CONTRASTING PURULENT AND NON-PURULENT MIDDLE EAR EFFUSIONS: A STUDY OF OTITIS MEDIA ETIOLOGY

BY

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ABBREVIATIONS

A. otitidis	*Alloiococcus otitidis*
AOM	Acute otitis media
bp	Base pair
CDC	Centers for Disease Control
°C	Degrees Celsius
dB	Decibel
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
EDTA	Ethylenediaminetetraacetic acid
FWD	Forward
GAS	Group A *Streptococcus*
H. influenzae	*Haemophilus influenzae*
IPD	Invasive pneumococcal disease
M	Molar
M. catarrhalis	*Moraxella catarrhalis*
MEE	Middle ear effusion
MEF	Middle ear fluid
mg	Milligram
mL	Milliliter
mM	Millimolar

mPCR	Multiplex polymerase chain reaction
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaOAc	Sodium acetate
ng	Nanogram
No.	Number
OM	Otitis media
OME	Otitis media with effusion
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PCV7	Pneumococcal conjugate vaccine (7-valent)
PCV13	Pneumococcal conjugate vaccine (13-valent)
rAOM	Recurrent acute otitis media
REV	Reverse
RNA	Ribonucleic acid
rpm	Revolutions per minute
RSV	Respiratory syncytial virus
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
S. pyogenes	*Streptococcus pyogenes*
SDS	Sodium dodecyl sulfate
Taq	*Thermus aquaticus*
TE	Tris-Ethylenediaminetetraacetic acid

TM	Tympanic membrane
TT	Tetanus toxin
U	Units
µg	Microgram
µL	Microliter
µM	Micromolar
URTI	Upper respiratory tract infection
US	United States

ABSTRACT

Otitis media (OM) is a very common childhood illness. OM is the reason for the majority of pediatric office visits and antibiotic prescriptions for children. 80% of children under the age of 3 will experience at least one episode of OM, and a portion of these children will experience multiple OM episodes by age 7. The economic burden associated with OM is estimated to be $3-5 billion annually. A small number of OM cases involve sequelae, such as acute mastoiditis, that can have fatal consequences if left untreated.

OM is a multifactorial disease, often involving mixed populations of viral and bacterial species. Understanding the prevalence of these organisms and their interspecies interactions will give insight into how best to treat cases of OM. This 5-year prospective study utilized multiplex polymerase chain reaction (mPCR) to evaluate the prevalence of 3 classic otopathogens (*Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*) as well as the fastidious organisms *Alloiococcus otitidis* in middle ear fluid obtained from children undergoing routine tympanostomy tube placement at Wake Forest University Baptist Medical Center from July 2009 – March 2014.

In our patient population, when fluid obtained was purulent in nature, the most often identified organism was *H. influenzae*. These purulent fluids contained mostly single organisms rather than mixed populations, and only a small percentage of purulent fluids yielded no results. Analysis of the non-purulent fluids obtained revealed different results. *H. influenzae* and *A. otitidis*

were the most prevalent organisms identified. These non-purulent fluids contained evidence of more equal proportions of single and polymicrobial infections. Unlike the purulent effusions, half of the non-purulent samples yielded no results.

Statistical analysis of our data failed to reveal a correlation between the 3 classic otopathogens; however, a positive correlation was observed between *M. catarrhalis* and *A. otitidis*. A positive correlation was observed between the presence of purulent effusions and *H. influenzae*. Children with a history of previous infections were also more likely to harbor *H. influenzae*. Although the identification of *S. pneumoniae* was low in our patient population, a positive correlation was observed between pneumococcus and children under the age of 3. Our data also suggests that *A. otitidis* may be able to more effectively persist in the middle ear of patients.

CHAPTER I.

INTRODUCTION

Otitis Media

Otitis media (OM) is a disease condition involving inflammation of the middle ear. OM is a very common childhood illness that is responsible for the majority of pediatric office visits as well as antibiotic prescriptions for children [1-5]. Current estimates suggest that 80% of children will have experienced at least one episode of OM before 3 years of age [6, 7]. By 7 years of age, 40% of these children will have experienced 6 or more recurrences of OM [8]. Annual direct cost estimates associated with OM approach $3-5 billion [9-11]; indirect costs such as loss of productivity and lost work days by caregivers drive this estimate higher [12, 13]. An often overlooked aspect of OM burden is that a small number of OM cases will transition into sequelae with fatal consequences. In 2013, ~2400 deaths were reported worldwide due to complications from OM [14]. These complications are most often mastoid and intracranial infections occurring in developing countries.

Categories of OM

Two predominant subtypes of OM are acute otitis media (AOM) and otitis media with effusion (OME). Pneumatic otoscopy (visually inspecting the tympanic membrane (TM)) is the standard procedure for diagnosing and distinguishing subtypes of OM. This process allows for the assessment of the contour of the TM (normal, bulging, retracted, etc.), its color and translucency,

and its mobility (increased, decreased, etc.) [15].

AOM

AOM represents inflammation of the middle ear coinciding with signs and symptoms of active infection. These symptoms include an opaque bulging TM, the presence of middle ear effusion (otorrhea), ear pain (otalgia), and erythema of the TM [15-17]. Most AOM occurs between 6 and 24 months of age, with the peak incidence falling between 9 and 15 months [18]. According to clinical practice guidelines, a diagnosis of AOM is appropriate when children present with moderate to severe bulging of the TM or new onset of otorrhea not due to otitis externa. It is also appropriate when children present with mild bulging of the TM and recent onset of otalgia or intense TM erythema. Middle ear effusion must be present for AOM to be clinically diagnosed [15].

OME

OME is described as the accumulation of a glue-like fluid behind an intact TM in the absence of symptoms of acute inflammation [19, 20]. OME is most prevalent in children 6 months to 4 years of age [21], with rates of disease dropping drastically by age 7, possibly due to the maturation of the eustachian tube [22]. Otoscopically, the TM usually maintains a translucent appearance and is found in a normal or retracted position (in contrast to the opaque bulging TM of AOM) [17, 23]. The most common symptom reported during cases of OME is hearing loss (due to fluid accumulation) [22], with an average hearing loss of ~27dB [24]. The hearing impairment and discomfort associated with OME may

have long-term linguistic, developmental, and behavioral consequences if the disorder persists for a long duration [20, 25-31], although the extent of these effects can be variable and is a controversial topic [20].

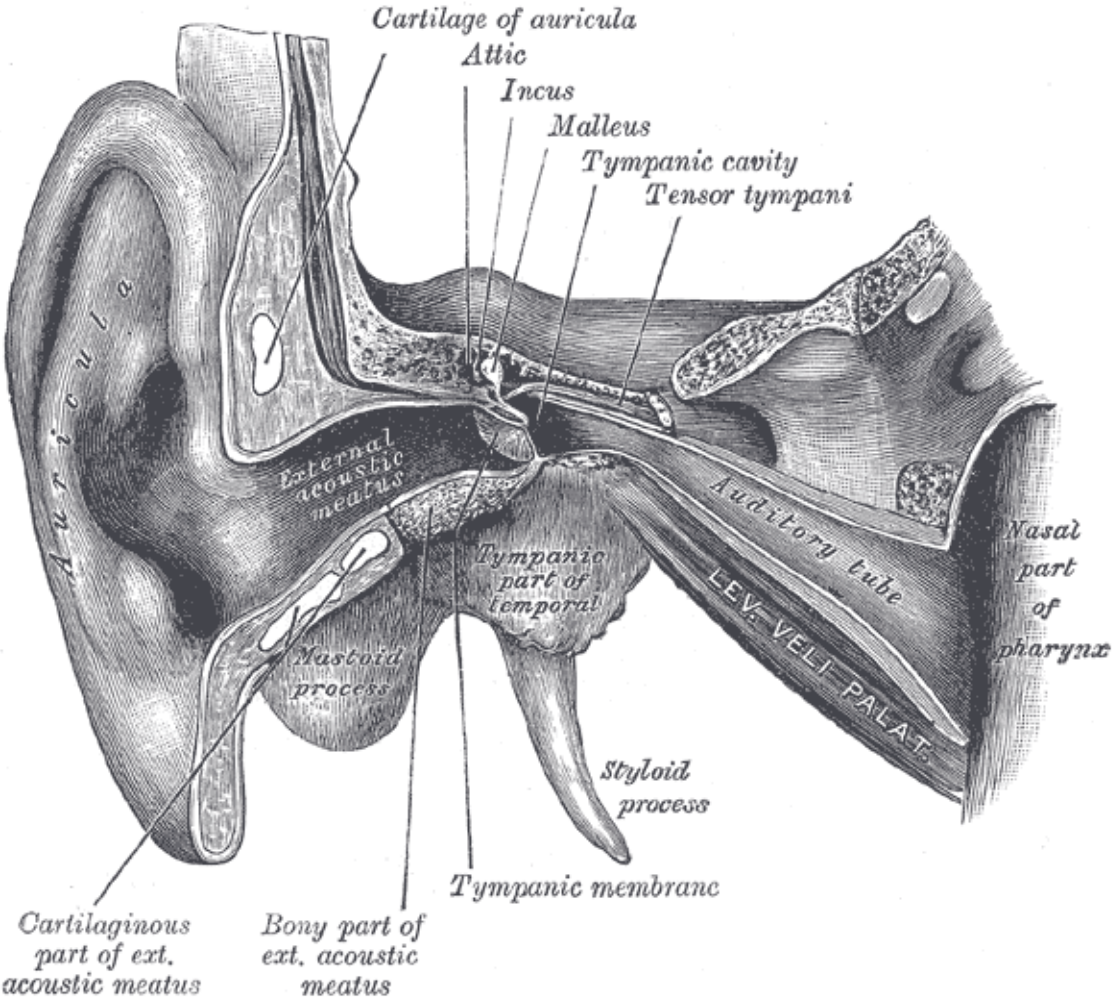
Contributing Factors to OM Onset

Eustachian Tube Dysfunction

The eustachian tube (auditory tube) is an ear canal that connects the nasopharynx with the middle ear cavity (Figure 1). It is involved in regulating pressure within the middle ear space, equalizing it with air pressure found outside of the body. It is also responsible for proper drainage and clearance of middle ear secretions into the nasopharynx [32]. If these functions are not carried out properly, conditions arise that are conducive to the onset of OM. A common pattern of OM disease progression seen in children is as follows: 1) congestion of the eustachian tube mucosa (due to an antecedent upper respiratory tract infection) or some other physical abnormality results in obstruction of the tube; 2) negative middle ear pressure develops, allowing for the aspiration of potential pathogens from the nasopharynx into the middle ear cavity; 3) fluid accumulation occurs in the middle ear due to lack of proper effusion clearance; 4) pathogens proliferate in the middle ear secretions, ultimately resulting in symptoms associated with OM [32].

The anatomy of the pediatric eustachian tube, along with an immature immune system, is partly responsible for why children are prone to developing OM. A child's eustachian tube is shorter and narrower than the eustachian tube

Figure 1. Diagram of the Eustachian Tube [33]


Cartilage of auricula
Attic
Incus
Malleus
Tympanic cavity
Tensor tympani
Auricula
External acoustic meatus
Auditory tube
Nasal part of pharynx
Tympanic part of temporal
LEV. VELI PALAT.
Mastoid process
Styloid process
Tympanic membrane
Cartilaginous part of ext. acoustic meatus
Bony part of ext. acoustic meatus

of an adult [34]. The shorter tube length allows nasopharyngeal commensals/pathogens to more easily access the middle ear cavity, and the narrow nature of the tube makes it more prone to blockage, ultimately leading to eustachian tube dysfunction and the generation of negative middle ear pressure.

Another characteristic of the pediatric eustachian tube that plays a role in the development of OM is the way that it is arranged in a child's skull. While the angle of an adult's eustachian tube from nasopharynx to middle ear lies at a relatively steep 45° compared to the horizontal plane, a child's eustachian tube lies at an almost horizontal 10° [35]. This angle, in conjunction with a shorter tube length, results in less stringent conditions required for organism ascension into the middle ear space.

Upper Respiratory Tract Infections

Evidence suggests that up to 97% of children will experience one or more viral upper respiratory tract infections (URTI) between the ages of 6 months and 3 years, with a small percentage of these children experiencing >12 URTI episodes in 1 year [36]. OM can be concomitantly diagnosed in ~30-70% of URTI cases [36, 37]. Several different upper respiratory viruses have been identified coincident with episodes of OM. The most prevalent viruses are adenovirus, coronavirus, influenza virus, rhinovirus, and respiratory syncytial virus (RSV) [36-42], although the hierarchy of these viruses differs between specific research studies and location.

These viral infections facilitate the onset of OM by producing an

environment in the upper respiratory tract that is conducive to bacterial colonization as well as ascension into the middle ear space. Factors involved include increased mucus production, ciliary dysmotility, impaired neutrophil and β-defensin function, and mucosal inflammation [17, 43-48]. The resultant eustachian tube dysfunction and hindered bacterial clearance mechanisms can lead to prolonged disease duration.

Bacterial Otopathogens

During the early 1900s, Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) was the most common pathogen associated with OM, specifically AOM [49]. Since then, the prevalence of GAS has significantly decreased and a handful of other bacterial species have become predominant OM pathogens: *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae*, and *Moraxella catarrhalis*, all of which represent commensal bacteria residing in the nasopharynx.

Simultaneous carriage of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* can be common in children [50, 51]. Interactions between these bacteria affect which organisms will persist in the nasopharynx and cause disease [52-56]. The nature of these bacterial interactions is constantly evolving and can be dependent on which species are present in a given polymicrobial environment. As an example, in a study involving 212 children ranging from 6 months to 3 years of age, *S. pneumoniae* colonization was negatively associated with *H. influenzae* colonization. However, if *H. influenzae* and *M. catarrhalis* were

colonized together, the association with *S. pneumoniae* colonization shifted to a positive value [50].

In experiments utilizing a chinchilla model of OM, other relationships have been identified between the common otopathogens. *H. influenzae* has been shown to facilitate the persistence and antimicrobial resistance of *M. catarrhalis* via protection within a mixed biofilm structure (aggregation of cells attached to a surface and encased in an extrapolymeric matrix [57]) [58]. Also, *S. pneumoniae* is afforded some level of passive protection against antimicrobial treatment during middle ear infection when *H. influenzae* is present, although systemic pneumococcal disease incidence was decreased in this same setting [59]. These examples demonstrate that the synergistic or antagonistic relationships between organisms found in the nasopharynx and middle ear play a role in determining disease outcome.

Another bacterium that is gaining attention because of its association with OM is *Alloiococcus otitidis*. The identification of this organism in cases of OM has increased in the recent past because of the advent of polymerase chain reaction (PCR) and other molecular approaches to examine OM etiology. Because of its slow growth (2-5 days at 37°C [60]), *A. otitidis* was often missed or overlooked as an otopathogen when standard bacterial culturing techniques were used to determine pathogens present in cases of OM. The role of this organism in AOM and OME is being investigated.

Treatment

Antibiotics

Although the majority of OM cases resolve spontaneously if given enough time [13, 61], up to 98% of AOM cases in the United States are treated with antibiotics [62]. Recent clinical guidelines for the management of AOM recommend amoxicillin as the initial drug of choice, assuming that the patient has not received amoxicillin treatment within the past 30 days. In cases where infections are unresponsive to amoxicillin treatment, antibiotics with additional β-lactamase coverage, such as amoxicillin/clavulanate, should be used. Patients with allergies to penicillin should be administered cephalosporin treatment [15].

Much like with AOM, the majority of OME cases will spontaneously resolve themselves within 3 months to 1 year (63%-90% [61, 63-65]). Because the administration of antibiotics has not been demonstrated to significantly improve OME outcomes, current clinical guidelines for OME management and treatment suggest a 3-month “watch and wait” period of patient observation [23]. During this time, a child’s level of hearing loss and language/speech development may be monitored so that necessary surgical treatment options can be identified. In patients that are averse to surgery, a single course of antibiotics may be prescribed at the clinician’s discretion.

Tympanostomy Tube Placement

A surgical treatment option for combatting OM is the insertion of tympanostomy tubes (ventilation tubes; ear tubes). These tubes, approximately

1/20th of an inch in width, are introduced into a child's tympanic membrane to help ventilate the middle ear space. This form of surgery has become the most common ambulatory surgery performed on children in the United States [66]. Annually, ~670,000 children under the age of 15 years old undergo tympanostomy tube placement [67]. By 3 years of age, 1 in 15 children (6.8%) will have tympanostomy tubes; this number is increased by more than 2-fold with daycare attendance [68].

Pediatricians may recommend ear tube placement for a variety of reasons, such as the presence of persistent middle ear effusion, frequent ear infections, and ear infections that fail to respond to antimicrobial therapies. Tympanostomy tubes not only provide a mechanism for middle ear fluid drainage but also alleviate pressure accumulation against the tympanic membrane. Placement of tubes has been shown to significantly improve hearing [69], reduce effusion prevalence [70], and reduce the incidence of recurrent AOM [66], thus greatly improving patient and caregiver quality of life.

Vaccine Development

Efforts to decrease the prevalence of invasive pneumococcal disease (IPD) have resulted in the development of several vaccines throughout the years (Table I). A successful strategy in vaccine creation against IPD has been to conjugate pneumococcal polysaccharide components with a protein carrier molecule such as modified diphtheria toxin (CRM197). In 2000, a 7-valent pneumococcal polysaccharide/protein conjugate vaccine (encompassing

Table I. History of Pneumococcal Vaccine Design [71, 72]

Manufacturer	Year	Valency Serotypes	Carrier Protein	Reference
Wyeth Pharmaceuticals	1994	6A and 23F	CRM197	[73]
	1995	6B, 14, 18C, 19F, and 23F	CRM197	[74]
	1996	4, 6B, 9V, 14, 18C, 19F, and 23F	CRM197	[75]
	2000	1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F	CRM197	[76]
	2008	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, and 19A	CRM197	[77-79]
Sanofi Pasteur	1994	19F	DT	[80]
		6B, 14, 19F, and 23F	TT	[81]
	1996	3, 4, 6B, 9V, 14, 18C, 19F, and 23F	TT	[82]
		3, 4, 6B, 9V, 14, 18C, 19F, and 23F	DT	[82]
	1997	3, 4, 6B, 9V, 14, 18C, 19F, and 23F	TT or DT	[83]
	2004	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F	TT or DT	[84]
Merck Sharp & Dohme	1994	14	OMP	[85]
		6B, 14, 19F, and 23F	OMP	[86]
	1996	4, 6B, 9V, 14, 18C, 19F, and 23F	OMP	[87]
	2011	4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, and 33F	CRM197	[88, 89]
GlaxoSmithKline	2001	6B, 14, 19F, and 23F	PD	[90]
	2006	1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F	PD	[91]
	2009	1, 4, 5, 6B, 7F, 9V, 14, 18C-TT, 19F-DT, and 23F; 8-on-PD	PD or DT or TT	[92]

capsular serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) was released by Wyeth Laboratories for widespread use in the United States [93]. This vaccine, designated Prevnar, was successful in reducing the prevalence of IPD [94]. It also had an effect on the etiology of OM.

Briefly, shortly after vaccine introduction a decrease in the prevalence of *S. pneumoniae* in cases of OM was observed. The decrease in pneumococcus coincided with an increase in the identification of *H. influenzae* in OM cases [95, 96]. This phenomenon was short-lived, however. In research done 6-8 years after the initial introduction of Prevnar for widespread use, it was observed that the prevalence of *S. pneumoniae* had again risen to a level on par with if not higher than that seen with *H. influenzae*. Research also revealed that the increase in pneumococcal presence was due to serotypes not covered in the 7-valent vaccine [97-99].

To address the need for better pneumococcal serotype coverage, a 13-valent iteration of the vaccine called Prevnar 13 was released in 2010 by Wyeth Laboratories for use in the US [93]. This vaccine incorporated the serotypes of the original Prevnar formulation as well as 6 new emerging pneumococcal serotypes (1, 3, 5, 6A, 7F, and 19A). Although the full ramifications of Prevnar 13 introduction on the etiology of OM have not been fully realized, some reports suggest that the new vaccine is having a positive effect on preventing pneumococcal disease [17, 100-103].

Purpose of Experimentation

The five-year prospective study presented here was designed to examine the prevalence of otopathogens in middle ear fluid obtained from children undergoing tympanostomy tube placement at Wake Forest Baptist Medical Center from July 2009 through March 2014. The experimental design has allowed us to investigate not only which pathogens are present in cases of OM in our geographic region but also the polymicrobial nature of these infections. Another aspect of OM that this research may give insight into is the effect of Prevnar 13 on the etiology of OM in our patients. As has been mentioned, the Prevnar 13 vaccine was released for widespread use during the middle of our study timeframe, allowing us to evaluate results in potential pre- and post-vaccination populations.

CHAPTER II.

ONE THIRD OF MIDDLE EAR EFFUSIONS FROM CHILDREN UNDERGOING TYMPANOSTOMY TUBE PLACEMENT HAD MULTIPLE BACTERIAL PATHOGENS

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ABSTRACT

Background

Because previous studies have indicated that otitis media may be a polymicrobial disease, we prospectively analyzed middle ear effusions of children undergoing tympanostomy tube placement with multiplex polymerase chain reaction for four otopathogens.

Methods

Middle ear effusions from 207 children undergoing routine tympanostomy tube placement were collected and classified by the surgeon as acute otitis media (AOM) for purulent effusions and as otitis media with effusion (OME) for non-purulent effusions. DNA was isolated from these samples and analyzed with multiplex polymerase chain reaction for *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Alloiococcus otitidis*, and *Moraxella catarrhalis*.

Results

119 (57%) of 207 patients were PCR positive for at least one of these four organisms. 36 (30%) of the positive samples indicated the presence of more than one bacterial species. Patient samples were further separated into 2 groups based on clinical presentation at the time of surgery. Samples were categorized as acute otitis media (AOM) if pus was observed behind the tympanic membrane. If no pus was present, samples were categorized as otitis media with effusion (OME). Bacteria were identified in most of the children with AOM

(87%) and half the children with OME (51%, $p<0.001$). A single bacterial organism was detected in middle ear effusions from children with AOM more often than those with OME (74% versus 33%, $p<0.001$). *Haemophilus influenzae* was the predominant single organism and caused 58% of all AOM in this study. *Alloiococcus otitidis* and *Moraxella catarrhalis* were more frequently identified in middle ear effusions than *Streptococcus pneumoniae*.

Conclusions

Haemophilus influenzae, *Streptococcus pneumoniae*, *Alloiococcus otitidis*, and *Moraxella catarrhalis* were identified in the middle ear effusions of some patients with otitis media. Overall, we found AOM is predominantly a single organism infection and most commonly from *Haemophilus influenzae*. In contrast, OME infections had a more equal distribution of single organisms, polymicrobial entities, and non-bacterial agents.

INTRODUCTION

Otitis media is one of the most common childhood diseases [104]. Acute otitis media (AOM) typically exhibits rapid-onset purulent middle ear effusion and symptoms of middle ear inflammation, including fever and otalgia [16], whereas otitis media with effusion (OME) exhibits non-purulent middle ear effusion in the absence of symptoms of acute infection [23]. Otitis media is the leading reason for pediatric office visits and for antibiotic prescriptions [1]. The economic burden of otitis media in the United States is estimated at $3-5 billion in direct annual costs [10, 11], and much higher if indirect costs such as lost working days and loss of productivity by family members caring for the sick are included [13].

A common treatment for frequent AOM and for persistent OME with hearing loss is the insertion of tympanostomy tubes. Insertion of tympanostomy tubes is the most common surgical procedure excluding circumcision for U.S. children ≤15 years of age [68, 105]. Parents of children who have undergone tympanostomy tube placement report an increased quality of life due to the elimination of AOM symptoms and improved hearing and speech [106].

The bacterial pathogens most commonly cultured from the middle ear effusions of children with AOM are *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* [97, 107]. Although routine bacterial cultures have been the conventional method to identify the etiology of AOM, approximately 35% of these bacterial cultures are negative [96, 108, 109]. Polymerase chain reaction (PCR) has detected bacterial DNA in culture-negative middle ear effusions [110, 111].

PCR not only provides a more sensitive method for identifying MEE pathogens, but it also allows for the identification of fastidious or slow-growing organisms. For example, *A. otitidis*, a pathogen first isolated from middle ear effusions of children with OME [60], was more recently identified with because its slow growth characteristics are not conducive to identification using conventional bacterial culture methods [108]. The sensitivity of PCR has led to a growing recognition that otitis media may be a polymicrobial disease [112]. Here we used a multiplex PCR to determine the prevalence of four known otopathogens (*H. influenzae*, *S. pneumoniae*, *A. otitidis*, and *M. catarrhalis*) in the middle ear effusions of children undergoing routine tympanostomy placement.

MATERIALS AND METHODS

Patients and Sample Collection

All children who had tympanostomy tube placement for clinical indications at Wake Forest School of Medicine from July 2009 through December 2010 and had middle ear fluid at the time of surgery were eligible. This study was reviewed and approved by the Wake Forest School of Medicine Institutional Review Board as an exempt study; no personal health information was collected and discarded samples of middle ear effusions were evaluated for bacterial pathogens.

Children who at the time of tympanostomy tube placement had middle ear effusions had that fluid aspirated into a sterile trap. Middle ear effusion samples were categorized as AOM or OME by the surgeon at the time of tympanostomy tube placement. AOM was defined as purulent fluid behind the tympanic

membrane, whereas OME was defined as non-purulent fluid behind the tympanic membrane. All middle ear effusion samples were kept at room temperature and transported to the research laboratory within 2 hours. Samples were then refrigerated at 4°C until DNA isolation.

Isolation of DNA

For the few effusions that were very viscous, they were placed in Lysing Matrix D tubes (MP Biomedical, Solon, OH) with 500µL of 1X TE buffer (pH 7.5). The lysing tubes were processed in a FastPrep FP120 homogenizer (Thermo Electron Corporation, Milford, MA) for 40 seconds on a setting of 6.0. Processed samples were centrifuged at 12000rpm for 5 minutes and 200µL of supernatant were used as the starting material for genomic DNA extraction.

DNA was isolated from middle ear effusions using a conventional genomic DNA extraction protocol. First, 200µL of effusion were treated with 15µL of Mutanolysin (10U/µL; Sigma, St. Louis, MO) and 21µL of Lysozyme (20mg/mL; Amresco, Solon, OH) and then incubated at 37°C for 1 hour. Second, samples were treated with 55µL of 10% SDS (EMD, Gibbstown, NJ) and 68µL of RNAse A (Sigma, St. Louis, MO) and then incubated at 37°C for 1 hour. Third, samples were treated with 10µL of Proteinase K (10mg/mL; Amresco, Solon, OH) and then incubated at 37°C for 1 hour. Fourth, samples were treated with 55µL of 5M NaCl (Sigma, St. Louis, MO) and 50µL of prewarmed (to 60°C) 10% Hexadecyltrimethyl-ammonium bromide (Sigma, St. Louis, MO) and then incubated at 60°C for 20 minutes. Fifth, samples were mixed with 470µL of

Phenol:Chloroform:Isoamyl Alcohol (25:24:1; Sigma, St. Louis, MO) and transferred to prespun Phase Lock Tubes (2mL, heavy; 5 Prime, Gaithersburg, MD). Tubes were centrifuged for 5 minutes at 12000rpm, and the upper (aqueous) layer from each sample was transferred to a sterile 1.5mL microcentrifuge tube. Aqueous layers were mixed with 50µL of 3M NaOAc (Sigma, St. Louis, MO) and 470µL of ice cold 100% Ethanol (Warner-Graham, Cockeysville, MD). Samples were incubated at -20°C for a minimum of 30 minutes. Precipitated samples were centrifuged for 30 minutes at 12000rpm at 4°C. Supernatants were discarded and pellets washed with 1mL of 70% Ethanol. Pellets were allowed to completely dry and were resuspended in a final volume of 100µL of dH_2O.

Polymerase Chain Reaction

A multiplex PCR procedure was performed to simultaneously detect *H. influenzae*, *S. pneumoniae*, *A. otitidis*, and *M. catarrhalis* with minor modifications of the methods described by Hendolin et al [113]. Briefly, PCR extension steps were performed at 65°C with HotMasterMix (5 Prime, Gaithersburg, MD) and using the primers listed in Table II. *H. influenzae* 86-028, *S. pneumoniae* TIGR4, *A. otitidis* SS1337, and *M. catarrhalis* 7169 were used as positive controls for PCR (Figure 2). Results were visualized using agarose gel electrophoresis (2.5% agarose, 100 volts for 3 hours) and ethidium bromide staining (500ng/mL final ethidium bromide concentration).

Chart Review

Table II. Primers Used in Multiplex PCR

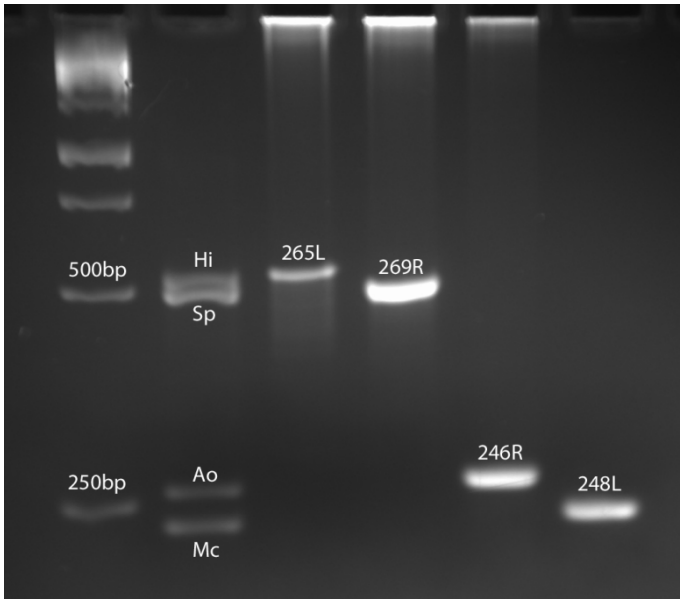
Table II.
Primers Used in Multiplex PCR

Primer Name	Nucleotide Sequence	Predicted Amplicon Size[a]
H. influenzae FWD	5'-CGTATTATCGGAAGATGAAAGTGC-3'	525 base pairs
S. pneumoniae FWD	5'-AAGGTGCACTTGCATCACTACC-3'	484 base pairs
A. otitidis FWD	5'-GGGGAAGAACACGGATAGGA-3'	264 base pairs
M. catarrhalis FWD	5'-CCCATAAGCCCTGACGTTAC-3'	237 base pairs
Universal REV	5'-CTACGCATTTCACCGCTACAC-3'	

[a]When the designated FWD primer is used in conjunction with the Universal REV primer

Figure 2. Multiplex PCR Results

Multiplex PCR products were electrophoresed on 2.5% agarose. Hi, Haemophilus influenzae control band, expected size 525bp; Sp, Streptococcus pneumoniae control band, expected size 484bp; Ao, Alloiococcus otitidis control band, expected size 264bp; Mc, Moraxella catarrhalis control band, expected size 237bp; 265L, 269R, 246R, and 248L, experimental samples; bp, base pair


500bp
Hi
Sp
265L
269R
250bp
Ao
Mc
246R
248L

The demographic characteristics, current medications, and past medical history were all determined by chart review performed at the time of surgery without collecting any personal health identifiers. The pediatric otolaryngologist used a standardized clinical form that elicited all of this clinical information by history and/or by documentation from the primary care provider. Previous antibiotics were documentation of any course of antibiotics within 6 months of surgery.

Analyses

The frequency of demographic characteristics, clinical characteristics, or microbiologic results of children with AOM and OME middle ear effusions were compared using chi-square analyses or Fisher's exact tests. For the children who had middle ear effusions in both ears, the results from each ear were combined so that all children with one or two middle ear effusions had one result. STATA 8.1 was used for all statistical analyses.

RESULTS

The study population comprised 207 children undergoing tympanostomy tube placement for clinical indications at Wake Forest School of Medicine from July 2009 to December 2010. Two-thirds of the study population was male, half were 1-3 years of age, and 60% were Caucasian (Table III). Children with AOM at tympanostomy were more likely to be younger, to have had previous ear infections, or to have been treated with antibiotics in the previous 6 months than

Table III. Child Demographics

Table III.
Child Demographics

	Total	Purulent Effusions	Nonpurulent Effusions	
	No. of Children (column %) (n = 207)	No. of Children (column %) (n = 38)	No. of Children (column %) (n = 169)	p-value
Age				
<1	33 (16)	5 (13)	28 (17)	0.03
1-3	103 (50)	25 (66)	78 (46)	
>3	69 (33)	7 (18)	62 (37)	
Gender				
Male	139 (67)	21 (55)	118 (70)	0.09
Female	68 (33)	17 (45)	51 (30)	
Race				
White	123 (60)	29 (76)	94 (56)	0.09
Black	44 (21)	5 (13)	39 (23)	
Hispanic	36 (17)	3 (8)	33 (20)	
Other	2 (1)	0 (0)	2 (1)	
Currently* on Antibiotics[a]				
Yes	26 (13)	7 (18)	19 (11)	0.27
No	179 (86)	30 (79)	149 (88)	
Currently* on Allergy Medicines[b]				
Yes	44 (21)	8 (21)	36 (21)	1.00
No	160 (77)	30 (79)	130 (77)	
Previous Ear Infections				
Yes	145 (70)	33 (87)	112 (66)	0.008
No	57 (28)	4 (11)	53 (31)	
Previously on Antibiotics[c]				
Yes	100 (48)	26 (68)	74 (44)	0.007
No	107 (52)	12 (32)	95 (56)	
Adenoidectomy				
Yes	16 (8)	0 (0)	16 (9)	0.046
No	186 (90)	38 (100)	148 (88)	
Previous Ear Tubes				
Yes	49 (24)	6 (16)	43 (25)	0.21
No	153 (74)	32 (84)	121 (72)	
Cleft Palate				
Yes	9 (4)	1 (3)	8 (5)	1.00
No	189 (91)	37 (97)	152 (90)	

*At time of tympanostomy tube placement
[a]Current antibiotics include: Amoxicillin, Amoxicillin + Clavulanic Acid, Azithromycin, Cefdinir, Cefpodoxime, Cefprozil, Sulfamethoxazole
[b]Current allergy medicines include: Budesonide, Cetirizine, Clemastine, Diphenhydramine, Fexofenadine, Fluticasone, Levocetirizine, Loratadine, Mometasone, Montelukast, Olopatadine
[c]Previous antibiotics include: Amoxicillin, Amoxicillin + Clavulanic Acid, Azithromycin, Cefdinir, Cefpodoxime, Cefprozil, Ceftriaxone, Cefuroxime, Ciprofloxacin, Clindamycin, Sulfamethoxazole

children with OME at tympanostomy. In contrast, children with OME were more likely to have had an adenoidectomy than children with AOM.

Effusions from 119 (57%) of 207 children were PCR positive for *H. influenzae, S. pneumoniae, A. otitidis,* and/or *M. catarrhalis*. Of these 119 PCR positive samples, 36 (30%) had 2-4 bacterial species detected (Figure 3, Tables IV and V). Bacteria were identified in 33 (87%) of 38 children with AOM as compared to 86 (51%) of 169 children with OME ($p<0.001$). Single bacterial species were identified in the majority of children with AOM and a minority of children with OME (74% versus 33%, $p<0.001$). Identifying multiple bacterial pathogens was similar for children with AOM and OME (13% versus 18%, $p=0.64$), whereas not identifying any of the four bacterial pathogens was less common for AOM than OME (13% versus 49%, $p<0.001$).

For middle ear effusions with a single bacterial species identified, the etiology for children with AOM differed significantly from those with OME ($p<0.001$). *H. influenzae* accounted for 79% of the isolates from children with AOM, whereas three bacteria accounted for the majority of isolates for children with OME: *H .influenzae* for 36%, *A. otitidis* for 40% and *M. catarrhalis* for 20%. *S. pneumoniae* was detected in 5% of all isolates for children with AOM and OME. *Alloiococcus otitidis* was identified either as a single organism or as a polymicrobial component in 18% of AOM and 25% of OME. All four bacteria were identified in at least one of the middle ear effusions with AOM or OME.

Figure 3. Flowchart of PCR Analysis (July 2009 – December 2010)

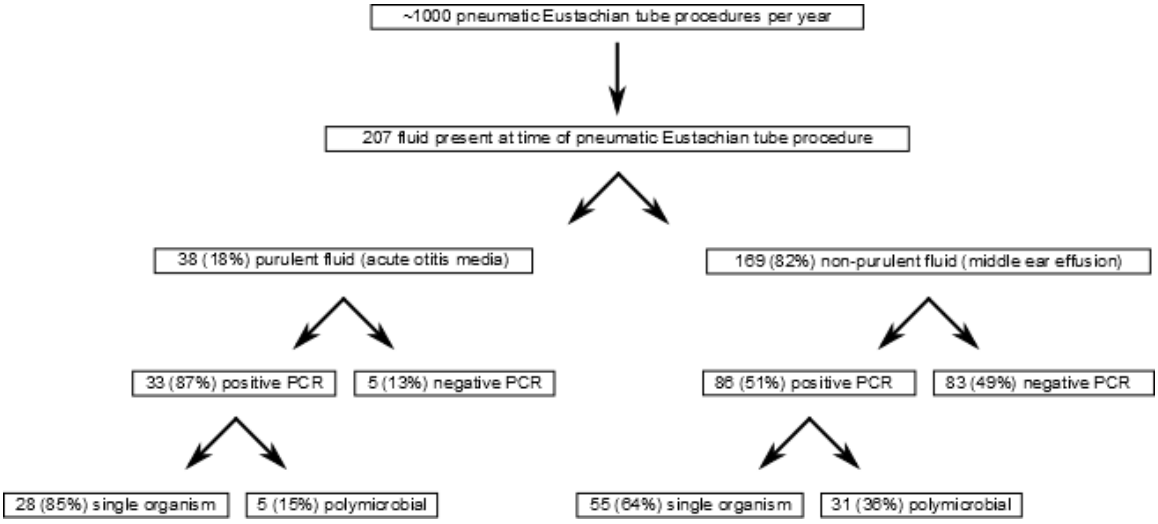

~1000 pneumatic Eustachian tube procedures per year
207 fluid present at time of pneumatic Eustachian tube procedure
38 (18%) purulent fluid (acute otitis media)
169 (82%) non-purulent fluid (middle ear effusion)
33 (87%) positive PCR
5 (13%) negative PCR
86 (51%) positive PCR
83 (49%) negative PCR
28 (85%) single organism
5 (15%) polymicrobial
55 (64%) single organism
31 (36%) polymicrobial

Table IV. Incidence of Bacterial DNA in Middle Ear Effusions

Table IV.
Incidence of Bacterial DNA in Middle Ear Effusions

Presence of Bacterial DNA	Purulent Effusions No. of Children (column %) (n = 38)	Nonpurulent Effusions No. of Children (column %) (n = 169)	p-value
Haemophilus influenzae			
Single organism	22 (58)	20 (12)	<0.001
Polymicrobial component	3 (8)	20 (12)	
None	13 (34)	129 (76)	
Streptococcus pneumoniae			
Single organism	1 (3)	2 (1)	0.79
Polymicrobial component	1 (3)	6 (4)	
None	36 (95)	161 (95)	
Alloiococcus otitidis			
Single organism	2 (5)	22 (13)	0.40
Polymicrobial component	5 (13)	21 (12)	
None	31 (82)	126 (75)	
Moraxella catarrhalis			
Single organism	3 (8)	11 (7)	0.95
Polymicrobial component	4 (11)	18 (11)	
None	31 (82)	140 (83)	
Overall			
Single organism	28 (74)	55 (33)	<0.001
Polymicrobial	5 (13)	31 (18)	
No Bacteria Detected	5 (13)	83 (49)	

*Percent totals may not add up to 100% due to rounding error.

Table V. Combinations of Bacterial DNA in Middle Ear Effusions

Table V.
Combinations of Bacterial DNA in Middle Ear Effusions

Organism Identified	Purulent Effusions			Nonpurulent Effusions		
	No. of Patients (n = 38)	Percentage[a]	95% Confidence Intervals	No. of Patients (n = 169)	Percentage[b]	95% Confidence Intervals
Haemophilus influenzae	22	58	41-74	20	12	8-18
Streptococcus pneumoniae	1	3	0.07-15	2	1	0.01-3
Alloiococcus otitidis	2	5	1-19	22	13	8-19
Moraxella catarrhalis	3	8	1-19	11	7	4-12
H. influenzae *S. pneumoniae*	0	0		2	1	0.4-5
H. influenzae *A. otitidis*	1	3	0.07-15	8	5	2-9
H. influenzae *M. catarrhalis*	0	0	0.07-15	7	4	2-8
S. pneumoniae *A. otitidis*	0	0		1	1	0.01-3
S. pneumoniae *M. catarrhalis*	0	0		1	1	0.01-3
A. otitidis *M. catarrhalis*	2	5	1-19	9	5	2-9
H. influenzae *S. pneumoniae* *A. otitidis*	0	0		2	1	0.1-4
H. influenzae *S. pneumoniae* *M. catarrhalis*	0	0		0	0	
H. influenzae *A. otitidis* *M. catarrhalis*	1	3	0.07-15	1	1	0.01-3
S. pneumoniae *A. otitidis* *M. catarrhalis*	0	0		0	0	
H. influenzae *S. pneumoniae* *A. otitidis* *M. catarrhalis*	1	3	0.07-15	0	0	
None detected	5	13	3-26	83	49	41-57

[a]Percentage of purulent effusion isolates only (rounded to nearest whole number)
[b]Percentage of nonpurulent effusion isolates only (rounded to nearest whole number)

DISCUSSION

Using a multiplex PCR to simultaneously detect four bacterial otopathogens in the middle ear effusions obtained from children undergoing routine tympanostomy tube placement, we found distinct bacterial profiles for AOM and OME. Bacteria were identified in most of the children with AOM (87%) and half the children with OME (51%, $p<0.001$). A single bacterial organism was detected in middle ear effusions from patients with AOM more often than those with OME (74% versus 33%, $p<0.001$). *Haemophilus influenzae* was the predominant single organism and caused 58% of all AOM in this study. *Alloiococcus otitidis* and *Moraxella catarrhalis* were more frequently identified in middle ear effusions than *Streptococcus pneumoniae*.

Overall, we found AOM is predominantly a single organism infection, whereas, OME infections had a more equal distribution of single organisms, polymicrobial entities, and non-bacterial agents.

H. influenzae was the predominant bacterial species identified, comprising 66% (25 of 38) of middle ear effusions with AOM and 24% (40 of 169) of middle ear effusions with OME. We observed a higher proportion of samples with *H. influenzae* and a lower proportion with *S. pneumoniae* than what has been previously reported. Kaur et al., using a similar multiplex PCR approach on AOM middle ear effusion, detected *H. influenzae* in 31% of children who were on antibiotic prior to sample acquisition and 39% in children who had not undergone antibiotic treatment [108]. However, Kaur et al. utilized specifically culture-

negative MEE, a distinction we have not made with the samples obtained for our research. In other work using culture-negative AOM MEE samples, Xu et al. observed an *H. influenzae* percentage of 24% [114].

While the levels of *H. influenzae* in our patients appear to be higher than what others have seen, we observed the opposing trend with respect to *S. pneumoniae*. *S. pneumoniae*, regarded as one of the 3 most prevalent bacterial contributors to OM infection, was identified in only 2 of 38 AOM patients (~5%) and 8 of 169 OME patients (~5%). In the studies listed above by Kaur et al. and Xu et al., *S. pneumoniae* was identified in around 38%-57% of culture-negative AOM MEE samples, percentages that are considerably higher than what was observed in our study [108, 114].

A number of factors may explain our finding that *S. pneumoniae* was detected in only 5% of all study subjects. Published studies of pneumococcal prevalence in otitis media report a wide incidence range. Brook et al. used standard culture based techniques to detect *S. pneumoniae* in approximately 16% of OME effusions [115]. Post et al. used PCR to detect *S. pneumoniae* in OME effusions at a higher percentage of approximately 30% [116]. Research conducted by Hendolin et al. detected *S. pneumoniae* in only 8% of cases examined [113]. We used the same multiplex PCR primers used by Hendolin et al. Kaur et al. used these PCR primers to identify *S. pneumoniae* in approximately 57% of effusions from children with AOM [108], and this data does not suggest that our PCR assay has a low sensitivity for detection of pneumococcus.

The low *S. pneumoniae* incidence found in our study might be explained by the effectiveness of pneumococcal conjugate vaccination. A heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in 2000, and since its acceptance for widespread use there has been a shift in the incidence of otitis media pathogens. In the years immediately following PCV7 introduction, *H. influenzae* emerged as the most common AOM isolate [95, 96]. More recently, *S. pneumoniae* serotypes not included in the PCV7 vaccine have been increasingly isolated from AOM cases [97]. In 2010, a new modified pneumococcal conjugate vaccine was introduced to combat the emergence of *S. pneumoniae* serotypes not included in the original PCV7 vaccine. It contained components from 13 serotypes of *S. pneumoniae* (7 serotypes included in the PCV7 vaccine along with 6 recently emerging serotypes). Our findings are consistent with improved pneumococcal vaccine prevention in children enrolled in this study.

Another interesting result was the high incidence of *A. otitidis* in our OME samples (approximately 25%, single and co-infections included). *A. otitidis* was first identified in OME samples by Faden et al [60]. It is a Gram-positive organism that exhibits very slow growth, making it very difficult to identify through standard culture techniques. It has been thought of as solely an OME pathogen; however, it is being increasingly recognized as a pathogen in AOM [108, 117, 118]. Our findings support what has been reported previously in OME cases [113]. The *A. otitidis* results further demonstrate the importance of using methods other than standard culture for the identification of fastidious

otopathogens.

CONCLUSIONS

In conclusion, the etiology of OM appears to revolve around disease type (AOM or OME). A multiplex PCR approach may be used to identify specific bacterial DNA species in effusions from children experiencing OM. The PCR procedures can overcome the obstacles of culturing fastidious organisms, and may offer a more sensitive and time efficient method for evaluating middle ear effusions. While our approach targeted 4 organisms, the method could be adapted for the identification of additional microorganisms.

Our criterion for separating AOM cases from OME cases was the presence of pus behind the tympanic membrane at the time of tympanostomy tube placement. The results of our research clearly show that this single easily observable patient difference was sufficient to categorize disease condition into 2 distinct populations. Moreover, our results indicate that when AOM is observed there is usually a single bacterial etiology. Culture or PCR analysis of pus at tympanostomy tube placement may be especially useful in guiding antibiotic therapy.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTIONS

RCH participated in the design of the study, experimental procedures, data analysis, and manuscript preparation. DJK and AKE participated in the design of the study, sample collection surgeries, data analysis, and manuscript preparation. TRP, KAP, and WES participated in the design of the study, data analysis, and manuscript preparation. SDR participated in the design of the study, experimental procedures, data analysis, and manuscript preparation. All authors gave final approval of the completed manuscript.

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CHAPTER III.

OTOPATHOGENS DETECTED IN MIDDLE EAR FLUID OBTAINED DURING TYMPANOSTOMY TUBE INSERTION: CONTRASTING PURULENT AND NON-PURULENT EFFUSIONS

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ABSTRACT

Otitis media is a prominent disease among children. Previous literature indicates that otitis media is a polymicrobial disease, with *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Alloiococcus otitidis* and *Moraxella catarrhalis* being the most commonly associated bacterial pathogens. Recent literature suggests that introduction of pneumococcal conjugate vaccines has had an effect on the etiology of otitis media. Using a multiplex PCR procedure, we sought to investigate the presence of the aforementioned bacterial pathogens in middle ear fluid collected from children undergoing routine tympanostomy tube placement at Wake Forest Baptist Medical Center during the period between January 2011 and March 2014. In purulent effusions, one or more bacterial organisms were detected in ~90% of samples. Most often the presence of *H. influenzae* alone was detected in purulent effusions (32%; 10 of 31). In non-purulent effusions, the most prevalent organism detected was *A. otitidis* (26%; 63 of 245). Half of the non-purulent effusions had none of these otopathogens detected. In purulent and non-purulent effusions, the overall presence of *S. pneumoniae* was lower (19%; 6 of 31, and 4%; 9 of 245, respectively) than that of the other pathogens being identified. The ratio of the percentage of each otopathogen identified in purulent vs. non-purulent effusions was >1 for the classic otopathogens but not for *A. otitidis*.

INTRODUCTION

Otitis media (OM) is a leading cause for outpatient visits as well as antibiotic prescriptions for children [1-5]. It is estimated that by 3 years of age 80% of children will have experienced at least one case of OM [6, 7], and 40% of these children will experience six or more recurrences by 7 years of age [8]. Annual direct costs associated with OM are estimated to approach $3-5 billion [9-11], and indirect costs which include loss of productivity and lost working days by family members drive this value higher [12, 13].

Distinct from our previous work [119], this study focuses on the prevalence of four otopathogens (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Alloiococcus otitidis*, and *Moraxella catarrhalis*) in middle ear fluid collected from children undergoing tympanostomy tube placement at Wake Forest Baptist Medical Center (Winston-Salem, NC, USA) from January 2011 until March 2014, after the introduction of the pneumococcal conjugate vaccine PCV13.

MATERIALS AND METHODS

Subjects and Sample Collection

Children who had tympanostomy tube placement at Wake Forest Baptist Medical Center from January 2011 through March 2014 and had middle ear fluid at the time of surgery were eligible. Discarded samples of middle ear effusions were evaluated for bacterial pathogens, as described below.

Study specimens were collected from middle ears that had effusions at the

time of tympanostomy tube placement. Fluid was aspirated into a sterile trap. Specimens were categorized as purulent or non-purulent fluid behind the tympanic membrane by the otolaryngologist at the time of tympanostomy tube placement. Fluids visually observed by the pediatric otolaryngologist to have a whitish appearance with a milky or mucoid consistency were designated as purulent. All middle ear effusion samples were kept at room temperature and transported to the research laboratory within 2 hours and then refrigerated at 4°C until DNA isolation. Samples were processed within 5 days of refrigeration.

Isolation of DNA

DNA was extracted from middle ear effusions as described previously [119]. Briefly, middle ear effusions were incubated with various degradation components (mutanolysin, lysozyme, sodium dodecyl sulfate (SDS), RNase, proteinase K) to disrupt bacterial cell walls, remove RNA, and degrade proteins. Remaining DNA was phenol:chloroform:isoamyl alcohol extracted and ethanol precipitated. Resuspended DNA precipitates were used as template material in the multiplex PCR procedure.

Polymerase Chain Reaction

A multiplex PCR procedure created by Hendolin et al [113] was used with minor modification to simultaneously detect *H. influenzae*, *S. pneumoniae*, *A. otitidis*, and *M. catarrhalis* in middle ear effusions, as previously described [119]. The modification mentioned above was that the polymerase used in the procedure (5 PRIME HotMasterMix) required an extension temperature of 65ºC

rather than the often used 72ºC associated with Taq polymerase.

Chart Review

This study was reviewed and approved by the Wake Forest School of Medicine Institutional Review Board. Sex, age, medications, clinical characteristics and prior antimicrobial treatments were collected from the subject record without identifiable information. Although identifiable information was not collected, administrative data reveals that the average age of children who had tympanostomy tubes placed during this study period was 2.7 years (range of 0-18 years). The demographic characteristics, current medications, previous antibiotic use, and past medical history were all determined by medical record review using a standardized clinical form. The medical record typically contained notes about ear infections from the primary care provider as well as clinic visits to the otolaryngologist.

Analyses

We compared the demographic characteristics, clinical characteristics, or microbiologic results of children with purulent and non-purulent middle ear effusions by using chi-square analyses or Fisher's exact tests. For children with bilateral middle ear effusions, the results from each ear were combined so that each child had one result. The unadjusted odds ratio and 95% confidence intervals were calculated for each variable. A multivariate logistic regression analysis was performed to compute the odds of a purulent infection as compared to a non-purulent infection for each of four otopathogens and age groups. We

lacked sufficient power to analyze other variables. STATA 12.1 (College Station, TX) was used for all statistical analyses.

RESULTS

A total of 276 children had middle ear fluid collected at the time of tympanostomy tube placement from January 2011 through March 2014. Half were 1-3 years of age, two-thirds were male, and 58% were White (Table VI). Children with purulent effusions were more likely to be 1-3 years of age, to have a history of ear infections, to have been on antibiotics within the past 6 months, and to be on antibiotics at the time of surgery than children with non-purulent effusions.

Overall, 149 (54%) of 276 children had middle ear fluid that were PCR positive for the presence of *H. influenzae*, *S. pneumoniae*, *A. otitidis*, and/or *M. catarrhalis*. Of these 149 PCR positive samples, 117 (79%) identified a single organism and 32 (21%) were polymicrobial (Figure 4).

The three classic otopathogens were more likely to be identified as either a single organism or a polymicrobial component in purulent than non-purulent effusions (Tables VII and VIII). This pattern was observed for *H. influenzae* (52% vs. 18%, $p<0.001$), *S. pneumoniae* (19% vs. 4%, $p<0.001$) and *M. catarrhalis* (26% vs. 12%, $p=0.04$) but not for *A. otitidis* (23% vs. 26%, $p=0.71$).

A single organism was identified in 61% percent (19 of 31) of all purulent effusions (Table 2), most frequently *H. influenzae* followed by *M. catarrhalis*. *S. pneumoniae* and *A. otitidis* were identified as single organisms in a minority of

Table VI. Subject Demographics

		Total (n=276)	Purulent Effusions (n=31)	Non-purulent Effusions (n=245)	
		No. of Children (Column %*)	No. of Children (Column %*)	No. of Children (Column %*)	Unadjusted Odds Ratio (95% Confidence Interval)
Age	<1 year	32 (12)	8 (26)	24 (10)	Reference
	1-3 years	136 (49)	20 (65)	116 (47)	0.5 (0.2-1.3)
	>3 years	108 (39)	3 (10)	105 (43)	0.09 (0.02-0.35)
Gender	Female	94 (34)	11 (35)	83 (34)	Reference
	Male	182 (66)	20 (65)	162 (66)	0.9 (0.4-2.0)
Race	White	158 (58)	24 (77)	134 (56)	Reference
	Black	65 (24)	5 (16)	60 (25)	0.47 (0.17-1.28)
	Hispanic/Other	49 (18)	2 (6)	47 (20)	0.24 (0.05-1.04)
Currently** on Antibiotics[a]	No	245 (89)	23 (74)	222 (91)	Reference
	Yes	31 (11)	8 (26)	23 (9)	3.4 (1.3-8.4)
Previously on Antibiotics[b]	No	144 (52)	6 (19)	138 (56)	Reference
	Yes	132 (48)	25 (81)	107 (44)	5.4 (2.1-13.6)
Any Antibiotics[c]	No	138 (50)	4 (13)	134 (55)	Reference
	Yes	138 (50)	27 (87)	111 (45)	8.1 (2.8-24.0)
Currently** on Allergy Medicines^	No	214 (78)	27 (87)	187 (76)	Reference
	Yes	62 (22)	4 (13)	58 (24)	0.5 (0.2-1.4)
Previous Ear Infections	No	95 (34)	1 (3)	94 (38)	Reference
	Yes	175 (63)	30 (97)	145 (59)	21.6 (2.9-160.9)
Adenoidectomy	No	253 (92)	30 (97)	223 (91)	Reference
	Yes	23 (8)	1 (3)	22 (9)	0.34 (0.04-2.6)
Previous Ear Tubes	No	195 (71)	25 (81)	170 (69)	Reference
	Yes	81 (29)	6 (19)	75 (31)	0.5 (0.2-1.4)
Cleft Palate	No	256 (93)	31 (100)	225 (92)	Reference
	Yes	20 (7)	0 (0)	20 (8)	--[#]

*Percent totals may not add up to 100% due to rounding error or lack of patient reporting

**At time of tympanostomy tube placement

[a]Current antibiotics include: Amoxicillin, Amoxicillin + Clavulanic Acid, Azithromycin, Cefdinir, Cefixime, Clindamycin, Sulfamethoxazole + Trimethoprim

[b]Previous antibiotics include: Amoxicillin, Amoxicillin + Clavulanic Acid, Ampicillin, Azithromycin, Cefdinir, Cefixime, Cefpodoxime, Cefprozil, Ceftibuten, Ceftriaxone, Cefuroxime, Ciprofloxacin + Dexamethasone, Clindamycin, Penicillin, Sulfamethoxazole + Trimethoprim

[c]Any antibiotics: Combination of results from patients currently or previously on antibiotics

^Current allergy medicines include: Budesonide, Cetirizine, Diphenhydramine, Fexofenadine, Fluticasone, Loratadine + Pseudoephedrine, Montelukast, Prednisone

[#]Odds ratio cannot be computed

Figure 4. Flowchart of PCR Analysis (January 2011 – March 2014)

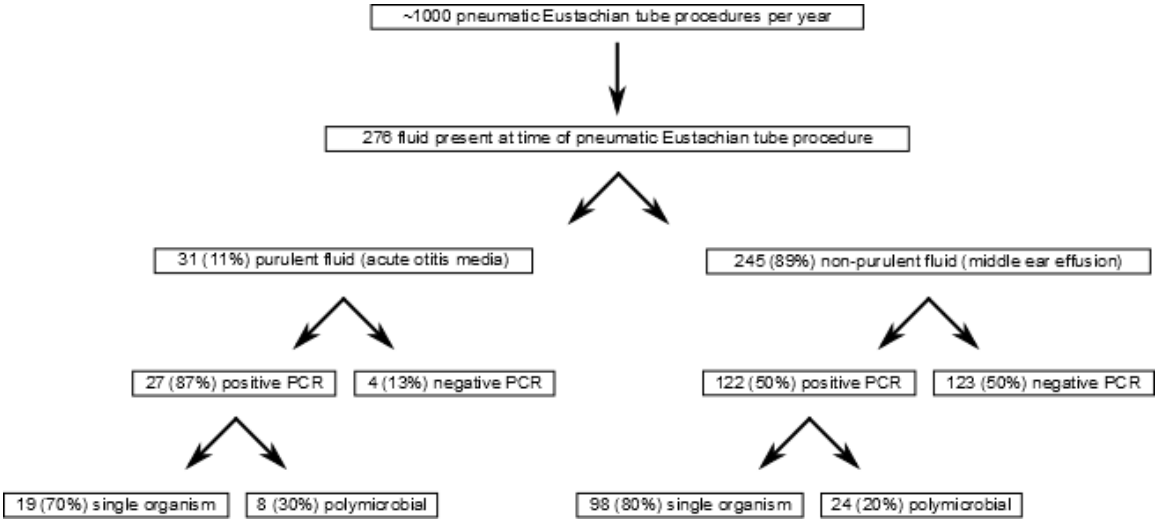
~1000 pneumatic Eustachian tube procedures per year
276 fluid present at time of pneumatic Eustachian tube procedure
31 (11%) purulent fluid (acute otitis media)
245 (89%) non-purulent fluid (middle ear effusion)
27 (87%) positive PCR
4 (13%) negative PCR
122 (50%) positive PCR
123 (50%) negative PCR
19 (70%) single organism
8 (30%) polymicrobial
98 (80%) single organism
24 (20%) polymicrobial

Table VII. Bacterial DNA Identified in Middle Ear Fluid Based on Effusion Type

		Purulent Effusions (n=31)	Non-purulent Effusions (n=245)	
		No. of Children (Column %*)	No. of Children (Column %*)	p-value
Haemophilus influenzae	Single Organism	10 (32)	30 (12)	<0.001
	Polymicrobial Component	6 (19)	15 (6)	
	None	15 (48)	200 (82)	
Streptococcus pneumoniae	Single Organism	3 (10)	7 (3)	0.002
	Polymicrobial Component	3 (10)	2 (1)	
	None	25 (81)	236 (96)	
Alloiococcus otitidis	Single Organism	2 (6)	46 (19)	0.07
	Polymicrobial Component	5 (16)	17 (7)	
	None	24 (77)	182 (74)	
Moraxella catarrhalis	Single Organism	4 (13)	15 (6)	0.07
	Polymicrobial Component	4 (13)	15 (6)	
	None	23 (74)	215 (88)	
Overall	Single Organism	19 (61)	98 (40)	<0.001
	Polymicrobial Component	8 (26)	24 (10)	
	None	4 (13)	123 (50)	

*Percent totals may not add up to 100% due to rounding error

Table VIII. Comparison of Purulent and Non-purulent Bacterial DNA Prevalence

	Purulent Effusions (P)	Non-purulent Effusions (N-P)	
	No. of Patients (%) (n = 31)	No. of Patients (%) (n = 245)	Fold Difference (P vs. N-P)
Haemophilus influenzae	16 (52)	45 (18)	2.9
Streptococcus pneumoniae	6 (19)	9 (4)	4.75
Moraxella catarrhalis	8 (26)	30 (12)	2.2
Alloiococcus otitidis	7 (23)	63 (26)	0.88

purulent effusions. All four otopathogens contributed to the 26% of polymicrobial purulent effusions.

A single organism was identified in 40% (98 of 245) of non-purulent effusions (Table VII). *A. otitidis* accounted for almost half the single organisms identified and *H. influenzae* accounted for about a third, with *S. pneumoniae* and *M. catarrhalis* being identified less frequently. Overall, 10% of non-purulent effusions were polymicrobial, containing *H. influenzae*, *A. otitidis*, and *M. catarrhalis* with similar frequency and infrequently harboring *S. pneumoniae*.

H. influenzae was identified 2.9x more frequently in purulent effusions than non-purulent effusions (52% vs. 18%); *S. pneumoniae* was identified 4.8x more frequently in purulent effusions than non-purulent effusions (19% vs. 4%); *M. catarrhalis* was identified 2.2x more frequently in purulent effusions than non-purulent effusions (26% vs. 12%). In contrast, *A. otitidis* was present in relatively equal amounts in purulent effusions and non-purulent effusions (23% vs. 26%) (Table VIII) In adjusted and unadjusted multiple logistic regression analyses (Table IX), predictors of purulent as opposed to non-purulent middle ear fluid were *H. influenzae*, *S. pneumoniae*, and age <1 year.

DISCUSSION

This prospective study evaluated the presence of four otopathogens (*H. influenzae*, *S. pneumoniae*, *A. otitidis*, and *M. catarrhalis*) in middle ear fluids obtained from children undergoing routine tympanostomy tube placement at Wake Forest Baptist Medical Center from January 2011 through March 2014.

Table IX. Factors Associated with Purulent As Opposed To Non-purulent Middle Ear Effusions

Variable		Unadjusted Odds Ratio (95% Confidence Interval)	Adjusted Odds Ratio (95% Confidence Interval)
Organism	*Haemophilus influenzae*	4.7 (2.2-10.3)	4.3 (1.8-10.1)
	Streptococcus pneumoniae	6.3 (2.1-19.1)	5.6 (1.5-20.2)
	Alloiococcus otitidis	0.8 (0.3-2.1)	1.1 (0.4-2.9)
	Moraxella catarrhalis	2.49 (1.02-6.07)	2.0 (0.8-5.6)
Age	<1 year	Reference	Reference
	1-3 years	0.5 (0.2-1.3)	0.8 (0.3-2.2)
	>3 years	0.09 (0.02-0.35)	0.20 (0.04-0.90)

Our major findings were: (1) analyses of purulent effusions most often yielded the identification of a single bacterial organism rather than a polymicrobial mixture; (2) the most prevalent single organism identified in purulent effusions was *H. influenzae*; (3) ~90% of purulent effusions indicated the presence of one or more of the otopathogens; (4) *A. otitidis* was the most common organism identified in non-purulent effusions; (5) half of the non-purulent effusion samples indicated the absence of all of the otopathogens being evaluated; (6) the overall prevalence of *S. pneumoniae* in effusion samples was low; (7) *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* all occurred ≥2-fold more prevalently in purulent effusions than non-purulent effusions; however, *A. otitidis* occurred equally among purulent and non-purulent effusions.

Our results obtained with purulent effusions correlate with a potentially developing trend in AOM etiology. Historically, the bacterial organisms most commonly identified as AOM pathogens have been *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* [120-123]. After introduction of the heptavalent pneumococcal conjugate vaccine PCV7 in 2000, *H. influenzae* briefly became the most prevalent otopathogen identified in cases of OM [95, 96]. Shortly thereafter, serotypes of *S. pneumoniae* not covered in the initial conjugate vaccine design became prevalent in OM cases and were identified in proportions equaling that of *H. influenzae* [97-99]. In 2010, a new vaccine incorporating PCV7 *S. pneumoniae* serotypes as well as 6 new emerging serotypes was released for widespread usage in the United States. Recent data based on results from Rochester, NY detailing the prevalence of bacteria causing AOM in

North America in 2012 suggest that a shift in OM bacterial etiology may again be occurring [17]. Briefly, *H. influenzae* has reemerged as the predominant AOM pathogen, followed by *M. catarrhalis*. The prevalence of *S. pneumoniae* in cases of AOM has decreased to levels below those of the other two common otopathogens. The purulent effusion data obtained in our study revealing high levels of *H. influenzae* and *M. catarrhalis* in conjunction with low levels of *S. pneumoniae* are similar to the results described by Casey and Pichichero [17] and Zhao et al [102]. Notably, our subject population underwent tympanostomy tube placement for clinical indications so that most would have recurrent or chronic OM infection and may differ from studies of children from outpatient pediatric clinics, which could include first episodes of otitis media. Also, our designation of purulence based on visual subjectivity rather than molecular quantification represents a potential limitation to this study.

A. otitidis is a slow-growing organism that has been historically overlooked as an OM pathogen due to the use of standard bacterial culture techniques when evaluating organisms present in OM middle ear fluid. Because *A. otitidis* grows poorly in standard culture, the increasing use of PCR to identify the etiology of otitis media has resulted in an increased recognition of its presence in middle ear fluid. Our finding that *A. otitidis* is the most prevalent organism in non-purulent effusions is consistent with what has been seen in other recent literature [103, 118, 119].

As was seen in our purulent effusions, *S. pneumoniae* was also the least identified organism in the non-purulent effusion samples. The overall low

prevalence of *S. pneumoniae* in middle ear fluid in our study is consistent with what is being reported in current OM literature [17, 100-103, 119]. Taken together, our results and current literature may suggest that the pneumococcal conjugate vaccines have significantly reduced the ability of *S. pneumoniae* to cause recurrent OM.

Another interesting finding lies in the different frequency of identifying each organism when purulent effusions are compared with non-purulent effusions. *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* were detected ≥2.2x more frequently in purulent effusions than non-purulent effusions. Because OME can persist for several months after AOM [17], decreased frequency of identification of these pathogens from non-purulent effusions may be partly explained by bacterial clearance following antimicrobial treatment and/or clearance by human immune defense mechanisms.

Although the above differences were apparent for the 3 most common otopathogens, *A. otitidis* results follow a distinct pattern. The fact that *A. otitidis* percentages remained stable in cases of purulent and non-purulent effusions may suggest that the organism is a commensal. Another possible explanation is that *A. otitidis* is able to more effectively persist in the middle ear than the three classic otopathogens. According to the literature, strains of *A. otitidis* lack β-lactamase and can exhibit intermediate levels of resistance to β-lactam antimicrobials as well as cephalosporins [124]. Given that current OM treatment guidelines recommend amoxicillin (a β-lactam) as the first drug of choice, the resistance observed in *A. otitidis* would decrease the efficacy of this drug. Given

that *A. otitidis* contains no β-lactamase, a combination of amoxicillin and clavulanate would also be less effective against this organism than the classic otopathogens. One could speculate that the described resistances to common OM antimicrobial treatments could facilitate middle ear persistence.

CONCLUSIONS

The work presented here describes the prevalence of 4 bacterial otopathogens in middle ear fluid obtained from children undergoing tympanostomy tube placement. Our results suggest that *H. influenzae* has surpassed *S. pneumoniae* as the most common pathogen identified in cases of purulent effusions, a result that may have possible implications in antimicrobial treatment guidelines. Although *S. pneumoniae* was infrequently identified, the odds of a purulent versus non-purulent infection were significantly associated with identification of *H. influenzae*, *S. pneumoniae*, and with age <1 year.

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CHAPTER IV.

DISCUSSION

Statistical Findings

The impetus for the work presented was the identification of otopathogens in middle ear fluid obtained from patients undergoing routine tympanostomy tube placement at Wake Forest Baptist Medical Center. Patients involved belonged to one of two concurrent time frames (July 2009 – December 2010; January 2011 – March 2014). Chapters II and III detail the experimental findings associated with each individual cohort. When the data from the entire project were combined and analyzed statistically, a number of correlations were observed. Statistical analyses were completed with the program "R", a free software environment for statistical computing and graphics (www.r-project.org).

H. influenzae Relationship with Purulent Effusions and Recurrence

Statistical analysis revealed a positive correlation between purulent effusions and the presence of *H. influenzae* ($p < 0.001$). Given that the most prevalent organism identified in the purulent effusions (our surrogate for AOM infection) from each patient cohort was *H. influenzae*, this result was not surprising. Many examples in OM literature describe *H. influenzae* as a very prominent AOM pathogen.

Also of interest was the fact that *H. influenzae* was positively correlated with patients that had experienced previous ear infections ($p = 0.0011$). If a child experiences 3 episodes of AOM in a 6 month period or 4 episodes in a 1 year

period, the AOM is designated as recurrent AOM (rAOM) [15]. An association or prevalence of *H. influenzae* in cases of rAOM has been described [125].

Association between Otopathogens

Previous reports investigating bacterial interactions during nasopharyngeal colonization in children have yielded disparate results. As mentioned in the introduction, Pettigrew et al. evaluated the prevalence of the 3 classic otopathogens (*H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*) in nasopharyngeal swabs collected from healthy children. Repeated measures logistic regression models predicting *S. pneumoniae* colonization revealed that *H. influenzae* is negatively associated with pneumococcus; however, when *H. influenzae* was present with *M. catarrhalis*, the correlation with *S. pneumoniae* is positive [50].

In a separate report, Jacoby et al. examined nasopharyngeal microbial interactions in >900 samples from Aboriginal and non-Aboriginal children under 2 years of age. Statistical modelling performed on this data set revealed a positive correlation among all 3 of the pathogens being evaluated regardless of which pathogens were being paired [52]. Taken together, these examples demonstrate that there is variation in the nature of nasopharyngeal microbial interactions.

In our patient population, statistical analysis failed to identify a positive or negative correlation between the classic otopathogens. This lack of correlation could be a function of factors associated with our geographical location (otopathogen strain variants or genetic differences between regional populations,

as examples). Another possible explanation is that the low number of *S. pneumoniae*-positive middle ear fluids that we obtained failed to reach a level high enough to facilitate statistical significance given the parameters being tested.

Interestingly, a positive correlation was identified between *M. catarrhalis* and *A. otitidis* ($p < 0.05$). Although previous research has not specifically evaluated the microbial interactions between these two species during nasopharyngeal colonization or otitis media, the innate β-lactam resistances observed in these organisms may play a role in their coexistence. *M. catarrhalis* exhibits a β-lactamase that can be secreted to the surrounding environment; *A. otitidis* exhibits intermediate resistance to β-lactams independent of a β-lactamase. In an environment exposed to amoxicillin (the preferred antibiotic for OM treatment), these two organisms would be better adapted for survival than a β-lactam sensitive organism such as *S. pneumoniae*, thus allowing them to be identified together more frequently.

Impact of Age on Presence of S. pneumoniae

Despite the limited number of patient samples that were positive for pneumococcus, our results yielded a statistically significant negative correlation ($p = 0.0369$) between the presence of *S. pneumoniae* and children that were >3 years of age. This finding was not unexpected. According to the 2013 Active Bacterial Core Surveillance Report from the Centers for Disease Control and Prevention (CDC), as well as other published literature, children under the age of

2 years old are at the highest risk for *S. pneumoniae* infections [126-129].

Underlying viral infections represent one of the reasons for the *S. pneumoniae*/age correlation. As discussed in Chapter I, viral URTIs play a role in disrupting bacterial clearance mechanisms such as proper ciliary function, regulation of mucus production, appropriate neutrophil and β-defensin function, and control of mucosal inflammation. These viral infections are most prevalent in children under 3 years of age, making children in this age group more susceptible to *S. pneumoniae* infection. Another contributing factor is the immature nature of the infant immune system. Infants and children less than 2 years of age fail to mount a robust immune response to T-cell-independent antigens, such as the polysaccharides found in the capsule of *S. pneumoniae* [130-132], also hindering the process of pneumococcal clearance.

Alloiococcus otitidis Persistence

Chapter III introduced the topic of *A. otitidis* persistence in cases of OM. The hypothesized persistence mechanism was the inherent antimicrobial resistances to β-lactams and extended spectrum cephalosporins (both of which are first and second line treatments for OM) observed with *A. otitidis* [124]. Another possible explanation for the perceived alloiococcal persistence is the ability of the organism to form biofilms.

Biofilms are defined as a sessile community of organisms encased in an extrapolymeric matrix and attached to a natural or abiotic surface [57, 133]. It is believed that the majority of bacterial species have the ability to form these 3-

dimensional communal structures and that this way of life represents the normal state of growth for these organisms in nature [134]. Biofilms pose a significant clinical health risk because the organisms within them are inherently tolerant to host immune defenses and may be upwards of 1000x more resistant to common antibiotics [135].

There is clear evidence that biofilms are associated with OM. It has been demonstrated that the 3 classic otopathogens (*H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*) all have the ability to form biofilms *in vitro* and *in vivo* [58, 59, 136-148]. These OM biofilm structures have been observed in experiments utilizing a chinchilla model of middle ear infection and more notably in human middle ear mucosal samples. Also, bacterial DNA and mRNA have been observed in culture-negative middle ear fluids from OM patients [149], supporting the idea that live bacteria are attached to the middle ear mucosa during these infections. Although the biofilm-forming capabilities of *A. otitidis* have not been experimentally examined, one could hypothesize that the observed ability of *A. otitidis* to persist in the middle ear could be due to biofilm formation.

Impact of Prevnar 13 on OM Etiology

The results of our experimentation were inconclusive concerning the impact of vaccination on OM etiology. Samples from our first patient cohort (July 2009 – December 2010) were obtained prior to the introduction of Prevnar 13 for widespread use. Although the patient medical history information recorded at the time of surgery did not include prior vaccinations, it is estimated that ~92% of

children are vaccinated with PCV annually [150]. Thus, it would be a fair hypothesis that the majority of the members of the first patient cohort were vaccinated with the original Prevnar (7-valent) formulation.

Samples from our second patient cohort (January 2011 – March 2014) were obtained after Prevnar 13 had officially been released for widespread use in the US. Based on the vaccine coverage statistics mentioned above, it is a fair assumption that the majority of these patients received the newer, more comprehensive vaccine formulation. When results from the patient populations were compared, no statistical differences were observed in the prevalences of *S. pneumoniae* and *H. influenzae*, two organisms that have been shown to be affected by PCV administration.

One explanation for the lack of differences between the patient populations is that the original Prevar vaccinations introduced in 2000 may be very effective in combatting the capsular serotypes of *S. pneumoniae* in our geographic region. This hypothesis would also explain the overall low levels of *S. pneumoniae* identified in our study. Our experimentation didn't focus on the serotypes of *S. pneumoniae* being identified. The true impact of vaccination on our patient populations could be determined by adding this experimental variable to future studies, as will be touched upon in the following section.

Future Directions

The goal of biomedical research should be to utilize experimental findings to drive the discovery and implementation of more effective disease therapies.

This five-year prospective study has revealed information regarding the presence of otopathogens in children who have experienced or are experiencing OM. Elucidating the identity and prevalence of these pathogens allows for the administration of more specific and effective treatment. As an example, the use of a more narrow spectrum of antibiotic will help ensure efficacy as well as decrease the chances of resistance development.

To gain a more thorough understanding of the multiple factors involved in the bacterial pathogenesis of OM, our research should be expanded. A relatively easy addition to the current protocols would be to implement multiplex PCR parameters that could evaluate the four otopathogens described in this work as well as other less prevalent bacterial species. As an example, primer sets could be designed and used for the identification of Group A *Streptococcus* or *Pseudomonas aeruginosa*, both of which have been implicated in cases of OM. Also, unpublished data from the laboratory of Dr. Edward Swords (Wake Forest School of Medicine) has demonstrated that *Haemophilus. parainfluenzae* has the ability to cause OM in a chinchilla model of infection, suggesting that it may be prudent to start targeting this organism for evaluation in patient samples.

In a similar vein, mPCR parameters could be utilized that allow for the identification of viral species that may be present during OM due to coincident URTIs, such as RSV, adenovirus, coronavirus, influenza virus, or rhinovirus. Implementing these primer sets would give insight into the viral-bacterial interactions occurring during the disease process, possibly allowing for the introduction of specific antiviral therapies for reducing incidence of disease.

While increasing organism identification through the addition of more diverse mPCR primer sets will yield valuable information concerning otopathogen presence, taking a more in-depth look at the strains identified will also enhance our understanding of OM disease. After culturing organisms found in MEF samples, processes such as serotyping or sequencing could be done to elucidate strain subtypes associated with OM in our region. These findings could reveal potential vaccine deficiencies (lack of serotype coverage as has been seen in PCVs) as well as which strain subtypes are positively or negatively correlated with each other.

The experimentation described in the body of this work utilized MEF from a single point in the time course of a patient's disease. Sampling each patient's MEF from multiple points over the course of OM would allow for the examination of trends associated with disease. This type of analysis combined with more stringent organism/strain identification would give the clearest picture of what is occurring during OM in our patients.

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