences from the 1NP organisms.

Initial identification of the cultured isolates from 1NP (now identified as *S. oralis* and *S. salivarius*) using the sequence of their gyrB gene proved unsuccessful, as no alignments were found in the BLAST database. However, re-examination some months later allowed identification, as the genome of these organisms had been sequenced in the meantime. The HMP and other organisations are currently sequencing and making available the complete genomes of human colonising bacteria. This means that in future research, this gene could prove to be a useful tool for analysis.

Table 3.3 Information obtained from the sequencing of cultured isolates. Table includes information on sequencing primer, most likely species, the number of bases run through BLAST, gaps present and the percentage the sequence aligned with the database sequence.

Sample	Organism	ID gene	Primers	Sequencing primer	BLAST Identification	Bases compared	Gaps	Identifies
1RE	A	gyrb	gyrase b f/r	gyraseb reverse	*Staphylococcus epidermidis*	129	0	100%
8RE	A	16S	AO/COM2R	702R	*Alloiococcus otitidis*	171	0	100%
3LE	A	16S	63f/DG74	1195R	*Alloiococcus otitidis*	432	0	100%
6LE	A	16S	AO/COM2R	702R	*Alloiococcus otitidis*	130	0	100%
1NP	A	gyrb	gyrase b f/r	gyraseb reverse	*Streptococcus oralis*	160	0	96%
1NP	B	gyrb	gyrase b f/r	gyraseb reverse	*Streptococcus salivarius*	74	0	92%
2NP	A	16S	MS/COM2R	702R	*Moraxella catarrhalis*	166	0	100%
3NP	A	16S	TO/COM2R	702R	*Turicella otitidis*	378	0	100%
26NP	A	16S	63f/DG74	1195R	Uncultured *Dolosigranulum*	433	1	99%
9LM	A	16S	63f/DG74	1195R	*Alloiococcus otitidis*	349	0	100%
26RM	A	16S	63f/DG74	1195R	*Staphylococcus* species	466	0	99%
29RM	A	16S	63f/DG74	1195R	*Staphylococcus aureus/haemolyticus*	292	0	100%
25CLE	A	16S	63f/DG74	1195R	*Staphylococcus auricularis*	220	2	95%
25CRE	A	16S	63f/DG74	1195R	*Staphylococcus auricularis*	304	1	99%
25CRE	B	16S	63f/DG74	1195R	*Staphylococcus epidermidis*	240	2	99%
26CLE	A	16S	63f/DG74	1195R	*Alloiococcus otitidis*	240	3	98%
26CRE	A	16S	63f/DG74	1195R	*Alloiococcus otitidis*	180	7	96%
25CNP	A	16S	63f/DG74	1195R	*Corynebacterium pseudodiphtheriticum*	271	0	99%
25CNP	B	16S	63f/DG74	1195R	*Dolosigranulum pigrum*	263	5	98%
25CNP	C	16S	63f/DG74	1195R	*Moraxella catarrhalis*	320	0	99%
26CNP	A	16S	63f/DG74	1195R	*Dolosigranulum pigrum*	405	0	99%
26CNP	B	16s	63f/DG74	1195R	Uncultured bacterium clone	343	0	100%

As previously stated, the 16S sequence of *M. catarrhalis* and *M. nonliquefaciens* are very similar. The sequences isolated from the organisms under the labels 2NP-A and 25CNP-C, when aligned with the BLAST database, were initially identified as *M. nonliquefaciens*. Recognition of their alignment with *M. catarrhalis* only occurred when the Moraxella taxid was selected for in the search parameters. Gram stain results indicated that both organisms were gram negative diplococcus. This is consistent with *M. catarrhalis*, as *M. nonliquefaciens* is rod shaped. This provided definitive evidence of their true species identity.

The plates culturing the NP samples were more densely populated than those of the OE samples. This demonstrates the more extensive colonisation of this area. Slower growing organisms, like *A. otitidis* which was observed to take a minimum of four days to grow, may have therefore been undetectable due to faster growing organisms of the sampled sites. OE samples were not as regularly densely populated, possibly allowing time for *A. otitidis* to grow without being overrun by other species.

Of the six species targeted by the second step of nested PCR, only *A. otitidis, T. otitidis* and *M. catarrhalis* were cultured. This is not surprising as *S. pneumoniae* is a very fragile bacterium, and has a tendency to autolyse when grown to stationary phase (Saha et al. 2011). *H. influenzae* is also sensitive to environmental stresses, such as those endured during storage of the samples. *C. auris* is less prevalent than *A. otitidis,* but is also slow growing, and therefore may have been overcome by other fast growing organisms if it were present in the sample.

Considering the low number of samples cultured, the amount of samples positive for *A. otitidis* may suggest that the antibiotics used on OM patients are ineffective against this species. However, without full antibiotic prescription reports, antibiotic susceptibility profiles, and further culture data, this hypothesis cannot be substantiated. Further, low sample numbers decreases the strength any p values this data set may produce. Therefore statistical significance calculations between the six species of interest isolated through culture vs. non-culture techniques were not completed.

3.3 Bacterial DNA Isolation

Of the 42 OM patients involved in this study, bacterial genomic DNA was isolated directly from 67 OE swabs, 43 MEEs and 33 NP swabs. MEE samples were unable to be collected in every OM patient because prior to the surgical procedure, the child is anesthetized using a gas. The gases ventilate the middle ear and can displace previously present exudates, therefore making collection impossible (T. Cecire, personal communication, January 13th, 2012). Of 36 control patients, 32 NP swabs and 64 OE samples were directly extracted.

After extraction, DNA was re-suspended in 20µL of TE before 2µL was used for DNA quantification analysis using a ND-1000 spectrophotometer (Nanodrop). Table 3.4 depicts the typical spread of Nanodrop results for OM patients, while Table 3.5 is an example of those from control participants. DNA concentration is given in ng/µL.

The spectrophotometer measures the absorption of the sample at 230nm, 260nm, and 280nm. The 260/230 absorption ratio depicts the levels of nucleic acids to phenol in the sample. Pure DNA samples should have a 260/230 ratio of 1.8. Purity of the DNA sample is determined by the ratio of absorption at 260nm and 280nm. A 260/280 ratio of 1.8 – 2 is optimal for minimal non-DNA contamination.

In comparison to the NP, the biomass of OE microbiota is much less. Potentially because of this, the purity ratios in the OE samples were not optimal. Further, samples may also contain human DNA. All samples were analysed however, and PCR results obtained. This suggests non-DNA contaminants predominantly did not inhibit cycling reactions, but DNA may have contained undetectable levels of some species and were therefore recorded as negative.

Table 3.4 Nanodrop results for a subset of OM patients.

Sample ID	ng/µL	A260	260/230	260/280
33LE	107.68	2.154	0.76	1.50
33RE	95.53	1.911	0.61	1.62
15RE	40.32	0.806	0.61	1.55
15LE	78.75	1.575	0.64	1.45
32LE	102.01	2.040	0.60	1.49
32RE	237.01	4.740	0.62	1.49
32NP	392.57	7.851	1.84	1.82
15NP	115.19	2.304	1.14	1.76
33NP	566.83	11.337	1.55	1.86
21NP	1311.10	26.224	1.05	1.66
24NP	717.18	14.344	1.45	1.81
22NP	812.12	16.242	2.24	1.94
21LM	2689.2	53.785	1.5	1.61
33LM	1926.90	38.538	2.15	1.84
33RM	187.24	3.745	1.24	1.77
32LM	3860.10	77.202	1.37	1.56
18RM	405.26	8.105	1.68	1.78
18LM	1444.10	28.884	1.41	1.76

Table 3.5 Nanodrop results for a subset of control patients.

Sample ID	ng/µL	A260	260/230	260/280
31CNP	4396.90	87.939	1.80	1.74
29CNP	667.78	13.356	1.66	1.81
24CNP	894.47	17.889	1.39	1.74
19CNP	410.72	8.214	2.06	1.90
20CNP	496.16	9.923	1.85	1.85
1CNP	678.57	13.571	1.76	1.83
2CNP	1001.80	20.037	1.52	1.70
3CNP	2358.20	47.166	1.41	1.73
5CNP	581.75	11.635	1.49	1.76
6CNP	614.67	12.293	1.48	1.79
31CLE	194.31	3.886	0.64	1.46
31CRE	49.84	0.997	0.58	1.57
29CRE	123.47	2.469	0.66	1.39
29CLE	122.00	2.440	0.60	1.49
24CRE	87.84	1.757	0.60	1.46
24CLE	330.69	6.614	0.88	1.61
19CLE	112.08	2.242	0.65	1.42
19CRE	115.50	2.310	0.77	1.44
20CLE	37.25	0.745	0.64	1.45
20CRE	36.97	0.739	0.65	1.39

A selection of DNA samples were run on a 1% (w/v) agarose gel using TAE buffer for 30 minutes at 100V. This was to determine the amount of genomic DNA isolated, as the Nanodrop spectrophotometer also measures the rRNA which is isolated. Figure 3.2 demonstrates the typical spread of gel results for these DNA samples. The OE samples DNA concentration is much less than the other two, to the point that it cannot be visualised using gel electrophoresis. Nanodrop quantitation results showed DNA was present in the samples, and therefore these quantitation results were used.

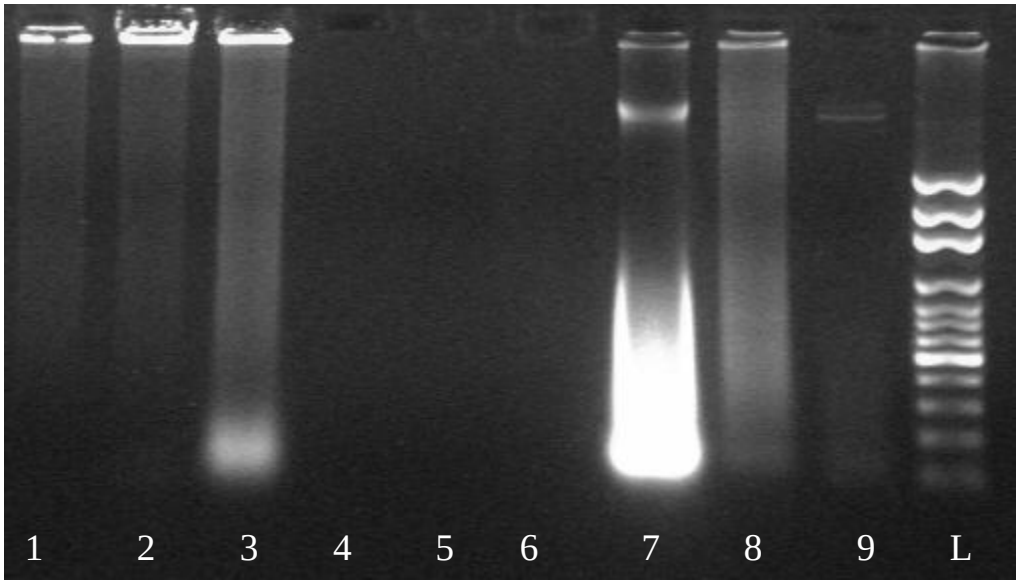


Figure 3.2 1% (w/v) TAE agarose gel of DNA extracted directly from samples. Lanes 1-3 contain MEE samples, 4-6 OE samples, and 7-9 NP samples. L = 100bp ladder (Invitrogen).

3.4 Culture independent extraction efficiency

The efficiency of the direct DNA extraction technique was assessed by mixing cultured organisms of interest on a blood agar plate, running a swab over this mix, storing it as we would any other sample, then extracting via the direct swab extraction method. The technique did not appear successful with *S. pneumoniae* and *H. influenzae* and, as often seen in my results from cultured DNA, many non-specific bands were present (Figure 3.3). *T. otitidis* and *Moraxella* spp. negatives also both had small amounts of contamination.

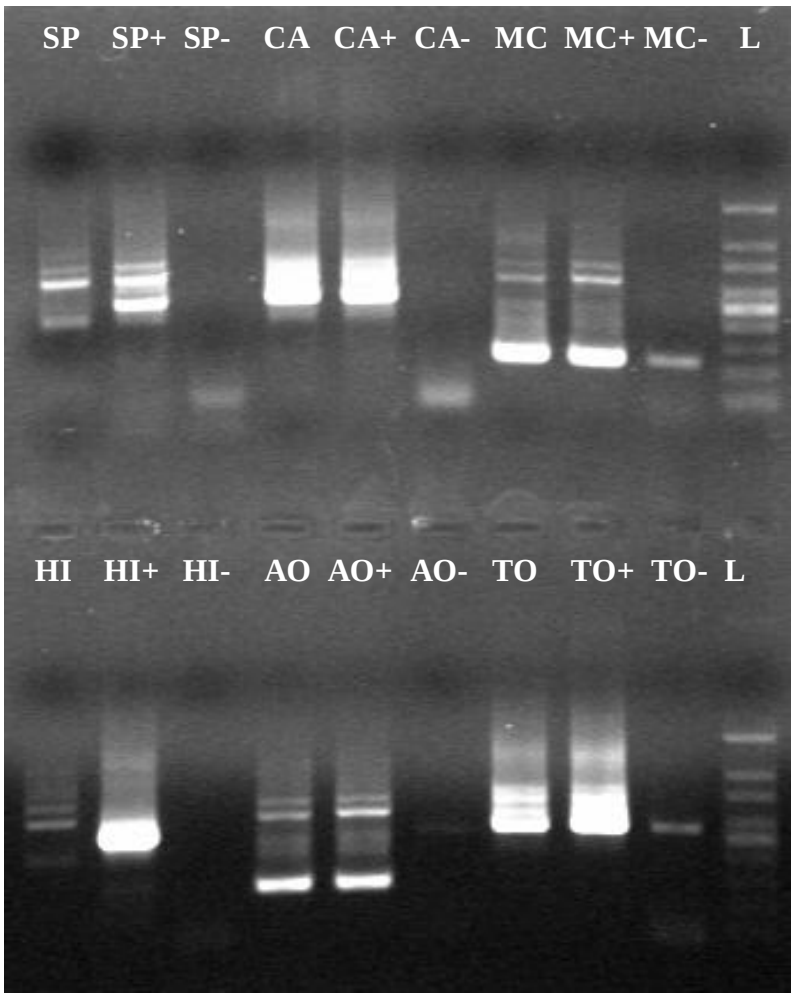


Figure 3.3 Results using the direct DNA extraction protocol on a swab positive for all species of interest, followed by use of the nested PCR protocol, visualised on a 1% (w/v) TAE agarose gel. Samples were either negative of all bacteria (-), positive using DNA from cultured organisms (+), or positive from direct DNA extraction from swab.

The culture plates that the *S. pneumoniae* and *H. influenzae* were taken off had both died, due to the fragility of these species in culture. To ensure our procedure was able to detect all species, samples that had previously tested positive using the direct extraction method were sequenced. Sequencing identified that positive results were from the species of interest, and therefore the direct extraction procedure had successfully lysed the cells from these bacteria of interest. Therefore, use of this extraction procedure went ahead.

3.5 Multiplex PCR

Multiplex PCR relies on the specificity of forward primers and the subsequent ability to distinguish different sized PCR products, often through gel electrophoresis. Initial multiplex attempts, using the primer concentrations stated

by Hendolin et al (1997) appeared successful, encouraging continuation (Figure 3.4) (Hendolin et al. 1997).

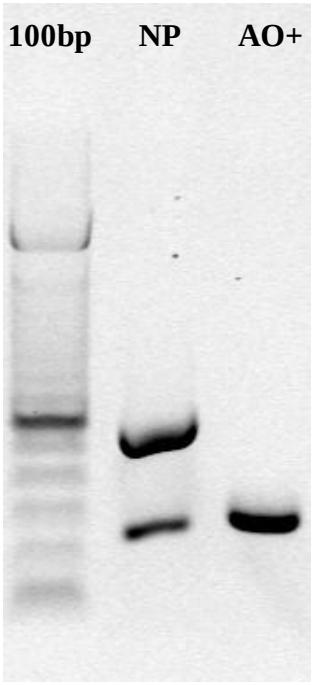


Figure 3.4 Initial multiplex results. The two bands from the nasopharyngeal (NP) sample were isolated separately and sent for sequencing. Sequencing results identified the products as *H. influenzae* (100%), and *M. catarrhalis* (99%), BLAST alignment can be found in Appendix V, section 2).

Reproduction of these results initially successful results was unable to be achieved (Figure 3.5). Nor was the incorporation of the other two primers for bacterium not studied by Hendolin et al (1997) successful, despite the separation into two multiplex reactions of three primers, then three multiplexes of two primers.

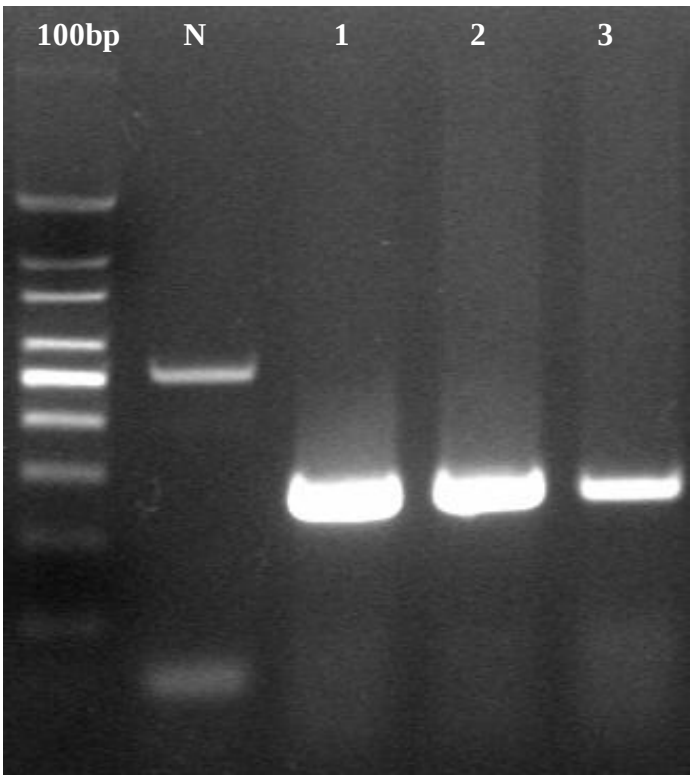


Figure 3.5 Multiplex reactions with single PCR products, despite the presence of multiple forward primers. Slight band in the negative. Reactions contained the specific primers for, and DNA of: Reaction 1: *A. otitidis* and *S. pneumoniae*, reaction 2: *A. otitidis* and *H. influenzae*, reaction 3: *A. otitidis*, *S. pneumoniae*, *H. influenzae*.

Further complicating issues stem from the CA and TO primers designed using primrose technology. These had an overlapping region, leading to their PCR products being extremely similar sizes and therefore the inability to use them in the same multiplex reaction. These complications and inability to optimise the Hendolin et al (1997) protocol led to separation of the reactions.

3.6 Gradient PCR

To find the correct temperature for use in the first round of the nested PCR, and other reactions using the universal 16S primers, a gradient PCR was run using annealing temperatures between 50°C and 60°C. Two different DNA concentrations were also tested for efficiency. These concentrations were 30ng/µL (600 ng/20µL reaction) and 2.5ng/µL (50 ng/20µL reaction).

Results showed all reactions at the lower concentration of DNA gave PCR products, despite the Tm of COM2R being 50.9°C (Figure 3.6). Of the higher DNA concentration reactions, the samples that did not work were at annealing temperatures of 52.8°C, 56°C, and 59.8°C. Reactions between these temperatures

did work however, so why these failed is not clear. Looking at these results, 55°C was chosen as an appropriate first round annealing temperature.

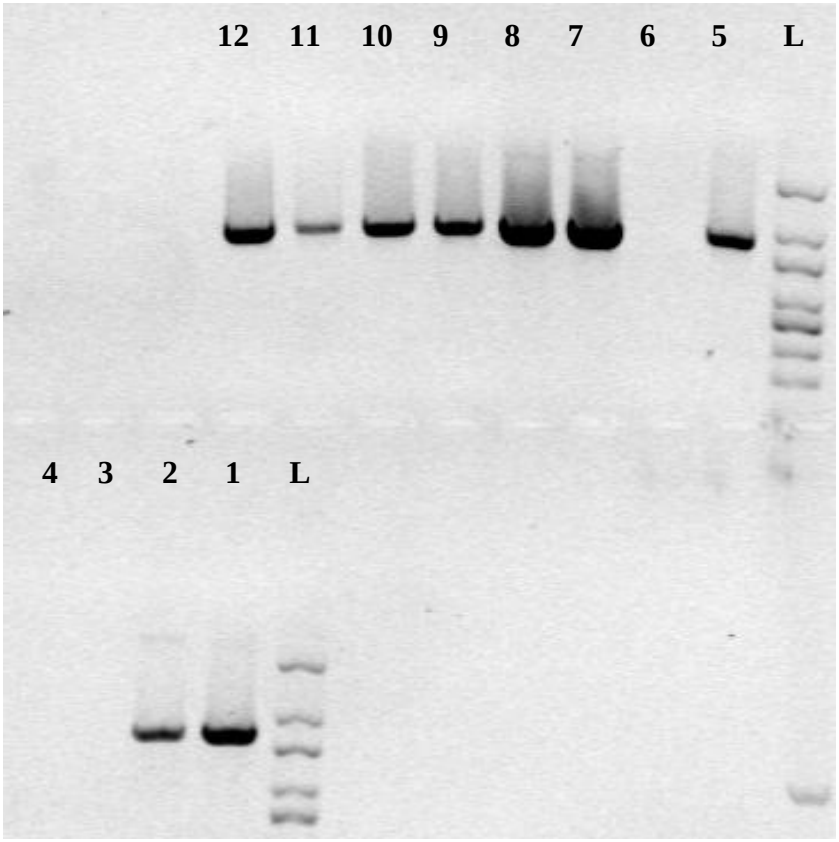


Figure 3.6 Gradient gel at two different DNA concentrations. 1-6 had 600ng/20µL of DNA added, while 7 – 12 had 50ng/20µL added. Annealing temperatures were as follows: 1 and 7: 50°C, 2 and 8: 50.9°C, 3 and 9: 52.8°C, 4 and 10: 56°C, 5 and 11: 58.5°C, 6 and 12: 59.8°C.

Hendolin et al (1997) used the annealing temperature of 66°C for their four specific primers for *A. otitidis*, *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* (Hendolin et al. 1997). The TO primer designed for this current study was able to sufficiently anneal at this temperature, however the CA primer could not. This led to the use of a gradient PCR to determine the highest temperature that CA was functional at.

The CA primer was designed using Primrose technology, and had a Tm of 52.8°C. Using 702R as the reverse primer, a gradient between 54°C and 66°C was set up to test CA primer functionality. The results are depicted in Figure 3.7. 61°C was the temperature that was decided upon for the CA primer. Non-specific activity was tested for in the other primers at this temperature, with none found. All second round nested PCRs were subsequently completed at an annealing temperature of 61°C.

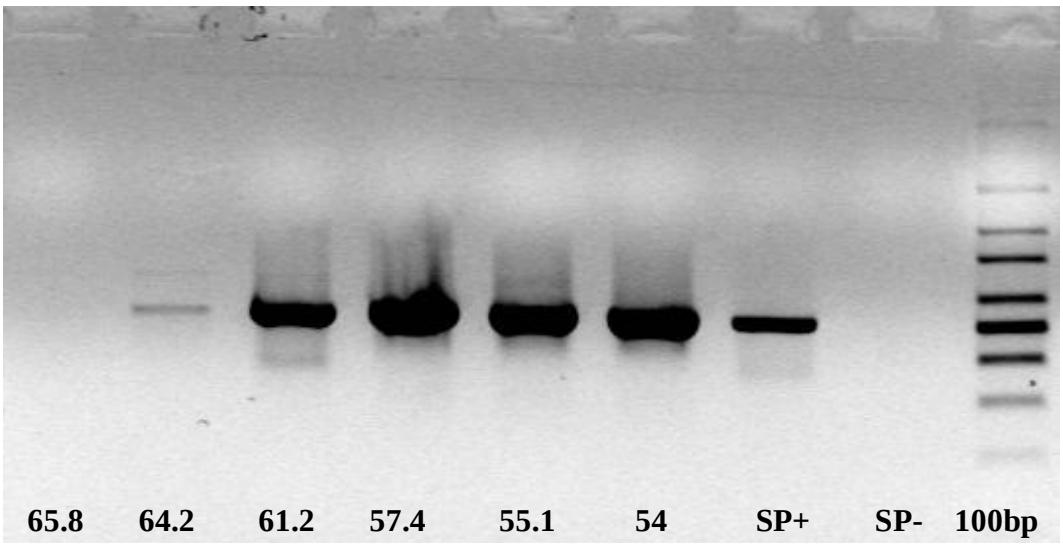


Figure 3.7 Gradient gel of *C. auris* and *S. pneumoniae*. First six lanes contain *C. auris* primer with *C. auris* DNA, annealing temperatures stated below running lane in °C. Final three lanes contain a positive and negative control using SP primer (Tm of 66°C, run at 66°C), and 100bp.

3.7 Nested PCR

3.7.1 Cycling parameters

The correct number of cycles for both the initial universal round and then the specific round was investigated. 30 cycles for each round of the nested PCR was initially used, resulting in 60 cycles all up. This large number of rounds was used to pick up on even the smallest amount of DNA of our species of interest, but led to the first round products being visible on a gel after the second round of PCR, and non-specific banding (see Figure 3.8). Therefore, the number of first round cycles was decreased to 25. With 25 cycles in the first round and 30 cycles in the second, many non-specific bands were still seen. These were only removed when the number of second round cycles was also decreased to 25 (see Figure 3.9). Specificity was further increased by the addition of a touchdown protocol to the amplification cycling.

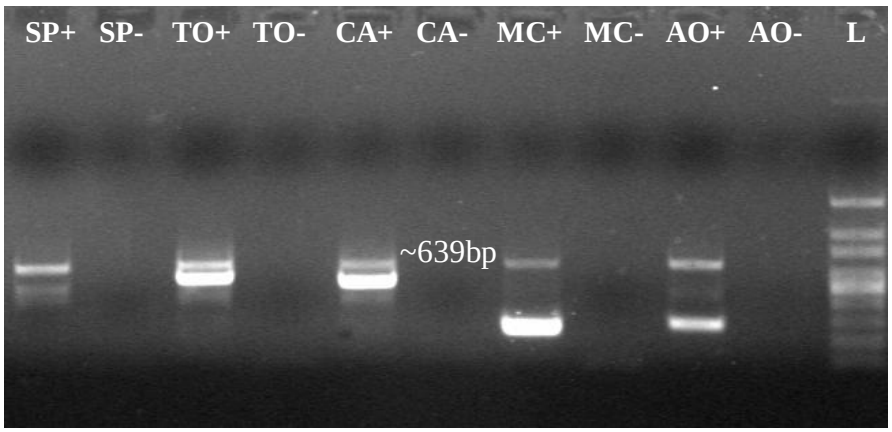


Figure 3.8 The nested PCR products after 30 rounds of amplification in the first and second cycling steps, and with the addition of 100ng/20µL of DNA. Smaller products in each reaction correspond to the species specific forward primers and 702R amplification. No bands in negatives, but products from first round primer 63f and second round reverse primer 702R clearly visible (639bp). L: 100bp ladder (Invitrogen).

3.7.2 Primer concentrations

Initially, the first round primer concentrations were the same as the concentrations used for our second round primers. This meant that often products could be seen in the gel after the second, possibly due to first round primer carry over upon transfer of first round PCR products to the second round reagents. This led to a need for a decrease in first round primer concentration.

Initial concentrations: 0.4µM of both COM2R and 63f.

Results of this can be seen in Figure 3.8.

Final concentrations: 0.1µM of COM2R and 0.05µM of 63f.

Results of this can be seen in Figure 3.9.

3.7.3 DNA concentration added to PCR

Initially, 100ng of DNA was added to the first round reactants. Unfortunately, non-specific products, possibly from first round primer carry over, were distinguishable under UV visualisation of the EtBr stained gel after the subsequent specific cycle. This complicated interpretation of results, and increased the likelihood that PCR inhibiting contaminants could influence results (as seen in Figure 3.6). This, and results seen in Figure 3.8, led to the decrease in DNA addition to 50ng/20µL, which decreased the number of non-specific bands and eased interpretation of results (Figure 3.9).

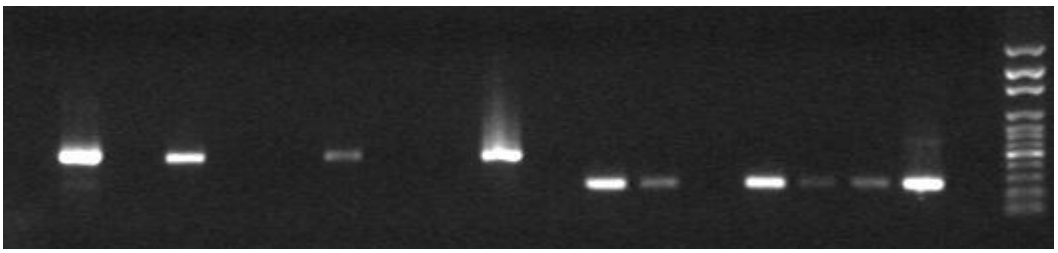


Figure 3.9 Gel of the products from final reagents and parameters of use in the nested protocol. There is only one product band after the decrease in amplification cycles, first round primer concentrations and amount of DNA added.

3.7.4 Interpretation of results

The difference between a positive and negative result was subjective. Often there were very faint bands which may represent a minute number of these bacteria in the sample, background levels of contamination, or residual first round primer products. Therefore, only clear positives were recorded as so. As such, negative results may be due to the lack of sufficient DNA templates after direct extraction, or because the DNA contained PCR inhibiting contaminants.

3.7.5 DNA quantification using gel electrophoresis

After a PCR product had been prepared for sequencing through the use of the rAPid Alkaline Phosphatase protocol, 5µL of the product was run on a 1% (w/v) TAE agarose gel to obtain a crude estimate of the concentration of DNA. Figure 3.10 is an example of how this was put to use.

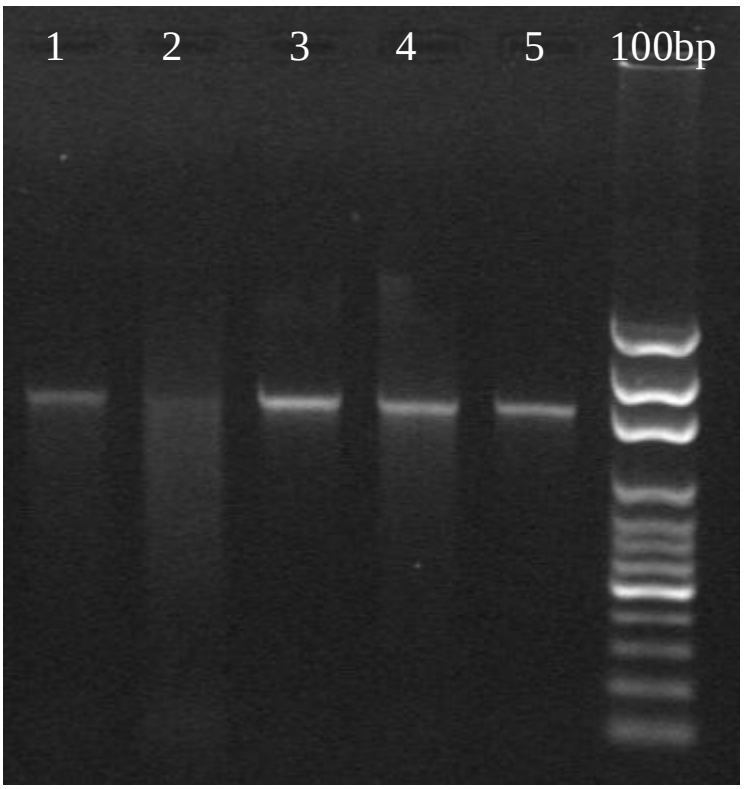


Figure 3.10 Gel of PCR products that had been subject to the rAPid Alkaline Phosphatase protocol. The band closest in size to the PCR products is 1,500bp's and 58ng/5µL. Therefore, DNA concentration estimates are as follows; 1: 15ng, 2: ~0, 3: 30ng, 4: 30ng, 5: 28ng, all /5µL.

3.8 Non-culture based results

Positive results for the bacteria of interest were indicated by the presence of a PCR product of correct size after the second specific round of PCR, as visualised by gel electrophoresis, and confirmed by the sequencing of a subset of samples. Sequencing results can be seen in Appendix V, section 3. Table 3.6 and Figure 3.11 represent the results derived from all patients in both study groups when using the direct swab extraction method and nested PCR protocol. Figure 3.12 displays results with site and group sampled on the x axis.

Table 3.6 Prevalence of bacteria of interest in all study participants, expressed as a percentage of samples analysed.

Samples	Site	*A. otitidis*	*C. auris*	*T. otitidis*	*Moraxella* spp.	*S. pneumoniae*	*H. influenzae*	Sample n.
OM	NP	9%	12%	6%	70%	24%	48%	33
Control	NP	6%	6%	3%	88%	22%	50%	32
OM	MEE	16%	9%	21%	44%	9%	51%	43
OM	OE	58%	13%	34%	33%	1%	18%	67
Control	OE	64%	14%	38%	58%	2%	30%	64

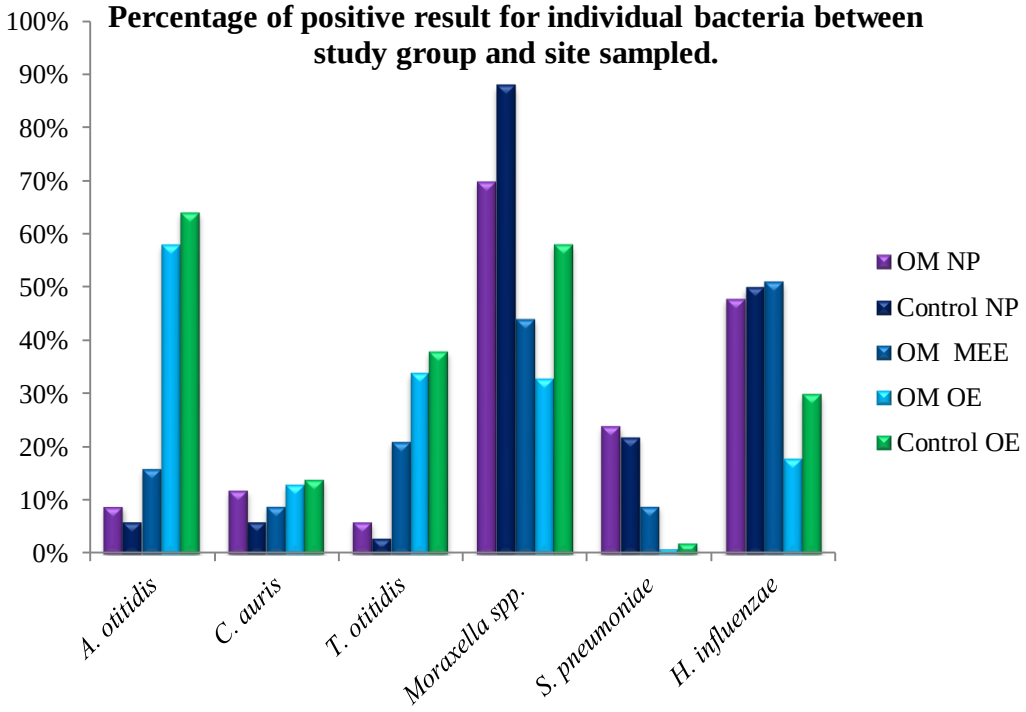


Figure 3.11 Bar graph of bacteria of interest with bacterial species on the x axis, expressed as a percentage of samples analysed. Differences between bacterial colonisation of the studied sites are more substantial than differences between study groups. *Moraxella* spp. prevalence is high across all study variables.

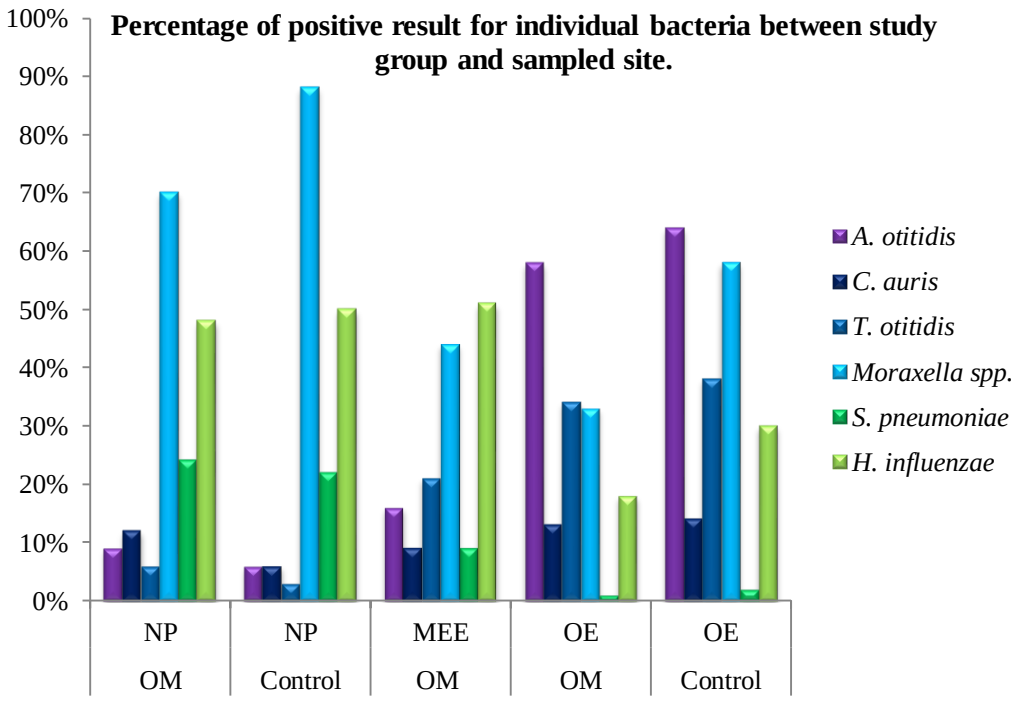


Figure 3.12 Bar graph of bacteria of interest with site and group analysed on the x axis, expressed as a percentage of samples analysed. The results between study groups (eg. NP OM vs. NP Control) are comparable, whereas between study sites (eg. NP OM vs. NP OE) many variations can be seen.

3.8.1 Statistical significance between study groups

H_0 - There is no difference in colonisation of the six bacterium of interest when comparing otitis free controls and otitis media patients.

Statistical significance between control and OM patient bacterial prevalence was calculated using the chi-squared calculation on Statistica software (p values presented in Table 3.7). Information on statistical significance calculations can be found in Appendix VI.

Table 3.7 Table of p values when comparing bacterial prevalence between study groups, in both the nasopharynx (NP) and outer ear canal (OE). Statistical significance was only seen between control and OM patients *Moraxella* spp. colonisation of the OE.

Bacteria	NP p value	OE p value
A. otitidis	0.6674	0.3903
C. auris	0.4136	0.9167
H. influenzae	0.9028	0.1129
Moraxella spp.	0.0809	0.0023
S. pneumoniae	0.8208	0.974
T. otitidis	0.5728	0.7052

The null hypothesis is rejected only in the case of OE colonisation of *Moraxella* spp..

3.8.2 Statistical significance between body sites studied

H_0 - There is no difference in colonisation of the six bacteria of interest between the outer ear canal and nasopharynx.

To compare the NP colonisation with that of the OE (which includes the left ear canal and right ear canal), only the patients with all three of those sites analysed were included in the analysis. This removed the problem of missing data in the statistical significance calculations. A positive result in the OE was counted if one or both ears had infection, and a negative if neither had the bacterium. This meant only one result was considered for the ear in each patient as is necessary for the use of the McNemar's test. 30 OM patients and 29 control patients were used in this second analysis, the bacterial prevalence in this subset of patients is presented in Table 3.8.

Table 3.8 Percentages of bacterial prevalence in the NP and OE in a subset of participants. Sample n=number of participants included in study

	A. otitidis	*C. auris*	*T. otitidis*	*Moraxella spp.*	*S. pneumoniae*	*H. influenzae*	Sample n
OM NP	10%	13%	3%	73%	27%	47%	30
Control NP	7%	3%	3%	90%	21%	48%	29
OM OE	67%	23%	43%	43%	0%	30%	30
Control OE	66%	21%	59%	69%	0%	48%	29

In this subset of participants, some variation can be seen when compared with results looking at all participants. These include the complete lack of *S. pneumoniae* in the OE in this subset, the increase in *T. otitidis* prevalence of the OE, the increase in *Moraxella* spp. in the control OE, but decrease in the OE of OM patients, and the increase in *C. auris* in the OE of both study groups. This subset was not used for statistical analysis between study groups as missing data was not a factor in those analyses.

For the analysis comparing the NP and OE colonisation within study groups, the McNemar's test for statistical significance was used. Appendix VI details how the statistical analysis was undertaken. P values are presented in Table 3.9.

Table 3.9 Statistical significance between the colonisation of each individual bacteria when comparing the NP with the OE prevalence. Study groups (control and OM patients) were considered separately. Control (CNTL) n=29, OM n=30.

Bacteria	Control OEvsNP p value	OM OEvsNP p value
A. otitidis	0.0433	0.0704
C. auris	0.0000	0.0002
T. otitidis	0.0050	0.0004
Moraxella spp.	0.0005	0.3827
S. pneumoniae	NA	NA
H. influenzae	1.0000	0.1904

The null hypothesis is rejected in the control study group with *A. otitidis*, *C. auris*, *Moraxella* spp., and *T. otitidis*. Rejection of the null hypothesis in the OM group occurs with the OE commensals *C. auris* and *T. otitidis*.

The use of NP bacterial prevalence to predict the MEE bacterial profile could not be assessed in this research. This is because only 23 OM patients had their MEEs analysed, with only 13 of these having both tested. In the NP, only 2, 3, and 2 of these 23 patients tested positive for *A. otitidis*, *C. auris*, and *T. otitidis* in one or both effusions (respectively). This is not a large enough n to use the McNemar's

test in determining the true value of NP sampling in pathogen prediction. A larger sampling population would be required.

3.8.3 Bacteria of interest prevalence results

Using p values to determine statistical significance, and prevalence percentages of all the sample sites and study groups, the following results were determined:

A. otitidis showed no statistically significant differences in colonisation between control and OM patients in either the NP (6% and 9% respectively) or the OE (64% and 58% respectively). The MEE had a higher prevalence of *A. otitidis* than the NP, but was lower than that of the OE. In the subset of participants, a statistically significant difference in *A. otitidis* colonisation between the NP and OE sites was found in the control study group (p=0.0433), but not in the OM group. This indicates that OM patients NP and OE are not dissimilar at a statistically significant level, despite the 10% vs. 67% colonisation proportion for the NP and OE respectively. Because this statistical significance is not present in the OM group, the assumption could be made that *A. otitidis* colonised the NP post-OM, as the NP has become accessible to it. This would indicate that *A. otitidis* colonises both areas in a more similar manner in children with OM.

C. auris colonisation showed no statistically significant difference when comparing controls with OM patients in either study site (NP or OE). In the subset of participants, colonisation in the control OE (21%) was more substantial than that of the control NP (3%), to a statistically significant level (p=0.000). Further, colonisation in the OM groups OE (23%) was also statistically significant over the NP (13%) (p=0.002). These results would indicate *C. auris* colonises the OE preferentially over the NP in both OM-sufferers and OM-free individuals. Substantial colonisation in the MEE by *C. auris* was not noted.

The OE was seen to host *T. otitidis* more frequently than the NP samples in both study groups. Using the subset of participants, this difference was statistically significant (p=.005, and p=.0004, respectively). Of the three OE bacteria, *T. otitidis* was present in the MEE in the highest number of patients (21%). The study groups did not show statistical significance in either body site. A further look into the NP shows it is largely absent of *T. otitidis*, but OM patient NPs did have double the prevalence of controls when looking at all participants colonisation.

Moraxella species showed statistical significance between the colonisation of OM patients and controls in the colonisation of the OE (p=.002). This is unusual as it was the control OE that had more extensive colonisation than the OE of OM patients. *M. catarrhalis* is a commensal of the NP, and *M. nonliquefaciens* of the upper respiratory tract. How this bacterium got into the control OE in high numbers is unknown. No statistical significance was seen between OM patients and controls in the NP.

Colonisation of the MEE by *Moraxella* spp. was less than that seen in the NP, but more than that seen in the OE of OM patients. In the subset of participants, statistically significant differences were found between the colonisation pattern of the control OE and NP, but not the OM patients. This indicates the OM patient colonisation by *Moraxella* spp. between body sites is more similar than the control participants. This may demonstrate the increased propensity for cross over in bacterial colonisation of OM patients due to periods of ear drum perforation or increased permeability.

S. pneumoniae was the least prevalent of the three NP pathogens in both study groups and all the body sites. In the MEE, *S. pneumoniae* was less prevalent than both *A. otitidis* and *T. otitidis*. No statistical significance was found between controls and OM patients in the sites analysed. When looking at the subset of participants, none were positive for this bacterium in the OE. This led to the inability to determine statistical significance using the McNemar's test, as no results were registered for one of the variables. However, it could be assumed that the NP is the preferred site of colonisation.

H. influenzae did not show statistically significant differences between the study groups in either body site. MEE colonisation by *H. influenzae* was the highest of all bacteria analysed, and was in a similar proportion to *H. influenzae* colonisation in the NP of both study groups. Control OE samples tested positive for *H. influenzae* more frequently than those from the OM patients (30% and 18% respectively). This difference is unexpected as this bacterium is a commensal of the NP, and no known passage between these two areas is known in OM-free children. In the participant subset, colonisation of the control OE and NP was the same. No statistically significant differences between the studied sites were seen in either study group.

3.9 The polymicrobial status of studied sites and groups within the six bacteria of interest.

Of the samples that were tested using non-culture techniques and the nested PCR protocol, a number were positive for more than one of the six bacteria of interest. This is to be expected as OM can be a polymicrobial infection. Table 3.10 details the polymicrobial status of the different sample sites and between study groups. Overall across all study sites and participant groups, 71 samples were positive for only one bacterium, compared to 128 which were positive for two or more. 40 samples were negative for all studied bacterium.

Across all study variables, positives for *Moraxella* spp. alone were regularly found. The NP of OM patients was the most regular host site of *Moraxella* spp., while the OM patient OE demonstrated the least. This bacterium was also a constituent in the other two polymicrobial populations that dominated the flora of OM patients NP. These were the *H. influenzae* and *Moraxella* spp. duo, and all three NP pathogens together. This last population was also in high proportions of control patient NPs, demonstrating the capacity for asymptomatic carriage of these three bacteria.

H. influenzae and *Moraxella* spp. appear frequently together across all sampling variables, predominantly in the NP. However, surprisingly large proportions of this duo were found in the OE also. Two interesting points to note are the lack of colonisation by *S. pneumoniae* alone, the only bacteria to fail to do so, and the high proportion of *H. influenzae* only populations in MEEs.

Of the OE samples, *A. otitidis* was found alone in high proportion of both study groups. Populations of the *A. otitidis* and *T. otitidis* duo were found only in the OE canal, with OM patients having 3.83 times the frequency of this pair compared to controls. This means OM patients are 3.83 times more likely to be colonised by these two bacteria together than OM-free controls. A similar increase can be seen in the colonisation of the *A. otitidis* and *C. auris* duo in OM patients, with a 2.87 increase in frequency when compared to the control group. Interestingly, control participants tended to have these two bacterium plus *Moraxella* species more regularly than just the two OE bacteria.

Table 3.10 Percentages of samples that showed either singular, or polymicrobial species. Percentages allow comparisons between sites. The numbers of samples that were positive for the specified bacteria are presented in brackets. Bacterial species have been abbreviated; AO, *A. otitidis*; CA, *C. auris*; TO, *T. otitidis*; HI, *H. influenzae*; MS, *Moraxella* species; SP, *S. pneumoniae*.

Bacterial species present						MEE OM	NP OM	NP Control	OE OM	OE Control
AO						0%	3.03% (1)	0%	14.93% (10)	14.06% (9)
	CA					0%	0%	0%	2.99% (2)	0%
		TO				2.33% (1)	0%	0%	2.99% (2)	1.56% (1)
			HI			20.93% (9)	9.09% (3)	3.13% (1)	1.49% (1)	0%
				MS		13.95% (6)	21.21% (7)	28.13% (9)	4.48% (3)	9.37% (6)
AO	CA					0%	0%	0%	4.48% (3)	1.56% (1)
AO		TO				0%	0%	0%	11.94% (8)	3.12% (2)
AO			HI			6.96% (3)	0%	0%	1.49% (1)	0%
AO				MS		0%	0%	6.25% (2)	4.48% (3)	12.50% (8)
AO					SP	0%	0%	0%	0%	1.56% (1)
	CA			MS		2.33% (1)	3.03% (1)	0%	0%	0%
	CA				SP	0%	3.03% (1)	0%	0%	0%
		TO	HI			0%	0%	0%	0%	1.56% (1)
		TO		MS		4.65% (2)	0%	0%	0%	3.12% (2)
			HI	MS		4.65% (2)	15.15% (5)	28.13% (9)	1.49% (1)	7.81% (5)
				MS	SP	2.33% (1)	6.06% (2)	9.38% (3)	0%	0%
AO	CA	TO				0%	0%	0%	1.49% (1)	4.69% (3)
AO	CA		HI			0%	0%	0%	0%	1.56% (1)
AO		TO	HI			0%	3.03% (1)	0%	1.49% (1)	0%
AO			HI	MS		2.33% (1)	3.03% (1)	0%	5.97% (4)	4.69% (3)
AO		TO		MS		0%	0%	0%	7.46% (5)	7.81% (5)
	CA	TO	HI			0%	0%	0%	0%	1.56% (1)
	CA		HI	MS		0%	6.06% (2)	3.13% (1)	0%	0%
	CA	TO		MS		0%	0%	0%	1.49% (1)	0%
		TO	HI	MS		4.65% (2)	0%	0%	1.49% (1)	0%
		TO		MS	SP	0%	3.03% (1)	0%	0%	0%
		TO	HI		SP	2.33% (1)	0%	0%	0%	0%
			HI	MS	SP	2.33% (1)	12.12% (4)	12.50% (4)	1.49% (1)	0%
AO		TO	HI	MS		0%	0%	0%	1.49% (1)	7.81% (5)
AO	CA	TO		MS		0%	0%	0%	1.49% (1)	0%
AO	CA	TO	HI	MS		4.65% (2)	0%	0%	1.49% (1)	4.69% (3)
AO	CA	TO	HI	MS	SP	2.33% (1)	0%	0%	0%	0%
No positives						23.26% (10)	12.12% (4)	9.38% (3)	23.88% (16)	10.93% (7)
Total number of samples						43	33	32	67	64

3.10 Culture vs. non-culture results

The NP, MEE and OE are known anatomical sites for polymicrobial populations. The six bacteria this research has studied are not the be all and end all of colonisation of these areas. The culture results further describe the species present in these sites (Section 3.2.2). Of the samples analysed by culture, only the MEEs were also able to be analysed using non-culture methods. Comparisons between these two methods in regards to the MEEs were therefore able to be made.

3 of the MEE cultures yielded no bacterial growth. The results by PCR of these samples agreed in 2 of those cases, but results for the culture negative sample from 6RM gave positives in *T. otitidis*, *S. pneumoniae*, and *H. influenzae* using non-culture techniques. 29RM, which grew a *Staphylococcus* spp, was positive for 5 of the 6 bacteria of interest using non-culture techniques. This organism had grown and taken over the plate overnight, possibly inhibiting these other species from growing. 26RM, which was positive for *S. epidermidis*, yielded positives for *T. otitidis* and *M. catarrhalis*. This means that these bacterium were present in these samples, but failed to culture for any number of reasons.

The species diversity is further reflected in the complexity of the Sau-PCR molecular profiles (see section 3.12). Culture results provide a rough indication of the organisms responsible for the banding patterns seen in the Sau-PCR fingerprints, as they further demonstrate the complex and polymicrobial nature of these sites.

3.11 *S. pneumoniae* prevalence and vaccination status

The PCV7 vaccine history and *S. pneumoniae* colonisations of participants was assessed. Of the 78 children involved in this study, 27 had complete PCV7 immunizations, 4 had incomplete vaccinations, while 47 were not vaccinated. Children with incomplete vaccination profiles were excluded from the analysis.

2/12 (16%) vaccinated OM patients had *S. pneumoniae* in their NP (with one of these also having it in their MEE), whilst 4/19 (21%) of non-vaccinated OM patients were positive for the bacteria (see Table 3.11). This demonstrates a slight decrease in the prevalence of *S. pneumoniae* in vaccinated patients. In addition, none of the OEs of vaccinated OM patients were positive for *S. pneumoniae*,

while 2% of non-vaccinated participants tested positive. Contrasting results to these were found when looking at the MEE data.

Table 3.11 PCV7 vaccination status in patients with OM, and the presence or absence of *S. pneumoniae* in the respective body sites.

OM Patients	*S. pneumoniae*			%+ve
OM OE	Present	Absent	Total	
Vaccinated	0	21	21	0%
Non-vaccinated	1	41	42	2%
Total	1	62	63	
OM NP	Present	Absent	Total	
Vaccinated	2	10	12	16%
Non-vaccinated	4	15	19	21%
Total	6	25	31	
OM MEE	Present	Absent	Total	
Vaccinated	2	6	8	25%
Non-vaccinated	2	29	31	6%
Total	4	35	39	

2/8 (25%) vaccinated OM patients tested positive for this bacterium in their MEE, while 2/31 (6%) of non-vaccinated OM patients were positive. This would suggest an increased likelihood of *S. pneumoniae* colonisation in the MEE when vaccinated against it, however, the limited number of vaccinated OM patients who also had their MEE tested limits these results. More vaccinated participants would need to be tested to confirm this. Data on serotype or antibiotic resistance profiles were not completed.

6/13 (46%) vaccinated control patients tested positive for *S. pneumoniae* in their NP. All but one of the control patients who tested positive for *S. pneumoniae* in this site were vaccinated against it (see Table 3.12). This may indicate the replacement of *S. pneumoniae* serotypes in the NP of control patients. Serotypical information on the positive results would need to be undertaken to confirm this statement.

Table 3.12 PCV7 vaccination status in control participants, and the presence or absence of *S. pneumoniae* in the respective body sites.

Control	*S. pneumoniae*			
Control OE	Present	Absent	Total	%+ve
Vaccinated	1	23	24	4%
Non-vaccinated	0	36	36	0%
Total	1	59	60	
Control NP	Present	Absent		
Vaccinated	6	7	13	46%
Non-vaccinated	1	16	17	5%
Total	7	23	31	

The numbers of participants who had been vaccinated were markedly lower than those who had not been. This led to a smaller number of results for the vaccinated group, possibly limiting the strength of these results.

3.12 Sau-PCR Microbial Community Assessment

Genomic DNA was digested with either SauAI3 or MboI restriction enzymes. Subsequent amplification cycles with Sau primers yielded banding patterns representative of the number of GATC cut sites plus an additional dinucleotide or single nucleotide (e.g. SauC primer elongates those with GATCC) present throughout the digested bacterial DNA. Comparisons between the fingerprint of different samples gives an indication of similarities and/or differences in bacterial diversity. SauC and SauA primers were used most extensively. Figure 3.13 demonstrates the difference between the two primers on the same DNA from mixed cultures. The SauC primer yielded a larger number of bands with reasonable product yields (as approximated by band brightness) and was therefore used the most.

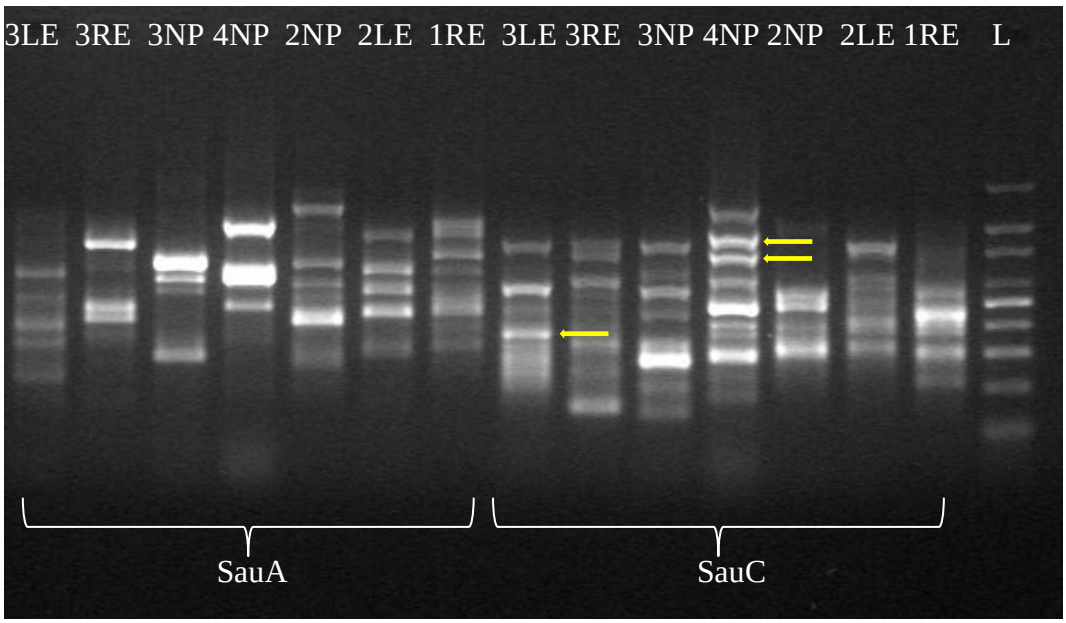


Figure 3.13 Banding patterns from Sau-PCR using both SauA and SauC primers. DNA used was from cultured organisms of both NP and OE samples from OM patients. L = 100 base pair ladder (Invitrogen). Yellow arrows indicate the bands that are of the same size as those seen in the fingerprint of the isolated organisms of interest.

Isolated cultures of the six species of interest were digested to determine banding patterns (see Figure 3.14). Three bands appeared quite regularly throughout these profiles. These were approximately 500, 390, and 260 base pairs (bps) in size and may represent a stretch of DNA with a large copy number. *A. otitidis* had a much brighter band at 260bps than the other samples, as did *C. auris* at 390bps. *H. influenzae* had two distinctive large bands at approximately 900bps and 780bps, which the others lacked. Similar bands as those mentioned above can be seen in the SauC profiles of the cultured samples of 4NP (*H. influenzae* large bands) and 3LE (*C. auris* small band) in Figure 3.13. This may indicate the presence of these bacteria in those samples; however this is was not confirmed.

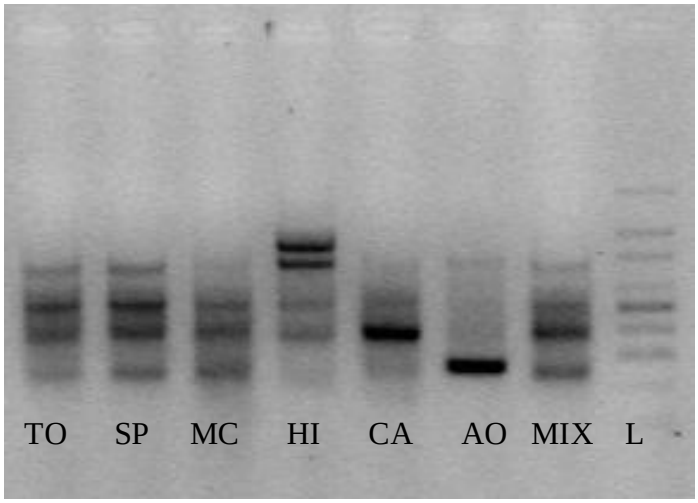


Figure 3.14 Sau-PCR of DNA from cultured isolates of bacteria of interest using SauC primer. L = 100bp ladder (Invitrogen).

Fingerprinting profiles of DNA from mixed culture yielded different profiles than that of DNA from direct swab extraction of the same site (Figure 3.13 vs. Figure 3.15). Culture banding profiles appear indicative of a larger amount of DNA from fewer culturable organisms (Figure 3.13). DNA from the direct extraction procedure is representative of more organisms but with less DNA from each to digest (Figure 3.15). Therefore the brightness of the bands (DNA yield) and number of bands is decreased. Furthermore, some digested DNA may not be amplified to a sufficient level for visualisation. Therefore, samples from culture are indicative of a smaller number of species, but have more significant DNA amplification.

In an example of Sau-PCR banding patterns potential use in identifying organisms present in mixed samples, the ~260bp band present in the *A. otitidis* banding profile is present in all the OE fingerprints presented in Figure 3.15. This could indicate the presence of this bacterium in these samples, and indeed all three of these OE samples were positive for *A. otitidis* using the nested PCR protocol (Appendix IV contains the raw data from all participants). Other bacteria had less unique profiles, so assumptions on their presence could not be made as easily.

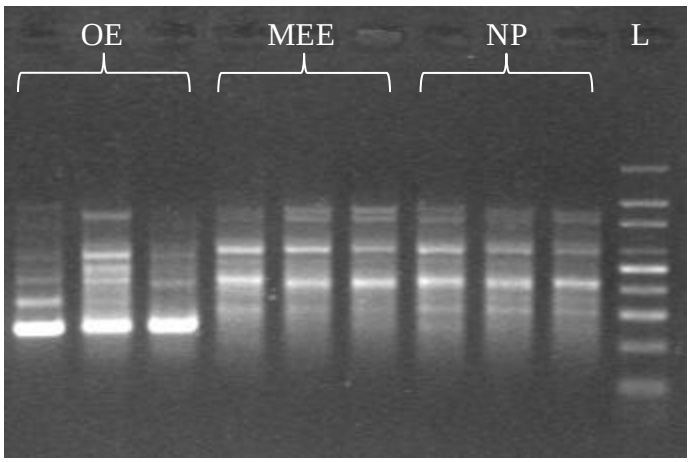


Figure 3.15 Sau-PCR results using SauC primer with DNA extracted directly from OM patient swabs of the NP, OE and MEE. Sample order is as follows: N, 28LE, 17RE, 4LE, 6RM, 27RM, 21LM, 12NP, 16NP, 14NP, L = 100bp ladder (Invitrogen).

Looking at the direct swab extraction DNA digests in Figure 3.15, fingerprints from MEEs resembled each other quite closely, as did samples from the NP. Furthermore, the resemblance continued between these two sites, but did not extend to the OE fingerprint profile.

More differences were seen between the Sau-PCR profiles of control patients when comparing DNA from the OE and the NP, than were seen between the same sites in OM patients (Figure 3.15 vs. Figure 3.16). These differences may be a reflection of the antibiotic treatments the OM group had previously been administered. However, a complete antibiotic profile would be necessary to substantiate this hypothesis.

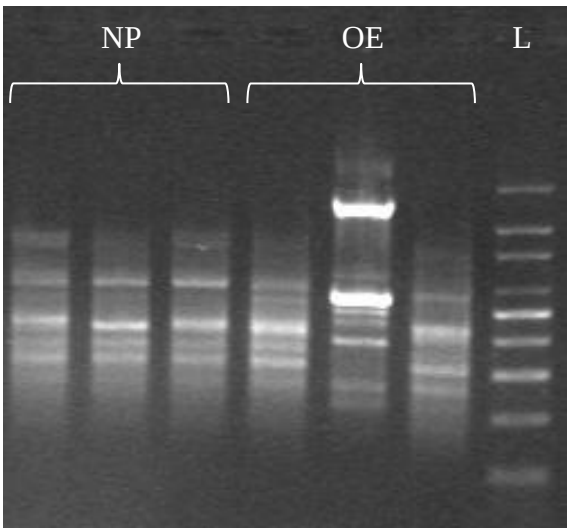


Figure 3.16 Sau-PCR banding pattern using SauC primer with DNA from direct DNA extraction from control samples of the NP and OE. Sample order is as follows: 8CNP, 12CNP, 23CNP, 23CLE, 24CLE, 3CLE. L = 100bp ladder (Invitrogen).

Sau-PCR profiles using DNA from direct extraction may have contained human DNA in unknown quantities. As such, some of the bands present in the banding pattern may be representative of the human participant DNA, and not be microbiota related.

More specific identification of the most prevalent species is theoretically possible through cloning of the DNA products of the highest yield, however was not achieved in this study. The inability to clone the Sau-PCR products meant attempts to quickly and efficiently identify dominant species in the sample was unable to be completed.

Specific highly sequenced genes with a large number of alignments in public databases (such as 16S) were not selected for before the Sau-PCR digest. This whole genome approach provided more information when comparing the fingerprints of the organisms. However, the brightest bands may have been from a DNA sequence not yet covered extensively in databases such as BLAST. This is a possibility for why no matches were found for the cloned DNA sequence.

Chapter 4: Discussion

4.1 Previous findings on bacterial prevalence (culture and non-culture techniques)

The prevalence of *A. otitidis* and the three NP pathogens has been documented in OM patients. One study looked at the prevalence of *H. influenzae*, *S. pneumoniae*, *A. otitidis*, and *M. catarrhalis* in the NP and MEE of OME patients who were undergoing ventilation tube insertion (Takada et al. 2003). Table 4.1 demonstrates their results when using both culture and non-culture based techniques. *A. otitidis* failed to culture, however was detected in 28.9% of MEEs and 10.7% of NP specimens using non-culture techniques. This reinforces the use of culture-independent techniques when studying slow growing organisms, such as *A. otitidis*, *T. otitidis*, and *C. auris*.

Table 4.1 Table of prevalence of bacteria expressed as a percentage of total samples (Takada et al. 2003).

Bacteria	MEE %	NP %
S. pneumoniae	4.8%	8.9%
H. influenzae	7.2%	12.0%
M. catarrhalis	3.6%	30.4%
A. otitidis	28.9%	10.7%

It is interesting to note that the proportion of positives for *A. otitidis* in the MEE is higher than those positive in the NP. This would indicate that the source of *A. otitidis* for these infections would be elsewhere (Takada et al. 2003). *M. catarrhalis* was the most prevalent bacteria in the NP; however had the least prevalence in the MEE.

Other studies have also found *A. otitidis* in high proportions of MEEs. Guvenc et al (2010) used culture techniques and found *A. otitidis* more frequently than other major nasopharyngeal pathogens in MEEs (Guvenc et al. 2010). Another study cultured *A. otitidis* from 48.27% of MEEs, providing further evidence that this bacterium is present in the middle ear cavity during episodes of OM (Martinez & Macias 2008). Both studies looked at patients with a more chronic form of infection (OME), which *A. otitidis* has been suggested to play more of a role in (Leskinen et al. 2002).

Harimaya et al (2006) looked at the prevalence of *A. otitidis* using culture-independent techniques, and compared study groups of otitis-prone and non-otitis-prone children (all participants were OM sufferers) (Harimaya et al. 2006). Of the MEE samples from non-otitis-prone children, 13.8% were positive for *A. otitidis*, while 64% of otitis prone children were positive. This difference in colonisation showed statistical significance ($p<0.001$). In addition, *A. otitidis* was found in higher proportions of NP specimens from the otitis prone children (29.4%) when compared to non-otitis prone (2.6%). This difference also showed statistical significance ($p<0.001$). Results such as these further provide evidence for the potential role of *A. otitidis* in the chronicity of OM.

Looking at the differences between OM-free controls and OM patients, the colonisation of the NP and MEE of Turkish children has been compared (Eser et al. 2009). A 2-fold increase was found when looking at the NP colonisation of *S. pneumoniae* and *H. influenzae* between the two study groups. This difference was not found to be statistically significant. Furthermore, the presence of bacterial pathogens in the NP was not indicative of the pathogens presence in the child's MEE (Eser et al. 2009).

Looking at healthy individuals, the prevalence of bacterial species in 70 volunteers between the ages of 19 and 22 has been assessed using culture independent techniques (De Baere et al. 2009). Nasopharyngeal pathogens were found in the NP at a rate of 14% for *H. influenzae*, 34% for *M. catarrhalis* and 9% for *S. pneumoniae*. This study reports that *A. otitidis* was completely absent from the NP, while OE positives for this bacterium were detected in 83% of samples.

C. auris and *T. otitidis* were not targeted in the non-culture techniques of this past study. However, in a cultured subset of 10 OE samples, 5 grew *T. otitidis*, while 2 grew *C. auris* (De Baere et al. 2009). 9 of the 10 cultured samples grew ≥2 species, with the one exception being a pure culture of *A. otitidis*. This further cements the polymicrobial status of these anatomical sites.

In addition, culture independent research has found no NP pathogens in the OE of 24 healthy individuals (predominantly adults) (Frank et al. 2003). This disagrees with the study by De Baere et al (2009) who report a carriage rate in the OE of 6% for *H. influenzae* (De Baere et al. 2009). As previously referred to in the introductory sections, Frank et al (2003) found the OE commensal bacteria *A.*

otitidis, *T. otitidis*, *S. auricularis*, and *C. auris* to be the four most prevalent species of the OE (Frank et al. 2003).

Comparisons between culture and PCR based results demonstrate the increased sensitivity of the latter. Evidence provided by De Baere et al (2009) showed that out of 10 samples, 9 tested positive by PCR for *A. otitidis*, while only 5 of these were positive when using culture (De Baere et al. 2009). Further use of culture techniques have shown *T. otitidis* and *C. auris* to be the two most prevalent coryneforms from the OE and the cerumen of healthy adults, with *A. otitidis* the third most prevalent bacteria overall (Stroman et al. 2001). Due to the high frequency of positives, these authors considered this to demonstrate that these bacteria are part of the normal OE flora.

Organisms hypothesised to play an 'interfering' role in the colonisation of OM pathogens have been observed using metagenomic non-culture based techniques. These bacteria include the genus' *Corynebacterium* and *Dolosigranulum* who, when present, are thought to compete with pathogenic species for colonisation, possibly leading to a decrease in OM frequency (Laufer et al. 2011).

Looking specifically at *T. otitidis* colonisation in OM sufferers, 153 MEEs from 112 patients were cultured and assessed for presence of this bacterium. 9 exudates tested positive from 7 different patients (Gomez-Garces et al. 2004). 5 of these were isolated from patients who had a well-documented history of infection, and predominantly had a visible perforation of the ear drum.

Other bacteria isolated in this past study include all three of the known OM pathogens studied here (proportions not published), *S. aureus, Pseudomonas aeruginosa* and enterobacteria (Gomez-Garces et al. 2004). In addition, cultured bacteria from a recent study found commensal bacteria of the skin *Staphylococcus auricularis* and *Staphylococcus epidermidis* in the NP of sufferers of Chronic Suppurative OM (CSOM) (Chang et al. 2011).

Earlier investigations into the bacterial prevalence in New Zealand children with OME include the study of a group of Auckland children in the 1990s using culture techniques on 105 MEEs (67 patients) (Watson et al. 1996). Specifically looking at nasopharyngeal pathogens, *H. influenzae* was found in 16% of MEEs, *S. pneumoniae* in 8%, and *M. catarrhalis* in 12% (Watson et al. 1996).

Further research in New Zealand looking at four of the bacteria of interest found the following percentages of positives in 98 MEE samples: 81% for *A. otitidis*, 35% for *S. pneumoniae*, 18% for *H. influenzae*, and 27.5% for *M. catarrhalis* (Cecire et al. 2009). This New Zealand study went further to explain the polymicrobial nature of OM infections (Cecire et al. 2009). Of the 98 participants, 41% tested positive for one of the four bacteria they studied, 29% for two, 16% for three and 4% for all four. The majority therefore were positive for more than one species.

4.2 Comparisons with results of this study

4.2.1 Cultured species

The cultured species of this present study have previously been associated with the studied sites (Bogaert et al. 2011). Both *Dolosigranulum pigrum* and *Corynebacterium pseudodiphtheriticum* have been found in the URT of children suffering from an URTI. Results from this current study found *D. pigrum* in both of the control NP samples that underwent culture analysis, and *C. pseudodiphtheriticum* in one of these. Only one of the NP samples from OM patients tested positive for *Dolosigranulum* spp.. Laufer et al (2011) linked the colonisation of these bacteria to a decreased risk in developing OM (Laufer et al. 2011). Low numbers of cultured samples limit the ability of this current research to provide evidence towards this.

Unlike the study by Takada et al (2003), *A. otitidis* was the most regularly isolated bacterium through culture in this current study (Takada et al. 2003). This difference in cultivation could be because of the long incubation period used here, however the previous study does not state the length of their incubation for comparison. Five OE samples tested positive for this slow growing organism, as did one MEE across both the control and OM study groups. No obvious trend between colonisation of the study groups was observed in this organism.

Staphylococcus species were cultured from samples of the OE canal, in both the control and the OM patient samples. This is in agreement with the results from a 2003 study, which found these same bacteria in the OE of healthy adults, providing evidence that they are commensals of this anatomical site (Frank et al. 2003). Further, *S. epidermidis* and *S. auricularis* have been noted in the NP of sufferers of CSOM (Chang et al. 2011). The evidence put forward by Chang et al (2011) may indicate that these *Staphylococci* become opportunistic pathogens

after a primary ear infection (Chang et al. 2011). As none of these species were cultured in the NP of OM individuals in this current research, results here cannot substantiate this hypothesis.

This limited number of cultured samples gives us an indication of the bacteria found in the study sites beyond those of the six bacteria of interest. Indicating the microbial flora of New Zealand children agrees with the flora isolated in varying locations worldwide (Bogaert et al. 2011; Laufer et al. 2011). Further research using a larger number of cultured samples would provide further information into the extended number of species present. Antibiotic susceptibility profiles, which can only be conducted on cultured organisms, may further prove useful in determining future treatments of OM.

4.2.2 Bacterial prevalence

The results of this study show a number of differences to those from a previous culture based study (Takada et al. 2003). Despite the larger number of participants in the previous study, this difference in results may demonstrate the increased efficiency of DNA-based techniques in identifying small populations of non-culturable species in polymicrobial samples. It could also indicate the possibly for differences between global location, as the previous study was conducted in Japan (Takada et al. 2003).

The difference in *A. otitidis* prevalence between the anatomical sites, in this and previous studies, would suggest the bacterium colonises the MEE from the population in the OE, rather than the NP (Takada et al. 2003). 16% of MEEs tested in this current study were positive for *A. otitidis*. This is a larger proportion than were positive in the NP (9%). The higher prevalence in the MEE could be from OE contamination. Although due to the agreement with previous studies, this may be a true result (Takada et al. 2003).

Previously, non-otitis prone children with OM have shown positives for *A. otitidis* in 13.8% of children's MEE, a similar result to this study (Harimaya et al. 2006). This past study also found *A. otitidis* in similarly low proportions of OM patient NPs, with a statistically significant difference between *A. otitidis* colonisation in otitis-prone and non-otitis prone children ($p<0.001$). This current study found no such correlation between OM patients and OM-free controls as, despite the controls almost never having had an episode of OM, a 6% positive rate of

colonisation was found in the NP. This disagrees with the results from De Baere et al (2010), who found no OE commensal bacteria in the NP of healthy individuals (De Baere et al. 2009).

In comparison to the three NP pathogens, the number of positives for *A. otitidis* in the MEE was only higher than *S. pneumoniae*. This disagrees with the results seen in the study by Guvenc et al (2010) who found *A. otitidis* in a higher proportion than all three of the nasopharyngeal pathogens studied here (Guvenc et al. 2010). Furthermore, a previous study found *A. otitidis* in 48.27% of cultured MEEs from Spanish patients suffering from OME (Martinez & Macias 2008). This is much higher than the 16% of non-culture based positives this current research found. Potential reasons behind this discrepancy include: the potential for global differences; the possibility that the DNA extraction technique was not as efficient on bacteria present in a mucosal layer/biofilm.

C. auris colonisation showed no significant difference in colonisation between control and OM patients. When looking at all patients, the prevalence appeared to be similarly low between all study sites and groups. However, statistically significant differences were seen in the subset of participants used to compare the OE colonisation with that of the NP. Substantial colonisation in the MEE by *C. auris* did not appear to be present, with 9% of MEE showing positive results. This is the same proportion, but not the same patients, as *S. pneumoniae*.

At 21%, *T. otitidis* had the highest prevalence in the MEE of all the OE bacteria. This is much more substantial colonisation than that seen in the NP with positive results in 3% of control NP, and 6% of OM patients (not statistically significant). The OE was seen to host *T. otitidis* more frequently than the NP, with statistically significant differences in between these anatomical sites in both the controls ($p= 0.0050$) and OM patients ($p=0.0004$). With these results, it could be concluded the *T. otitidis* in the MEE originated from the OE and may be an example of this OE commensal acting as an opportunistic pathogen during a primary episode of OM.

T. otitidis was positive in the OE of 38% and 34% of controls and OM patients, respectively. Stroman et al (2001), who looked at the bacteria colonising the healthy OE of adults, found both *T. otitidis* and *C. auris* to colonise at much higher proportions than in this current research (Stroman et al. 2001). This discrepancy may be due to the older age of the participants in the earlier study, and may provide evidence against their pathogenicity.

S. pneumoniae has a lowered carriage rate in adults. This is thought to be because of an immune response against it (Garcia-Rodriguez & Martinez 2002). *T. otitidis* and *C. auris* have a higher carriage rate in adults, as seen when comparing the results of this study to those of previous research on adults (Stroman et al. 2001). This may indicate there is no immune response against colonisation of these bacteria, and therefore they may simply be part of the normal flora. At present, no research has been published on the immune response against these organisms. Further research into this prospect is necessary for substantiation.

Gomez-Garces et al. (2004) has previously cultured *T. otitidis* in 9 out of 153 MEEs (Gomez-Garces et al. 2004). This is a much lower proportion than the 21% of positives recorded here. Furthermore, this current research found *T. otitidis* in the MEE more often than *A. otitidis*. This is unexpected as *A. otitidis* has been known to be the sole bacteria present in high proportions of MEEs, while *T. otitidis* is not (Martinez & Macias 2008).

De Baere et al (2009) found *H. influenzae, A. otitidis, M. catarrhalis*, and *S. pneumoniae* in lower proportions in the healthy NP than were found in this current research, although the order of prevalence in their results agrees with this present study (De Baere et al. 2009). These authors had similar trouble with multiplex PCR optimization and therefore also separated their species specific reactions.

M. catarrhalis has previously been recorded as the most prevalent of the three nasopharyngeal pathogens in the NP (Takada et al. 2003). The results of this current study agree with this notion, showing *Moraxella* spp. in high proportions of NP samples. Further, this study found a high colonisation rate of the NP in both OM patients and OM-free controls, indicating just as many children are colonised by this bacterium asymptomatically, as symptomatically.

H. influenzae demonstrated no significant differences when comparing the study groups. This is in contrast to the study by Eser et al (2009) which found twice the numbers of both *H. influenzae* and *S. pneumoniae* in patients with OME than infection free controls (not statistically significant) (Eser et al. 2009). Whether this is of biological significance remains to be seen.

H. influenzae was more prevalent in the NP than the OE in both study groups. This agrees with the high nasopharyngeal carriage rate of this organism through-

out the globe (Garcia-Rodriguez & Martinez 2002). Surprisingly, increased positives were found in the control group OEs when compared to the OM patient OEs, although not to a statistically significant level. Further, *Moraxella* spp. results showed statistically significant differences between the colonisation of control and OM patients in the OE ($p=0.0023$). This was the only difference between the control and OM patient groups to reach statistical significance but, surprisingly, it was the control OE that demonstrated the larger number of positive. These results are highly unexpected as these bacteria are significantly linked to episodes of OM, which are absent in the control group.

Theoretically, an increase in the amount of *Moraxella* spp. and *H. influenzae* in the OE would be expected in OM patients, as they may experience periods of perforation or increased permeability of the ear drum during inflammation. Both of which could allow bacteria in the MEE a passage into the OE. This passage should be absent in control participants.

Further, a previous study looking at the OE of healthy volunteers found no NP pathogens (Frank et al. 2003), whilst another found *H. influenzae* in much smaller proportions than reported here (De Baere et al. 2009). This discrepancy between results could be explained by the older age of the previous studies participants. The current research found the majority of healthy OE samples positive for *Moraxella* species, and 30% positive for *H. influenzae*, which may indicate these species are commensal bacteria of the OE as well as the NP.

Of all six bacteria, *H. influenzae* gave the most positives in the MEE at 51%. This is in contrast to a previous study which found *A. otitidis* to be the most prevalent species of the MEE by culture (Takada et al. 2003). This result would indicate that *H. influenzae* has played a role in over half the episodes of infection in the New Zealand children involved in this study.

The culture-independent results of this study show the majority of bacteria in higher proportions than those in a past study of New Zealand children. *S. pneumoniae* colonisation was the only exception, in which only 9% of MEEs were positive in this study, compared to 8% of total samples in the previous study (Watson et al. 1996). *S. pneumoniae* had the lowest colonisation percentage of the three known pathogens. This decrease could indicate the efficacy of the immunisation protocol. However, the immunization results reported here, and in

other studies, would suggest immunisation has not played a significant role in this decrease (Kuo et al. 2011).

A study of the Australian Aboriginal population also noted the absence in *S. pneumoniae* colonisation (Stuart et al. 2003). Stuart et al (2003) analysed their samples using culture techniques, and thought the decreased numbers of *S. pneumoniae* may have been due to antibiotic use. The use of non-culture based techniques in this current study was intended to minimise the influence of previously used antibiotics on results. Whether this was successful is unknown.

4.2.3 Polymicrobial infections

The polymicrobial nature of OM has been well documented. Results of a previous study looking at the polymicrobial nature of MEEs in New Zealand children contrast with the results reported here (Cecire et al. 2009). Cecire et al (2009) found *A. otitidis* in the majority of samples, and the NP pathogens alone in very few. This current study found 21% of MEE had *H. influenzae* alone, and 21% had *Moraxella spp.* alone.

Further, the previous study found *S. pneumoniae* colonisation either as the sole pathogenic agent, or with *A. otitidis*, to be higher than that of both other NP bacteria. This is in direct contrast to the current study, which found no instances of *S. pneumoniae* colonising without one or both of the other NP bacteria and no colonisation of the *A. otitidis* and *S. pneumoniae* duo in the MEE. It is important to note that the primer used for *M. catarrhalis* in the previous study was the same as used for this current research. Therefore, these previous results, specified as *M. catarrhalis*, identified this genus with the same specificity as this research.

In vitro, it has been observed that *H. influenzae* and *Moraxella* spp. can co-exist in a mutually beneficial manner (Armbruster et al. 2010). This polymicrobial combination was the most observed of the control NP samples, and the second equal most observed in the NP of OM patients, after *Moraxella* spp. alone. Further, all sampled sites and groups had at least one patient with this polymicrobial status. This suggests these bacteria do co-exist readily. However, associations with OM cannot be made based on these results as the control NP was populated at close to double the frequency of the NP of OM patients.

4.2.4 Limitations to non-culture based results

Small numbers of participants, lack of antibiotic resistance profiles, no information on viral prevalence, or the presence of a biofilm, create limitations to the results of this current research. Further, the extraction technique was not tested for its efficiency on middle ear effusions, and therefore may have been biased or less efficient at lysing bacteria in a mucus or biofilm layer.

Results may have been influenced by the necessity to freeze the samples before analysis. Samples were frozen for storage purposes, and if they had been extracted or cultured shortly after collection, results may have varied. The true impact of this storage in the samples is unknown.

The small number of cultured samples made the strength of statistical significance calculations in comparing culture and non-culture based techniques for bacterial identification pointless, and therefore these calculations were not completed.

Some studies have looked specifically at a type of OM, for example, Ashhurst-Smith et al (2007) looked specifically at children with OME in relation to *A. otitidis* prevalence (Ashhurst-Smith et al. 2007). This current study combined a number of different forms of OM, and not just the chronic or the acute forms separately, when comparing to OM-free controls. This could have had some influence on the results.

4.3 Previous findings on *S. pneumoniae* prevalence and vaccination status

A modest 6-7% decrease in OM episodes has been seen in children vaccinated with PCV7 (Binks et al. 2011). A recent study by Laufer et al (2011) used Roche/454 Life Science pyrosequencing to take an in-depth look into the bacterial colonisation of the NP in children with an URTI (Laufer et al. 2011). 43.5% of the children in their study population were culture positive for *S. pneumoniae*, despite 88% of these children having complete PCV7 vaccination status. Data from the proportion of the immunised children that were positive for *S. pneumoniae* was not presented.

A study of 292 patients in the USA found no statistically significant differences between the *S. pneumoniae* colonisation and vaccination status in children, though a slight decrease in colonisation rate was present in vaccinated children (Poetker

et al. 2005). In this study, which utilized culture techniques, 4% of vaccinated children were positive for *S. pneumoniae* colonisation of the MEE, while positives were found in 7.1% of non-vaccinated children (all children were OM sufferers). The same trend was found in colonisation of the OE (2.5% vs. 6.5%, respectively).

4.3.1 Comparisons with PCV7 vaccination and *S. pneumoniae* prevalence in this study

14 control participants and 13 OM patients were fully vaccinated. This represents a modest decrease in the number of children vaccinated who have OM, as 38% of controls were vaccinated, compared to 30% of OM patients. This is slightly higher than the 6-7% decrease of OM episodes previously reported in vaccinated children (Binks et al. 2011).

The limited influence the PCV7 vaccination seems to have had on *S. pneumoniae* colonisation, and in preventing OM, is in agreement with the results of past studies (Binks et al. 2011; Laufer et al. 2011). Episodes of OM due to non-vaccinated serotypes, and other pathogenic species, have increased since the introduction of the PCV7 vaccine (Vergison 2008). Serotypical information from the positive results in this current research was not collected.

This current research found *S. pneumoniae* present in 16% of vaccinated NPs of patients with OM, and 21% of non-vaccinated OM patients. This slight decrease in vaccinated participants agrees with previous results from the OE and MEE (Poetker et al. 2005). However, when looking at the MEE of the OM patient group, 25% of vaccinated patients tested positive, compared to 6% of non-vaccinated patients. The results from the control study group also suggest a positive relationship between vaccination and *S. pneumoniae* colonisation, with 46% of vaccinated control NPs being positive, compared to 5% of non-vaccinated.

The patient numbers involved in this current study are not substantial enough to determine the true effects of the PCV7 vaccine throughout the New Zealand population. Therefore, statistical significance between patients of different vaccination status was not determined. Further research would be necessary to determine the true effect vaccination has had on the pneumococcal status of New Zealand children.

4.4 Sau-PCR

Sau-PCR has never been completed on samples of the OE, NP or MEE before. Comparisons to previous results are therefore unable to be made. Furthermore, the paper by Corich et al (2005) used this technique on cultured isolates of bacteria, none of which having consequence to this study (Corich et al. 2005).

Corich et al (2005) suggests use of the Sau-PCR protocol on virtually any culturable organism (Corich et al. 2005). This current study demonstrates the efficacy of use on mixed environmental samples not from culture. The potential for organism identification through comparisons of unique banding patterns is also not to be overlooked.

4.5 Conclusions

Moraxella spp. colonised the OE of control participants more regularly than the OE of OM patients (58% vs. 33%). This difference was found to be statistically significant (p= 0.0023). *H. influenzae* also demonstrated a number of positives in the OE of control participants. How these bacteria got into the OE in a large number of participants without an episode of OM is unknown.

Differences between the colonisation of the outer ear canal and nasopharyngeal body sites were found at a statistically significant level in control participants with *A. otitidis* (p=0.0433), *C. auris* (p=0.0000), *T. otitidis* (p=0.0050) and *Moraxella* spp. (p=0.0005). Of the same body sites in otitis media patients, only the colonisation of *C. auris* (p=0.0002) and *T. otitidis* (p=0.0004) were found to be statistically significant. As the OM group displays less statistically significant differences in colonisation between anatomical sites, it could be assume there is an increased likelihood for bacteria to get into the OE from the NP or vice versa during episodes of OM.

The colonisation of the NP by the three OE commensals is low in both study groups. Statistically significant differences between the OE and NP can be seen in both study groups for all three bacteria, except *A. otitidis* in the OM group. This may demonstrate the increased propensity for its establishment in the NP after an episode of OM, as this site has become accessible to it.

H. influenzae and *Moraxella* species were detected the most of all species in the MEE. *S. pneumoniae* had the lowest prevalence of all the nasopharyngeal

pathogens in all sites tested. Vaccination status does not appear to be an explanation for this.

Culture results yielded little information of the six species of interest. Commensal bacteria of the upper respiratory tract, some with the potential for limiting OM occurrence were cultured. These results indicate that New Zealand children have similar colonisation to those of worldwide studies.

Sau-PCR results indicated the similarities and differences between the body sites in terms of species diversity (represented by an increased or decreased number of bands in the gel profile). Isolated species profiles which had a more unique banding pattern allowed for an estimation of their presence in DNA samples from direct extraction. Cloning experiments failed to yield information on the most dominant PCR products.

4.6 Future Research

Further research using a larger study group to look into the differences in colonisation between OM-patients and OM-free controls is necessary.

Information that includes the carriage rates of the potential 'interfering' organisms *Dolosigranulum pigrum* and *Corynebacterium pseudodiphtheriticum* could be useful in determining the role of these bacteria, whose colonisation has been associated with a decrease in OM frequency.

Information on the antibiotics participants have been prescribed prior to sample collection, as well as antibiotic susceptibility profiles on cultured bacteria, would increase understanding on how to treat these infection in New Zealand children.

Determination of an immune response against the two OE commensals *C. auris* and *T. otitidis* may provide further insight into the pathogenic status of these organisms, as would complete genome sequencing to determine the presence of pathogenic genes. Determination of the genes that are 'switched on' using cDNA analysis could provide additional insight into pathogenic abilities.

Further research into vaccine efficiency using larger patient numbers is required.

Information on the dynamics of the biofilm formed during OM in the NP and the MEE could increase the understanding of infection, and provide further insight for more effective treatments.

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Appendix I: Ethics information

The following information sheets and consent forms were given to the parents of the children undergoing the operations pertaining to their AOM, OME, or RAOM. Parents of control patients who were undergoing non-middle ear infection related operations were given the control version of the information and consent forms. Version 2 of these documents was approved by the Northern Y Regional Ethics committee, as well as the University of Waikato Ethics committee.

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Information Sheet for Patients
Version 2 (05/05/2011)

Project Title

Do bacteria of the external ear canal contribute to otitis media in children?

Principal Investigator

Rebecca White (Master of Science student)
Molecular Genetics Laboratory (C.2.03)
Department of Biological Sciences
The University of Waikato
Day Ph: (07) 838 4466 Evening Phone: 027 3669156

Introduction

You/your child are invited to participate in a study that will investigate the influence that outer ear bacteria have on middle ear disease.

Participation and Confidentiality

- Your child's participation is entirely voluntary.
- If you agree to allow your child to take part you are free to withdraw your child from the study at any time, without having to give a reason and this will in no way effect your child's future health care.
- There will be neither cost nor payment towards your child's participation.
- No material which could personally identify your child will be used in any reports on this study.
- Results will be kept confidential throughout the duration of the study and stored in a secure location after completion of the study.
- If you would like to know the results of the study, the research student or the supervisor will give you a copy of the final report once the report is finished or published, or discuss the results with you in person.

Project Purpose

Middle ear diseases are among the most common diseases found in infants and children. The influence of outer ear bacteria on the recurrence, progression and outcome of the disease is currently uncertain and controversial. Normal pathogens of middle ear infections come from the nasopharynx, however some external ear canal bacteria have been found in middle ear fluids during infection (see diagram 1). This research looks to profile the bacteria of the nasopharynx and outer ear canal to determine a relationship between the bacteria present in these areas and middle ear disease prevalence.

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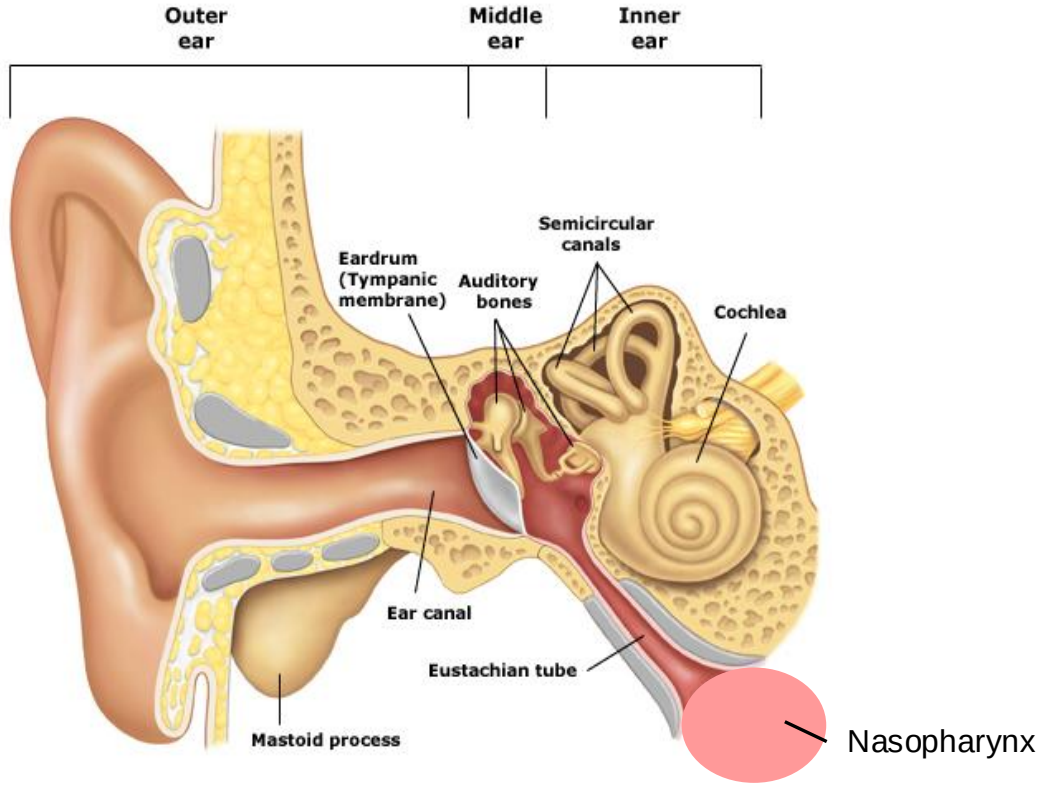

Diagram 1 – Indicates the three main areas of study:

- The nasopharynx, where bacteria involved in middle ear infection normally reside. These remain in a non-pathogen state until a viral attack (such as the common cold), when they can become pathogenic.
- The middle ear, where infection occurs.
- And the outer ear or external ear canal. This is where the potential pathogens reside, and the area of most interest in this research.

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Project Method

During the surgical procedures, a health practitioner will take a swab of your child's external ear canal. A nasopharyngeal sample will be retrieved by insertion of a fine swab through the nose. If your child is having grommets inserted, a sample of your child's middle ear fluid will also be taken. This will not result in an increased time in theatre, nor will it influence recovery time. These samples will then be transported to the Molecular Genetics Laboratory at the University of Waikato for -80° storage until processing can begin. Bacteria from these samples will then be cultured or identified through molecular genetics techniques. These techniques will only be looking at the DNA of the bacterial species in your child's samples, not your child's DNA.
Approximately 200 patients will be involved in this study. These will be either patients admitted to Waikato Hospital for surgery due to middle ear disease (cases) or patients admitted to hospital with no significant history of middle ear disease (controls).

Benefits and Risks

The results of this proposed study could potentially improve the understanding of the factors that cause the recurrence of middle ear disease. Antibiotic therapy currently used to treat this disease could potentially be improved if a better understanding of the bacteria involved is achieved.
No side effects or risks are anticipated in this study.

Definition of Scientific Terms

Pathogens – disease causing micro organism
Nasopharynx – the area that joins the nasal cavity with the throat.
Nasopharyngeal – The mucosal layer of the nasopharynx.

Declaration

In the unlikely event of a physical injury as a result of your child's participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act 2001. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention compensation. This depends on a number of factors. ACC usually provides only partial reimbursement of costs and expenses. Therefore, there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will effect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

Statement of Ethical Approval

This project is has received ethical approval from the Northern Y Regional Ethics Committee. If you have any questions or concerns regarding your rights as a participant in this study you may wish to contact a Health and Disability Advocate of Mid and Lower North Island on 0800 42 36 38.

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Consent form
Version 2 13/06/2011

Project: Do commensal bacteria of the external ear canal contribute to middle ear infection in children?

Research Student:
Rebecca White
Supervisor: Dr. Ray T. M. Cursons
Location: Molecular Genetics Lab (C2.03), the University of Waikato
Ph: (07) 838 4466 ext 8482

I have read and I understand the information sheet for caregivers volunteering their children to take part in the study looking to identify the potential role of external ear bacteria in middle ear disease. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

Yes/No

I understand that taking part in this study is completely voluntary and that I may withdraw my child from the study at any time and this will in no way effect their future health care.

Yes/No

I understand that the participation of my child in the study is confidential and that no material which could identify them will be used in any reports on this study.

Yes/No

I have had time to consider whether to allow my child to take part. I know whom to contact if my child has any side effects from the study.

Yes/No

I understand the ACC compensation provisions for this study.

Yes/No

I wish to receive a copy of the results or to discuss the outcomes of the study with the researcher.

Yes/No

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I.....hereby consent to my

child, ..., taking part in this study.

Date: .....

Signature: .....

Project explained by: .....

Project role: .....

Signature: .....

Date: .....

Contact details (please feel free to contact the researchers if you have any questions about this study):

Rebecca White
Location: Molecular Genetics Lab (C2.03), the University of Waikato
Ph: (07) 838 4466 ext 8482
Email: rkw14@waikato.ac.nz

Dr. Ray T. M. Cursons
Location: Molecular Genetics Lab (C2.03), the University of Waikato
Ph: (07) 838 4466 ext 8482
Email: r.cursons@waikato.ac.nz

Dr. Tony Cecire
Location: Anglesea Clinic, Cnr Anglesea and Thackeray Streets, Hamilton.
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Information Sheet for Control Patients of Middle ear infection study
Version 2 (05/05/2011)

Project Title
Do bacteria of the external ear canal contribute to otitis media in children?

Principal Investigator

Rebecca White (Master of Science student)
Molecular Genetics Laboratory (C.2.03)
Department of Biological Sciences
The University of Waikato
Day Ph: (07) 838 4466 Evening Phone: 027 3669156

Introduction

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Participation and Confidentiality

- Your child's participation is entirely voluntary.
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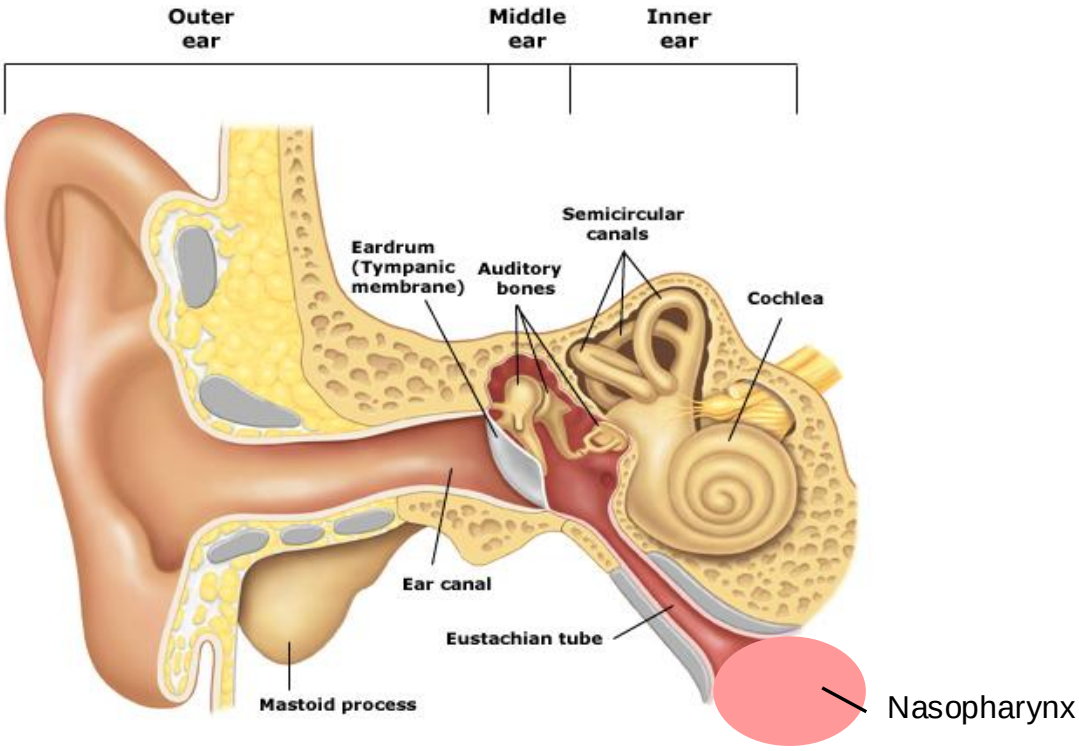

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- The nasopharynx, where bacteria involved in middle ear infection normally reside. These remain in a non-pathogen state until a viral attack (such as the common cold), when they can become pathogenic.
- The middle ear, where infection occurs.
- And the outer ear or external ear canal. This is where the potential pathogens reside, and the area of most interest in this research.

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Project Method

During your child's surgical procedure, a health practitioner will take a swab of your child's external ear canal. A nasopharyngeal sample will also be retrieved by insertion of a fine swab through the nose. These painless samples will be taken when your child is under anaesthetic, in order to decrease any discomfort they may feel. This will not result in an increased time in theatre, nor will it influence recovery time. These samples will then be transported to the Molecular Genetics Laboratory at the University of Waikato for -80° storage until processing can begin. Bacteria from these samples will then be cultured or identified through molecular genetics techniques. These techniques will only be looking at the DNA of the bacterial species in your child's samples, not your child's DNA. Approximately 200 patients will be involved in this study. These will be either patients admitted to Waikato Hospital for surgery due to middle ear disease (cases) or patients admitted to hospital with no significant history of middle ear disease, as in the case of your child (controls).

Benefits and Risks

The results of this proposed study could potentially improve the understanding of the factors that cause the recurrence of middle ear disease, and although will not benefit your child directly, may help in the treatment of this prolific disease. Antibiotic therapy currently used to treat this form of infection could potentially be improved if a better understanding of the bacteria involved is achieved.
No side effects or risks are anticipated in this study.

Definition of Scientific Terms

Pathogens – disease causing micro organism
Nasopharynx – the area that joins the nasal cavity with the throat.
Nasopharyngeal – The mucosal layer of the nasopharynx.

Declaration

In the unlikely event of a physical injury as a result of your child's participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act 2001. ACC cover is not automatic and your case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention compensation. This depends on a number of factors. ACC usually provides only partial reimbursement of costs and expenses. Therefore, there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will effect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator.

Statement of Ethical Approval

This project has received ethical approval from the Northern Y Regional Ethics Committee. If you have any questions or concerns regarding your rights as a participant in this study you may wish to contact a Health and Disability Advocate of Mid and Lower North Island on 0800 42 36 38.

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Consent form
Version 2 13/06/2011

Project: Do commensal bacteria of the external ear canal contribute to middle ear infection in children?

Research Student:
Rebecca White
Supervisor: Dr. Ray T. M. Cursons
Location: Molecular Genetics Lab (C2.03), the University of Waikato
Ph: (07) 838 4466 ext 8482

I have read and I understand the information sheet for caregivers volunteering their children to take part in the study looking to identify the potential role of external ear bacteria in middle ear disease. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

Yes/No

I understand that taking part in this study is completely voluntary and that I may withdraw my child from the study at any time and this will in no way effect their future health care.

Yes/No

I understand that the participation of my child in the study is confidential and that no material which could identify them will be used in any reports on this study.

Yes/No

I have had time to consider whether to allow my child to take part. I know whom to contact if my child has any side effects from the study.

Yes/No

I understand the ACC compensation provisions for this study.

Yes/No

I wish to receive a copy of the results or to discuss the outcomes of the study with the researcher.

Yes/No

Department of Biological Sciences
School of Science & Engineering
The University of Waikato
Private Bag 3105
Hamilton
New Zealand
Phone +64 7 838 4148

I.....hereby consent to my

child, ..., taking part in this study.

Date: .....

Signature: .....

Project explained by: .....

Project role: .....

Signature: .....

Date: .....

Contact details (please feel free to contact the researchers if you have any questions about this study):

Rebecca White
Location: Molecular Genetics Lab (C2.03), the University of Waikato
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Appendix II: Patient Information

Patient information was obtained that was relevant to the study. 42 OM patients were included in this study, as well as 36 control patients. Control patient #1C did not have their colonisation data included in analysis, due to OM history. Some patients had both culture and direct DNA analysis, while others had one or the other.

Note two control patients that had a history of OM were not excluded as this episode had occurred in the years previous to sampling. Further, controls with an unspecified history of OM did not present with any symptoms of infection at the time of sampling, and were deemed appropriate for use as controls by the health practitioner.

The following abbreviations were used in this section:

R me – Right middle ear effusion

L me – Left middle ear effusion

RE – Right outer ear canal

LE – Left outer ear canal

NP – Nasopharyngeal sample

Transoral – sample taken via the oral cavity

Transnasal – Sample taken via the nasal cavity

TAH – Tonsils and adenoid hypertrophy.

Flat tymps – Flat tympanometry.

Ads – Adenoidectomy.

Tonsils – Tonsillectomy.

Grommets – Ventilation tube insertion.

UTD – Immunisations up to date.

OD – Immunisations over due by the months specified.

VT – Ventilation tube insertion (Grommets).

NS – Not specified.

Table AII.1 Patient information for the otitis media study group.

Patient	Sex	Age (months)	Diagnosis/Procedure	MEE sampled?	Effusion status	History	Ethnicity	PCV7 dose	Imms
1	F	38	OME	R me	mucoid	nil previous	Maori	0	UTD
				L me	dry				
2	M	59	OME	R me	mucoid	Grommets,Tonsils	Maori	0	UTD
				L me	mucoid	Ads 2007			
3	M	52	NS	No	-	NS	NZ Euro	0	UTD
4	M	91	NS	R me	NS	NS	NZ Euro	0	UTD
5	M	58	OME	R me	bloody	nil	NZ Euro	0	UTD
			Grommets/Tonsils/Ads	L me	dry				
6	F	11	RAOM	R me	mucoid	nil previous	Maori	0	Unimmunised
				L me	mucoid				
7	M	14	RAOM	R me	dry	nil previous	NZ Euro	4	UTD
				L me	dry				
8	M	44	OME	No	mucopurulent	previous VT x2	NZ Euro	0	UTD
9	M	11	RAOM	R me	serous	nil previous	NZ Euro	4	UTD
				L me	serous				
10	M	45	OME,TAH	R me	mucopurulent	previous VTx1	NZ Euro	0	UTD
				L me	mucopurulent				
11	F	17	RAOM	R me	dry	nil previous	NZ Euro	4	UTD
				L me	dry				
12	M	14	RAOM	R me	mucoid	nil previous	Maori	4	UTD
				L me	mucoid				
13	M	42	OME	R me	mucoid	previous VTx1	NZ Euro	0	UTD
				L me	mucoid				

Table AII.2 Patient information for the otitis media study group continued.

Patient	Sex	Age (months)	Diagnosis/Procedure	MEE sampled?	Effusion status	History	Ethnicity	PCV7 dose	Imms
14	M	51	OME	R me	mucoid	NS	NZ Euro	0	UTD
				L me	mucoid				
15	M	46	Grommets	R me	mucoid	nil previous	NZ Euro	0	UTD
			Adenoids	L me	dry				
16	M	37	Bilateral Grommets	R me	dry	nil previous	Maori	4	UTD
				L me	dry	flat tymps			
17	M	43	Bilateral Grommets	R me	dry	nil previous	Maori	0	UTD
			rAOM	L me	dry				
18	F	73	OME	R me	mucoid	nil previous	NZ Euro	0	UTD
				L me	mucoid				
19	F	18	RAOM	R me	mucoid	NS	NZ Euro	0	UTD
				L me	dry				
20	F	68	OME	R me	mucoid	Grommets x1 pair	NZ Euro	0	UTD
				L me	dry				
21	M	32	OME	R me	mucoid	NS	NZ Euro	0	UTD
				L me	mucoid				
22	F	37	OME	R me	mucoid	NS	Maori	0	UTD
				L me	mucoid				
23	F	34	OME	R me	dry	grommets x1	NZ Euro	4	UTD
				L me	dry				
24	F	44	OME	R me	mucoid	NS	NZ Euro	0	UTD
				L me	mocoid				
25	F	14	rAOM	R me	dry	nil previous	NZ Euro	4	UTD
			Bilateral Grommets	L me	dry	rAOM			

Table AII.3 Patient information for the otitis media study group continued.

Patient	Sex	Age (months)	Diagnosis/Procedure	MEE sampled?	Effusion status	History	Ethnicity	PCV7 dose	Imms
26	M	17	OME	R me	mucoid	RAOM + OME	NZ Euro	4	UTD
			Bilateral Grommets	L me	mucoid	flat tymps			
27	M	39	OME	R me	mucopus	RAOM + OME	NZ Euro	0	UTD
			Grommets/Tonsils/Ads	L me	mucopus	flat tymps			
28	M	50	OME	R me	dry	OME	NZ Euro	0	UTD
			Grommets/Adenoids	L me	dry	flat tymps			
29	F	11	Bilateral Grommets	R me	perforation+discharge	nil previous	Maori	4	UTD
			rAOM	L me	dry				
30	M	18	Bilateral Grommets	R me	dry	nil previous	Maori	4	UTD
			rAOM	L me	dry				
31	M	21	Bilateral Grommets	R me	dry	nil previous	NZ Euro	4	UTD
			rAOM	L me	dry				
32	F	42	OME	R me	dry	nil	NZ Euro	0	UTD
			Grommets/Ads/Tonsils	L me	mucoid				
33	M	17	Bilateral Grommets	R me	mucoid	nil previous	NZ Euro	1	INCOMPLETE
			rAOM	L me	mucoid				
34	M	34	OME	R me	mucoid	nil previous	Maori	3	UTD
			Grommets/Ads	L me	mucoid				
35	F	33	OME	R me	scant	nil previous	NZ Euro	4	UTD
				L me	mucopurulent				
36	F	45	Gs endo Ads	R me	seropu	nil previous	NZ Euro	0	UTD
			rAOM	L me	mucoid				
37	M	59	OME, ATH	R me	mucoid	nil	NZ Euro	0	UTD
				L me	mucoid				

Table AII.4 Patient information for the otitis media study group continued.

Patient	Sex	Age (months)	Diagnosis/Procedure	MEE sampled?	Effusion status	History	Ethnicity	PCV7 dose	Imms
38	M	56	OME	R me	mucoid	previous rAOM	NZ Euro	0	UTD
				L me	mucoid	in infancy			
39	F	45	AOM	R me	insufficient DNA		NZ Euro	4	UTD
			Grommets/Ads/Tonsils	L me	insufficient DNA				
40	M	110	OME,tonsillitis	R me	scant	NS	NZ Euro	0	UTD
				L me	mucoid				
41	M	60	OME	R me	insufficient DNA	NS	Maori	0	UTD
			Grommets	L me	insufficient DNA				
42	M	94	OME	R me	dry	previous VTx2	Maori	0	UTD
				L me	mucoid				

Table AII.5 Patient information for the control study group.

Sample	Sex	Age (months)	Sites sampled	History	Ethnicity	PCV7 doses	Immunization
1C	M	45	RE	4-5 AOM in 3 years	NZ Euro	0	UTD
			LE	**Excluded from study**			
			NP	**due to OM history**			
2C	M	40	RE	nil previous	NZ Euro	4	UTD
			LE				
			NP				
3C	M	55	RE	nil	NZ Euro	0	UTD
			LE				
			NP				
4C	M	25	RE	previous AOMx1	Indian	4	UTD
			LE				
			NP				
5C	M	2	RE	nil	Euro	0	UNIMMUNISED
			LE				
			NP				
6C	M	61	RE	NS	NZ Euro	0	UTD
			LE				
			NP				
7C	M	68	RE	NS	Maori	0	UTD
			LE				
			NP				
8C	M	28	RE	NS	Cook Island Maori	4	UTD
			LE				
			NP				

Table AII.6 Patient information for the control study group continued.

Sample	Sex	Age (months)	Sites sampled	History	Ethnicity	PCV7 doses	Immunization
9C	M	29	RE	NS	NZ Euro	4	UTD
			LE				
			NP				
10C	F	18	RE	nil	Somali	4	UTD
			LE	nil AOM			
			NP				
11C	M	13	RE	nil	Maori	3	OD 15mnths
			LE	nil AOM			
			NP				
12C	M	33	RE	nil	Maori	4	UTD
			LE				
			NP				
13C	M	42	RE	nil previous	Euro	4	UTD
			LE				
			NP				
14C	M	48	RE	NS	Maori	0	UTD
			LE				
			NP				
15C	M	23	RE	nil	Polynesian	0	UNIMMUNISED
			LE				
			NP				
16C	M	46	RE	NS	NZ Euro	0	UTD
			LE				
			transoral NP				

Table AII.7 Patient information for the control study group continued.

Sample	Sex	Age (months)	Sites sampled	History	Ethnicity	PCV7 doses	Immunization
17C	F	53	RE	NS	NZ Euro	0	UTD
			LE				
			transoral NP				
18C	M	34	RE	NS	NZ Euro	4	UTD
			LE				
			transoral NP				
19C	F	56	RE	NS	Maori	0	UTD
			LE				
			transoral NP				
20C	M	51	RE	NS	Other European	3	UTD
			LE				
			transoral NP				
21C	M	25	RE	NS	Maori	4	UTD
			LE				
			transoral nasoph				
22C	M	39	RE	NS	NZ Euro	4	UTD
			LE				
			transoral NP				
23C	M	49	RE	NS	NZ Euro	0	UNIMMUNISED
			LE				
			transoral NP				
24C	M	35	RE	NS	Maori	4	UTD
			LE				
			NP				

Table AII.8 Patient information for the control study group continued.

Sample	Sex	Age (months)	Sites sampled	History	Ethnicity	PCV7 doses	Immunization
25C	M	43	RE	nil previous	Maori-Poly	0	UTD
			LE				
			NP				
26C	M	48	RE	nil previous	Euro	0	UTD
			LE				
			NP				
27C	M	15	RE	nil previous	Maori	0	UTD
			LE				
			NP				
28C	M	18	RE	nil previous	Euro	4	UTD
			LE				
			NP				
29C	F	73	RE	no previous	NZ Euro	0	UTD
			LE				
			NP				
30C	M	47	RE	previous VTx1	NZ Euro	0	UTD
			LE				
			NP				
31C	F	98	RE	NS	NZ Euro	0	UTD
			LE				
			transoral NP				
32C	F	80	RE	nil previous	NZ Euro	0	UTD
			LE				
			transnasal NP				

Table AII.9 Patient information for the control study group continued.

Sample	Sex	Age (months)	Sites sampled	History	Ethnicity	PCV7 doses	Immunization
33C	M	84	RE	nil previous	Maori	0	UTD
			LE				
			transnasal NP				
34C	F	32	RE	nil previous	NZ Euro	4	UTD
			transnasal NP				
35C	F	44	RE	NS	NZ Euro	4	UTD
			LE				
			NP				
36C	F	52	RE	NS	Maori	0	UTD
			LE				
			NP				

Appendix III: *M. catarrhalis* and *M. nonliquefaciens* alignment.

The primer used in the study by Hendolin et al (1997) targets an area of the 16S ribosomal gene in the *Moraxella* species that is complimentary to both *M. catarrhalis* and *M. nonliquefaciens*. The 16S alignment of these two species is listed below, demonstrating the significant similarities between them both. Genbank accession numbers of sequences used: *M. catarrhalis*, U10876.1. *M. nonliquefaciens*: JN175343.1

```
MC      AAAAAATG-TCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAAC 59
MN      AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAAC 60
        * *   ** **************************************************

MC      GAAGTTAGGAAGCTTGCTTCTGATAC-TTAGTGGCGGACGGGTGAGTAATGCTTAGGAAT 118
MN      GATGAAGTCTAGCTTGCT--AGACGGATTAGTGGCGAACGGGTGAGTAATGCTTAGGAAT 118
        ** *      ********   **    ********* **********************

MC      CTGCCTAGTAGTGGGGGATAACTTGGGGAAACCCAAGCTAATACCGCATACGACCTACGG 178
MN      CTGCCTATTAGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGG 178
        ******* ************* * *******  * ************************

MC      GTGAAAGGGGGCTTTTAGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTGGTTGGT 238
MN      GTGAAAGGGGGCTTTTAGCTCTCGCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGT 238
        ************************* ************************ ******

MC      GGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACAC 298
MN      GGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACAC 298
        ************************************************************

MC      TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG-AATATTGGACAATG 357
MN      TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG 358
        ******************************************** **************

MC      GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCAC 417
MN      GGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCAC 418
        ************************************************************

MC      TTTAAGTGGGGAGGAAAAGCTTATGGTTAATACCCATAAGCCCTGACGTTACCCACAGAA 477
MN      TTTAAGTGGGGAGGAAAAGCTTATGGTTAATACCCATAAGCCCTGACGTTACCCACAGAA 478
        ************************************************************

MC      TAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC 537
MN      TAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATC 538
        ************************************************************

MC      GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTATTTAAGTCAGATGTGAAAGCCCCGG 597
MN      GGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTATTTAAGTCAGATGTGAAAGCCCCGG 598
        ************************************************************

MC      GCTTAACCTGGGAACTGCATCTGATACTGGATAACTAGAGTAGGTGAGAGGGGAGTAGAA 657
MN      GCTTAACCTGGGAACTGCATCTGATACTGGATAACTAGAGTAGGTGAGAGGGGAGTAGAA 658
        ************************************************************

MC      TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCT 717
MN      TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCT 718
        ************************************************************

MC      CCCTGGCATCATACTGACACTGAGGTGCGAAAGCGTGGGGTAGCAAACAGGATTAGATACC 777
MN      CCCTGGCATCATACTGACACTGAGGTGCGAAAGCGTGGGGTAGCAAACAGGATTAGATACC 778
        ************************************************************

MC      CTGGTAGTCCACGCCGTAAACGATGTCTACCAGTCGTTGGGTCTTTTAAAGACTTAGTGA 837
MN      CTGGTAGTCCACGCCGTAAACGATGTCTACCAGTCGTTGGGTCTTTTAAAGACTTAGTGA 838
        ************************************************************

MC      CGCAGTTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT 897
MN      CGCAGTTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT 898
        ************************************************************
MC      GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG 957
MN      GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG 958
        ************************************************************

MC      AACCTTACCTGGTCTTGACATAGTGAGAATCTTGCAGAGATGCGAGAGTGCCTTCGGGAA 1017
MN      AACCTTACCTGGTCTTGACATAGTGAGAATCTTGCAGAGATGCGAGAGTGCCTTCGGGAA 1018
        ************************************************************
```

Figure AIII.1 Alignment between the 16S sequence of *M. catarrhalis* and *M. nonliquefaciens*. Position of the MS forward primer is highlighted.

```
MC      TTCACATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC 1077
MN      TTCACATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC 1078
        ************************************************************

MC      CCGCAACGAGCGCAACCCTTTTCCTTAGTTACCAGCGACTCGGTCGGGAACTCTAAGGAT 1137
MN      CCGCAACGAGCGCAACCCTTTTCCTTAGTTACCAGCGACTCGGTCGGGAACTCTAAGGAT 1138
        ************************************************************

MC      ACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGA 1197
MN      ACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGA 1198
        ************************************************************

MC      CCAGGGCTACACACGTGCTACAATGGTTGGTACAAAGGGTTGCTACACAGCGATGTGATG 1257
MN      CCAGGGCTACACACGTGCTACAATGGTTGGTACAAAGGGTTGCTACACAGCGATGTGATG 1258
        ************************************************************

MC      CTAATCTCAAAAAGCCAATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC 1317
MN      CTAATCTCAAAAAGCCAATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC 1318
        ************************************************************

MC      GGAATCGCTAGTAATCGCAGATCAGAATGCTGCGGTGAATACGTTCCCGGGCCTTGTACA 1377
MN      GGAATCGCTAGTAATCGCAGATCAGAATGCTGCGGTGAATACGTTCCCGGGCCTTGTACA 1378
        ************************************************************

MC      CACCGCCCGTCACACCATGGGAGTTGATCTCACCAGAAGTGGTTAGCCTAACGCAAGAGG 1437
MN      CACCGCCCGTCACACCATGGGAGTTGATCTCACCAGAAGTGGTTAGCCTAACGCAAGAGG 1438
        ************************************************************

MC      GCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGA 1497
MN      GCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGA 1498
        ************************************************************

MC      ACCTGCGGTTGGAT--------- 1511
MN      ACCTGCGGTTGGATCACCTCCTT 1521
        **************
```

Figure AIII.2 Alignment between the 16S sequences of *M. catarrhalis* and *M. nonliquefaciens* continued.

Appendix IV: Bacterial prevalence results

Bacterial prevalence results attained from direct swab extraction and nested PCR protocol.

Table AIV.1 Bacterial prevalence in control patients. R: Removed from study, U: Unusable due to contamination or loss of sample, C: culture analysed.

Site	LE	LE	LE	LE	LE	LE	NP	NP	NP	NP	NP	NP	RE	RE	RE	RE	RE	RE
Bacteria	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO
Control 1C	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Control 2C	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0
Control 3C	1	0	0	1	0	1	U	U	U	U	U	U	1	0	0	1	0	0
Control 4C	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Control 5C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control 6C	1	0	0	0	0	1	0	0	0	1	0	0	1	0	0	1	0	0
Control 7C	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0
Control 8C	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0
Control 9C	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0
Control 10C	1	0	0	0	1	0	0	0	0	0	0	0	U	U	U	U	U	U
Control 11C	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0
Control 12C	1	0	0	1	0	0	0	0	1	1	1	0	1	0	0	1	0	0
Control 13C	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	1	0	1
Control 14C	0	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0
Control 15C	1	0	0	1	0	1	0	0	1	1	0	0	1	0	0	1	0	1
Control 16C	1	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	1
Control 17C	1	1	1	1	0	1	0	0	0	1	0	0	1	0	0	1	0	1
Control 18C	1	0	0	1	0	1	0	0	1	1	1	0	1	0	1	1	0	1
Control 19C	1	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0
Control 20C	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	0	0
Control 21C	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0
Control 22C	0	0	1	1	0	0	0	0	1	1	0	0	0	0	1	1	0	0
Control 23C	1	0	1	1	0	1	0	1	1	1	0	1	1	0	1	1	0	1
Control 24C	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	0	0	1
Control 25C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Control 26C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
Control 27C	0	0	0	1	0	1	0	0	0	1	1	0	0	0	0	1	0	0
Control 28C	0	0	1	1	0	0	0	0	1	1	1	0	1	0	1	1	0	0
Control 29C	1	1	1	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0
Control 30C	1	1	1	0	0	0	0	1	1	1	0	0	1	0	0	0	0	0
Control 31C	1	0	0	0	0	0	1	0	0	1	0	0	1	1	0	0	0	1
Control 32C	1	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0
Control 33C	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Control 34C	0	0	0	0	0	0	0	0	1	1	1	0	U	U	U	U	U	U
Control 35C	0	0	1	0	0	1	0	0	0	1	1	0	0	1	1	0	0	1
Control 36C	1	1	1	1	0	1	0	0	1	1	0	0	1	0	1	1	0	1
Total of positives	20	5	10	19	1	13	2	2	16	28	7	1	21	4	9	18	0	11

Table AIV.2 Bacterial prevalence results for the OM Patient group. Where no data is included in the table indicates the sample was not analysed (invariably because it was not collected). Cultured samples are indicated with a C.

Site	LE	LE	LE	LE	LE	LE	LM	LM	LM	LM	LM	LM	NP	NP	NP	NP	NP	NP	RE	RE	RE	RE	RE	RE	RM	RM	RM	RM	RM	RM
Bacteria	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO
OM Patient 1	0	0	1	1	1	0	0	0	0	0	0	0	C	C	C	C	C	C	C	C	C	C	C	C						
OM Patient 2	C	C	C	C	C	C							C	C	C	C	C	C	1	0	0	0	0	1	0	0	0	1	0	0
OM Patient 3	C	C	C	C	C	C							C	C	C	C	C	C	C	C	C	C	C	C						
OM Patient 4	1	0	0	1	0	0							C	C	C	C	C	C	0	0	0	0	0	0	0	0	0	1	0	0
OM Patient 5	1	0	0	0	0	1							1	0	1	0	0	1	1	0	1	0	0	1	0	0	0	0	0	1
OM Patient 6	C	C	C	C	C	C	0	0	1	0	0	0	C	C	C	C	C	C	C	C	C	C	C	C	0	0	1	0	1	1
OM Patient 7	1	0	0	0	0	1							0	0	0	0	0	0	1	0	0	0	0	0						
OM Patient 8																			C	C	C	C	C	C	0	0	0	0	0	0
OM Patient 9	1	0	0	0	0	0							0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
OM Patient 10	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
OM Patient 11	1	0	0	0	0	0							1	0	0	0	0	0	1	0	0	0	0	0						
OM Patient 12	0	0	0	0	0	0							0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OM Patient 13	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OM Patient 14							0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
OM Patient 15	0	0	0	0	0	0							0	0	0	0	0	0	0	0	0	0	0	0						
OM Patient 16	0	0	0	0	0	0							0	1	1	1	0	0	0	0	0	0	0	0						
OM Patient 17	1	0	0	1	0	1							0	0	1	1	0	0	1	0	0	1	0	1						
OM Patient 18	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
OM Patient 19	0	0	0	0	0	0							0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OM Patient 20																			1	0	0	0	0	0	0	0	0	0	0	0
OM Patient 21	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	0	0
OM Patient 22	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
OM Patient 23	C	C	C	C	C	C							C	C	C	C	C	C	C	C	C	C	C	C						
OM Patient 24	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0

Table AIV.3 Bacterial prevalence results for the OM Patient group continued.

Site	LE	LE	LE	LE	LE	LE	LM	LM	LM	LM	LM	LM	NP	NP	NP	NP	NP	NP	RE	RE	RE	RE	RE	RE	RM	RM	RM	RM	RM	RM
Bacteria	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO	AO	CA	HI	MS	SP	TO
OM Patient 25													0	0	1	0	0	0												
OM Patient 26	1	0	0	1	0	1	0	0	1	1	0	1	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	1
OM Patient 27	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	0	0	0	0	1	1	0
OM Patient 28	1	0	0	0	0	1							0	0	0	1	1	0	1	0	0	1	0	1						
OM Patient 29													0	0	0	1	0	0	0	0	1	1	0	0	1	1	1	1	0	1
OM Patient 30	0	0	0	0	0	0							0	0	0	1	0	0	0	0	0	0	0	0						
OM Patient 31	1	0	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	1	0	0
OM Patient 32	1	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	0	0	1	1	0	0	0	1						
OM Patient 33	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	1	1	0	0	0	0	0	1	0	0	1	1	0	1
OM Patient 34	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	1	1	1	1	0	1
OM Patient 35	1	1	0	0	0	0	1	1	1	1	1	1	0	1	0	1	0	0	1	0	1	1	0	0						
OM Patient 36	1	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	1	1	1	0
OM Patient 37	1	0	1	1	0	0	1	0	1	1	0	0	0	0	1	1	1	0	0	1	0	0	0	0						
OM Patient 38	1	0	1	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	1	0	0	0	0	1	0	0	0	1	0	0
OM Patient 39	0	0	1	1	0	1							0	0	1	1	1	0	0	1	0	1	0	1						
OM Patient 40	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	1	0	1	1	0	1	0	1						
OM Patient 41	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0						
OM Patient 42	1	1	0	0	0	0							0	0	0	1	0	0	1	0	0	0	0	1						
Total positives	19	3	7	12	1	12	4	2	13	9	1	3	3	4	16	23	8	2	20	6	5	10	0	11	3	2	9	10	3	6

Appendix V: Sequencing results

Electropherogram results are provided in a subset of samples.

Section 1

Sequencing results for organisms isolated in pure culture either on 10% Columbia blood agar or 10% Columbia chocolate agar. Results are presented as the list from the BLAST database, detailing the possible organisms the DNA sequence could correspond to.

Section 2

The PCR products after a multiplex reaction, isolated separately and sequenced for identification.

Section 3

A subset of samples that tested positive for the species of interest using the nested PCR method were sequenced for accuracy assurance. 702R was used as the sequencing primer before the sequences were aligned using BLAST. This section details the alignment efficiency of the sequenced DNA with that of the BLAST database.

Section 1: Results from sequencing species isolated from cultured samples.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF269920.1	Staphylococcus epidermidis strain SR1 clone step.1031f05	366	366	100%	3e-98	100%
CP000029.1	Staphylococcus epidermidis RP62A, complete genome	366	366	100%	3e-98	100%
AE015929.1	Staphylococcus epidermidis ATCC 12228, complete genom	366	366	100%	3e-98	100%
AM295250.1	Staphylococcus carnosus subsp. carnosus TM300 complet	185	185	94%	8e-44	85%
AP012054.1	Streptococcus pasteurianus ATCC 43144 DNA, complete g	150	150	77%	3e-33	84%

Figure AV.1 BLAST results for sequence isolated from sample 1RE-A.

AY189558.1	Uncultured Alloiococcus sp. clone EarCan031 16S ribosom	370	370	97%	2e-105	100%
AY189559.1	Uncultured Alloiococcus sp. clone EarCan032 16S ribosom	370	370	97%	2e-105	100%
AY189560.1	Uncultured Alloiococcus sp. clone EarCan033 16S ribosom	370	370	97%	2e-105	100%
AY957475.1	Alloiococcus otitis 16S ribosomal RNA gene, partial seque	370	370	97%	2e-105	100%
AF193887.1	Alloiococcus otitis 16S ribosomal RNA gene, partial seque	370	370	97%	2e-105	100%

Figure AV.2 BLAST results for sequence isolated from sample 8RE-A, after *Alloiococcus* taxid was specified in search conditions.

AY189553.1	Uncultured Alloiococcus sp. clone EarCan026 16S ribosomal RNA gene, p	806	806	100%	0.0	100%
AY957475.1	Alloiococcus otitis 16S ribosomal RNA gene, partial sequence	806	806	100%	0.0	100%
GQ020609.1	Uncultured bacterium clone nbu271g04c1 16S ribosomal RNA gene, par	804	804	99%	0.0	100%
AB680896.1	Alloiococcus otitis gene for 16S rRNA, partial sequence, strain: NBRC 15	802	802	100%	0.0	99%
AY189533.1	Uncultured Alloiococcus sp. clone EarCan006 16S ribosomal RNA gene, p	802	802	100%	0.0	99%

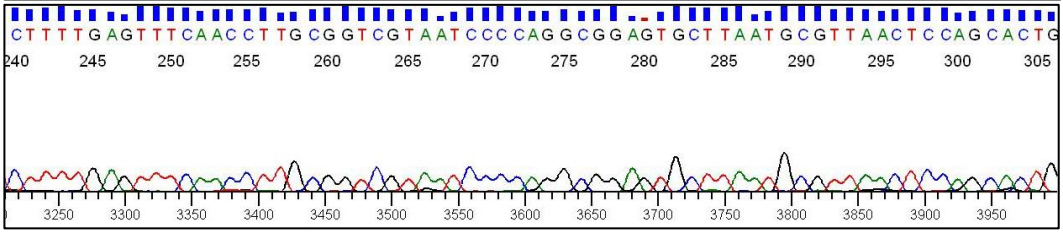


Figure AV.3 BLAST results for 3LE-C, with a sample of the electropherogram data.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB680896.1	Alloiococcus otitis gene for 16S rRNA, partial sequence, s	254	254	100%	1e-64	100%
JF235976.1	Uncultured bacterium clone ncd2732c02c1 16S ribosomal	254	254	100%	1e-64	100%
JF235943.1	Uncultured bacterium clone ncd2731h11c1 16S ribosomal	254	254	100%	1e-64	100%
JF235863.1	Uncultured bacterium clone ncd2730e05c1 16S ribosomal	254	254	100%	1e-64	100%

Figure AV.4 BLAST results for sequence isolated from sample LE6-A.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FR720602.1	Streptococcus oralis Uo5 complete genome sequence	377	377	83%	2e-101	96%
CP002176.1	Streptococcus pneumoniae 670-6B, complete genome	276	276	84%	7e-71	88%
CP002121.1	Streptococcus pneumoniae AP200, complete genome	276	276	84%	7e-71	88%
FQ312027.1	Streptococcus pneumoniae OXC141 genome	276	276	84%	7e-71	88%

Figure AV.5 BLAST results for sequence isolated from sample 1NP-A.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
CP002888.1	Streptococcus salivarius 57.I, complete genome	193	193	100%	3e-46	89%
FR873481.1	Streptococcus salivarius CCHSS3 complete genome	187	187	100%	2e-44	88%
FR873482.1	Streptococcus salivarius JIM8777 complete genome	178	178	96%	1e-41	88%

Figure AV.6 BLAST results for sequence isolated from sample 1NP-B.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN175343.1	Moraxella nonliquefaciens strain CIP 68.36 16S ribosomal	307	307	100%	1e-80	100%
JF240120.1	Uncultured bacterium clone ncd2796h03c1 16S ribosomal	307	307	100%	1e-80	100%
JF240116.1	Uncultured bacterium clone ncd2796g06c1 16S ribosomal	307	307	100%	1e-80	100%
JF240113.1	Uncultured bacterium clone ncd2796g02c1 16S ribosomal	307	307	100%	1e-80	100%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN175343.1	Moraxella nonliquefaciens strain CIP 68.36 16S ribosomal	329	329	100%	3e-91	100%
HM152563.1	Moraxella nonliquefaciens strain CCUG 348 16S ribosoma	329	329	100%	3e-91	100%
CP002005.1	Moraxella catarrhalis RH4, complete genome	329	7628	100%	3e-91	100%
FM881879.2	Uncultured Moraxella sp. partial 16S rRNA gene, clone NT	329	329	100%	3e-91	100%

Figure AV.7 BLAST results for sequence isolated from sample 2NP-A when searching the whole database (top), and when search parameters are set to only *Moraxella* (taxid:475) (bottom).

GQ059844.1	Uncultured bacterium clone nbw13f01c1 16S ribosomal RN	795	795	100%	0.0	100%
GQ059842.1	Uncultured bacterium clone nbw13e11c1 16S ribosomal RI	795	795	100%	0.0	100%
NR_026120.1	Turicella otitidis strain 234/92 16S ribosomal RNA, partia	795	795	100%	0.0	100%
JF204228.1	Uncultured bacterium clone ncd2332a04c1 16S ribosomal	789	789	100%	0.0	99%

Figure AV.8 BLAST result for sequence isolated from sample NP3-A

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF927887.1	Uncultured Dolosigranulum sp. clone ASD1 16S ribosomal	379	379	100%	3e-102	100%
JF014043.1	Uncultured bacterium clone ncd156b10c1 16S ribosomal R	379	379	100%	3e-102	100%
JF240103.1	Uncultured bacterium clone ncd2796e12c1 16S ribosomal	379	379	100%	3e-102	100%
JF240102.1	Uncultured bacterium clone ncd2796e10c1 16S ribosomal	379	379	100%	3e-102	100%

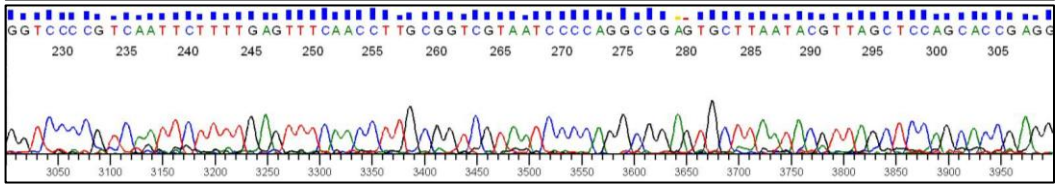


Figure AV.9 BLAST data on the best alignments with the sequence isolated from 26NP-A and electropherogram data from the sequencing results.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB680896.1	Alloiococcus otitis gene for 16S rRNA, partial sequence, strain: NBRC 15	645	645	100%	0.0	100%
JF235976.1	Uncultured bacterium clone ncd2732c02c1 16S ribosomal RNA gene, pa	645	645	100%	0.0	100%
JF235943.1	Uncultured bacterium clone ncd2731h11c1 16S ribosomal RNA gene, pa	645	645	100%	0.0	100%
JF235863.1	Uncultured bacterium clone ncd2730e05c1 16S ribosomal RNA gene, pa	645	645	100%	0.0	100%

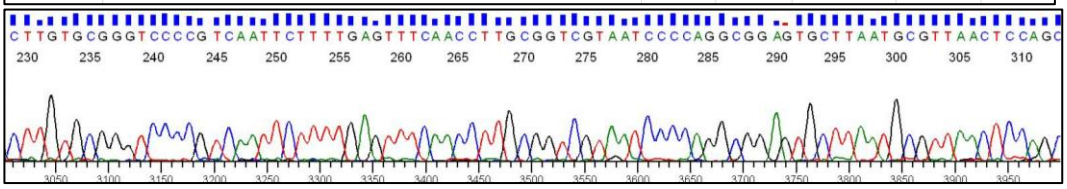


Figure AV.10 BLAST results from DNA sequence isolated from organism 9LM-A, including a sample of electropherogram results.

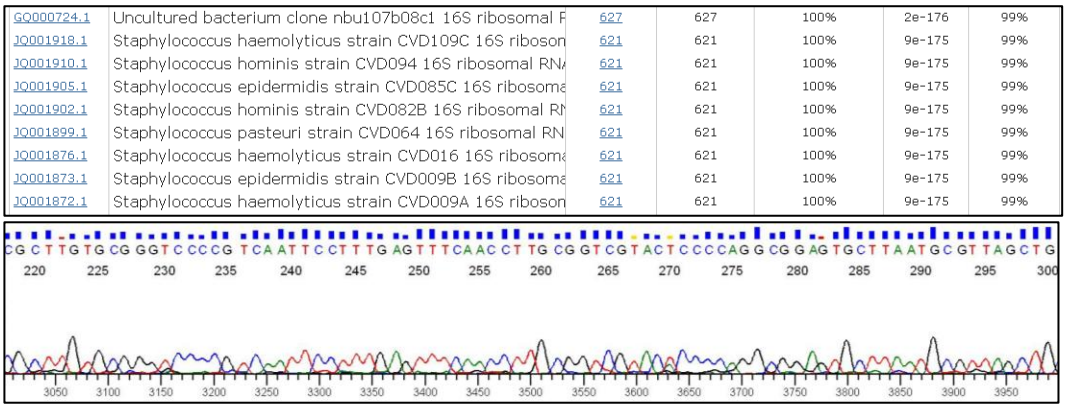


Figure AV.11 Electropherogram data and BLAST results on the organisms corresponding to the DNA sequence isolated from organism 26RM-A.

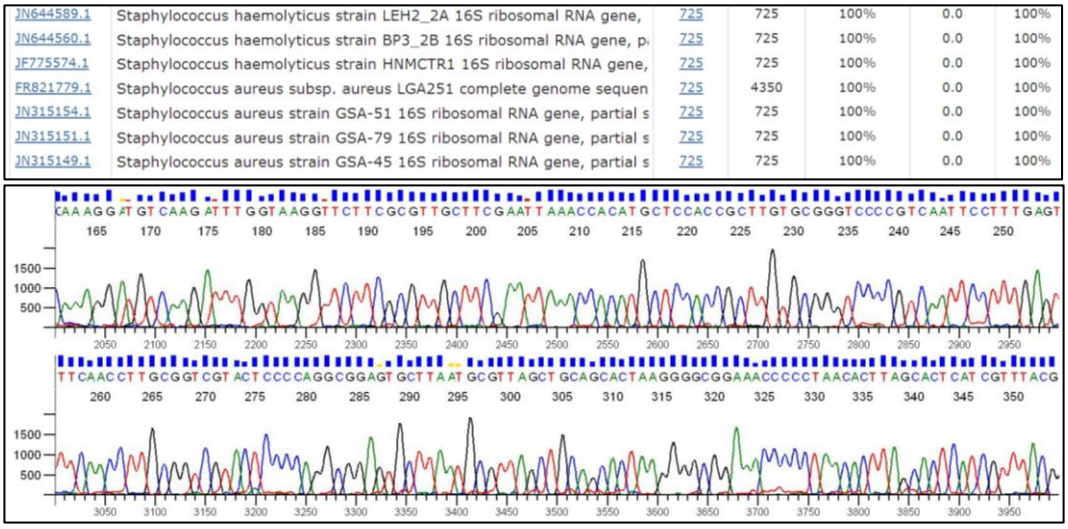


Figure AV.12 Sample of the electropherogram data from 29RM-A sequencing and BLAST results of the organisms the sequence aligned with.

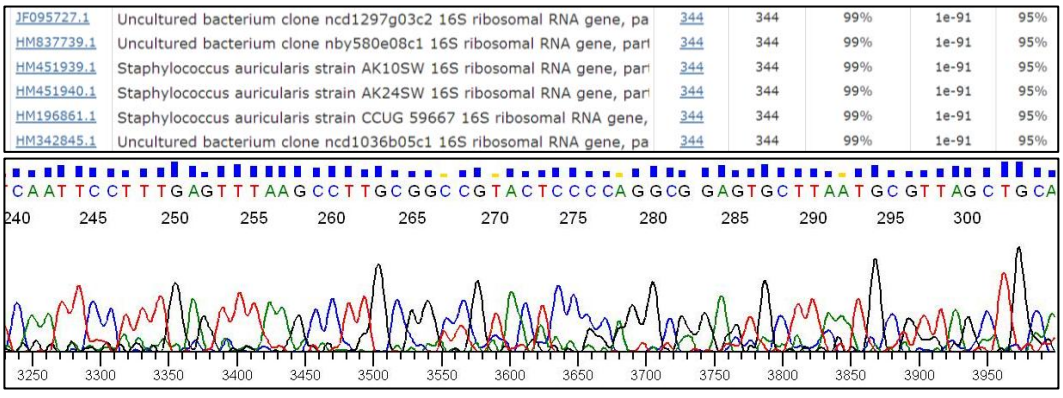


Figure AV.13 BLAST results for sequence isolated from sample 25CLE-A. Electropherogram data shows a low signal to noise ratio, accounting for the lack of 100% identification with BLAST sequences.

JF122649.1	Uncultured bacterium clone ncd1409c12c1 16S ribosomal RNA gene, pa	556	556	100%	2e-155	99%
JF104325.1	Uncultured bacterium clone ncd1319f12c1 16S ribosomal RNA gene, par	556	556	100%	2e-155	99%
JF095727.1	Uncultured bacterium clone ncd1297g03c2 16S ribosomal RNA gene, pa	556	556	100%	2e-155	99%
HM837739.1	Uncultured bacterium clone nby580e08c1 16S ribosomal RNA gene, par	556	556	100%	2e-155	99%
HM451939.1	Staphylococcus auricularis strain AK10SW 16S ribosomal RNA gene, par	556	556	100%	2e-155	99%
HM451940.1	Staphylococcus auricularis strain AK24SW 16S ribosomal RNA gene, par	556	556	100%	2e-155	99%

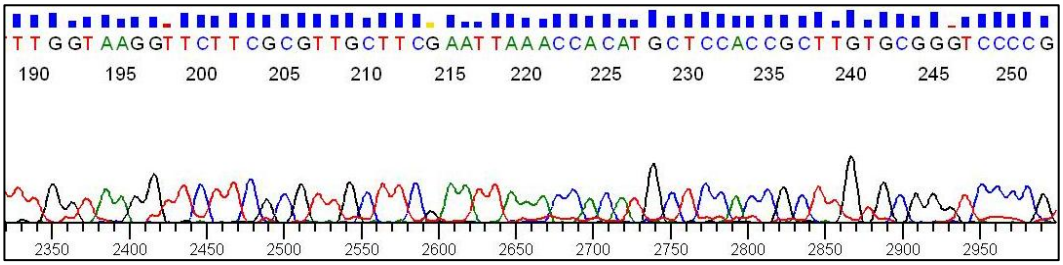


Figure AV.14 Sample of the electropherogram data from 25CRE-A sequencing and BLAST results of the organisms the sequence aligned with.

GQ037011.1	Uncultured bacterium clone nbu387d06c1 16S ribosomal RNA gene, p	433	433	100%	3e-118	99%
GQ053297.1	Uncultured bacterium clone nbw1084f06c1 16S ribosomal RNA gene,	433	433	100%	3e-118	99%
JN571721.1	Lysinibacillus sphaericus strain HKG 148 16S ribosomal RNA gene, pa	429	429	100%	4e-117	99%
JN571717.1	Staphylococcus sp. HKG 144 16S ribosomal RNA gene, partial sequen	429	429	100%	4e-117	99%
JN571716.1	Staphylococcus epidermidis strain HKG 143 16S ribosomal RNA gene,	429	429	100%	4e-117	99%
JN571715.1	Staphylococcus epidermidis strain HKG 142 16S ribosomal RNA gene,	429	429	100%	4e-117	99%

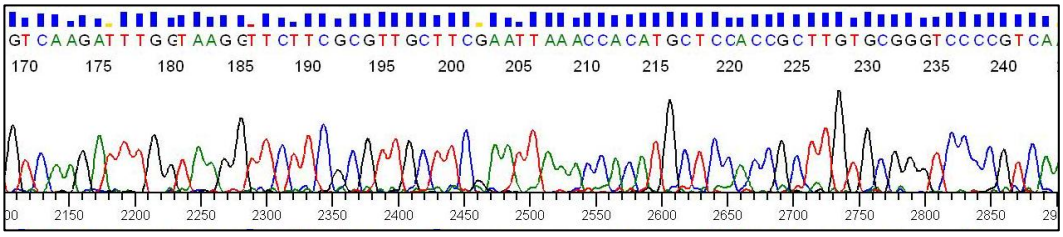


Figure AV.15 BLAST results for sequence isolated from sample 25CRE-B.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB680896.1	Alloiococcus otitis gene for 16S rRNA, partial sequence, strain: NBRC 15	279	279	99%	3e-72	96%
JF235976.1	Uncultured bacterium clone ncd2732c02c1 16S ribosomal RNA gene, pa	279	279	99%	3e-72	96%
JF235943.1	Uncultured bacterium clone ncd2731h11c1 16S ribosomal RNA gene, pa	279	279	99%	3e-72	96%
JF235863.1	Uncultured bacterium clone ncd2730e05c1 16S ribosomal RNA gene, pa	279	279	99%	3e-72	96%
JF232216.1	Uncultured bacterium clone ncd2671e10c1 16S ribosomal RNA gene, pa	279	279	99%	3e-72	96%

Figure AV.16 BLAST results for sequence isolated from sample 26CRE-A.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB680896.1	Alloiococcus otitis gene for 16S rRNA, partial sequence, strain: NBRC 15	407	407	100%	2e-110	98%
JF235976.1	Uncultured bacterium clone ncd2732c02c1 16S ribosomal RNA gene, pa	407	407	100%	2e-110	98%
JF235943.1	Uncultured bacterium clone ncd2731h11c1 16S ribosomal RNA gene, pa	407	407	100%	2e-110	98%
JF235863.1	Uncultured bacterium clone ncd2730e05c1 16S ribosomal RNA gene, pa	407	407	100%	2e-110	98%
JF232216.1	Uncultured bacterium clone ncd2671e10c1 16S ribosomal RNA gene, pa	407	407	100%	2e-110	98%

Figure AV.17 BLAST results for sequence isolated from sample 26CLE-A.

JF235171.1	Uncultured bacterium clone ncd2719e02c1 16S ribosomal RNA gene,	490	490	100%	2e-135	99%
JF235166.1	Uncultured bacterium clone ncd2719d05c1 16S ribosomal RNA gene,	490	490	100%	2e-135	99%
JF235162.1	Uncultured bacterium clone ncd2718g03c1 16S ribosomal RNA gene,	490	490	100%	2e-135	99%
JF235157.1	Uncultured bacterium clone ncd2718f03c1 16S ribosomal RNA gene, p	490	490	100%	2e-135	99%
NR_042137.1	Corynebacterium pseudodiphtheriticum strain CIP103420T, (ATCC107	490	490	100%	2e-135	99%

Figure AV.18 BLAST results for sequence isolated from sample 25CNP-A.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF195322.1	Uncultured bacterium clone ncd2263d01c1 16S ribosomal RNA gene,	462	462	100%	4e-127	98%
JF211607.1	Uncultured bacterium clone ncd2440c11c2 16S ribosomal RNA gene,	457	457	100%	2e-125	98%
AB680900.1	Dolosigranulum pigrum gene for 16S rRNA, partial sequence, strain:	455	455	100%	7e-125	98%
JF014552.1	Uncultured bacterium clone ncd163b10c1 16S ribosomal RNA gene, p	455	455	100%	7e-125	98%
JF014539.1	Uncultured bacterium clone ncd163d01c1 16S ribosomal RNA gene, p	455	455	100%	7e-125	98%

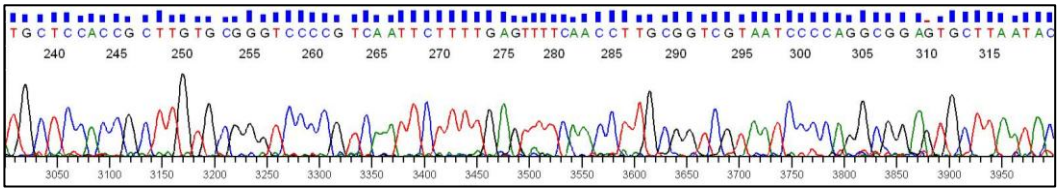


Figure AV.19 Electropherogram data and BLAST results of organisms corresponding to the DNA sequence isolated from 25CNP-B.

a) Search parameters set to search all:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN175343.1	Moraxella nonliquefaciens strain CIP 68.36 16S ribosomal RNA gene,	586	586	100%	3e-164	99%
JF893610.1	Uncultured Moraxella sp. clone C057 16S ribosomal RNA gene, partia	586	586	100%	3e-164	99%
JF240120.1	Uncultured bacterium clone ncd2796h03c1 16S ribosomal RNA gene,	586	586	100%	3e-164	99%
JF240111.1	Uncultured bacterium clone ncd2796f12c1 16S ribosomal RNA gene,	586	586	100%	3e-164	99%

b) Search parameters set to align only with *Moraxella* (taxid:475):

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN175343.1	Moraxella nonliquefaciens strain CIP 68.36 16S ribosomal	586	586	100%	3e-168	99%
JF893610.1	Uncultured Moraxella sp. clone C057 16S ribosomal RNA	586	586	100%	3e-168	99%
HM152563.1	Moraxella nonliquefaciens strain CCUG 348 16S ribosoma	586	586	100%	3e-168	99%
CP002005.1	Moraxella catarrhalis RH4, complete genome	586	2346	100%	3e-168	99%
AF443790.1	Moraxella nonliquefaciens isolate 96127/99 16S ribosoma	586	586	100%	3e-168	99%

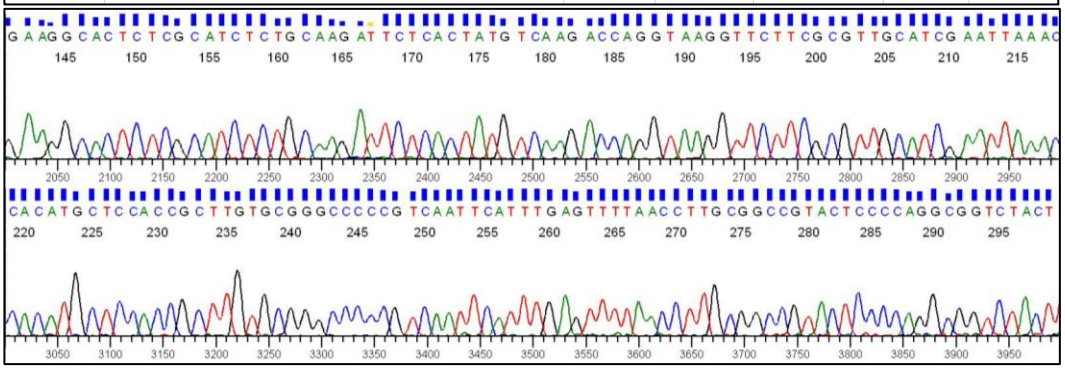


Figure AV.20 Electropherogram data and BLAST alignment results of organisms corresponding to the DNA sequence isolated from 25CNP-C. a) BLAST alignment results with no taxid specified. B) BLAST alignment results when specifying *Moraxella* (taxid:475) in search parameters.

JF233918.1	Uncultured bacterium clone ncd2699a08c1 16S ribosomal RNA gene, pa	749	749	100%	0.0	100%
JF233525.1	Uncultured bacterium clone ncd2690d10c1 16S ribosomal RNA gene, pa	749	749	100%	0.0	100%
JF233485.1	Uncultured bacterium clone ncd2689g09c1 16S ribosomal RNA gene, pa	749	749	100%	0.0	100%
JF233415.1	Uncultured bacterium clone ncd2692d09c1 16S ribosomal RNA gene, pa	749	749	100%	0.0	100%
AB680900.1	Dolosigranulum pigrum gene for 16S rRNA, partial sequence, strain: NB	745	745	100%	0.0	99%

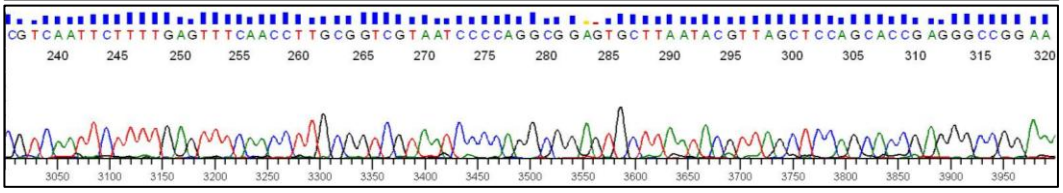


Figure AV.21 BLAST results from the sequence isolated from organism 26CNP-A, including a sample of the electropherogram results.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JF012962.1	Uncultured bacterium clone ncd140d01c1 16S ribosomal RNA gene, part	800	800	100%	0.0	100%
JF012849.1	Uncultured bacterium clone ncd138d12c1 16S ribosomal RNA gene, part	800	800	100%	0.0	100%
JF012814.1	Uncultured bacterium clone ncd138b12c1 16S ribosomal RNA gene, part	800	800	100%	0.0	100%
JF012804.1	Uncultured bacterium clone ncd138c12c1 16S ribosomal RNA gene, part	800	800	100%	0.0	100%
JF012741.1	Uncultured bacterium clone ncd137a08c1 16S ribosomal RNA gene, part	800	800	100%	0.0	100%

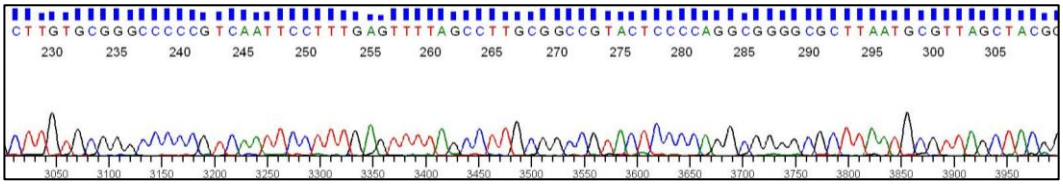


Figure AV.22 BLAST results for the DNA sequence isolated from organism 26CNP-B, along with electropherogram data indicating accuracy of sequence. Only uncultured species matched this sequence, despite a clear electropherogram.

Section 2: Sequencing results from multiplex PCR products

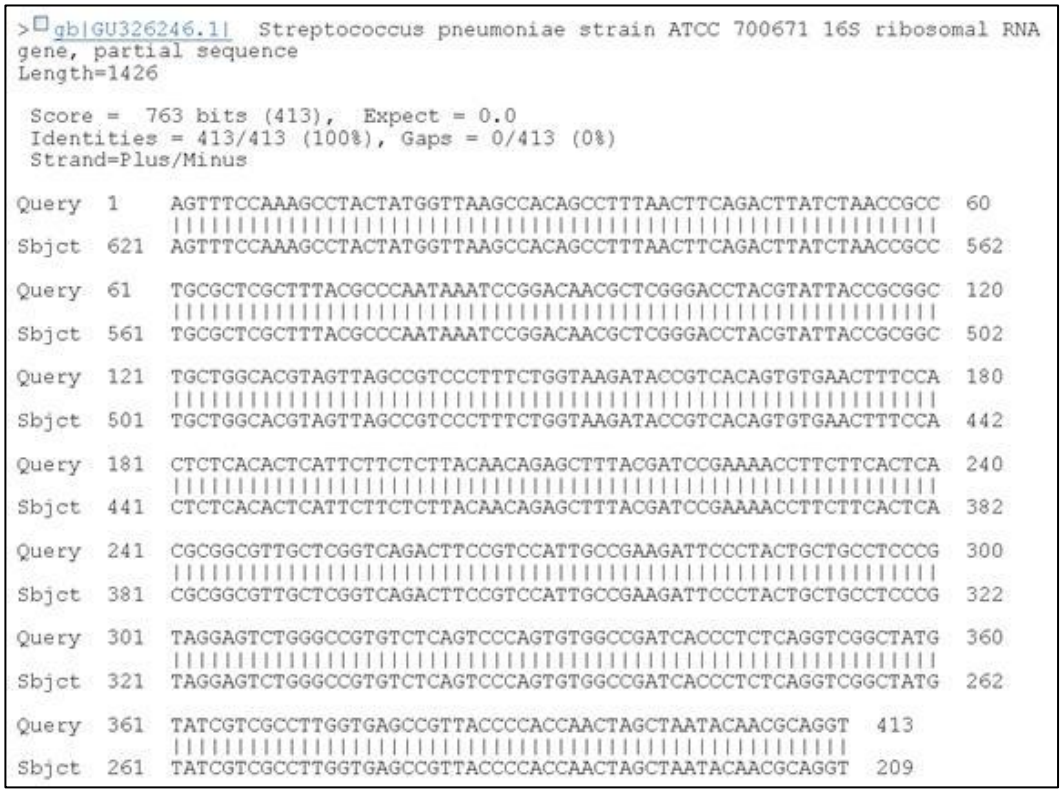
```
>gb|U10876.1|MCU10876  Moraxella catarrhalis ATCC 25238 16S rRNA gene
Length=1511

 Score =  268 bits (145),  Expect = 5e-73
 Identities = 147/148 (99%), Gaps = 0/148 (0%)
 Strand=Plus/Minus

Query  1    TCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGCTTTCACATCTGACTTAAATGAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  628  TCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGCTTTCACATCTGACTTAAATAAC  569

Query  61   CACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568  CACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCTGTATTACCG  509

Query  121  CGGCTGCTGGCACAGAGTTAGCCGGTGC  148
            ||||||||||||||||||||||||||||
Sbjct  508  CGGCTGCTGGCACAGAGTTAGCCGGTGC  481
```

Figure AV.23 BLAST alignment of 237bp PCR product from multiplex PCR. Results indicate 99% identity with *M. catarrhalis*.

```
>gb|JN227837.1|  Haemophilus influenzae strain M19478 16S ribosomal RNA gene,
partial sequence
Length=1462

 Score =  835 bits (452),  Expect = 0.0
 Identities = 452/452 (100%), Gaps = 0/452 (0%)
 Strand=Plus/Minus

Query  1    ACCCAGTCTGAAATGCAATTCCCAGGTTAAGCCCGGGGCTTTCACACCTCACTTAAATAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621  ACCCAGTCTGAAATGCAATTCCCAGGTTAAGCCCGGGGCTTTCACACCTCACTTAAATAA  562

Query  61   CCGCCTGCGTGCCCTTTACGCCCAGTTATTCCGATTAACGCTCGCACCCTCCGTATTACC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561  CCGCCTGCGTGCCCTTTACGCCCAGTTATTCCGATTAACGCTCGCACCCTCCGTATTACC  502

Query  121  GCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTATTTAACGTCAATTTGATGTGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  GCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGTATTTAACGTCAATTTGATGTGC  442

Query  181  TATTAACACATCAACCTTCCTCAATACCGAAAGAACTTTACAACCCTAAGGCCTTCTTCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  TATTAACACATCAACCTTCCTCAATACCGAAAGAACTTTACAACCCTAAGGCCTTCTTCA  382

Query  241  TTCACGCGGCATGGCTGCGTCAGGGTTCCCCCCATTGCGCAATATTCCCCACTGCTGCCT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  TTCACGCGGCATGGCTGCGTCAGGGTTCCCCCCATTGCGCAATATTCCCCACTGCTGCCT  322

Query  301  CCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  CCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGC  262

Query  361  TAGAGATCGCAGGCTTGGTAGGCCTTTACCCCACCAACTACCTAATCCCACTTGGGCTCA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  TAGAGATCGCAGGCTTGGTAGGCCTTTACCCCACCAACTACCTAATCCCACTTGGGCTCA  202

Query  421  TCCTATGGCATGCGGCCTCTCAGTCCCGCACT  452
            ||||||||||||||||||||||||||||||||
Sbjct  201  TCCTATGGCATGCGGCCTCTCAGTCCCGCACT  170
```

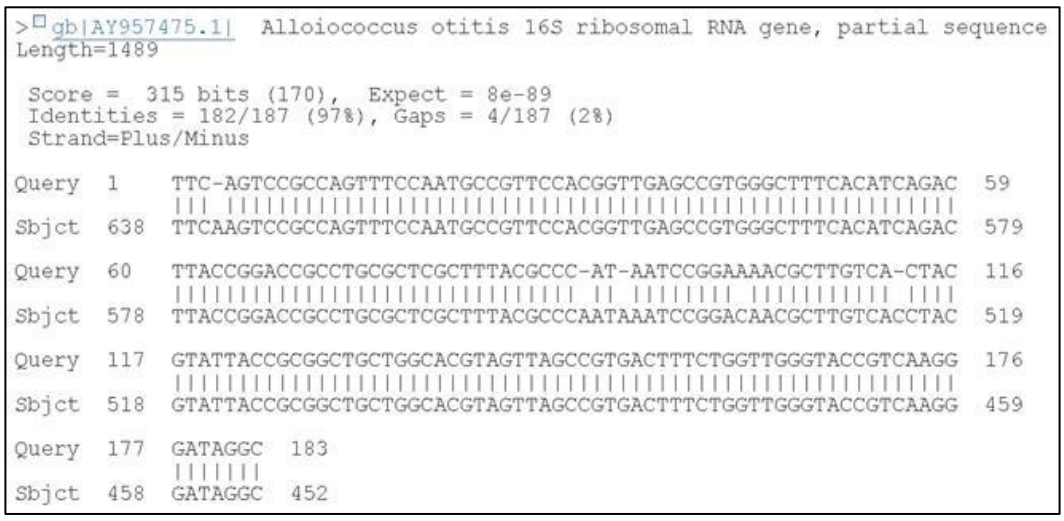
Figure AV.24 BLAST alignment of 525bp PCR product from multiplex PCR. Results indicate a 100% match with *H. influenzae*.

Section 3: Sequencing results from PCR products after second amplification round of nested PCR



```
>gb|GU326246.1|  Streptococcus pneumoniae strain ATCC 700671 16S ribosomal RNA
gene, partial sequence
Length=1426

 Score =  763 bits (413),  Expect = 0.0
 Identities = 413/413 (100%), Gaps = 0/413 (0%)
 Strand=Plus/Minus

Query  1    AGTTTCCAAAGCCTACTATGGTTAAGCCACAGCCTTTAACTTCAGACTTATCTAACCGCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621  AGTTTCCAAAGCCTACTATGGTTAAGCCACAGCCTTTAACTTCAGACTTATCTAACCGCC  562

Query  61   TGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561  TGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGTATTACCGCGGC  502

Query  121  TGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGATACCGTCACAGTGTGAACTTTCCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501  TGCTGGCACGTAGTTAGCCGTCCCTTTCTGGTAAGATACCGTCACAGTGTGAACTTTCCA  442

Query  181  CTCTCACACTCATTCTTCTCTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  CTCTCACACTCATTCTTCTCTTACAACAGAGCTTTACGATCCGAAAACCTTCTTCACTCA  382

Query  241  CGCGGCGTTGCTCGGTCAGACTTCCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  CGCGGCGTTGCTCGGTCAGACTTCCGTCCATTGCCGAAGATTCCCTACTGCTGCCTCCCG  322

Query  301  TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATG  262

Query  361  TATCGTCGCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATACAACGCAGGT  413
            ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  TATCGTCGCCTTGGTGAGCCGTTACCCCACCAACTAGCTAATACAACGCAGGT  209
```

Figure AV.25 Alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using SP specific forward primer. This DNA was obtained from sample 27RM.



```
>gb|AY957475.1|  Alloiococcus otitis 16S ribosomal RNA gene, partial sequence
Length=1489

 Score =  315 bits (170),  Expect = 8e-89
 Identities = 182/187 (97%), Gaps = 4/187 (2%)
 Strand=Plus/Minus

Query  1    TTC-AGTCCGCCAGTTTCCAATGCCGTTCCACGGTTGAGCCGTGGGCTTTCACATCAGAC  59
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  638  TTCAAGTCCGCCAGTTTCCAATGCCGTTCCACGGTTGAGCCGTGGGCTTTCACATCAGAC  579

Query  60   TTACCGGACCGCCTGCGCTCGCTTTACGCCC-AT-AATCCGGAAAACGCTTGTCA-CTAC  116
            |||||||||||||||||||||||||||||| || |||||||| |||||||||| ||||
Sbjct  578  TTACCGGACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGTCACCTAC  519

Query  117  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTTGGGTACCGTCAAGG  176
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  518  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGACTTTCTGGTTGGGTACCGTCAAGG  459

Query  177  GATAGGC  183
            |||||||
Sbjct  458  GATAGGC  452
```

Figure AV.26 Alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using *A. otitidis* specific forward primer. This DNA was obtained from sample 7CLE. Electropherogram demonstrated a low signal to noise ratio, accounting for gaps in the sequencing.

```
>gb|JN175343.1|  Moraxella nonliquefaciens strain CIP 68.36 16S ribosomal RNA
gene, complete sequence
Length=1521

 Score =  361 bits (195),  Expect = 1e-96
 Identities = 199/201 (99%), Gaps = 0/201 (0%)
 Strand=Plus/Minus

Query  2    CCCATCTCAACTACTCTAGTTATCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGC  61
            ||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  651  CCCCTCTCACCTACTCTAGTTATCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGC  592

Query  62   TTTCACATCTGACTTAAATAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAAC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  591  TTTCACATCTGACTTAAATAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAAC  532

Query  122  GCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTG  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  531  GCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTG  472

Query  182  GGTAACGTCAGGGCTTATGGG  202
            |||||||||||||||||||||
Sbjct  471  GGTAACGTCAGGGCTTATGGG  451
```

Figure AV.27 Alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using MS specific forward primer. This DNA was obtained from sample 7CNP. Note the identical nature of the alignment to that of *Moraxella catarrhalis* (see Figure AV.28).

```
>emb|AM263480.1|  Moraxella catarrhalis partial 16S rRNA gene, clone P4.20
Length=603

 Score =  361 bits (195),  Expect = 1e-100
 Identities = 199/201 (99%), Gaps = 0/201 (0%)
 Strand=Plus/Minus

Query  2    CCCATCTCAACTACTCTAGTTATCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGC  61
            ||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499  CCCCTCTCACCTACTCTAGTTATCCAGTATCAGATGCAGTTCCCAGGTTAAGCCCGGGGC  440

Query  62   TTTCACATCTGACTTAAATAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAAC  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  TTTCACATCTGACTTAAATAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAAC  380

Query  122  GCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTG  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  GCTTGCACCCTCTGTATTACCGCGGCTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGTG  320

Query  182  GGTAACGTCAGGGCTTATGGG  202
            |||||||||||||||||||||
Sbjct  319  GGTAACGTCAGGGCTTATGGG  299
```

Figure AV.28 Alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using MS specific forward primer. This DNA was obtained from sample 7CNP.Note the identical nature of the alignment to that of *Moraxella nonliquefaciens* (see Figure AV.27).

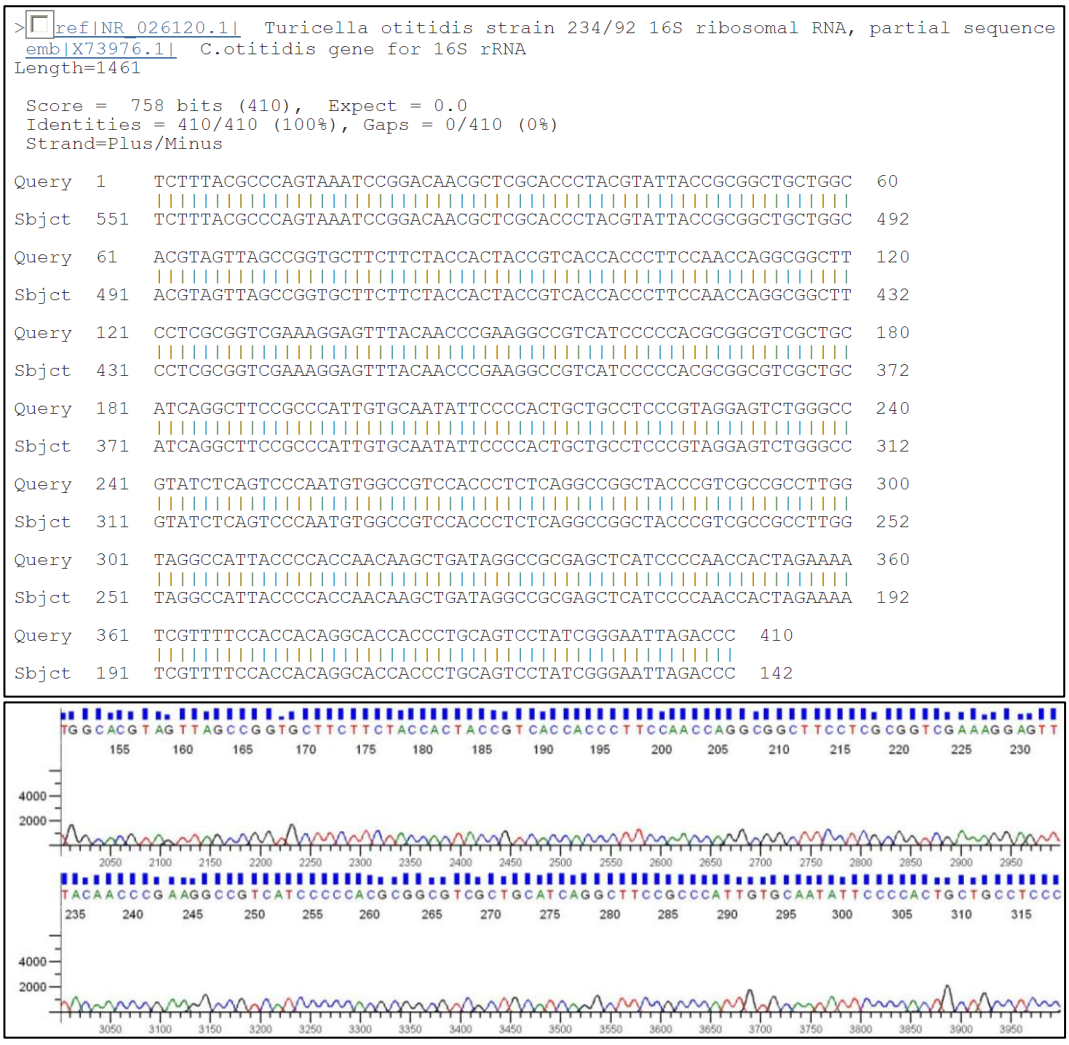
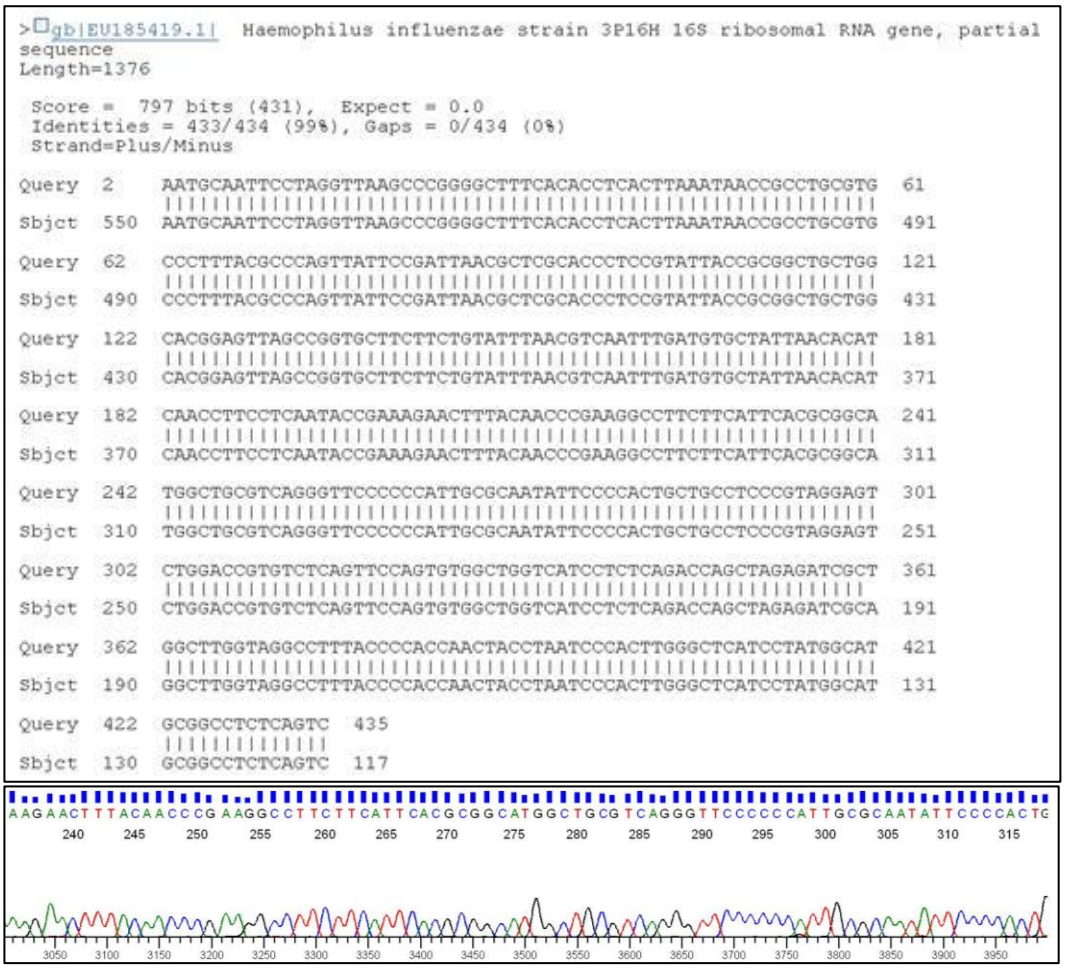


Figure AV.29 Sample 18CLE - alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using TO specific forward primer. Electropherogram data gives an indication of sequence clarity.



>gb|EU185419.1| Haemophilus influenzae strain 3P16H 16S ribosomal RNA gene, partial sequence
Length=1376

Score = 797 bits (431), Expect = 0.0
Identities = 433/434 (99%), Gaps = 0/434 (0%)
Strand=Plus/Minus

```
Query  2    AATGCAATTCCTAGGTTAAGCCCGGGGCTTTCACACCTCACTTAAATAACCGCCTGCGTG  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  550  AATGCAATTCCTAGGTTAAGCCCGGGGCTTTCACACCTCACTTAAATAACCGCCTGCGTG  491

Query  62   CCCTTTACGCCCAGTTATTCCGATTAACGCTCGCACCCTCCGTATTACCGCGGCTGCTGG  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  490  CCCTTTACGCCCAGTTATTCCGATTAACGCTCGCACCCTCCGTATTACCGCGGCTGCTGG  431

Query  122  CACGGAGTTAGCCGGTGCTTCTTCTGTATTTAACGTCAATTTGATGTGCTATTAACACAT  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  430  CACGGAGTTAGCCGGTGCTTCTTCTGTATTTAACGTCAATTTGATGTGCTATTAACACAT  371

Query  182  CAACCTTCCTCAATACCGAAAGAACTTTACAACCCGAAGGCCTTCTTCATTCACGCGGCA  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  370  CAACCTTCCTCAATACCGAAAGAACTTTACAACCCGAAGGCCTTCTTCATTCACGCGGCA  311

Query  242  TGGCTGCGTCAGGGTTCCCCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGT  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  310  TGGCTGCGTCAGGGTTCCCCCCATTGCGCAATATTCCCCACTGCTGCCTCCCGTAGGAGT  251

Query  302  CTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGAGATCGCT  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  250  CTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGAGATCGCA  191

Query  362  GGCTTGGTAGGCCTTTACCCCACCAACTACCTAATCCCACTTGGGCTCATCCTATGGCAT  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  190  GGCTTGGTAGGCCTTTACCCCACCAACTACCTAATCCCACTTGGGCTCATCCTATGGCAT  131

Query  422  GCGGCCTCTCAGTC  435
            ||||||||||||||
Sbjct  130  GCGGCCTCTCAGTC  117
```


Figure AV.30 Sample 17CLE - alignment to BLAST database of sequencing results from PCR products of the second step of the nested PCR using HI specific forward primer. Electropherogram results indicate sequence clarity.

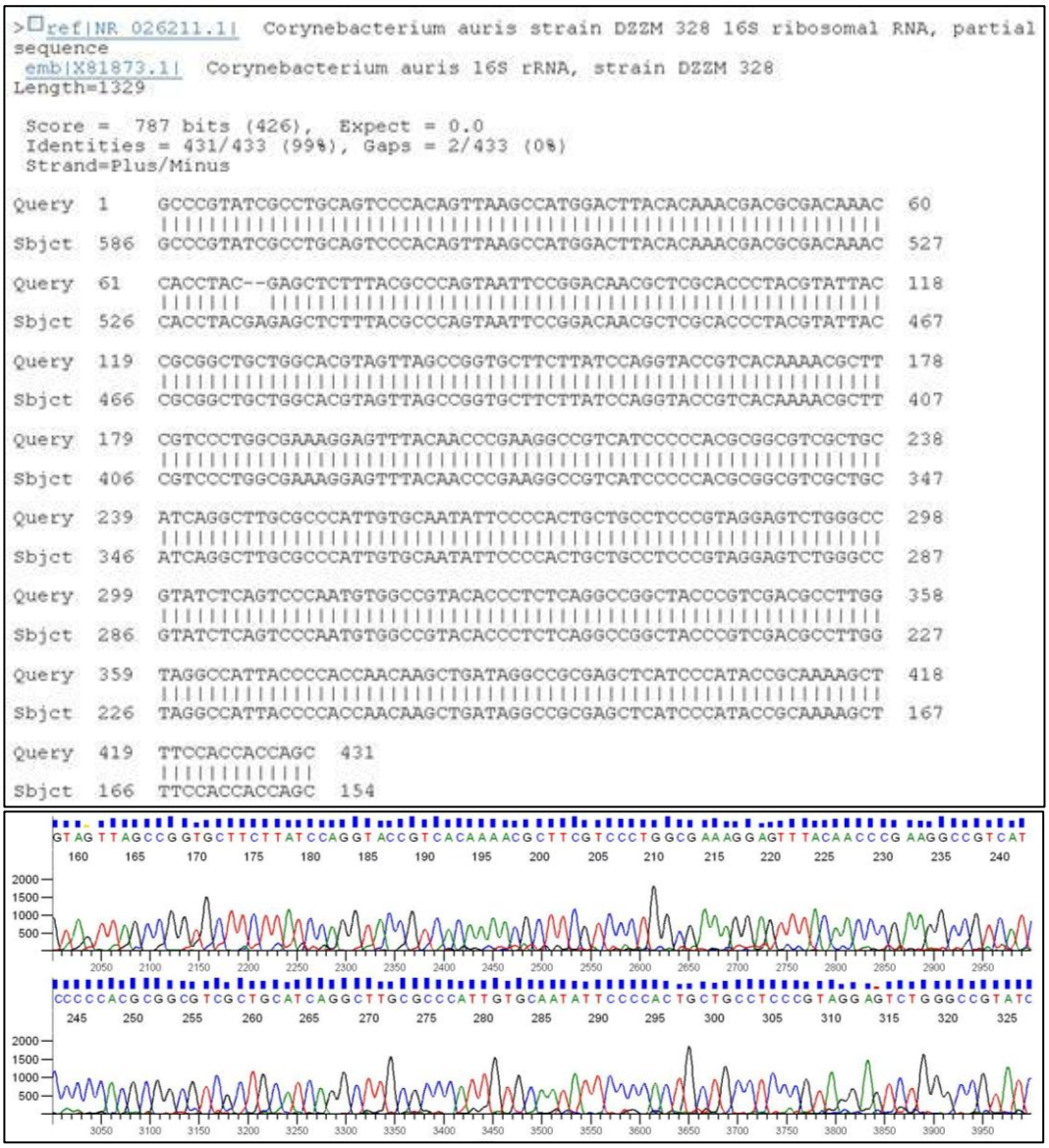


Figure AV.31 Sample 23CNP - Alignment to BLAST database of sequence from PCR products of the second step of the nested PCR using CA specific forward primer. Electropherogram results indicating clarity of the sequence.

Appendix VI: Statistics

The following are the statistical reports from the software program Statistica. The chi-squared test p values were used when comparing the outer ear and nasopharyngeal cavities between the study groups, while the McNemar's test p values were used when comparing the studied sites within the study groups.

	OM NPvsOE AO		
	OE AO Abs	OE AO Pres	Row Totals
NP AO Abs	9	18	27
Percent of total	30.000%	60.000%	90.000%
NP AO Pres	1	2	3
Percent of total	3.333%	6.667%	10.000%
Column totals	10	20	30
Percent of total	33.333%	66.667%	
Chi-square (df=1)	0.00	p=1.0000	
V-square (df=1)	0.00	p=1.0000	
Yates corrected Chi-square	.42	p= .5186	
Phi-square	0.00000		
Fisher exact p, one-tailed		p= .7488	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	3.27	p= .0704	
Chi-square (B/C)	13.47	p= .0002	

	OM OEvs.NP CA		
	OM OE CA Abs	OM OE CA Pres	Row Totals
OM NP CA Abs	18	6	24
Percent of total	64.286%	21.429%	85.714%
OM NP CA Pres	3	1	4
Percent of total	10.714%	3.571%	14.286%
Column totals	21	7	28
Percent of total	75.000%	25.000%	
Chi-square (df=1)	0.00	p=1.0000	
V-square (df=1)	0.00	p=1.0000	
Yates corrected Chi-square	.39	p= .5329	
Phi-square	0.00000		
Fisher exact p, one-tailed		p= .7077	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	13.47	p= .0002	
Chi-square (B/C)	.44	p= .5050	

	OM OEvs.NP TO		
	OM OE TO Abs	OM OE TO Pres	Row Totals
OM NP TO Abs	17	12	29
Percent of total	56.667%	40.000%	96.667%
OM NP TO Pres	0	1	1
Percent of total	0.000%	3.333%	3.333%
Column totals	17	13	30
Percent of total	56.667%	43.333%	
Chi-square (df=1)	1.35	p= .2448	
V-square (df=1)	1.31	p= .2528	
Yates corrected Chi-square	.02	p= .8912	
Phi-square	.04509		
Fisher exact p, one-tailed		p= .4333	
two-tailed		p= .4333	
McNemar Chi-square (A/D)	12.50	p= .0004	
Chi-square (B/C)	10.08	p= .0015	

	OM OEvs.NP MS		
	OM OE MS Abs	OM OE MS Pres	Row Totals
OM NP MS Abs	8	0	8
Percent of total	26.667%	0.000%	26.667%
OM NP MS Pres	9	13	22
Percent of total	30.000%	43.333%	73.333%
Column totals	17	13	30
Percent of total	56.667%	43.333%	
Chi-square (df=1)	8.34	p= .0039	
V-square (df=1)	8.06	p= .0045	
Yates corrected Chi-square	6.11	p= .0135	
Phi-square	.27807		
Fisher exact p, one-tailed		p= .0042	
two-tailed		p= .0044	
McNemar Chi-square (A/D)	.76	p= .3827	
Chi-square (B/C)	7.11	p= .0077	

	OM OEvs. NP HI		
	OM OE HI Abs	OM OE HI Pres	Row Totals
OM NP HI Abs	14	2	16
Percent of total	46.667%	6.667%	53.333%
OM NP HI Pres	7	7	14
Percent of total	23.333%	23.333%	46.667%
Column totals	21	9	30
Percent of total	70.000%	30.000%	
Chi-square (df=1)	5.00	p= .0254	
V-square (df=1)	4.83	p= .0279	
Yates corrected Chi-square	3.37	p= .0663	
Phi-square	.16667		
Fisher exact p, one-tailed		p= .0323	
two-tailed		p= .0457	
McNemar Chi-square (A/D)	1.71	p= .1904	
Chi-square (B/C)	1.78	p= .1824	

Table VI.1 Statistica report tables showing input values and p values when comparing colonisation of the nasopharynx and the outer ear canal in the OM patient study group. The McNemar's Chi-square was the p value used here.

	Control HI NPvs.OE		
	CNTL OE HI Abs	CNTL OE HI Pres	Row Totals
Control NP HI Abs	11	4	15
Percent of total	37.931%	13.793%	51.724%
Control NP HI Pres	4	10	14
Percent of total	13.793%	34.483%	48.276%
Column totals	15	14	29
Percent of total	51.724%	48.276%	
Chi-square (df=1)	5.81	p= .0159	
V-square (df=1)	5.61	p= .0179	
Yates corrected Chi-square	4.16	p= .0415	
Phi-square	.20036		
Fisher exact p, one-tailed		p= .0199	
two-tailed		p= .0268	
McNemar Chi-square (A/D)	0.00	p=1.0000	
Chi-square (B/C)	.13	p= .7237	

	Control AO NPvs.OE		
	CNTL OE AO Abs	CNTL OE AO Pres	Row Totals
Control NP AO Abs	10	17	27
Percent of total	34.483%	58.621%	93.103%
Control NP AO Pres	0	2	2
Percent of total	0.000%	6.897%	6.897%
Column totals	10	19	29
Percent of total	34.483%	65.517%	
Chi-square (df=1)	1.13	p= .2877	
V-square (df=1)	1.09	p= .2961	
Yates corrected Chi-square	.09	p= .7700	
Phi-square	.03899		
Fisher exact p, one-tailed		p= .4212	
two-tailed		p= .5320	
McNemar Chi-square (A/D)	4.08	p= .0433	
Chi-square (B/C)	15.06	p= .0001	

	Control MS NPvs.OE		
	CNTL OE MS Abs	CNTL OE MS Pres	Row Totals
Control NP MS Abs	2	1	3
Percent of total	6.897%	3.448%	10.345%
Control NP MS Pres	7	19	26
Percent of total	24.138%	65.517%	89.655%
Column totals	9	20	29
Percent of total	31.034%	68.966%	
Chi-square (df=1)	1.98	p= .1589	
V-square (df=1)	1.92	p= .1662	
Yates corrected Chi-square	.56	p= .4533	
Phi-square	.06845		
Fisher exact p, one-tailed		p= .2200	
two-tailed		p= .2200	
McNemar Chi-square (A/D)	12.19	p= .0005	
Chi-square (B/C)	3.13	p= .0771	

	Control TO NPvs.OE		
	CNTL OE TO Abs	CNTL OE TO Pres	Row Totals
Control NP TO Abs	12	16	28
Percent of total	41.379%	55.172%	96.552%
Control NP TO Pres	0	1	1
Percent of total	0.000%	3.448%	3.448%
Column totals	12	17	29
Percent of total	41.379%	58.621%	
Chi-square (df=1)	.73	p= .3925	
V-square (df=1)	.71	p= .4008	
Yates corrected Chi-square	.03	p= .8586	
Phi-square	.02521		
Fisher exact p, one-tailed		p= .5862	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	7.69	p= .0055	
Chi-square (B/C)	14.06	p= .0002	

	Control CA NPvs.OE		
	CNTL OE CA Abs	CNTL OE CA Pres	Row Totals
Control NP CA Abs	22	6	28
Percent of total	75.862%	20.690%	96.552%
Control NP CA Pres	1	0	1
Percent of total	3.448%	0.000%	3.448%
Column totals	23	6	29
Percent of total	79.310%	20.690%	
Chi-square (df=1)	.27	p= .6032	
V-square (df=1)	.26	p= .6095	
Yates corrected Chi-square	.54	p= .4615	
Phi-square	.00932		
Fisher exact p, one-tailed		p= .7931	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	20.05	p= .0000	
Chi-square (B/C)	2.29	p= .1306	

Table VI.2 Statistica report tables showing input values and p values for comparisons between the colonisation of the nasopharynx and the outer ear canal in the control group. The McNemar's Chi-square was the p value used here.

	Outer ear AO		
	AO Y	AO N	Row Totals
Patient ears	38	29	67
Percent of total	29.008%	22.137%	51.145%
Control ears	41	23	64
Percent of total	31.298%	17.557%	48.855%
Column totals	79	52	131
Percent of total	60.305%	39.695%	
Chi-square (df=1)	.74	p= .3903	
V-square (df=1)	.73	p= .3922	
Yates corrected Chi-square	.46	p= .4963	
Phi-square	.00563		
Fisher exact p, one-tailed		p= .2483	
two-tailed		p= .4754	
McNemar Chi-square (A/D)	3.21	p= .0731	
Chi-square (B/C)	1.73	p= .1886	

	Outer ear TO		
	TO Y	TO N	Row Totals
Patient ears	23	44	67
Percent of total	17.557%	33.588%	51.145%
Control ears	24	40	64
Percent of total	18.321%	30.534%	48.855%
Column totals	47	84	131
Percent of total	35.878%	64.122%	
Chi-square (df=1)	.14	p= .7052	
V-square (df=1)	.14	p= .7063	
Yates corrected Chi-square	.04	p= .8445	
Phi-square	.00109		
Fisher exact p, one-tailed		p= .4222	
two-tailed		p= .7195	
McNemar Chi-square (A/D)	4.06	p= .0438	
Chi-square (B/C)	5.31	p= .0212	

	Outer ear SP		
	SP Y	SP N	Row Totals
Patient ears	1	66	67
Percent of total	.763%	50.382%	51.145%
Control ears	1	63	64
Percent of total	.763%	48.092%	48.855%
Column totals	2	129	131
Percent of total	1.527%	98.473%	
Chi-square (df=1)	.00	p= .9740	
V-square (df=1)	.00	p= .9741	
Yates corrected Chi-square	.46	p= .4964	
Phi-square	.00001		
Fisher exact p, one-tailed		p= .7403	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	58.14	p= .0000	
Chi-square (B/C)	61.13	p= .0000	

	Outer ear CA		
	CA Y	CA N	Row Totals
Patient ears	9	58	67
Percent of total	6.870%	44.275%	51.145%
Control ears	9	55	64
Percent of total	6.870%	41.985%	48.855%
Column totals	18	113	131
Percent of total	13.740%	86.260%	
Chi-square (df=1)	.01	p= .9167	
V-square (df=1)	.01	p= .9170	
Yates corrected Chi-square	.02	p= .8814	
Phi-square	.00008		
Fisher exact p, one-tailed		p= .5585	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	31.64	p= .0000	
Chi-square (B/C)	34.39	p= .0000	

	Outer ear MS		
	Column 1	Column 2	Row Totals
Patient ears	21	46	67
Percent of total	16.031%	35.115%	51.145%
Control ears	37	27	64
Percent of total	28.244%	20.611%	48.855%
Column totals	58	73	131
Percent of total	44.275%	55.725%	
Chi-square (df=1)	9.30	p= .0023	
V-square (df=1)	9.22	p= .0024	
Yates corrected Chi-square	8.25	p= .0041	
Phi-square	.07096		
Fisher exact p, one-tailed		p= .0020	
two-tailed		p= .0028	
McNemar Chi-square (A/D)	.52	p= .4705	
Chi-square (B/C)	.77	p= .3799	

	Outer ear HI		
	Column 1	Column 2	Row Totals
Patient ears	12	55	67
Percent of total	9.160%	41.985%	51.145%
Control ears	19	45	64
Percent of total	14.504%	34.351%	48.855%
Column totals	31	100	131
Percent of total	23.664%	76.336%	
Chi-square (df=1)	2.51	p= .1129	
V-square (df=1)	2.49	p= .1143	
Yates corrected Chi-square	1.90	p= .1677	
Phi-square	.01919		
Fisher exact p, one-tailed		p= .0837	
two-tailed		p= .1501	
McNemar Chi-square (A/D)	17.96	p= .0000	
Chi-square (B/C)	16.55	p= .0000	

Table VI.3 Statistica report tables of input values for statistical significance calculations between the study groups within the outer ear study site. The Chi-square with 1 degrees freedom (df=1) was the p value used here.

	Nasopharyngeal SP		
	SP Y	SP N	Row Totals
Patient NP	8	25	33
Percent of total	12.308%	38.462%	50.769%
Control NP	7	25	32
Percent of total	10.769%	38.462%	49.231%
Column totals	15	50	65
Percent of total	23.077%	76.923%	
Chi-square (df=1)	.05	p= .8208	
V-square (df=1)	.05	p= .8222	
Yates corrected Chi-square	.00	p= .9458	
Phi-square	.00079		
Fisher exact p, one-tailed		p= .5273	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	7.76	p= .0054	
Chi-square (B/C)	9.03	p= .0027	

	Nasopharyngeal TO		
	TO Y	TO N	Row Totals
Patient NP	2	31	33
Percent of total	3.077%	47.692%	50.769%
Control NP	1	31	32
Percent of total	1.538%	47.692%	49.231%
Column totals	3	62	65
Percent of total	4.615%	95.385%	
Chi-square (df=1)	.32	p= .5728	
V-square (df=1)	.31	p= .5758	
Yates corrected Chi-square	.00	p= .9782	
Phi-square	.00489		
Fisher exact p, one-tailed		p= .5117	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	23.76	p= .0000	
Chi-square (B/C)	26.28	p= .0000	

	Nasopharyngeal HI		
	HI Y	HI N	Row Totals
Patient NP	16	17	33
Percent of total	24.615%	26.154%	50.769%
Control NP	16	16	32
Percent of total	24.615%	24.615%	49.231%
Column totals	32	33	65
Percent of total	49.231%	50.769%	
Chi-square (df=1)	.01	p= .9028	
V-square (df=1)	.01	p= .9035	
Yates corrected Chi-square	.02	p= .8998	
Phi-square	.00023		
Fisher exact p, one-tailed		p= .5500	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	.03	p= .8597	
Chi-square (B/C)	0.00	p=1.0000	

	Nasopharyngeal CA		
	CA Y	CA N	Row Totals
Patient NP	4	29	33
Percent of total	6.154%	44.615%	50.769%
Control NP	2	30	32
Percent of total	3.077%	46.154%	49.231%
Column totals	6	59	65
Percent of total	9.231%	90.769%	
Chi-square (df=1)	.67	p= .4136	
V-square (df=1)	.66	p= .4172	
Yates corrected Chi-square	.15	p= .6973	
Phi-square	.01028		
Fisher exact p, one-tailed		p= .3511	
two-tailed		p= .6724	
McNemar Chi-square (A/D)	18.38	p= .0000	
Chi-square (B/C)	21.81	p= .0000	

	Nasopharyngeal AO		
	AO Y	AO N	Row Totals
Patient NP	3	30	33
Percent of total	4.615%	46.154%	50.769%
Control NP	2	30	32
Percent of total	3.077%	46.154%	49.231%
Column totals	5	60	65
Percent of total	7.692%	92.308%	
Chi-square (df=1)	.18	p= .6674	
V-square (df=1)	.18	p= .6698	
Yates corrected Chi-square	.00	p= .9714	
Phi-square	.00284		
Fisher exact p, one-tailed		p= .5149	
two-tailed		p=1.0000	
McNemar Chi-square (A/D)	20.48	p= .0000	
Chi-square (B/C)	22.78	p= .0000	

	Nasopharyngeal MS		
	MS Y	MS N	Row Totals
Patient Nasopharynx	23	10	33
Percent of total	35.385%	15.385%	50.769%
Control Nasopharynx	28	4	32
Percent of total	43.077%	6.154%	49.231%
Column totals	51	14	65
Percent of total	78.462%	21.538%	
Chi-square (df=1)	3.05	p= .0809	
V-square (df=1)	3.00	p= .0833	
Yates corrected Chi-square	2.08	p= .1488	
Phi-square	.04688		
Fisher exact p, one-tailed		p= .0735	
two-tailed		p= .1305	
McNemar Chi-square (A/D)	12.00	p= .0005	
Chi-square (B/C)	7.61	p= .0058	

Table VI.4 Statistica report tables of input values for determining statistical significance between study groups within the nasopharyngeal study site. The Chi-squared with 1 degrees of freedom (df=1) was the p value used here.