Epidemiology and Pathogenesis of *Moraxella catarrhalis* Colonization and Infection

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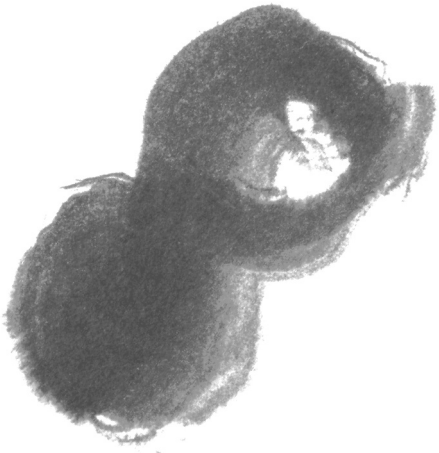

Chapter 1

General introduction, aim and outline of the thesis

General introduction

Moraxella catarrhalis is part of the normal bacterial flora in the nasopharynx of children, although over the past two decades, it has emerged as a significant bacterial pathogen and not simply a commensal colonizer. The bacterium rapidly colonizes the nasopharynx soon after birth and many factors affect nasopharyngeal carriage of this human-specific pathogen, including for example, the presence of siblings, day-care attendance and respiratory illness. Otitis media (OM) is a particularly important respiratory illness during early childhood and the primary reason for children to visit a physician. The most common bacterial species cultured from the nasopharynx of children during OM episodes are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *M. catarrhalis*, either as single pathogens or as co-cultures, with the patterns of nasopharyngeal colonization by microorganisms being important determinants for OM disease. There is increasing information regarding the biological mechanisms facilitating *M. catarrhalis*-mediated colonization and disease development, with most publications stressing the importance of bacterial adherence as an essential step in this process.

Treatment of OM is not always appropriate, and the long-term overuse of antibiotics in OM reduces the effectiveness of treatment and places children at an increased risk of antibiotic-resistant infections. Because there is currently no ideal treatment for OM, an alternative to the use of antibiotics is vaccination. To date, several *M. catarrhalis* vaccine candidates have been described, which may be effective in reducing the burden of OM disease. The challenge in identifying potential vaccine candidates for *M. catarrhalis* lies in identifying antigens that are able to generate an appropriate immune response to prevent colonization and infection. However, there is no licensed vaccine available against *M. catarrhalis*, and none of the putative vaccine candidates so far described have progressed to clinical trials.

Aim and outline of the thesis

The aim of this thesis is to help understand the epidemiology and pathogenesis of *M. catarrhalis* colonization and infection, with special emphasis on isolate variation, the humoral immune response and novel immunologically relevant vaccine candidates of *M. catarrhalis*.

In this respect, **Chapter 2** provides a detailed description of our current knowledge regarding *M. catarrhalis* as a human pathogen and the treatment and management of this organism. The subsequent seven chapters are then divided into two main themes, relating to the dynamics of *M. catarrhalis* colonization and infection (**Chapters 3 – 7**) and the humoral immune response (**Chapter 8 – 9**).

In **Chapter 3**, determinants of *M. catarrhalis* colonization in healthy Dutch children during the first months of life were defined, whereas in **Chapter 4**, pulsed-field gel electrophoresis, PCR and PCR-RFLP are used to determine the genotypic relationship between colonizing *M. catarrhalis* strains of healthy children, and to study virulence gene diversity. Further, co-colonization with *H. influenzae* was also investigated. In **Chapter 5**, the age-related genotypic and phenotypic differences in *M. catarrhalis* isolates from children and adults presenting with respiratory disease were investigated. **Chapter 6** gives an overview of a prospective study that looked into the differences in the presence of bacteria and viruses in the middle ear and nasopharynx of children diagnosed with recurrent acute otitis media (rAOM) and chronic otitis media with effusion (COME). The role of the major bacterial pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, in the absence or presence of 15 respiratory viruses, was studied in detail. In fact, previous research has shown that bacterial co-colonization does lead to increased colonization and infection, with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* being the predominant co-colonizing bacterial organisms associated with OM. However, group A *Streptococcus* (GAS) may also be included as one of the most frequent causative agents of OM, especially with reference to the increasing importance of GAS in OM disease in Eastern Europe. Therefore, in **Chapter 7**, studies were performed to investigate the effect of *M. catarrhalis* co-culture on GAS gene expression, particularly with respect to the expression of GAS virulence genes.

Relatively little is known about the development of the humoral immune response to *M. catarrhalis* surface antigen vaccine candidates within the first few years of life in healthy and sick children. In this respect, the studies presented in **Chapter 8 and 9** were established in order to study the humoral immune response generated against the currently identified surface antigens of *M. catarrhalis* (**Chapter 8 and 9**) and *S. pneumoniae* (**Chapter 9**). The relationship between colonization and immune response was also investigated.

Finally, **Chapter 10** gives a summary of the research presented within this thesis and discusses the findings with respect to *M. catarrhalis* vaccine development.

Chapter 2

Moraxella catarrhalis: a human pathogen

Adapted from:

Suzanne J.C. Verhaegh and John P. Hays. Chapter 80 *Moraxella*. In: Liu, D. (ed), Molecular Detection of Human Bacterial Pathogens. 2011. Taylor & Francis CRC Press, Boca Raton, FL, USA. In press.

The genus *Moraxella*

The genus *Moraxella* belongs to the family *Moraxellaceae*, which includes the closely related genera *Acinetobacter* and *Psychrobacter*. The *Moraxella* genus itself currently contains 15 different species, including *M. lacunata*, *M. atlantae*, *M. boevrei*, *M. bovis*, *M. canis*, *M. caprae*, *M. catarrhalis*, *M. caviae*, *M. cuniculi*, *M. equi*, *M. lincolnii*, *M. nonliquefaciens*, *M. osloensis*, *M. ovis* and *M. saccharolytica* [1]. Recently, *M. pluranimalium* has been described as a new species of the genus *Moraxella* after its isolation from sheep and pigs [2]. Although all of these 16 species have been classified within the genus *Moraxella*, the classification of the *Moraxellaceae* family is still not definitive but continuously evolving. For example, molecular systematic studies (of which 16S ribosomal RNA (rRNA) gene sequencing has long been the 'gold standard') has led to the re-classification of *Riemerella anatipestifer*, *Psychrobacter phenylpyruvicus* and *Oligella urethralis*, which were formally known as *Moraxella anatipestifer*, *Moraxella phenylpyruvica* and *Moraxella urethralis*, respectively [1, 3].

Of the 16 *Moraxella* species, *M. caprae*, *M. caviae*, *M. cuniculi*, *M. equi*, *M. lincolnii*, *M. ovis*, *M. saccharolytica*, *M. boevrei*, *M. bovis* and *M. pluranimalium* are only associated are only associated with diseases in animals. Occasionally however, some *Moraxella* species are associated with human disease. Specifically, *M. lacunata* has been associated with chronic conjunctivitis, arthritis and endocarditis in humans, [4-8] whilst *M. nonliquefaciens* has been known to cause bacterial infection (botryomycosis) and inflammation (endophthalmitis) [9-11]. *M. atlantae* is an unusual and only rarely isolated species that has been associated with bacteremia in humans, [12-13] whilst *M. osloensis* rarely causes infections but has been associated with bacteremia and endophthalmitis [14-16]. Interestingly, *M. canis* is an upper airway commensal bacterium in cats and dogs, and has been considered non-pathogenic for humans even although it has been isolated from humans in a few cases [17-18]. The single most important exception to this '*Moraxella*-animal disease' relationship is *M. catarrhalis*, an organism which is a strictly human pathogen and causes a range of human diseases.

Classification, biology and epidemiology of *Moraxella catarrhalis*

M. catarrhalis (formally known as *Branhamella catarrhalis*) has undergone several name changes in the past 100 years [19-20]. It was first described at the end of the nineteenth century when it was named *Micrococcus catarrhalis*, and it was later changed to *Neisseria catarrhalis* because of its similarity in phenotype and ecological niche to

species of *Neisseria* [21]. In 1970, the bacterium was transferred to a new genus, *Branhamella*, based on differences between *N. catarrhalis* and the type species of the genus *Neisseria* and six other species of this genus [19]. In 1984, *Branhamella catarrhalis* was reassigned to the genus *Moraxella* as *Moraxella (Branhamella) catarrhalis* [22]. *M. catarrhalis* is now the most widely accepted and currently preferred name for this bacterial species.

M. catarrhalis is an oxidase-positive, Gram-negative diplococcus, which grows well on both blood and chocolate agars, as well as several selective agar media. Optimal growth is achieved at a temperature of 35°C and may be enhanced in an atmosphere of 5% carbon dioxide. Colonies are generally non-hemolytic, gray to white, opaque and smooth, and can be pushed along the agar surface without losing colonial integrity, the so-called 'hockey puck sign' [23-24].

M. catarrhalis is an important colonizer of the respiratory tract. Until recently, *M. catarrhalis* was thought to be a harmless commensal. However, it is now recognized as one of the more important bacterial pathogens associated with both upper and lower respiratory tract infections in humans. These upper and lower respiratory tract infections include acute, chronic and chronic suppurative otitis media and exacerbations of chronic obstructive pulmonary disease (COPD).

Acute otitis media (AOM) involves inflammation of the middle ear, including the eardrum, the inner ear, and the Eustachian tube. AOM is generally considered a bacterial infection, and bacteria may be isolated from the middle ear fluid (MEF) in approximately 80% of children with AOM. *Streptococcus pneumoniae* is reported to be the predominant bacterial species cultured in AOM disease, followed by *Haemophilus influenzae* and *M. catarrhalis.* However, this ranking is subject to some debate as there are still questions regarding which bacterial species plays the most important role in middle ear infection and therefore AOM [25]. Bacteria have been cultured from 30 to 50% of patients with chronic otitis media with effusion (COME), where *H. influenzae* predominates, and to a lesser extent, *S. pneumoniae* and *M. catarrhalis* [25-26]. Chronic suppurative otitis media (CSOM) is one of the most common chronic diseases of childhood in many underdeveloped countries (especially among the poor), and in certain populations of the developed nations. In contrast to AOM and COME, *Pseudomonas aeruginosa* is the most predominant species isolated, with a minor role for *M. catarrhalis* [27-28].

In contrast to the upper respiratory tract infections (URTI) associated with *M. catarrhalis* in children, *M. catarrhalis* in adults is mostly associated with lower respiratory tract infections (LRTI). COPD is a major LRTI and a considerable public health problem.

It is predicted to become the fifth leading cause of death worldwide in 2020 and the prevalence of, and mortality due to this disease are currently increasing [29]. Several lines of evidence developed in the last decade have established that *M. catarrhalis* is associated with exacerbations of COPD. For example, non-typeable *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* are the three predominant bacterial species isolated from sputum in 40 to 60% of acute exacerbations of COPD, with *M. catarrhalis* being isolated in 4-21% of sputa [30]. The high prevalence of bacterial colonization of the airways observed in COPD is generally related to the degree of airflow obstruction and cigarette smoking [31]. However, more research is needed to test the hypothesis that bacterial colonization accelerates the progressive decline in lung function seen in COPD (the vicious circle hypothesis), although neutrophilic airway inflammation and systemic inflammation are more intense in the presence of bacteria [30, 32].

Colonization and the immune response

Colonization with *M. catarrhalis* begins very early in life with a high percentage of infants being colonized, but a much lower percentage of adults being colonized (1-5%) [24]. In fact, a *M. catarrhalis* colonization peak is observed at 2 years of age, with both infants and children tending to acquire and eliminate a number of different strains [33-34]. Factors that have been particularly associated with an increased likelihood of *M. catarrhalis* colonization include the presence of siblings and day-care attendance [35]. Further, studies have shown that frequent colonization with *M. catarrhalis* increases the risk of otitis media (OM) and that patterns of colonization are important determinants of OM-related disease [24, 36-37].

In young infants, high antibody levels exist due to immunization by passively acquired maternal immunoglobulin G (IgG) antibodies, which are transferred to the fetus through the placenta. This passive immunity lasts until about six months after birth. In this period, the actively acquired humoral immune response is being established in which children are susceptible for *M. catarrhalis* colonization and infection. A significant fall in antibody concentration during the first 3 months of life from healthy children is observed and the IgG antibodies to *M. catarrhalis* remain at a low level in children below 1 year of age. However, from the age of 1 year, the immune response of the children increases slowly to reach maternal levels at the age of 10 years [38].

The development of mature IgG antibodies to *M. catarrhalis* is reflected in the age-related development of IgG subclass responses to *M. catarrhalis*. Usually, subclass 1 is the first antibody subclass to develop, and it is found after the first year of life,

where subclass 3 develops during the second and third years of life and is known to be elicited by bacterial outer membrane proteins (OMPs) [39-40]. The peak level of *M. catarrhalis* colonization (age of 2 years) and where children have a high susceptibility to *M. catarrhalis* infections coincides with the age range where the levels of *M. catarrhalis* IgG antibodies are low. This may indicate that the eradication of *M. catarrhalis* may be related to rising titers of *M. catarrhalis*-specific serum IgG1 and IgG3 after 2 years of age.

Pathogenesis

The recent recognition of *M. catarrhalis* as an important pathogen in both the upper and lower respiratory tract has resulted in an increased interest in both the bacterium's interaction with the human host and its antigenic composition.

Two distinct genetic lineages related to 3 different 16S rRNA types have been identified for *M. catarrhalis*, which differ phenotypically in their ability to resist the killing effect of human serum (sero-resistant versus sero-sensitive), and in their ability to adhere to human epithelial cells [41-42]. Further, the sero-resistant lineage of *M. catarrhalis* (16S rRNA type 1 lineage) has been reported to be more virulent than 16S rRNA types 2 and 3 (lineage 2 isolates) [42].

There is increasing information regarding the mechanisms facilitating *M. catarrhalis* colonization and infection of the respiratory tract. In particular, bacterial adherence to the respiratory mucosa is an essential step towards colonization of the human respiratory tract epithelium and the development of disease. Important adhesins responsible for the attachment of *M. catarrhalis* to host cells include the family of ubiquitous surface protein A (UspA) proteins UspA1 and UspA2, which have been extensively studied in *M. catarrhalis* and were initially described approximately 17 years ago [43]. The closely related UspA2H protein appears to be a hybrid protein that most closely resembles UspA1 at its N-terminal and UspA2 at its C-terminal region [44]. UspA1 and UspA2 acquire their adherence characteristics through multifunctional binding sites, which include domains that have the ability to attach to epithelial cells via cell-associated fibronectin, laminin and vitronectin [45]. Further, UspA1 includes a critical binding site for carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), which are expressed in various human tissues including respiratory epithelial cells [46]. The CEACAM-1 receptor-binding domain is located in the stalk region of UspA1 and consists of an extended, rod-like left-handed trimeric coiled-coil. The stalk region is capable of changing conformation after CEACAM-1 binding to allow a closer approach between

M. catarrhalis and the host cell membrane [47]. The stalk region of UspA1 and UspA2 share multiple amino acid repeats and distinct sequence 'motifs', a finding that suggests that the make-up of the UspA proteins might be interchangeable, possibly under the influence of immune pressure. This extensive sharing of sequences between UspA1 and UspA2 does not include the N- and C-terminal regions of these proteins [48-49]. The UspA stalk region contains one or two copies of the NINNY motif (which contains the epitope for the protective monoclonal antibody (MAb) 17C7), and the CEACAM binding motif (which contains the epitope for a separate anti-UspA1 antibody that also inhibits CEACAM binding) [49-50]. The exchange of repeats and motifs in UspA proteins may allow *M. catarrhalis* populations to exhibit various combinations of binding properties, which could help define the virulence of individual strains [49].

Another OMP that is important for bacterial attachment is the *Moraxella* immunoglobulin D-binding protein (MID), also referred to as Hag (human erythrocyte agglutinin), due to the ability of *M. catarrhalis* to agglutinate human erythrocytes and the unique immunoglobulin-binding properties [51-52]. The adhesive domain of MID is located in the 149 amino acid fragment $MID^{764-913}$ of *M. catarrhalis* strain Bc5 and promotes the attachment of bacteria to epithelial cells, whereas the IgD-binding domain is located within the 238 amino acid fragment $MID^{962-1200}$ of the same protein [51, 53]. Further, antibodies directed against $MID^{764-913}$ effectively inhibit attachment of the high MID-expressing isolates to alveolar epithelial cells, and animals immunized with $MID^{764-913}$ clear *M. catarrhalis* much more efficiently when compared to mice immunized with bovine serum albumin (BSA) [54].

M. catarrhalis has developed several mechanisms to evade the effect of human complement, allowing it to evade the host innate immune response. Three biochemical pathways activate the complement system: the classical pathway, the alternative pathway, and the mannose-binding lectin (MBL) pathway. The classical pathway of the complement system is initiated by the binding of the complement component (C)-1 complex to antibodies bound to an antigen on the surface of a bacterial cell, whereas the alternative pathway is initiated by the covalent binding of a small amount of C3b to the surface of a microbe and is activated by cleavage of C3 in plasma. Activation of these systems leads to a cascade of protein deposition on the bacterial surface, resulting in formation of the membrane attack complex (MAC) and opsonization of the pathogen, followed by phagocytosis [55-56].

M. catarrhalis has evolved mechanisms to interfere with the activation of the complement pathway, including the binding of complement inhibitor C4-binding protein

(C4BP; classical pathway) and by noncovalently binding C3 (alternative pathway). The *M. catarrhalis* OMPs UspA1 and UspA2 bind C4BP and C3, and UspA2 interferes with the proper formation of the MAC porin complex that inserts into bacterial outer membranes, due to the stable binding of vitronectin [57-60].

Another example of the ability of *M. catarrhalis* to evade the host immune system is phase variation and antigenic variation [61]. Phase variation in general refers to a reversible switch between an on-off expressing phase, resulting in variation in the level of expression of one or more OMPs between individual bacteria of a clonal population. Antigenic variation refers to the expression of functionally conserved OMPs within a clonal population of bacteria that are antigenically distinct [62]. As OMPs are found on the bacterial cell surface and may be exposed to the immune system, antigenic variation influences the efficacy of antibody-mediated defense mechanisms against the respective OMPs and hence against the bacterium *per se*. Currently, *M. catarrhalis* OMPs UspA1, UspA2 and Hag/MID are known to undergo phase variation, with antigenic variation reported in the target region of MAb 17C7 (a conserved UspA1 and UspA2 binding site), although this variation involves only minor amino acid changes [63-67]. Whether antigenic variation is actually a virulence mechanism for *M. catarrhalis*, is a subject of debate, as Meier *et al.* (2005) demonstrated that reduced or absent expression of UspA1, rather than antigenic variation, was responsible for non-reactivity with the MAb 17C7 [65].

Interestingly, *M. catarrhalis* releases outer membrane vesicles (OMVs), or 'blebs' from the surface of the cell during growth in various environments, including liquid culture, solid culture, and during biofilm formation. Blebs are thought to contain some of the underlying periplasm, together with OMPs and lipopolysaccharides (LPS) of the outer membrane layer [68]. The OMVs that are secreted by *M. catarrhalis* carry UspA1 and UspA2, and have been shown to interfere with successful complement activation against *H. influenzae* in mixed infections. Thus, OMVs from *M. catarrhalis* may contribute to the pathogenicity of pure and mixed *M. catarrhalis* populations by binding and depleting C3 in their immediate environment [69].

Another important component of the innate immune system are the transmembrane toll-like receptors (TLRs). TLRs are pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), small conserved molecular motifs found in microbes, for example bacterial LPS. The specific inflammatory responses induced by *M. catarrhalis* may be mediated by activation of these PRRs, with the inflammatory immune response to *M. catarrhalis* in respiratory epithelial cells being

mainly dependent on TLR2 [70]. This TLR2 inflammatory immune response is mediated by the cytokine interleukin (IL)-8, which has a critical role in regulating neutrophil and monocyte chemotaxis toward sites of infection. Further, the activation of protein kinase C (PKC) through nuclear factor (NF)-κB signaling pathways, which induces IL-8 release, has been found to be dependent on expression of UspA2 [71]. Additionally, IL-8 release is inhibited by UspA1-dependent interactions with CEACAM-1 [72].

A single highly homologous 32-amino acid sequence of *M. catarrhalis* UspA1 and UspA2 is also responsible for binding and neutralizing α1-antichymotrypsin. This serine protease inhibitor is not active anymore as an inhibitor when bound to *M. catarrhalis* UspA1 and UspA2. As a result, proteases disrupt the host tissue structure and provide bacteria with soluble substrates and extracellular matrix proteins for adhesion, which may be beneficial for bacterial colonization. *M. catarrhalis* α1-antichymotrypsin neutralization is therefore a novel microbial virulence mechanism [73].

Research has shown that co-colonization leads to increased colonization and infection, for example Verhaegh *et al.* (2010) showed that co-colonization of *M. catarrhalis* and *H. influenzae* was significantly more likely than single species colonization with either *M. catarrhalis* or *H. influenzae* [74-76]. Additionally, Armbruster *et al.* (2010) showed that quorum sensing during *M. catarrhalis* and *H. influenzae* co-culture positively influenced indirect pathogenicity in polymicrobial OM infections [77]. In fact, many pathogenic bacterial species use quorum sensing systems to coordinate their gene expression, in order to, for example, coordinate the formation of biofilm (as a response to the local bacterial population density). It is known that *M. catarrhalis* exists in biofilms and that it is able to prolong its survival through biofilm formation on mucosal surfaces in the nasopharynx. Biofilms are usually defined as microbial communities, surrounded by an extracellular polymeric substance (EPS) matrix that adhere to biological or non-biological surfaces [78]. Biofilm formation has been demonstrated for numerous pathogens and is clearly an important microbial survival strategy [79]. Several other pathogens associated with infections, including *H. influenzae* and *S. pneumoniae* in chronic OM, are also linked to biofilm formation. These bacteria benefit from this microbial community thanks to, for instance, the phenomenon of indirect pathogenicity, where *S. pneumoniae* is protected from the action of certain β-lactam antibiotics in the presence of BRO β-lactamase positive *M. catarrhalis*, and where *M. catarrhalis* protects *H. influenzae* due to binding and depleting C3 by release of OMVs [69, 80-81].

Studies have shown that several *M. catarrhalis* genes are associated with biofilm formation, for example *uspA1*, *uspA2*, *uspA2H* and *hag/mid*, although the exact role

of each of the resultant proteins in biofilm formation is still open to question. Further, Wang *et al.* (2007) showed that growth of *M. catarrhalis* in a biofilm results in increased expression of genes encoding a nitrate reductase, a nitrite reductase, and a nitric oxide reductase, which can function not only in energy generation but also in resisting the innate immune response [82].

Diagnosis

Recognition of the pathogenic status of *M. catarrhalis* means that the detection of *M. catarrhalis* in clinical specimens is now an important topic in disease management and diagnosis. In the routine clinical laboratory, the detection of *M. catarrhalis* tends to be based on conventional methods only, although a variety of molecular detection techniques are being used within research laboratories to investigate *M. catarrhalis* specific genes and virulence. These techniques include PCR and DNA hybridization-based bacterial detection methods, which are usually reliant on the specific detection and amplification of genes encoding for *M. catarrhalis* OMPs. For both research and diagnostic purposes, further details concerning the relatedness of *M. catarrhalis* isolates may be obtained by using one of several *M. catarrhalis* typing techniques. These epidemiological investigation techniques include pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as the most popular, as well as electrophoretic profiling of OMPs, iso-electric focusing of BRO β-lactamase enzymes and serological typing of LPS [83-85]. More recent developments include a *M. catarrhalis* micro-array assay that has been developed to study gene expression of *M. catarrhalis* under varying environmental conditions [82].

Despite being somewhat old-fashioned, time-consuming and unable to detect unculturable isolates, the conventional approaches for identifying *M. catarrhalis* (including selective culture media, Gram stain morphology and biochemical testing, for example the tributyrin disc test) are still extremely useful. The use of PCR tests directed against *M. catarrhalis* genes in the routine clinical microbiology laboratories is hindered by the lack of sensitivity, specificity and reproducibility data, as well as the lack of an accepted library of quality control *M. catarrhalis* isolates to test. Issues related to cost per unit specimen, as well as the fact that bacterial culture tends to be a 'catch-all' technique (rather than the more specific molecular techniques), also play a role in current decisions related to the implementation of molecular tests for *M. catarrhalis* in the routine clinical microbiology laboratory.

Treatment and management

M. catarrhalis is known to produce a BRO β-lactamase, an enzyme responsible for the resistance of *M. catarrhalis* to certain β-lactam antibiotics. Moreover, in the last 30 years, major changes have occurred in the incidence of β-lactamase production in *M. catarrhalis* isolates. BRO β-lactamase was derived from an, as yet unknown, Gram-positive bacterium, with its lipid modification being a remainder of its origin, and spread through the population mainly by horizontal transfer [86]. The first β-lactamase-producing isolate was detected in clinical specimens reported by Malmvall *et al.* in 1977, with a prevalence rate of 3.8% [87]. However, by 1980, 75% of *M. catarrhalis* isolates from the United States produced β-lactamase [88]. Recent studies have shown that thirty years after the first discovery of β-lactamase producing strains, over 95% of the clinical isolates worldwide produce a BRO β-lactamase [89].

All strains of *M. catarrhalis* are resistant to amoxicillin due to the production of BRO β-lactamase, but the discovery of the β-lactamase inhibitor clavulanic acid in the year 1972 and its co-administration with amoxicillin as amoxicillin/clavulanate still allows the continued use of β-lactam antibiotics to treat infections caused by *M. catarrhalis*. The efficacy of this combination has been demonstrated in a range of clinical trials, and since 1981, amoxicillin/clavulanate has been registered for the treatment of URTIs and other diseases [90]. Although amoxicillin is recommended as the initial antibacterial agent of choice for most children, it is shown that recurrent OM occurs more often in children originally treated with amoxicillin [91]. Further, the frequent use of antibiotics as therapy for AOM tends to result in a rise in antibiotic resistance, and increasing evidence is becoming available regarding the limited clinical efficacy of antibiotics in the successful treatment of AOM [92].

The American Academy of Pediatrics published a guideline in 2004 that addressed the diagnosis and treatment of AOM. This guideline recommended the use of watchful waiting as a potential strategy for the treatment of AOM, although rates of antibiotic prescription for AOM still vary to a great extent internationally [92-93].

An alternative to the use of antibiotics is vaccination. However, there is currently no licensed vaccine against *M. catarrhalis*, and to date, none of the putative vaccine candidates so far described have progressed to clinical trials. The challenge in identifying potential vaccine candidates for *M. catarrhalis* lies in identifying antigens that i) are able to generate an appropriate immune response to prevent colonization and infection and ii) are conserved among global strains. A further major obstacle in vaccine development has been the absence of a satisfactory animal model for *M. catarrhalis*-mediated

diseases, and consequently, problems associated with the identification and verification of the 'correlates of protection' against *M. catarrhalis* [24].

On a further note, effective vaccines against viruses and bacteria involved in AOM could have a dramatic impact on the overall incidence of this disease. However, the potential use of vaccines against for example non-typeable *H. influenzae* or *S. pneumoniae* (e.g. Prevnar™, Wyeth) may lead to a reduction in the incidence of these two pathogens in the etiology of AOM, possibly allowing *M. catarrhalis* to capitalize on the available niche and become the main bacterial AOM pathogen of the future.

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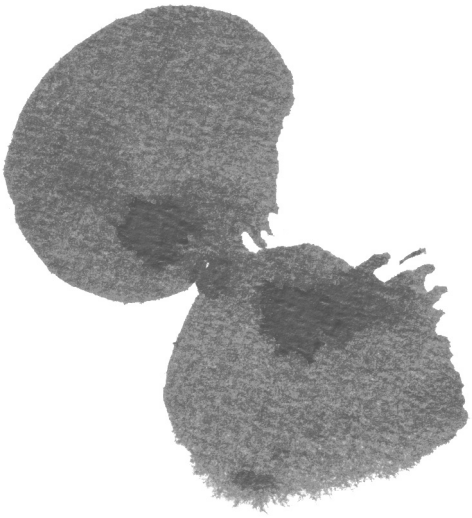
Dynamics of *Moraxella catarrhalis* colonization and infection



Chapter 3

Determinants of *Moraxella catarrhalis* colonization in healthy Dutch children during the first 14 months of life

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Abstract

Moraxella catarrhalis is an established bacterial pathogen, previously thought to be an innocent commensal of the respiratory tract of children and adults. The objective of this study was to identify significant risk factors associated with *M. catarrhalis* colonization in the first year of life in healthy Dutch children. This study investigated a target cohort group of 1,079 children forming part of the Generation R Study, a population-based prospective cohort study following children from fetal life until young adulthood, conducted in Rotterdam, The Netherlands. Nasopharyngeal swabs for *M. catarrhalis* culture were obtained at 1.5, 6 and 14 months of age, with all three swabs being available for analyses from 443 children. Data on risk factors possibly associated with *M. catarrhalis* colonization were obtained by questionnaire at 2, 6 and 12 months. *M. catarrhalis* colonization increased from 11.8% at age 1.5 months to 29.9% and 29.7% at 6 and 14 months, respectively. Two significantly important colonization risk factors were found: the presence of siblings and day-care attendance, which both increased the risk of being positive for *M. catarrhalis* colonization on two or more occasions within the first year of life. Colonization with *M. catarrhalis* was not associated with gender, educational level of the mother, maternal smoking, breast-feeding, or antibiotic use. Apparently, crowding is an important risk factor for early and frequent colonization with *M. catarrhalis* in the first year of life.

Introduction

Moraxella catarrhalis is an acknowledged respiratory tract pathogen [1]. The bacterium has the capacity to colonize the nasopharynx, and may be isolated in pure culture or together with other bacterial pathogens, e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae* and/or *Haemophilus influenzae* [2]. Although colonization does not always result in disease, it may be the first step towards invasive disease, and a source of horizontal spread of *M. catarrhalis* in the community [3]. Children are frequently colonized with *M. catarrhalis*, and frequent colonization with *M. catarrhalis* has been reported to increase the risk of otitis media (OM) [4]. Risk factors associated with *M. catarrhalis* colonization have been previously studied in several countries and age groups, and the findings have indicated that crowding and contact with children are risk factors for colonization, not only for *M. catarrhalis*, but also for *S. pneumoniae* and *H. influenzae* [5]. Other reported risk factors are genetics [6-7], smoking [8], socio-economic status [9], synergy and interference with other micro-organisms [10-11], frequency and location of sampling [12], season [12], gender [13], and vaccination [14]. However, medical and living conditions in The Netherlands may be quite different from those in other countries. For example, there is a restrictive prescription policy for antibiotic usage. These differences could lead to differences in *M. catarrhalis* epidemiology and colonization rate in The Netherlands, as compared with other countries.

The objective of this study was to assess risk factors for *M. catarrhalis* colonization in the first year of life in healthy Dutch infants within a geographically restricted environment (Generation R Focus cohort, living in Rotterdam, The Netherlands).

Materials and methods

This study was embedded in the Generation R Study, a population-based prospective cohort study following children from fetal life until young adulthood, conducted in Rotterdam, The Netherlands [15-16]. As part of the Generation R Study, detailed assessments of fetal and post-natal growth and development were conducted in 1,232 Dutch pregnant women and their children (Generation R Focus cohort), who were born between February 2003 and August 2005. The Medical Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands, has approved the study. Written informed consent was obtained from the parents of the children who were included. The mothers gave

birth to 1,244 infants, 138 of whom were excluded from the study analysis because consent was withdrawn after birth. Twins (*n*=27) were also excluded from the analysis because of genetic relatedness, leaving a final total of 1,079 infants. Infants visited the Generation R Focus cohort research center at 1.5 months (*n*=884), 6 months (*n*=882) and 14 months (*n*=863) after birth, resulting in 627 swabs being successfully taken at 1.5 months, 832 swabs at 6 months, and 757 swabs at 14 months. Seven hundred and fifty-eight infants attended all visits, and 443 provided three swabs for use in longitudinal analysis. Nasopharyngeal samples were taken using a sterile transport swab suitable for aerobes and anaerobes by a trained research nurse, using Amies transport medium. Infants treated with antibiotic in the preceding 48 hours were excluded from nasopharyngeal sampling [15-16]. Swabs were processed within the medical microbiology laboratory of the Erasmus MC, Rotterdam, The Netherlands, within 6 hours of sampling, using blood agar plates containing 5% sheep blood at an incubation temperature of 35°C in 5% CO_2 for 48 hours. Plates were examined daily for growth of *M. catarrhalis*.

Information related to determinants of colonization (birth weight, gestational period, gender, educational level of the mother, breast-feeding, maternal smoking (prenatal and postnatal), day-care attendance, presence of siblings, and antibiotic usage) was obtained from midwives, from hospital registries and by questionnaire at infant ages of 2, 6 and 12 months. Binary logistic regression analysis was used to determine significant associations between the parameters described above and *M. catarrhalis* colonization. Additionally, multivariate analysis was performed to adjust for confounding factors. All variables from the univariate analysis were included in the multivariate model. For all determinants the missing values were modelled as a separate category and thus adjusted for in all analyses. Statistical analyses were performed using the Statistical Package of Social Sciences version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Characteristics of the study participants are shown in Table 1. The prevalence of *M. catarrhalis* increased from 11.8% at the age of 1.5 months to 29.9% at the age of 6 months and 29.7% at the age of 14 months. The corresponding cumulative colonization rates were 12% (74/627), 22% (323/1459), and 25% (548/2216). The presence of siblings at the age of 1.5 months (adjusted OR (aOR) 2.36, 95% CI 1.37 – 4.06) and 14 months (aOR 1.73, 95% CI 1.02 – 2.91) and day-care attendance at the age of 6 months

Table 1. Characteristics of infants enrolled in the study (*n*=1,079)

Parameter	
Birth weight (kg)	3.509 (0.538)
Gestational period (weeks)	40.3 (37.1 - 42.1)
Gender, *n* (%)	
- Female	521 (48.3%)
- Male	558 (51.7%)
Mother's educational level, *n* (%)	
- Lower/intermediate	382 (35.9%)
- Higher	683 (64.1%)
Prenatal smoking mother, *n* (%)	
- No	885 (87.5%)
- Yes	127 (12.5%)
Postnatal smoking mother, *n* (%)	
- No	606 (87.1%)
- Yes	90 (12.9%)
Siblings, *n* (%)	
- No	583 (61.1%)
- Yes	371 (38.9%)
Day-care attendance, *n* (%)	
- No	164 (20.0%)
- Yes	654 (80.0%)
Breast-feeding at 2 months, *n* (%)	
- No	332 (33.5%)
- Yes	659 (66.5%)
Breast-feeding at 6 months, *n* (%)	
- No	714 (70.6%)
- Yes	297 (29.4%)
Moraxella catarrhalis at 1.5 months, *n* (%)	
- No	553 (88.2%)
- Yes	74 (11.8%)
Moraxella catarrhalis at 6 months, *n* (%)	
- No	583 (70.1%)
- Yes	249 (29.9%)
Moraxella catarrhalis at 14 months, *n* (%)	
- No	532 (70.3%)
- Yes	225 (29.7%)

Birth weights are presented as mean and standard deviation. Gestational period, with a skewed distribution, is presented with a median with a 95% range. All other values are presented as percentages. Incomplete data parameters included: educational level of the mother (*n*=14), prenatal smoking mother (*n*=67), postnatal smoking mother (*n*=383), siblings (*n*=125), day-care attendance (*n*=261), breast-feeding at 2 months (*n*=88) and 6 months (*n*=68), 1.5 month swab (*n*=452), 6 months swab (*n*=247), 14 months swab (*n*=322).

Table 2. Determinants of *M. catarrhalis* colonization in the first year of life

	M. catarrhalis 1.5 months		*M. catarrhalis* 6 months		*M. catarrhalis* 14 months	
	OR	aOR	OR	aOR	OR	aOR
Birth weight (kg)	1.13 (0.70 – 1.83)	1.08 (0.59 – 1.99	1.11 (0.83 – 1.48)	1.27 (0.76 – 2.14)	**1.59 (1.16 – 2.19)**	1.40 (0.76 – 1.35)
Gestational period (weeks)	0.99 (0.85 – 1.16)	0.96 (0.79 – 1.18)	1.04 (0.94 – 1.14)	1.02 (0.86 – 1.22)	**1.15 (1.03 – 1.28)**	1.09 (0.88 – 1.35)
Gender						
- Male	1.00	1.00	1.00	1.00	1.00	1.00
- Female	1.38 (0.84 – 2.25)	1.43 (0.86 – 2.38)	1.07 (0.79 – 1.44)	1.29 (0.85 – 1.97)	0.84 (0.61 – 1.16)	0.73 (0.45 – 1.21)
Educational level mother						
- Higher	1.00	1.00	1.00	1.00	1.00	1.00
- Lower/intermediate	0.76 (0.44 – 1.30)	0.65 (0.37 – 1.17)	0.75 (0.54 – 1.03)	0.92 (0.55 – 1.52)	0.75 (0.53 – 1.06)	1.39 (0.78 – 2.49)
Prenatal smoking mother	0.94 (0.41 – 2.16)	0.92 (0.34 – 2.51)	0.77 (0.47 – 1.25)	1.11 (0.44 – 2.81)	**0.54 (0.30 – 0.97)**	1.41 (0.50 – 3.97)
Postnatal smoking mother	0.76 (0.29 – 1.99)	0.81 (0.27 – 2.41)	1.01 (0.57 – 1.79)	1.08 (0.45 – 2.59)	0.91 (0.50 – 1.67)	0.91 (0.33 – 2.49)
Siblings	**2.40 (1.41 – 4.09)**	**2.36 (1.37 – 4.06)**	1.06 (0.76 – 1.48)	1.27 (0.81 – 2.00)	**1.61 (1.15 – 2.27)**	**1.73 (1.02 – 2.91)**
Day-care attendance	N.A.	N.A.	**2.75 (1.65 – 4.57)**	**2.57 (1.35 – 4.89)**	**2.38 (1.44 – 3.94)**	**5.39 (2.30 – 12.64)**
Breast-feeding at 2 months						
- Yes	1.00	1.00	1.00	1.00	1.00	1.00
- No	1.18 (0.69 – 2.01)	1.33 (0.76 – 2.35)	0.83 (0.59 – 1.16)	0.73 (0.43 – 1.23)	0.86 (0.60 – 1.24)	0.92 (0.49 – 1.71)
Breast-feeding at 6 months						
- Yes	N.A.	N.A.	1.00	1.00	1.00	1.00
- No			0.90 (0.64 – 1.27)	1.26 (0.75 – 2.10)	0.93 (0.65 – 1.33)	1.24 (0.67 – 2.32)
M. catarrhalis at 1.5 months	N.A.	N.A.	1.09 (0.58 – 2.02)	0.81 (0.41 – 1.60)	1.79 (0.98 – 3.27)	1.38 (0.63 – 3.02)
M. catarrhalis at 6 months	N.A.	N.A.	N.A.	N.A.	**1.52 (1.04 – 2.23)**	1.06 (0.61 – 1.84)

Values represent crude odds ratios (OR) and adjusted odds ratios (aOR) including 95% confidence intervals. Birth weight and gestational period were included in the models as continuous variables; all other parameters were included as binary variables. Significant ORs are shown in bold.

(aOR 2.57, 95% CI 1.35 – 4.89) and 14 months (aOR 5.39, 95% CI 2.30 – 12.64) were found to be significant risk factors for colonization with *M. catarrhalis*. However, gender, educational level of the mother, maternal smoking and breast-feeding were found to be non-significant (Table 2). Additionally, children who were colonized twice or more with *M. catarrhalis* were significantly more likely to have siblings (aOR 2.22, 95% CI 1.20 – 4.13) and attend day-care centers (aOR 5.84, 95% CI 1.67 – 20.41) (Table 3).

In this study, no antibiotics were given 48 hours before the swabs were taken. Of the 1,079 children enrolled in the study (all of whom were swabbed at least once), 93

Table 3. Determinants of frequent (≥2) *M. catarrhalis* colonization events in the first year of life

Parameter	OR	aOR
Birth weight (kg)	1.58 (0.87 – 2.87)	1.27 (0.60 – 1.41)
Gestational period (weeks)	1.14 (0.93 – 1.41)	1.08 (0.83 – 1.41)
Gender		
- Male	1.00	1.00
- Female	1.17 (0.67 – 2.06)	1.13 (0.62 – 2.04)
Breast-feeding at 6 months		
- Yes	1.00	1.00
- No	1.81 (0.91 – 3.58)	1.72 (0.82 – 3.60)
Educational level mother		
- Higher education	1.00	1.00
- Lower/intermediate education	0.86 (0.47 – 1.56)	1.17 (0.59 – 2.31)
Prenatal smoking mother		
- No	1.00	1.00
- Yes	0.93 (0.34 – 2.53)	1.22 (0.35 – 4.17)
Postnatal smoking mother		
- No	1.00	1.00
- Yes	0.74 (0.25 – 2.21)	0.82 (0.22 – 3.03)
Siblings		
- No	1.00	1.00
- Yes	**2.31 (1.29 – 4.14)**	**2.22 (1.20 – 4.13)**
Day-care attendance		
- No	1.00	1.00
- Yes	**6.55 (1.98 – 21.69)**	**5.84 (1.67 – 20.41)**

Values represent crude odds ratios (OR) and adjusted odds ratios (aOR) including 95% confidence intervals. Incomplete data parameters included: breastfeeding (n=10), educational level of the mother (n=3), prenatal smoking mother (n=14), postnatal smoking mother (n=60), siblings (n=18) and day-care attendance (n=54). Significant ORs are shown in bold.

children received antibiotics in the first 6 months after birth. Two hundred twenty-seven children received antibiotics between 6 and 12 months of age and 109 children received antibiotics in the first 12 months after birth. Two hundred and seventy-six children did not receive any antibiotics in their first year of life. There was no significant association between antibiotic use in the first 6 months after birth and *M. catarrhalis* colonization at 6 months, or antibiotic use in the first year of life and *M. catarrhalis* colonization at 14 months (data not shown).

Discussion

In this study of a large number of Dutch children, born in Rotterdam between February 2003 and August 2005, an increase in *M. catarrhalis* colonization prevalence was observed, from 11.8% at 1.5 months of age up to almost 30% at the ages of 6 and 14 months, where a 'plateau' phase of colonization was seen. The corresponding cumulative acquisition rates were 12%, 22%, and 25%, respectively.

The figure of 12% at 1.5 months is almost identical to that of an American study dating from 1997, although in this latter study, the cumulative colonization rates at the ages of 6 and 12 months were approximately 55% and 70%, respectively [4]. A reason for the difference in the cumulative colonization rate between The Netherlands and the USA is not available, although geographical and/or chronological differences in the genetic background of both bacteria and the human hosts cannot be ruled out. Interestingly, both studies indicated a peak in cumulative colonization rates at approximately 6 months of age (although this peak was much lower in the Rotterdam study). It appears, therefore, that geographically and/or chronologically distinct child populations remain susceptible to *M. catarrhalis* colonization up to approximately 6 months of age, but that after this time the cumulative acquisition and elimination rates of *M. catarrhalis* remain constant. This phenomenon may be due to the development of a more effective immune response against *M. catarrhalis* colonization, possibly as a consequence of children acquiring and eliminating genotypically distinct strains [17-18].

Of the environmental factors investigated in this study, only the presence of siblings within the family and day-care attendance were found to be significantly associated with the risk of acquiring *M. catarrhalis*. Moreover, both risk factors were also significantly associated with an increased frequency of colonization. Previous studies in France and Sweden have also identified both of these environmental factors as contributing signifi-

cantly to *M. catarrhalis* colonization [19-20]. From these findings, it appears likely that crowding is a global risk factor in facilitating *M. catarrhalis* colonization. Furthermore, this phenomenon is not only confined to the home environment but has also been found to be related to the hospital environment, as a recent publication indicated that multi-bed wards constituted a significant risk factor for patient-to-patient *M. catarrhalis* transmission in adults [21].

No significant association between the use of antibiotics and colonization with *M. catarrhalis* at 6 and 14 months of age was observed in this study. This means that there was no significant increase, or decrease, in colonization rates in children receiving antibiotics as compared with children who had received no antibiotics. In contrast, Varon *et al.* (2000) studied 629 children with respiratory tract infections in France, and cultured nasopharyngeal swabs before and after antibiotic treatment [19]. In this study, a significant decrease in the nasopharyngeal carriage of *M. catarrhalis* was observed after antibiotic treatment. However, the antibiotics used included amoxicillin-clavulanate, cefixime, erythromycin-sulfisoxazole, and cefpodoxime, antibiotics that are particularly effective against *M. catarrhalis*. In another study, Molstad *et al.* (1992) showed that increasing use of antibiotics was associated with an increase in β-lactam resistance in *M. catarrhalis* [22]. This effect may be reduced by enforcing strict national antibiotic prescription policies, as demonstrated by data obtained from Denmark [23]. Unfortunately, our study did not have access to individual antibiotic prescription patterns. Further, The Netherlands has a strict policy on (limiting) antibiotic usage and the number and type of antibiotics that may be prescribed, although the use of amoxicillin-clavulanic acid is increasing in children [24]. In any case, antibiotic resistance rates in bacteria obtained from the nasopharynx of children who have not previously been treated with antibiotics may be relatively high. For example, Faden *et al.* (1994) reported *M. catarrhalis* resistance rates of 90% for amoxicillin and 19% for trimethoprim-sulfamethoxazole in children who had not previously been treated with such antibiotics [25].

Prenatal and postnatal smoking was not found to be a significant risk factor for *M. catarrhalis* colonization, which is in contrast with the study of Brook and Gober (2008), who found that the nasopharynx of healthy children of smokers harbors a high number of pathogens that are similar to the bacterial flora found in their parents when compared with healthy children and non-smoking parents ($P<0.005$) [8]. In a previous study by Principi *et al.* (1999), it was shown that passive smoking was not a significant risk factor for *M. catarrhalis* colonization, which agrees with our findings if we consider that children whose parents smoke are likely to be passively exposed to cigarette smoke.

Breast-feeding was not a significant risk factor. Breast-feeding has been found to be beneficial in reducing the risk of respiratory infections, but appears not to be significant with respect to bacterial nasopharyngeal colonization rates [26-28].

In conclusion, the presence of siblings and day-care attendance are important independent risk factors for *M. catarrhalis* colonization in infants in The Netherlands. Consequently, crowding is an important risk factor for early (and frequent) colonization. The *M. catarrhalis* cumulative colonization rate increases until a peak is reached at 6 months of age, whereupon a colonization plateau is observed. This plateau could be a consequence of clearing of maternal antibodies or the development of a more effective immune response against the many different *M. catarrhalis* genotypes circulating in the first year of life, although this hypothesis requires further investigation.

Finally, this study was set up purely to investigate the genetic and environmental factors associated with nasopharyngeal carriage of respiratory bacterial pathogens in infancy. Although information about (presumed) OM episodes is available for this study, the diagnosis of OM was based on parental reports (postal questionnaires at the infant ages of 6, 12 and 24 months). Mothers were asked whether their children suffered from fever in the preceding period, whether this period of fever was accompanied by earache, and whether they had visited a general practitioner. The diagnosis of (presumed) OM required the presence of at least one period of fever accompanied by earache for which a doctor was visited. However, no association was found between the frequency of *M. catarrhalis* colonization and OM using these criteria (J.A.M. Labout, personal communication). Studies have shown that the use of parental reports for the definition of a clinical symptom may influence the outcome of epidemiological research [29-30]. In order to collect more definite data, OM should be diagnosed by a medical practitioner in future studies [4]. Additional studies will be necessary in order to investigate the effect of (multiple) colonization events on the prevalence of upper respiratory tract disease in infants.

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Transparency declaration

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Chapter 4

Colonization of healthy children by *Moraxella catarrhalis* is characterized by genotype heterogeneity, virulence gene diversity, and co-colonization with *Haemophilus influenzae*

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Abstract

The colonization dynamics of *Moraxella catarrhalis* were studied in a population comprising 1,079 healthy children living in Rotterdam, The Netherlands (the Generation R Focus cohort). A total of 2,751 nasopharyngeal swabs were obtained during four clinic visits timed to take place at 1.5, 6, 14 and 24 months of age, yielding a total of 709 *Moraxella catarrhalis* and 621 *Haemophilus influenzae* isolates. Between January 2004 and December 2006, approximate but regular 6-monthly cycles of colonization were observed, with peak colonization incidences occurring in the autumn/winter for *M. catarrhalis*, and winter/spring for *H. influenzae*. Co-colonization was significantly more likely than single-species colonization with either *M. catarrhalis* or *H. influenzae*, with genotypic analysis revealing no clonality for co-colonizing or single colonizers of either bacterial species. This finding is especially relevant considering the recent discovery of the importance of *H. influenzae* - *M. catarrhalis* quorum sensing in biofilm formation and host clearance. Bacterial genotype heterogeneity was maintained over the 3-year period of the study, even within this relatively localized geographical region, and there was no association of genotypes with either season or year of isolation. Furthermore, chronological and genotypic diversity in three immunologically important *M. catarrhalis* virulence genes (*uspA1*, *uspA2* and *hag/mid*) was also observed. This study indicates that genotypic variation is a key factor contributing to the success of *M. catarrhalis* colonization of healthy children in the first years of life. Furthermore, variation in immunologically relevant virulence genes within colonizing populations, and even within genotypically identical *M. catarrhalis* isolates, may be a result of immune evasion by this pathogen. Finally, the factors facilitating *M. catarrhalis* and *H. influenzae* co-colonization need to be further investigated.

Introduction

Moraxella catarrhalis is part of the normal bacterial flora in the nasopharynx of children, although over the past two decades, it has emerged as a significant bacterial pathogen and not simply a commensal colonizer [1]. Studies have shown that the bacterium rapidly colonizes the nasopharynx soon after birth and that the carriage rate of *M. catarrhalis* in healthy children varies between 7 and 36% [2]. However, in children with upper respiratory tract infections (URTI), including acute otitis media (AOM), the carriage rate increases to approximately 50% [3-5]. Otitis media itself is a particularly important URTI during early childhood and the primary reason for children to visit a physician [6-7]. Furthermore, in many countries, AOM is the most common reason for prescribing antibiotics [7-10] or to undergo surgery (for the placement of grommets) [11]. The most common bacterial species cultured from the nasopharynx of children during otitis media episodes are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *M. catarrhalis* and *Staphylococcus aureus*, either as single pathogens or as co-cultures [3, 5], with the patterns of nasopharyngeal colonization by micro-organisms being important determinants for otitis media disease [12-14].

With respect to *M. catarrhalis*, it has been shown that many factors affect nasopharyngeal carriage of this human-specific pathogen, including, for example, the presence of siblings, day-care attendance and respiratory illness [2, 12, 15-17]. Furthermore, there is increasing information regarding the biological mechanisms facilitating *M. catarrhalis*-mediated colonization and disease development, with most publications stressing the importance of bacterial adherence as an essential first step in this process. To date, several important *M. catarrhalis* adhesins have been described, including the ubiquitous surface proteins UspA1 and UspA2, and the hemagglutinin/*Moraxella* IgD-binding protein Hag/MID [18-19]. UspA1 and UspA2 acquire their adherence characteristics through multifunctional binding sites, which include domains that have the ability to attach to epithelial cells via cell-associated fibronectin, laminin and vitronectin [20]. Furthermore, UspA1 includes a critical binding site for carcinoembryonic antigen-related cell adhesion molecules, which are expressed in various human tissues including respiratory epithelia [21-22]. The *Moraxella* IgD-binding protein, also referred to as Hag (human erythrocyte agglutinin), is also important for host attachment. MID is an outer membrane protein (OMP) with specific affinity for soluble and cell-bound human IgD and is the only IgD-binding protein in *Moraxella* [23].

Several other virulence-associated genes have been identified that may be associated with colonization and infection of *M. catarrhalis*, including the OMPs copB, OMPCD, McaP, MhaB, MhaC, Msp22, Msp75, Msp78 and lipooligosaccharide (LOS) [24-31]. Furthermore, several of these virulence genes have also been associated with the induction of the humoral immune response, making them potential vaccine candidate genes. However, relatively little is known about the frequency and the extent of genotypic variation within these virulence genes, and in particular how this variation may be associated with colonization over time.

The aim of this study was to investigate the seasonal and yearly prevalence patterns of longitudinal colonization, genotypic variation and variation in virulence-associated genes of *M. catarrhalis* isolates colonizing children within a single and distinct geographical region. Using these data, we hoped to discover whether distinct isolate genotypes, and/or identical putative vaccine candidate gene types circulated during specific time periods in healthy colonized children. We also investigated the longitudinal prevalence of *M. catarrhalis* and *H. influenzae* within our cohort of children, looking for co-colonization trends within this age group. These results will further help our understanding of *M. catarrhalis* population dynamics and provide insights into the chronological diversity and functionality of virulence-associated genes for this bacterial pathogen.

Methods

Study population

This study was embedded within the Generation R Focus cohort, part of a population-based prospective cohort study from fetal life until young adulthood based in Rotterdam, The Netherlands (the Generation R Study) [32-33]. Within the Generation R Focus cohort, detailed assessments of fetal and postnatal growth and development were performed on 1,232 pregnant Dutch women. The mothers gave birth to 1,244 infants between February 2003 and August 2005, in Rotterdam, The Netherlands, of whom 138 were excluded from the study analysis because consent was withdrawn after birth. Twins were also excluded from the analysis because of genetic relatedness, leaving a total of 1,079 infants. The Medical Ethics Committee of the Erasmus MC approved the study. Written informed consent was obtained from the parents of all the participants.

Infants visited the Generation R Focus cohort research centre at 1.5, 6, 14 and 24 months after birth, resulting in 630 swabs being taken at 1.5 month, 787 swabs at 6

months, 717 swabs at 14 months and 617 swabs at 24 months between November 2003 and September 2007. From 623 children, at least 3 swabs were available for bacterial culturing.

Bacterial isolates

Bacterial isolates were cultured from nasopharyngeal swabs taken from infants at 1.5, 6, 14 and 24 months of age by a trained research nurse, using a sterile transport swab immersed in Amies transport medium. Swabs were cultured within 6 hours of sampling, using blood agar plates containing 5% sheep blood, chocolate agar and *Haemophilus*-selective agar at an incubation temperature of 35°C in 5% CO_2 for 48 hours. Plates were examined daily for the growth of *M. catarrhalis* and/or *H. influenzae*.

Genotyping

Genotyping of *M. catarrhalis* isolates was performed using PFGE and multilocus sequence typing (MLST). PFGE was also performed on *H. influenzae* isolates.

PFGE for *M. catarrhalis* was performed as detailed by Verduin *et al.* [34]. Briefly, *M. catarrhalis* plug digestions were performed using *Spe*I at 20 U per reaction and an electrophoresis protocol comprising a first block with a constant voltage of 6 V cm^{-1}, a pulse time from 3.5 to 25 seconds during the first 12 hours, followed by a second block of 8 hours where the pulse time increased linearly from 1 to 5 seconds. The PFGE protocol for *H. influenzae* was adapted from Moor *et al.* [35]. Digestions were performed using *Sma*I at 30 U per reaction and an electrophoresis protocol comprising a first block with a constant voltage of 6 V cm^{-1}, a pulse time from 6 to 8 seconds during the first 7 hours, followed by a second block of 17 hours where the pulse time increased linearly from 1 to 38 seconds. All PFGE patterns were analyzed using BioNumerics (Applied Maths), with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set at 1.5%. PFGE products between 48.5 and 339.5 kb for *M. catarrhalis* and 48.5 and 485 kb for *H. influenzae* were included in the band matching analysis.

MLST genotyping was performed to determine whether *M. catarrhalis* genotypes circulating in the Rotterdam area were similar to *M. catarrhalis* genotypes worldwide and to establish genetic relationships with other internationally recognized clones available in the *M. catarrhalis* MLST database. MLST was performed on 12 *M. catarrhalis* isolates that had been cultured serially on three separate occasions from four children. Briefly, PCR was performed to detect and sequence the *abcZ*, *adk*, *efp*, *fumC*, *glyRS*, *mutY*, *ppa* and *trpE* genes as described in the guidelines available at the MLST website (http://

mlst.ucc.ie/). A touchdown thermocycling program was used for *glyRS* and *adk*. The touchdown protocol used an initial annealing temperature of 70°C, which was reduced by 1°C per cycle over 15 PCR cycles. The following 20 cycles of amplification used an annealing temperature of 55°C. For *ppa*, *efp*, *abcZ* and *trpE*, a standard PCR protocol comprising an annealing temperature of 52°C and an elongation time of 2 minutes at 72°C for 25 cycles was used, whilst for *fumC* and *mutY* an annealing temperature of 55°C was used. PCR primer pairs were used for amplicon sequencing, except for the genes *glyRS*, *fumC* and *mutY*, which required separate sequencing primers (primers are available at http://mlst.ucc.ie/). Allelic profiles were defined and compared with the MLST types of isolates from different geographic regions available at http://mlst.ucc.ie/.

PCR screening of virulence genes

M. catarrhalis isolates were grown from the glycerol stock overnight at 37°C on blood agar plates. DNA was extracted using the MagNA Pure LC System (Roche Applied Science). PCR was performed to detect the *uspA1*, *uspA2*, *uspA2H*, *hag/mid* and *ompJ* genes and 16S rRNA and LOS types as described previously [36]. A touchdown thermocycling program was used for all PCRs except for *hag/mid* and LOS PCRs. The touchdown protocol used an initial annealing temperature of 70°C, which was reduced by 1°C per cycle over 15 cycles of PCR. The following 20 cycles of amplification used an annealing temperature of 55°C. All isolates negative for both *uspA2* and *uspA2H* genes were tested using primers uspA2end.f and uspA2end [36], which amplify a conserved region found at the 3'-end of both *uspA2* and *uspA2H* genes.

For the *hag/mid* gene PCR, a standard PCR protocol comprising an annealing temperature of 55°C and an extension time of 8 minutes at 68°C for 25 cycles was used. The LOS-typing PCR protocol was performed as described by Edwards *et al.* [37].

After amplification, 16S rRNA PCR products were digested using the enzymes *Fsp*BI (10 U) and *Hha*I (10 U) to identify the 16S rRNA types [36].

Virulence gene variation

Virulence gene variation was assessed using PCR-RFLP typing on three major *M. catarrhalis* virulence genes. These genes comprised the ubiquitous surface protein genes A1 and A2 (*uspA1* and *uspA2*), and the hemagglutinin/IgD-binding protein gene *hag/mid*. PCR products of these genes were digested using the restriction enzyme *Hae*III (*Bsu*RI), using 10 U per reaction mix, and incubated overnight at 37°C. The *Hae*III

restriction enzyme was chosen because its recognition site(s) is found within immunological important regions of the *uspA1*, *uspA2* and *hag/mid* genes.

PCR-RFLP patterns were analyzed using BioNumerics (Applied Maths), with gel lanes normalized against a 1 kb (*uspA2* and *hag/mid*) and 100 bp (*uspA1*) ladder and band tolerance set at 1.5%.

Data analysis

To evaluate potential predictors of colonization, logistic regression analysis was performed with general estimating equations using the logit link function as implemented in the GENMOD procedure in SAS version 9.1.3 (SAS Institute, 2002, SAS version 9.1.3.). Separate analyses were conducted for the binary dependent variables of presence or absence of either *M. catarrhalis* or *H. influenzae* in a repeated measures design. The repeated variable was children, each of which had 1-4 swabs in the study period. Predictor variables were the presence/absence of the non-target species, year and season. Seasons were defined as winter (December – February), spring (March – May), summer (June – August) and autumn (September – November). An independent correlation matrix structure among predictor variables was used but there were no differences in assessment of significances using exchangeable or unstructured correlation matrices. Two- and three-way interaction terms were not included in the final model because (i) all covariances between model variables in all analysis were low ($r<0.04$), (ii) exploratory analyses that excluded the repeated design showed interaction terms that did not contribute to the efficiency of the models and (iii) all, except one, of the 64 potential interaction terms for each target species were not significant.

Results

Bacterial isolates

A total of 709 *M. catarrhalis* isolates and 621 *H. influenzae* isolates were cultured from a total of 2,751 nasopharyngeal swabs obtained during the 3-year study period. The ages of the subjects when the cultures were taken are given in Table 1.

Population dynamics

Approximate, but regular 6-monthly cycles of peak colonization prevalence were observed over the 3-year study period for *M. catarrhalis*, with a less regular pattern ob-

Table 1. Number of *M. catarrhalis* and *H. influenzae* cultures used in this study

Age at collection (months)	*M. catarrhalis* *n*=709	*H. influenzae* *n*=621
1.5	73	44
6	240	194
14	212	227
24	184	156

Total number in study population = 2,751

served for *H. influenzae.* Peak seasonal prevalence differed between *M. catarrhalis* and *H. influenzae*, with *M. catarrhalis* prevalence highest in autumn and winter (odds ratio [OR] = 1.3 – 2.0 compared to spring or summer with $P<0.03$ for each comparison) and *H. influenzae* prevalence highest in winter and spring (OR = 1.5 – 1.9; $P<0.01$). Each species seasonal peak represented a significantly higher risk of carriage compared to non-peak seasons and the odds of a child being colonized by one of the two pathogens were significantly increased if the other species was present (OR = 1.5; $P<0.0001$). The peak in joint occurrences occurred in autumn. If the product of the individual species occurrence proportions is considered an indicator of the expected proportion of joint occurrences, chi-squared analyses showed significant excesses over expectations of joint occurrences for spring ($P<0.01$), summer ($P<0.01$), and autumn ($P=0.04$). Both pathogens were at or near their peak prevalence in 2006 and so the joint prevalence was highest that year at 10.8%. Conversely, the combined absence of both species in children was at its lowest in 2006 at 49.3%. For *M. catarrhalis*, there were significantly higher risks in 2006 and 2007 compared to 2004 and 2005 (OR = 1.5 – 2.1; $P<0.01$). In contrast, the risk for *H. influenzae* was highest in 2006 compared to other years (OR = 1.3 – 1.7; $P<0.03$) (Figure 1).

Genetic diversity of *M. catarrhalis* and *H. influenzae* isolates

A selection of 112 isolates were arbitrarily chosen for PFGE genotyping from the 709 *M. catarrhalis* isolates cultured in this study, including isolates from children colonized only once during the study period and serial isolates cultured from the same child. A high degree of genotypic heterogeneity was maintained over the entire study period. While 16S rRNA types 2 and 3 (lineage 2) comprised a distinct clade, heterogeneity of all markers was independent of year, season and serial colonization status (Figure 2).

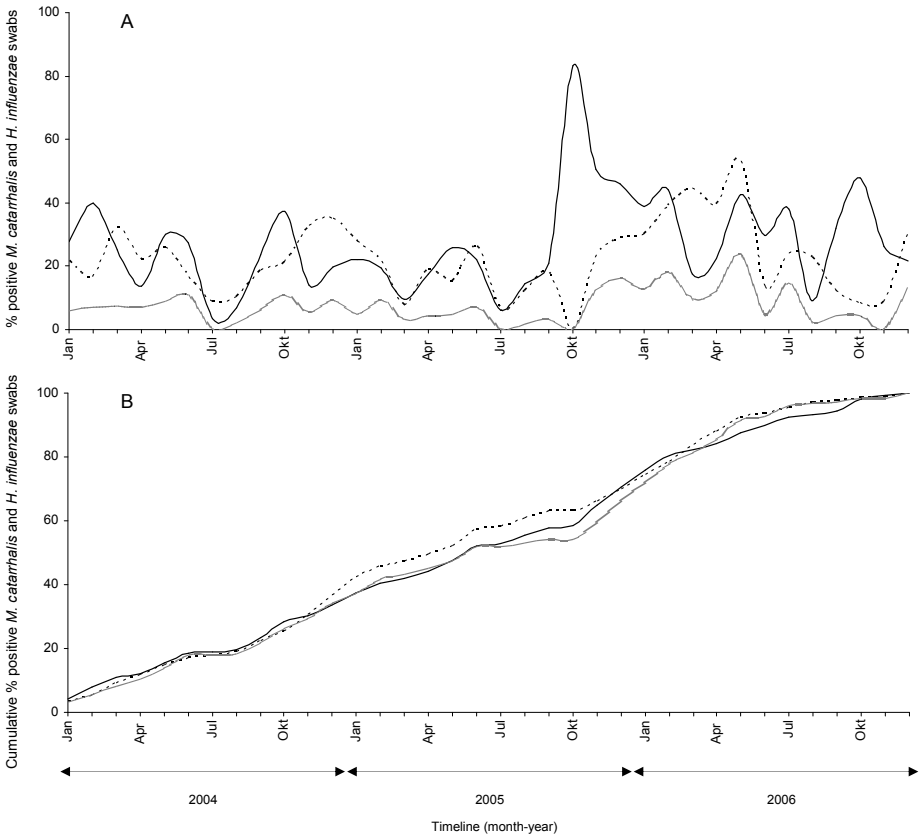
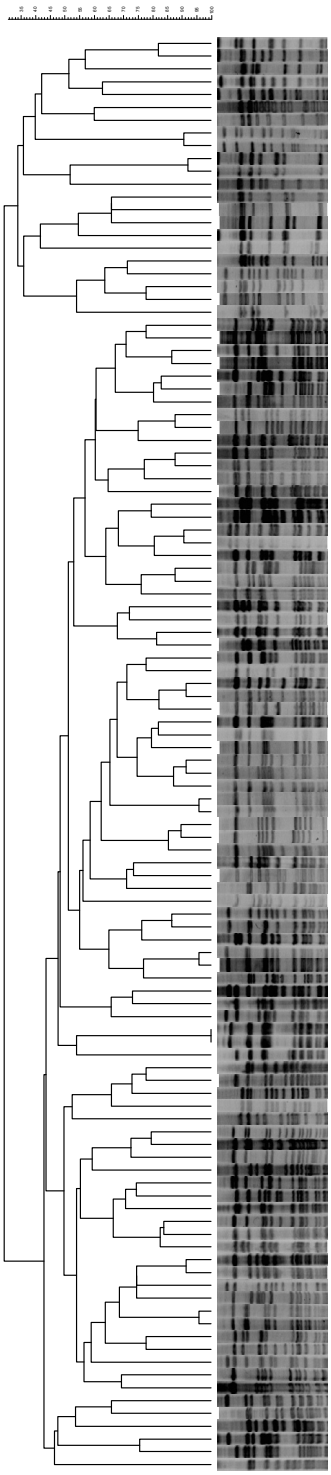


Figure 1. Colonization prevalence of *M. catarrhalis* and *H. influenzae* during 2004-2006. A. Proportion of *M. catarrhalis* (black line) and *H. influenzae* (dashed line) compared with the total number of swabs per month. The proportion of the joint occurrence of the two pathogens in each month compared with the total number of swabs taken per month is also shown (grey line). Note the approximate 6-monthly colonization cycle (from peak to peak), especially for *M. catarrhalis*. B. Cumulative percentage of *M. catarrhalis*-positive (black line) and *H. influenzae*-positive (dashed line) swabs recorded during the 3-year study period. Co-culture-positive swabs are also shown (grey line). The cumulative colonization burden in the study population increases at a constant rate for both pathogens and co-colonization over the 3-year study period.

MLST genotyping of 12 *M. catarrhalis* isolates was performed in order to determine the global context of colonizing isolates obtained from Rotterdam, The Netherlands. These 12 isolates originated from four different children that were colonized with *M. catarrhalis* on three separate occasions, and represented different PFGE genotypic clus-



Time period	Colonization events	16S type	Month	Year
4	1	2	10	2006
• 4	3	2	1	2007
4	3	2	10	2006
4	1	2	6	2006
4	3	2	4	2007
1	3	3	12	2004
• 3	3	2	6	2004
1	3	3	5	2004
3	3	3	3	2005
• 4	3	3	4	2007
1	3	2	9	2004
2	3	2	4	2005
4	3	2	5	2005
• 4	1	2	1	2007
• 4	3	2	4	2007
• 4	1	2	5	2006
• 3	1	2	8	2004
3	3	2	10	2006
• 4	3	2	12	2006
2	1	2	12	2003
4	1	2	3	2007
• 1	1	2	1	2004
• 4	3	1	4	2007
4	1	1	5	2007
3	1	1	9	2006
4	1	1	4	2007
4	1	1	11	2005
4	1	1	5	2007
• 4	3	1	11	2005
2	3	1	1	2006
4	3	1	4	2007
3	3	1	10	2006
2	3	1	5	2005
2	3	1	10	2005
• 2	3	1	1	2006
1	3	1	5	2005
2	1	1	8	2005
• 4	3	1	12	2005
4	3	1	6	2007
4	3	1	7	2007
2	1	1	12	2005
2	3	1	7	2005
• 2	3	1	6	2004
• 2	3	1	12	2005
2	1	1	12	2005
• 4	3	1	6	2007
3	3	1	6	2005
2	3	1	10	2005
4	3	1	11	2005
• 2	1	1	12	2003
4	3	1	12	2005
1	1	1	2	2004
• 4	3	1	7	2007
2	1	1	8	2005
• 2	3	1	12	2003
• 4	1	1	3	2007
2	1	1	2	2004
• 2	1	1	4	2004
2	3	1	1	2004
• 2	1	1	1	2004
1	3	1	12	2003
4	1	1	1	2007
• 2	1	1	12	2003
2	1	1	5	2004
2	1	1	12	2005
• 4	1	1	2	2007
1	3	1	12	2003
1	1	1	7	2004
1	3	1	1	2004
• 1	3	1	4	2004
3	1	1	12	2005
1	3	1	5	2005
4	1	1	4	2007
• 3	3	1	10	2006
4	1	1	11	2005
4	3	1	4	2006
• 3	1	1	5	2005
• 3	3	1	7	2006
3	3	1	10	2006
3	3	1	3	2006
• 4	3	1	1	2006
2	3	1	10	2004
• 3	3	1	2	2006
2	3	1	6	2004
• 2	3	1	5	2004
2	1	1	4	2005
3	3	1	5	2006
• 3	3	1	5	2005
3	3	1	5	2006
3	1	1	2	2005
3	3	1	6	2006
3	1	1	2	2006
1	3	1	3	2005
• 2	3	1	1	2006
4	3	1	12	2006
3	3	1	2	2006
• 2	1	1	2	2004
• 3	1	1	12	2004
• 3	3	1	1	2005
1	1	1	1	2005
2	1	1	3	2005
• 2	1	1	10	2005
• 2	1	1	11	2004
• 3	1	1	12	2004
• 3	3	1	1	2005
2	3	1	6	2005
3	3	1	1	2005
• 2	3	1	5	2004
• 2	1	1	11	2005
4	1	1	6	2005
• 4	1	1	5	2006
2	3	1	9	2004

Figure 2. Dendogram based on PFGE genotypic variation of 112 colonizing *M. catarrhalis* isolates obtained from 74 children aged 1.5-24 months during November 2003 and September 2007 in Rotterdam. Time period after birth when nasopharyngeal swabs were taken: 1, 6 weeks; 2, 6 months; 3, 14 months; 4, 24 months. Colonization events: 1, children colonized in a single sampling period only during the course of the study; 3, children colonized three times during the study period. 16S rRNA type: 1, lineage 1; 2/3, lineage 2. The month and year of isolate culture from the nasopharynx of children is also shown (year of peak prevalence underlined). Co-colonization with *H. influenzae* is indicated with asterisks (*). No clustering of serial isolates cultured from the same child, or co-colonizing (with *H. influenzae*) isolates, was observed.

ters. The Rotterdam isolates tested were found to be non-clonal in nature with many (8 out of 12) singleton MLST types, further indicating the genetic heterogeneity of *M. catarrhalis* isolates, even within a relatively restricted geographical region (Figure 3).

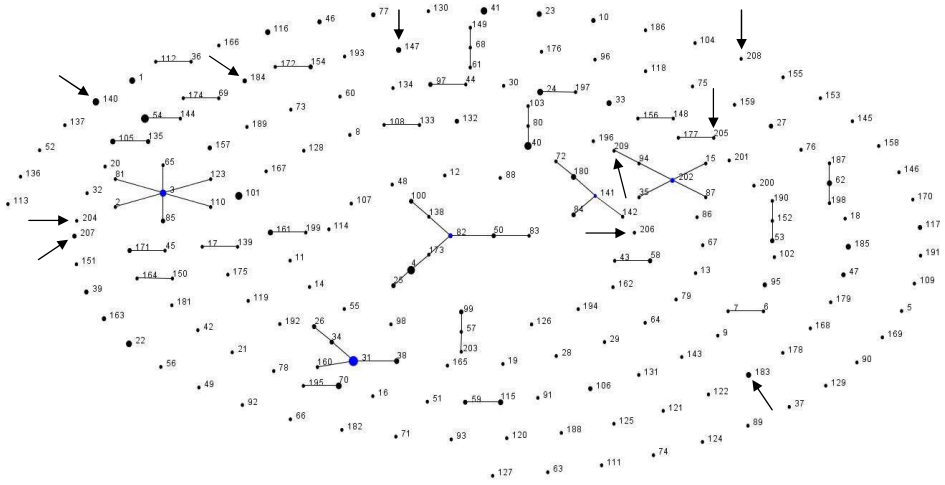


Figure 3. MLST genotyping of 12 *M. catarrhalis* isolates. The sequence types assigned to the isolates are indicated with black arrows, showing the non-clonal nature of the isolates. Each dot represents a distinct MLST type, and MLST types connected with a black line possess similar allelic profiles, possibly derived from a common ancestor.

Due to the significant association between *M. catarrhalis* and *H. influenzae* colonization, 36 *H. influenzae* isolates (including both co-colonizing and single-species isolations)

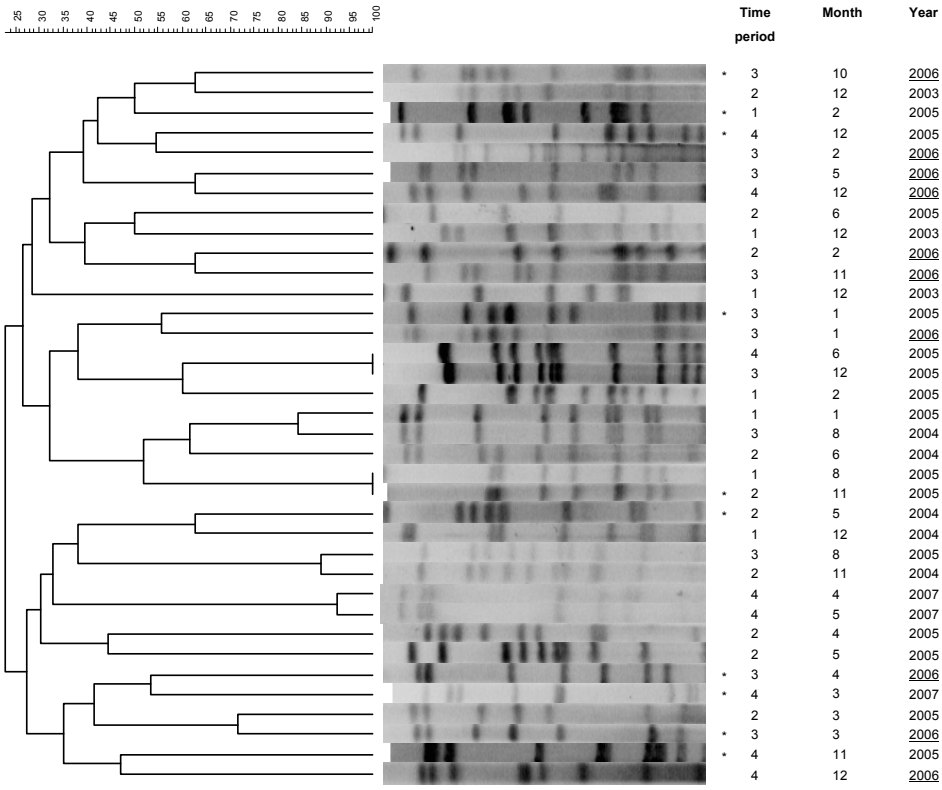


Figure 4. Dendogram of PFGE genotypic variation for 36 *H. influenzae* isolates obtained from 12 children in Rotterdam. Time period after birth when nasopharyngeal swabs were taken: 1, 6 weeks; 2, 6 months; 3, 14 months; 4, 24 months. The month and year of isolate culture from the nasopharynx of children is also shown (year of peak prevalence underlined). Co-colonization with *M. catarrhalis* is indicated with asterisks (*). Note the non-clonal nature of co-colonizing *H. influenzae* genotypes.

were chosen for PFGE genotyping to study their genetic diversity. No single *H. influenzae* genotype or clonal cluster was associated with co-colonization events (Figure 4).

PCR screening of virulence genes

PCR screening of the same 112 *M. catarrhalis* isolates for which PFGE was performed revealed an incidence of 97% (109/112) for *uspA1*, 80% (90/112) for *hag/mid*, 83% (85/102) for *uspA2*, 17% (17/102) for *uspA2H* and 100% (112/112) for *ompJ*. Ten isolates were found to be PCR-negative using previously published *uspA2*- and *uspA2H*-

specific primers, but positive using primers designed to amplify a conserved region of both *uspA2* and *uspA2H*. These 10 isolates were not included in subsequent *uspA2* and *uspA2H* prevalence calculations, though their inclusion as either *uspA2* or *uspA2H* would not have affected the significance of the results (Table 2).

Table 2. Prevalence of virulence genes for 112 *M. catarrhalis* isolates for which PFGE genotyping was performed

Virulence gene	Positive (%)	Negative (%)
uspA1	97	3
hag/mid	80	20
uspA2	83	17
uspA2H	17	83
ompJ	100	0

16S rRNA gene type analysis revealed an incidence of 80% for 16S rRNA type 1 (lineage 1), and a combined 20% for types 2 and 3 (lineage 2). All isolates that belonged to lineage 1 were associated with the presence of the *hag/mid* gene and OMPJ type 2.

Virulence gene diversity

PCR-RFLP was performed on amplified PCR products of the *hag/mid*, *uspA2* and *uspA1* virulence genes of 86 *M. catarrhalis* isolates to assess virulence gene diversity of colonizing *M. catarrhalis*. Figure 5 shows the diverse nature of the PCR-RFLP patterns obtained for *hag/mid*, *uspA2* and *uspA1* circulating between 2004 and 2006 in healthy children in Rotterdam, The Netherlands. No distinct clustering of PCR-RFLP patterns was observed during particular months or years, suggesting that many different virulence gene variants circulate within colonizing *M. catarrhalis* populations at any given time. This indicates that individual gene variation is as extensive as whole genome polymorphism.

Discussion

The colonization dynamics of *M. catarrhalis* and *H. influenzae* were studied in a population of 1,079 healthy children living in Rotterdam, The Netherlands. Over the 3-year study period, a trend towards a regular 6-monthly *M. catarrhalis* colonization cycle (peak to peak) was observed for *M. catarrhalis* (which was less evident for *H. influen-*

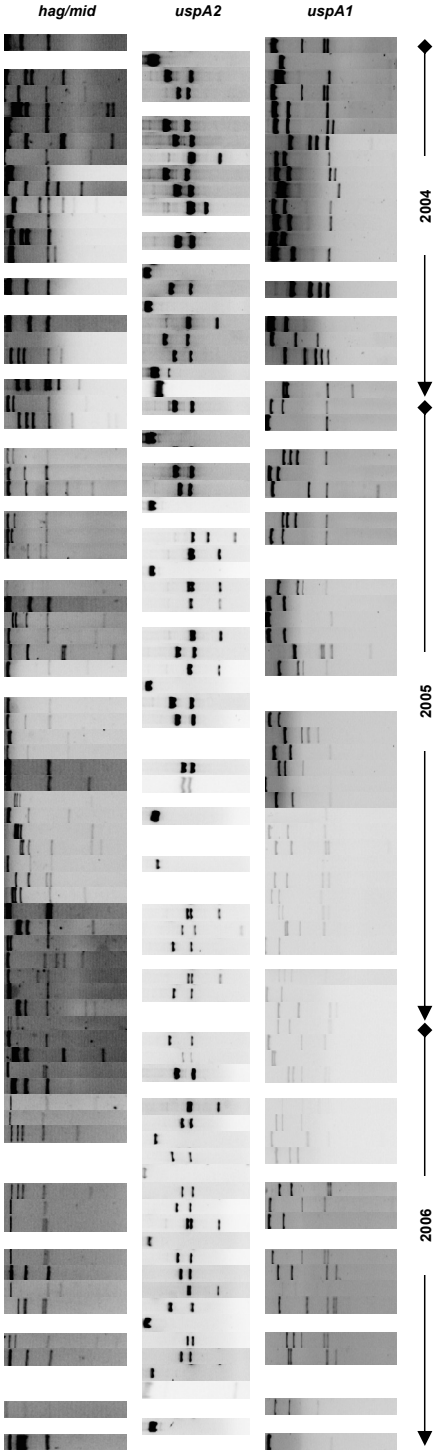

hag/mid
uspA2
uspA1
2004
2005
2006

Figure 5. Diversity of PCR-RFLP patterns obtained from the *hag/mid*, *uspA2* and *uspA1* virulence genes of 86 colonizing *M. catarrhalis* isolates cultured during the years 2004-2006 from healthy children in Rotterdam, The Netherlands. Note the diversity of restriction patterns, even within the same year, and that there is no linkage between genotype pattern combinations for the three immunologically relevant virulence genes tested.

zae), without a pronounced seasonal peak for either. This observation tends to contrast with other studies reporting a seasonal influence on *M. catarrhalis* colonization, although these studies also reported that higher detection rates of *M. catarrhalis* during the colder months could be a side effect of nasal hyper-secretion induced by viral illness or cold weather [15]. Marchisio *et al.* (2001) concluded that the seasonal influence (comparison between spring and autumn) on nasopharyngeal carriage of respiratory tract pathogens in healthy children was negligible [38]. However, their study was limited in describing seasonal variations due to lack of observations in other years, which would have allowed a better understanding of the dynamics of the individual pathogens. Our results suggest that colonization with *M. catarrhalis* (and to a lesser extent *H. influenzae*) occurs via regular cycles of colonization and clearance, with the apparent increase in *M. catarrhalis* and *H. influenzae* detection during the winter months probably being a consequence of increased viral respiratory infections, e.g. influenza, resulting in increased opportunity for secondary bacterial infection by bacterial pathogens. The link between seasonal virus infections and resultant secondary bacterial infections has been indicated by several publications [5, 39-40]. However, reports by Meijer *et al.* (2007) and Arkema *et al.* (2008), who collected clinical and virological data via the European Influenza Surveillance Scheme (EISS), concluded that the 2005-2006 and 2006-2007 influenza seasons in Europe were characterized by moderate clinical activity [41-42], and as a consequence may have resulted in less viral predisposition of the middle ear epithelium to bacterial infection.

At least two studies have suggested that the presence of a particular bacterial species during URTIs may create a more hospitable niche for co-colonization by a second distinct bacterial species [5, 43]. Armbruster *et al.* (2010) showed that *H. influenzae* promotes *M. catarrhalis* persistence within polymicrobial biofilms via inter-species quorum signaling, and that co-infection with both species promotes the increased resistance of biofilms to antibiotics and host clearance [43]. In fact, the process of biofilm formation has been demonstrated for numerous pathogens and is clearly an important microbial survival strategy.

In our present study, co-colonization between *M. catarrhalis* and *H. influenzae* was significantly more likely than colonization with either bacterial species alone. This is an interesting result, as (for example) competition for cellular binding sites may be expected to occur in the presence of two distinct bacterial species, although this hypothesis needs further study. In detail, both species can bind to carcinoembryonic antigen-related cell adhesion molecules (CEACAM)-1, which is a receptor for the OMPs UspA1 and P5 of *M. catarrhalis* and *H. influenzae*, respectively [44]. CEACAM-1 is a specific innate immune receptor and although the CEACAM-binding ligands of respiratory pathogens are structurally diverse, they target a common site on the receptor [45-46]. However, competition for binding sites could be outweighed by non-receptor-mediated factors associated with co-colonization events. For example, *M. catarrhalis* releases outer membrane vesicles (OMVs) or 'blebs' from its surface during growth in several environments, e.g. liquid culture, solid culture and biofilms. The OMVs that are secreted by *M. catarrhalis* carry UspA1 and UspA2, which are known to interfere with the activation of the complement cascade. Tan *et al.* (2007) showed that blebs carrying UspA1/A2 protected *H. influenzae* from complement-mediated killing, suggesting that *M. catarrhalis* may promote the survival of *H. influenzae* during co-colonization [47]. Deich & Hoyer (1982) demonstrated the generation and release of DNA-binding vesicles by *H. influenzae* [48], although this study did not mention the effect of *H. influenzae* blebs on the complement cascade. At this time, no other studies have reported the presence of *H. influenzae* blebs and their possible beneficial effect on other bacterial species in co-culture.

Whatever the exact cause of co-colonization dominance, neither co-colonizing *M. catarrhalis* or *H. influenzae* belonged to specific genotypic clones, suggesting that a ubiquitous system is facilitating the significant difference in co-colonization (versus single-species colonization) observed in this study. As recently described by Armbruster *et al.* (2010), the most likely (non-clonal genotype-related) ubiquitous system driving co-colonization events of these two bacterial pathogens appears to be the quorum sensing system [43]. However, complement-evading systems, e.g. via OMV-mediated protection, may also play a significant role.

Genotypic analysis revealed a high degree of diversity in colonizing *M. catarrhalis* isolates, an observation which is in agreement with previous studies showing that genotypic heterogeneity is typical of worldwide *M. catarrhalis* isolates colonizing both children and adults [36, 49-50]. Also, no significant association between genotype (cluster) and season, or year of isolation was observed, which is in contrast to a previous publication

by Levy *et al.* (2009) who reported that winter and spring season was a significant risk factor for clustering. However, Levy *et al.* (2009) studied transmission between patients within a relatively confined hospital setting [51], whereas the focus of our study was related to community transmission.

The prevalence of the virulence genes *uspA1*, *uspA2* and *hag/mid* within our Rotterdam isolates was very similar to that previously reported for global *M. catarrhalis* isolates [36]. These virulence genes encode OMPs that play an important role in bacterial adherence to human epithelia and complement resistance, as well as being immunogenic [19, 52-53]. This finding suggests that equilibrium has been reached with respect to virulence gene distribution in global *M. catarrhalis* isolates, as local geographical factors do not appear to generate significant differences in virulence gene prevalence.

Finally, a high degree of gene sequence diversity was identified in *uspA1*, *uspA2* and *hag/mid* virulence genes during the 3-year study period, with no apparent linkage between *uspA1*, *uspA2* and *hag/mid* banding patterns. Moreover, the PCR-RFLP method used was chosen to target restriction sites associated with immunologically important regions of the corresponding UspA1, UspA2 and Hag/MID proteins. The lack of intra-gene band pattern clustering and lack of inter-gene band pattern linkage indicate that these three immunologically relevant virulence genes may be experiencing immune pressure, each separately adapting to evade the human immune response. The consequence would be immune evasion and an increased ability to colonize human populations over time.

Conclusion

Regular 6-monthly cycles of *M. catarrhalis* and *H. influenzae* colonization peaks were observed during 2004-2006 in healthy children residing in Rotterdam, The Netherlands. Colonization was characterized by a high degree of *M. catarrhalis* genotypic diversity that was maintained over the 3-year study period, and was independent of year and season of isolation. Genetic diversity was observed in three immunologically important virulence genes, with no clustering of band pattern types during particular months or years. This high degree of whole-cell genotypic variation, coupled to independent (unlinked) virulence gene variation, is likely to be one of the factors ensuring the success of *M. catarrhalis* colonization events in the early years of life, facilitating immune evasion and further cycles of colonization.

The fact that the co-colonization prevalence was significantly greater than single-species prevalence alone for these two microbial pathogens, adds weight to the recently published findings indicating the importance of quorum sensing-mediated inter-species cooperation in biofilm maturation and increased resistance to host clearance. Our study provides the first evidence to indicate the importance of this quorum sensing interaction within the community setting.

Acknowledgments

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All authors declare that they have no conflicts of interest.

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Chapter 5

Age-related genotypic and phenotypic differences in *Moraxella catarrhalis* isolates from children and adults presenting with respiratory disease in 2001-2002

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Abstract

Moraxella catarrhalis is generally associated with upper respiratory tract infections in children and lower respiratory tract infections in adults. However, little is known regarding the population biology of isolates infecting these two age groups. To address this, a population screening strategy was employed to investigate 195 worldwide *M. catarrhalis* isolates cultured from children (<5 years of age) and adults (>20 years of age) presenting with respiratory disease in the years 2001-2002. Parameters compared included: genotype analysis; autoagglutination/biofilm forming ability; serum resistance; *uspA1*, *uspA2*, *uspA2H*, *hag* and *mcaP* incidence; *copB*/LOS/*ompCD*/16S rRNA types; and UspA1/Hag expression. A significant difference in biofilm formation (P=0.002), but not autoagglutination or serum resistance was observed, as well as significant differences in the incidence of *uspA2*- and *uspA2H*-positive isolates, and the distribution of LOS types (P<0.0001 and P=0.01, respectively). Further, a significant decrease in the incidence of Hag expression (for isolates possessing the *hag* gene) was observed in adult isolates (P=0.001). Both *uspA2H* and LOS type B were associated with 16S rRNA type 1 isolates only, and 2 surrogate markers (*copB* and *ompCD* PCR-RFLP types) for the 2 major *M. catarrhalis* 16S rRNA genetic lineages were identified. In conclusion, there are significant differences in phenotype and gene incidence between *M. catarrhalis* isolates from children and adults presenting with respiratory disease, possibly as a result of immune evasion in the adult age group. Our results should also be useful in the choice of effective vaccine candidates against *M. catarrhalis*.

Introduction

Moraxella catarrhalis has been associated with a number of respiratory-associated infections affecting both children and adults, including laryngitis [1], bronchitis [2] and pneumonia [3, 4]. However, the bacterium is mostly associated with upper respiratory tract infections in children e.g. otitis media [5], and lower respiratory tract infection in adults e.g. exacerbations of chronic obstructive pulmonary disease (COPD) [6].

M. catarrhalis populations may be sub-divided into two distinct genetic lineages, phenotypically characterized by (1) their ability to resist the destructive effect of human serum (i.e. complement resistant versus complement sensitive), and (2) differences in their ability to adhere to human epithelial cells [7, 8]. Recent research indicates that a population expansion (including the acquisition of virulence genes) probably occurred within the sero-resistant lineage of *M. catarrhalis* around the time of hominid expansion some 5 million years ago [8]. Further, the existence of these two subpopulations has implications for vaccine design, economics and vaccine development.

Several virulence-associated genes (and potential vaccine candidates), have been identified in *M. catarrhalis*, including the outer membrane proteins UspA1 [9], UspA2 [10], Hag [11], OMPCD [12], CopB [13] and lipooligosaccharide (LOS) [14]. All of these vaccine candidates have been associated with the production of antibody [15-20], although little is known about the frequency and expression of the corresponding genes in global isolates recovered from children and adults.

In this study, PFGE analysis, PCR screening methodologies, ELISA protein expression and phenotypic characterization studies were performed on a collection of worldwide *M. catarrhalis* isolates obtained from both children and adults presenting with respiratory disease. The study was set up to determine whether different *M. catarrhalis* populations infect these two age groups, and to provide preliminary incidence data that may be useful in the appraisal of current putative vaccine candidates against this pathogen.

Materials and methods

Bacterial isolates

Bacterial isolates comprised 195 *M. catarrhalis* isolates cultured between 2001 and 2002 from children and adults presenting with respiratory disease (GR Micro, London, UK).

Table 1. Geographical region of *M. catarrhalis* isolates

World region	Country	Number of isolates
North America	USA	20
	Canada	20
Western Europe	Belgium	4
	Germany	16
	Spain	20
Latin America	Brazil	14
	Ecuador	10
	Peru	5
	Venezuela	10
Far East	Japan	36
South Africa	South Africa	40

Table 2. Clinical source of *M. catarrhalis* isolates

Source	Number of isolates	
	Children	Adults
Ear fluid	14	1
Nasopharyngeal aspirate	27	13
Sinus	11	6
Blood	3	0
Throat	6	3
Unspecified	0	1
Sputum	33	61
Bronchoalveolar lavage	4	12

Approximately 40 random isolates from 5 world regions (11 countries) were included in the study (Tables 1 and 2). All isolates were cultured on Columbia blood agar at 37°C.

Autoagglutination assay

M. catarrhalis autoagglutination was measured according to Pearson *et al*. [21]. Briefly, isolates were grown overnight on brain heart infusion (BHI) agar, washed and resuspended in 4 ml of PBS to an OD_{405} of 1.0±0.1 (Pharmacia Biotech Novaspec II). The suspension was then allowed to stand undisturbed at room temperature. After 1 hour, 1

ml aliquots were taken from the surface, the OD_{405} measured, and the difference in the OD_{405} between the two time periods calculated.

Biofilm assay

Biofilm formation was assessed according to Pearson *et al*. [11]. *M. catarrhalis* isolates were grown in BHI broth overnight at 37°C, resuspended in BHI broth to approximately $2\text{-}4\times10^8$ cells ml^{-1} (OD_{600} approximately 0.2 using a Pharmacia Biotech Novaspec II spectrophotometer) and 2 ml volumes were pipetted into 24-well cell culture plates (Corning, COSTAR) in duplicate. Plates were incubated for 19 hours at 37°C, then the BHI broth was replaced with 2 ml 199 medium/Earle's balanced salt solution/crystal violet followed by incubation at room temperature for 15 minutes. After washing three times with deionized water, 2 ml of 95% ethanol was added and the plate gently rocked for 15 minutes. The absorbance of the ethanol solution was measured at 570 nm. Isolates whose duplicate absorbance's differed by >25% were re-tested and an average from four OD_{570} readings was taken.

Serum resistance assay

Serum resistance was determined using the culture-and-spot test [22]. A suspension of *M. catarrhalis* was made in PBS to an OD_{600} of 1.0±0.1 and 100 µl evenly spread over a Columbia blood agar plate. After drying, 50 µl of pooled human serum (obtained from 10 healthy adult volunteers) and 50 µl heat-inactivated serum (30 min at 56°C), were spotted onto the plate and the plate was incubated overnight at 37°C. Isolates were considered serum resistant (no effect on growth), intermediate (effect on growth visible but >50 colonies growing in active serum zone), or sensitive (<50 colonies growing in active serum zone). No effect on growth should be observed for the heat-inactivated (control) serum.

PFGE typing

Pulsed-field gel electrophoresis (PFGE) was performed as detailed by Verduin *et al.* [23]. Digestions were performed using *Spe*I at 20 U per reaction and the following electrophoresis protocol: the first block consisted of a constant voltage of 6 V cm^{-1}, with the pulse time increased from 3.5 to 25 seconds during the first 12 hours; it was followed by a second block of 8 hours where the pulse time increased linearly from 1 to 5 seconds. PFGE patterns were analyzed using GelCompar software (Applied Maths)

with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set at 1.5%. PFGE products between 48.5 and 339.5 kb were included in the analysis.

PCR screening of genes

Isolates were grown overnight at 37°C on blood agar plates and DNA extracted using the MagNA Pure LC system (Roche). PCR was performed to detect *uspA1*, *uspA2*, *uspA2H*, *hag*, *ompCD*, *copB*, *mcaP*, 16S rRNA type and LOS type (see Table 3 for primer sequences). A touchdown thermocycling program was used for all PCRs except for *hag* and LOS PCRs. The touchdown protocol used an initial annealing temperature of 70°C, which was reduced by 1°C per cycle over 15 cycles of PCR. The following 20 cycles of amplification used an annealing temperature of 55°C. All isolates negative for both *uspA2* and *uspA2H* genes were tested using primers uspA2end.f and uspA2end, which amplify a conserved region at the 3'-end of both *uspA2* and *uspA2H* genes.

Table 3. Primer sequences used in this study

Primer	Sequence (5'-3')	Gene
uspA2start	cgctgtaaccagtgccatga	*uspA2*
uspA2end	acgatagccagcaccgatag	*uspA2/UspA2H*
uspA2end.f	gacagcagacgccattacca	*uspA2/UspA2H*
MeierRTB2-10	ttgagccatagccaccaagtgc	*UspA2H*
MeierRTF1-9	ctgaatttgccaaaggtcat	*UspA2H*
MeierRTF1-8	cgttatgcactaaaagagcaggtc	*UspA1*
MeierRTB1-8	gcatctgaccagcttagaccaatc	*UspA1*
UspA1tl	tgtgagcaaatgactggc	*UspA1*
Pr406LOS	caaaagaagacaaacaagcagc	LOS
Pr408LOS	catcaaaaaccccctacc	LOS
Pr649LOS	atcctgctccaactgactttc	LOS
McCOPB2F	ggcgtgcgtgttgaccgttttg	*copB*
McCOPB2R	gtttggcaggcgataggcgacat	*copB*
Mcat.ompCD.f	acgcactggcaagaagctaga	*ompCD*
Mcat.ompCD.r	gacctgcaccaaccaagacat	*ompCD*
McatHag-2	gtcagcatgtatcatttttttaagg	*hag*
McatHagR4	tgagcggtaaatggtttaagtg	*hag*
EUB-R	agagtttgatcctggttcag	16S rRNA
EUB-L	ctttacgcccatttaatccg	16S rRNA
mcaP-f	cgcaataaagatcaccatgcttg	*mcaP*
mcaP-r	cgggatcccgctgacacattgcattgataaa	*mcaP*

For the *hag* gene PCR, a standard PCR protocol comprising an annealing temperature of 55°C and an extension time of 8 minutes at 68°C for 25 cycles was used. The LOS-typing PCR protocol was performed as previously described by Edwards *et al.* [24].

After amplification, 16S rRNA PCR products were digested with *Fsp*BI (10 U) and *Hha*I (10 U) according to the manufacturer's instructions. Gel electrophoresis revealed the following 16S rRNA types: type 1, *Fsp*BI 439/124 bp and *Hha*I 563 bp; type 2, *Fsp*BI 332/124/107 bp and *Hha*I 563 bp; type 3, *Fsp*BI 332/124/106 bp and *Hha*I 370/192 bp. The digestion method was based on the 16S rRNA type sequence results published by Bootsma *et al.* [7].

copB and *ompCD* PCR-RFLP

copB and *ompCD* PCR products were digested with *Rsa*I and *Bse*JI (*Bsa*BI). Five U of *Rsa*I (*copB*) or 5 U of *Bse*JI *(ompCD)* were used per reaction mix, incubated at 37°C or 65°C, respectively. The *copB* PCR-RFLP typing method was based upon sequence data published by Liu *et al*. (2006) but cannot distinguish between CopB types I and III [25]. Expected visible product sizes were: 374 and 157 bp for CopB type 0; 342 and 157 bp for CopB types I/III; 332 and 187 bp for CopB type II, and 519 bp for CopB type IV.

The restriction digestion site for *ompCD* PCR product (*Bse*JI) was situated within the first A549 cell-binding domain (amino acids 16-236) of OMPCD [26].

UspA1 and Hag ELISA

The presence or absence of UspA1 and Hag expression was assessed in *uspA1* and *hag* PCR-positive isolates using a standard ELISA methodology and specific anti-UspA1 MAb 24B5 and anti-Hag 5D2 antibodies (kindly supplied by Prof. E. Hansen, UT South-Western Medical Center at Dallas, Texas, USA). Briefly, *M. catarrhalis* isolates were grown overnight on Columbia blood agar at 37°C and resuspended in PBS to an OD_{600} of 0.5 (equivalent to approximately 5×10^8 cells ml^{-1}). One hundred microlitres of each suspension was then loaded into 96-well MaxiSorp ELISA plates (NUNC), and allowed to dry by overnight incubation at 37°C. The following morning, plates were washed four times with PBS, blocked in 150 µl PBS with 2% (w/v) BSA and 5% (w/v) sucrose for 2 hours at 37°C, and then re-washed four times in PBS with 0.05 % (v/v) Tween 20. One hundred microlitres of primary antibody was then added (at a 1:10 dilution of stock in PBS/BSA/sucrose for both 24B5 and 5D2) and incubated for 1 hour at 37°C, before being washed four times in PBS/Tween 20. One hundred microlitres of secondary antibody (1:5,000 dilution of stock of polyclonal goat anti-mouse for 24B5 or 1:10,000 dilution for 5D2

in PBS/BSA/sucrose) was added and incubated for 1 hour at 37°C before washing four times in PBS/Tween 20. Finally, 50 µl tetramethylbenzidine substrate was added to each well and color allowed to develop at room temperature; two PCR-negative isolates were included in triplicate in each ELISA plate. The geometric mean absorbance plus 4 times the standard deviation for the six negative control wells was calculated per plate and used as a negative cut-off value. Results were recorded as either positive or negative, dependent on this cut-off value.

Results

Autoagglutination and biofilm assays

No significant difference in autoagglutination ability was observed between *M. catarrhalis* isolates cultured from children and adults, from lower versus upper respiratory tract infection, or world region (unpaired *t*-test $P=0.5$ and $P=0.88$, Kruskal-Wallis test $P=0.31$). Also, autoagglutination ability was not significantly affected by the presence/absence of *hag*, *uspA1*, *uspA2* or *uspA2H* genes, *copB* type, 16S rRNA type, *ompCD* PCR-RFLP type, LOS type or ability to form a biofilm. However, only two isolates were found to be *uspA1* PCR-negative, which makes comparison rather difficult for this gene. Results from the biofilm assay revealed no significant difference between upper versus lower respiratory tract infection, or world region (unpaired *t*-test $P=0.94$ and Kruskal-Wallis test $P=0.18$). Further, biofilm-forming ability was not significantly affected by the presence/absence of the *hag* or *uspA1* genes, *copB* type, 16S rRNA type, *ompCD* PCR-RFLP type or LOS type. However, a significant difference in biofilm formation was observed between *M. catarrhalis* isolates cultured from children and adults ($P=0.002$) and between isolates carrying the mutually exclusive *uspA2* and *uspA2H* genes ($P<0.0001$) (Figure 1).

Serum resistance assay

In total, 120 isolates were found to be serum-resistant, 64 of intermediate resistant and 11 sensitive. The distribution between the age groups was 65 (66%) resistant, 28 (29%) intermediate and 5 (5%) sensitive for the <5 years age group, and 55 (57%) resistant, 36 (37%) intermediate and 6 (6%) sensitive for the >20 years age group (chi-squared test $P=1$).

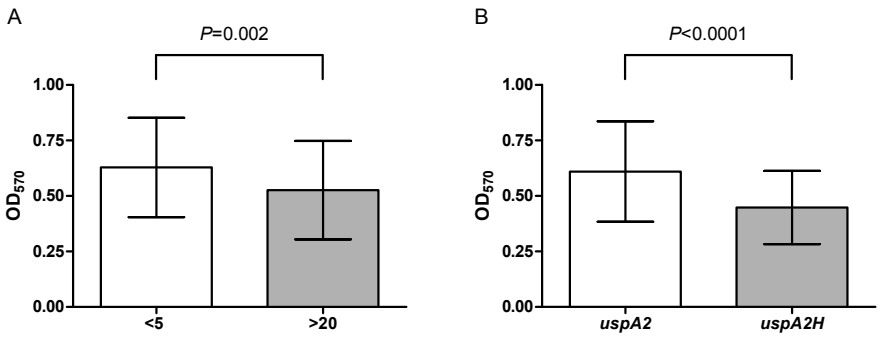

Figure 1. Difference in biofilm-forming ability (OD_{570}) with respect to age group (A) and presence of the mutually exclusive *uspA2* and *uspA2H* genes (B). Significant differences in biofilm-forming ability may be seen (*P* values = unpaired *t*-test). <5, isolates obtained from children less than 5 years of age; >20, isolates obtained from adults older than 20 years of age. *uspA2* / *uspA2H*, *uspA2* and *uspA2H* PCR-positive isolates.

PFGE and 16S RNA types

Analysis of PFGE patterns showed two major lineages (at 34% similarity) within the 195 isolates (data not shown), with 15/15 isolates in the first lineage comprising 16S rRNA types 2 and 3 only and 176/180 isolates in the second lineage being 16S rRNA type 1. No difference was observed between age groups and genotype lineage, or age group and 16S rRNA types. However, a significant difference was observed between 16S rRNA types versus upper or lower respiratory tract infection (Fisher's exact test $P<0.0001$). In particular, 62% (109/176) of type 1 isolates were cultured from lower respiratory tract samples (i.e. sputa and bronchial alveolar lavages), whilst 95% (18/19) of types 2 and 3 isolates were cultured from upper respiratory tract samples (i.e. nasopharyngeal aspirates, sinus fluids, ear fluids and throat swabs). Three type 1 isolates were also cultured from blood samples and one type 1 isolate was cultured from a sample of unknown origin. These isolates were not included in the analysis.

PCR screening of genes

PCR screening of the 195 *M. catarrhalis* isolates revealed an incidence of 99% (193/195) for *uspA1*, 90% (176/195) for *hag*, 78% (141/180) for *uspA2*, 22% (39/180) for *uspA2H* and 99% (194/195) for *mcaP*. The *copB* and *ompCD* genes were present in all the isolates screened. When divided into age groups, *uspA1* was found to be present in 99% (97/98) of child isolates and 99% (96/97) of adult isolates, and *hag* in 90% (88/98)

Table 4. Incidence of LOS types

	LOS type		
	A	B	C
<5	79	13	2
>20	61	27	2
16S type*	127/6/7	40/0/0	2/2/0

* = incidence 16S rRNA types 1/2/3 with respect to individual LOS types. A significant difference is observed between LOS types A and B and age group (Fisher's exact test P=0.01). All LOS B isolates and the great majority of LOS A isolates belonged to 16S rRNA type 1. <5, isolates from children; >20, isolates from adults

and 91% (88/97), respectively. Interestingly, *uspA2* was present in 95% (88/93) of child-associated but only 61% (53/87) of adult-associated isolates, whilst the mutually exclusive *uspA2H* was present in only 5% (5/93) of child-associated and 39% (34/87) of adult-associated isolates (Fisher's exact test P<0.0001). Fifteen isolates (8%) were found to be PCR-negative for *uspA2*- and *uspA2H*-specific PCRs, but positive using primers uspA2end.f and uspA2end. Although these isolates were not included in the above *uspA2* and *uspA2H* calculations, their inclusion as either *uspA2* or *uspA2H* would not have affected the significance of the results. All *uspA2H* PCR-positive isolates belonged to the 16S rRNA type 1, irrespective of age group and specimen source.

The majority of isolates belonged to LOS type A, representing 81% (79/98) and 63% (61/97) of child and adult isolates, respectively. The incidence of the LOS types is shown in Table 4. Within the age groups, 4% (4/98) and 7% (7/97) of isolates were untypeable (generated no PCR products). All negative PCRs were tested twice by PCR and were shown to contain DNA using a 16S rDNA PCR.

copB and *ompCD* PCR-RFLP

The distribution of CopB types was not significant between age groups, although there appeared to be a trend towards an increase in CopB types I/III (54/99) and a decrease in CopB type II (31/75) in adult-associated *M. catarrhalis* isolates. Further, CopB types I/III and II were almost exclusively associated with 16S rRNA type 1 (173/176 isolates), and CopB types 0 and IV were mainly associated with 16S rRNA types 2 and 3 (18/19 isolates).

Two PCR-RFLP patterns were observed for *ompCD*, comprising bands of approximately 200/100 bp (pattern 1), and 180/80 bp (pattern 2). All 18 *ompCD* PCR-RFLP pattern 2 isolates belonged to 16S rRNA types 2 and 3, with 176/177 of PCR-RFLP pattern 1 isolates belonging to 16S rRNA type 1.

UspA1 and Hag ELISA

The incidence of UspA1 and Hag expression in *uspA1* and *hag* PCR-positive isolates is shown in Table 5. A significant difference in Hag expression (but not UspA1 expression) between the child and adult age groups was observed (Fisher's exact test P=0.001).

Table 5. Incidence of *hag* and *uspA1* gene expression

	Hag		UspA1	
	positive	negative	positive	negative
<5	81 (92%)	7 (8%)	95 (98%)	2 (2%)
>20	64 (73%)	24 (27%)	93 (97%)	3 (3%)

Incidence of Hag and UspA1 expression (measured using ELISA) related to age group for global *M. catarrhalis* isolates PCR-positive for the *hag* and *uspA1* genes. A significant difference in the incidence of Hag, but not UspA1, expression may be seen between child and adult groups (Fisher's exact test P=0.001 and P=1, respectively). <5, isolates from children; >20, isolates from adults

Discussion

The ability to autoagglutinate has been linked to virulence in several Gram-negative bacterial species, including *Moraxella bovis* [27-29]. Further, the ability to autoagglutinate has been shown to be independent of the strain isolation site (i.e. lower versus upper respiratory tract), which was also indicated by our own results [30]. The loss of autoagglutination ability has previously been linked to the insertional inactivation of the hemagglutinin (*hag*) gene, but not the *uspA1* or *uspA2* genes, in *M. catarrhalis* isolate O35E [21, 31]. From our own population-based study, it appears that the *hag* gene may not be equally important in influencing autoagglutination ability in all isolates of *M. catarrhalis.*

Bacterial biofilms on the middle-ear mucosa are associated with chronic otitis media [32, 33]. Further, *M. catarrhalis* is an important etiological agent of otitis media in children that has been shown to be capable of forming such biofilms [11]. Published evidence suggests a role for both UspA1 and UspA2H in biofilm formation [11, 34]. From our results, only two isolates were found to be *uspA1*-negative by PCR, which makes comparisons between *uspA1*-positive and -negative populations in our study difficult. Further, we found that isolates positive for *uspA2* (a gene always found to be mutually exclusive of *uspA2H*) were better biofilm formers than isolates carrying the *uspA2H* gene, suggesting that *uspA2* could also have a role in biofilm formation. In

fact, several recently described genes have also been associated with biofilm formation, including *mcmA*, *mcaP* [35, 36] and *pil* genes for type-IV pili (TFP) [37, 38]. In this study, the gene *mcaP* was equally distributed within child and adult age groups; *mcmA*, however, was not tested, as only one sequence is available (GenBank accession no. EF017300), meaning that conserved primers could not be designed. Luke *et al*. (2007) recently showed that TFP play an important role in nasopharyngeal colonization of *M. catarrhalis* and that biofilm formation is enhanced by TFP expression; and that TFP genes are ubiquitous within *M. catarrhalis* [38]. So it seems likely that a combination of several genes is important in biofilm formation in *M. catarrhalis*. Whatever the true situation, our results indicate that *M. catarrhalis* isolates from children are better biofilm formers than isolates from adults, possibly related to differences in *hag* gene expression levels. The fact that no significant difference in biofilm formation was observed between isolates cultured from the upper and lower respiratory tract indicates that biofilm formation is probably not reliant on a particular cell type, unless that particular cell type or a particular receptor is present in both upper and lower respiratory tract tissues.

The distribution of serum-resistant and serum-sensitive isolates was similar to that previously reported by Schmitz *et al.* (2002), who reported a serum-sensitive incidence of 19% in 419 *M. catarrhalis* isolates collected during the 1997-1999 European SENTRY surveillance study [39]. It appears from our data that the incidence of serum resistance in clinical *M. catarrhalis* isolates has remained relatively constant during the period 1997-2002.

In this study, 99% of isolates possessed the *uspA1*, 78% *uspA2* and 22% *uspA2H* genes, an incidence similar to that previously reported by Meier *et al*. (99% *uspA1*, 79% *uspA2* and 21% *uspA2H*) [40]. However, significant differences in the incidence of *uspA2* (increased) and *uspA2H* (decreased) were observed between the child and adult groups. One possible scenario arising from our results is that children are initially immuno-naïve towards the various *M. catarrhalis* outer membrane proteins (and especially *uspA2*), but gain a repertoire of neutralizing antibodies over time. Non-naïve adults on the other hand would generally possess neutralizing antibodies (and in this scenario, especially anti-UspA2 antibodies), resulting in selection pressure for non-*uspA2*-expressing isolates. The reduced incidence of *uspA2* in adults could therefore be a consequence of immune evasion in *M. catarrhalis*. However, it should be noted that the actual presence of the *uspA2* or *uspA2H* gene does not necessarily indicate UspA2 or UspA2H expression [41]. At present there is unfortunately no antibody available that can specifically distinguish between the UspA2 and UspA2H proteins.

Three different LOS serotypes (A, B and C) have been described in *M. catarrhalis*, with a study by Vaneechoutte *et al*. (1990) indicating a worldwide incidence of 61.3% for type A, 28.8% for type B and 5.3% for type C, using an assortment of isolates obtained from children and adults. In that study, 4.6% of isolates remained unidentified [42]. A similar distribution of LOS types was found in our isolates, with the majority of isolates producing LOS type A in both children and adults, and 5.6% of isolates remaining untypeable. Interestingly, LOS type B was twice as prevalent in isolates from adults compared to children and was exclusively associated with 16S rRNA type 1 isolates. This result could also possibly be a consequence of immune evasion by adult-infecting *M. catarrhalis* isolates, and has, in any case, negative consequences with respect to the development of single-type LOS vaccines.

One of the major findings of this study was a statistically significant decrease in the incidence of *M. catarrhalis hag* gene expression (in isolates proven to possess the *hag* gene) in adult-associated versus child-associated isolates. In contrast, a significant difference in the incidence of the *hag* gene between child- and adult-associated *M. catarrhalis* isolates was not found. Hag expression appears therefore to be downregulated in *M. catarrhalis* isolates infecting adults, a phenomenon that could again possibly occur as part of an immune-evasion response. Hag (and indeed UspA1) have been previously shown to be immunogenic in children and adults [6, 17, 19, 20]. It is, however, possible that UspA1 and Hag expression *in vivo* may differ from that observed *in vitro*, meaning that further research is necessary to verify the clinical significance of our observations.

In this study, an attempt has been made to map genotypic and phenotypic differences occurring in *M. catarrhalis* isolates infecting children and adults. It was observed that the isolates from these two age groups are genetically diverse and are not associated with the two major genetic lineages of *M. catarrhalis* previously determined. However, our results do indicate that disease-causing isolates infecting children possess a greater biofilm-forming capacity, and that differences in the incidence and expression of outer membrane-associated virulence genes exist between child- and adult-associated *M. catarrhalis* isolates within a global context, possibly as a consequence of immune evasion. We also discovered two surrogate genetic markers that distinguish between isolates of 16S rRNA type 1, and types 2 and 3 (rRNA types associated with the two major *M. catarrhalis* lineages, as well as between upper and lower respiratory tract infections). Finally, from our data, it appears that vaccines directed against single immunogenic outer membrane proteins (e.g. UspA2, UspA2H, UspA1 and Hag) and/or LOS types alone may be unsuitable in preventing both child- and adult-associated *M. catarrhalis*

infections. A multivalent vaccine comprising a combination of immunogenic epitopes may provide better protection against *M. catarrhalis*-mediated disease.

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Chapter 6

Microbial analysis does not facilitate the differentiation of childhood recurrent acute otitis media (rAOM) from chronic otitis media with effusion (COME)

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In preparation

Abstract

Otitis media (OM) is one of the most frequent diseases in childhood. Although often self-limiting, in some cases recurrent acute otitis media (rAOM) or chronic otitis media with effusion (COME) occurs. In previous studies, the presence of bacteria and viruses was shown to be primarily associated with cases of (r)AOM, whereas COME is considered to be a mostly sterile condition. In this respect, we investigated whether microbiological infection patterns could be used to differentiate between rAOM and COME disease in children.

Children <6 years of age, suffering from rAOM (*n*=46) or COME (*n*=131), and scheduled for tympanostomy tube insertion were enrolled in a prospective study. Middle ear fluids (*n*=127) and nasopharyngeal samples (*n*=177) were collected during surgery for bacterial culture, PCR analysis for *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, as well as multiplex PCR to detect 15 respiratory viruses. Serotyping and genotyping analyses was also performed to further characterize the bacterial pathogens isolated.

The pattern of bacterial and viral pathogens in middle ear fluids did not significantly differ between patients suffering from rAOM or COME, with a single bacterial pathogen being primarily cultured. Seventy-seven per cent (*H. influenzae*) to 100% (*S. pneumoniae* and *M. catarrhalis*) of cultured bacteria were genetically identical between middle ear fluid and nasopharynx. However, in rAOM patients, nasopharyngeal carriage was dominated by colonization with a single bacterial pathogen, whereas in COME patients, colonization with 2 or 3 pathogens was significantly more often seen. In both patient groups, *H. influenzae* and rhinovirus were the predominant pathogens in the middle ear (20% and 44%, respectively) and nasopharynx (68% and 50%, respectively).

From our results, the common perception that rAOM is associated with recurrent episodes of microbiologically-mediated AOM, whereas COME is generally a sterile inflammation, should be reconsidered.

Introduction

Otitis media (OM) is one of the most frequent diseases during childhood and the most common reason for young children to visit a physician. In many countries, it is the most common reason for children to receive antibiotics or to undergo surgery [1-3]. In fact, the costs associated with OM disease are extremely high, not only due to the direct burden on healthcare systems *per se*, but also due to indirect effects associated with lost working days as parents stay at home in order to attend their sick children [4-6].

OM is the common denominator for a variety of middle ear diseases and can be divided into various categories, including acute otitis media (AOM) and otitis media with effusion (OME) [7-8]. AOM can be defined as the presence of middle ear effusion accompanied by signs of acute inflammation of the middle ear, such as otalgia, otorrhea, fever, and malaise or irritability of the child [3]. OME on the other hand, can either develop as a sequel to AOM, or develop *de novo*. The primary symptom of OME is hearing loss, due to the presence of middle ear fluid in the middle ear cavity, but in the absence of signs of acute inflammation [9]. Albeit often self-limiting, in 10-20% of the cases, OM results in chronic OME (COME) or recurrent AOM (rAOM) disease [10-11].

Although there is currently no universal standard for OM management, it may imply watchful waiting, antibiotic treatment, adenotomy, insertion of tympanostomy tubes or vaccination. AOM management involves, after initial observation and adequate analgesics, antibiotic treatment or, in case of recurrent infections, antibiotic prophylaxis or tympanostomy tube insertion. For OME, medical intervention is appropriate only if persistent clinical benefits can be achieved in the absence of spontaneous resolution. Therefore, healthy children with OME should be observed for at least 3 months before medical intervention is considered, in which case, surgical treatment involving the insertion of tympanostomy tubes, or adenoidectomy, is feasible [3].

Vaccination against OM diseases is currently very limited. For example, the heptavalent pneumococcal conjugate vaccine (implemented in the Dutch National Immunization Program in 2006), is primarily directed against pneumococcal invasive disease, offering only a limited protective effect against OM [12-15]. Therefore, the development of new OM medical interventions, such as antimicrobial drug design and vaccine development, are required in order to improve current OM treatment options [16-17]. In order to effectively target new therapies, a detailed understanding of the role of bacteria and viruses in facilitating OM disease is warranted [18].

A key element in the pathogenesis of OM is the nasopharynx, as this niche is the reservoir for bacterial pathogens related to middle ear infections [8]. Colonization of the nasopharynx with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (the 3 most important bacterial pathogens associated with OM disease) at an early stage, has been shown to predispose children to develop rAOM [19]. In addition, OM prone children have increased carriage rates of these bacterial pathogens compared to healthy controls [20-21]. In AOM, *S. pneumoniae* is the most frequently detected pathogen in middle ear fluid, followed by non-typeable *H. influenzae* and *M. catarrhalis* [22-23]. However, previous studies have indicated that this balance shifts towards *H. influenzae* and *M. catarrhalis* when AOM and COME are compared, although *S. pneumoniae* can still be present in the middle ear in a minority of these patients [24]. In general, fewer bacteria have been found in the middle ear of children suffering from COME compared to AOM.

Viral upper respiratory tract infections (URI) also predispose children to OM, as infection may cause Eustachian tube dysfunction, facilitates an increase in adherence of bacteria to epithelial cells, resulting in a rise in bacterial colonization of the nasopharynx and in a modulation of the host's immune function [25-27].

In this study, we investigated differences in the patterns of bacterial and viral colonization and infection in the middle ear and nasopharynx of children diagnosed with rAOM or COME. In particular, the contribution of the major bacterial pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in the absence or presence of 15 respiratory viruses was studied in relation to rAOM and COME disease. We demonstrate that clear-cut differences in the clinical presentation between rAOM and COME cannot be explained by differences in the microbial flora in the middle ear. We therefore conclude that the common perception that rAOM is caused by recurrent episodes of AOM associated with bacteria and/or viral infections, whereas COME is generally a sterile inflammation, should be reconsidered.

Materials and methods

Study design

This prospective clinical study comprised a cohort of children <6 years of age who suffered from rAOM or COME. The cohort was enrolled at a secondary and a tertiary hospital in Nijmegen, The Netherlands, from April 1st 2008 to July 1st 2009, and included children

suffering from rAOM or COME, as well as children who underwent surgery for the insertion of tympanostomy tubes, in addition to adenotomy, at the same surgical setting. Recurrent AOM was defined as 3 or more episodes of AOM in the last 6 months or 4 episodes in the last 12 months [28]. The COME patient population consisted of children who had experienced a period of persistent OM with effusion lasting longer than 3 months. Signs, symptoms, and results of audiometry and tympanometry, were documented prior to surgery by an ear nose and throat (ENT) specialist. Patients with a prior history of malignancy, organ transplantation or immune deficiency were excluded from participation, as well as patients with recent elective ear surgery or systemic infectious diseases. Patient characteristics and risk factors were identified using a questionnaire. Ethical permission was obtained from the Committee on Research Involving Human Subjects in January 2008 (CMO 2007/239, international trial register number: NCT00847756).

Clinical materials

Middle ear fluid was collected during surgery using a middle ear fluid aspiration system (Kuijpers Instruments) [29], whilst nasopharyngeal samples were obtained using a cotton wool swab (192C, Copan). Middle ear fluid was suspended in 2 ml saline and divided into aliquots for bacterial culture, bacterial PCR and viral PCR. The nasopharyngeal samples were used for bacterial culture and thereafter stored at -80°C in 1 ml of 80% glycerol for subsequent viral PCR analysis.

Microbiology

Middle ear fluid and the nasopharyngeal samples were cultured to determine the presence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Bacteria were cultured according to standard laboratory procedures [19] and stored at -80°C in the appropriate culture media containing an additional 15% or 50% glycerol (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, respectively). The less frequently described otopathogens *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus parainfluenzae*, *Pseudomonas aeruginosa* and *Alloicoccus otitidis* were also included in the analysis if found to be present during bacterial culture.

To further characterize the bacterial isolates, all *S. pneumoniae* and *H. influenzae* isolates were cultured overnight on blood agar and chocolate agar plates at 37°C supplemented with 5% CO_2, respectively. Pneumococcal isolates were serotyped using the Quellung reaction (Statens Serum Institute) and multiplex PCR was performed as previously described [30]. *H. influenzae* isolates were serotyped using slide aggluti-

nation according to the instructions of the manufacturer (Difco laboratories, Becton Dickinson) [31].

S. pneumoniae, *H. influenzae* or *M. catarrhalis* obtained from middle ear fluid, as well as the equivalent pathogen detected in the nasopharynx, were further analyzed using multilocus sequence typing (MLST). Genomic DNA was isolated using a Qiagen Genomic-tip 20/G Kit according to the manufacturer's instructions. To assign the strains to a sequence type (ST), 7 house-keeping genes were subjected to PCR amplification and DNA nucleotide sequencing. The primer sets were obtained from the MLST website (www.mlst.net). Fifty microliter reaction mixtures were prepared with 1.25 U Taq polymerase (Applied Biosystems), 1× Taq polymerase buffer (10 mmol/L Tris HCl [pH 8.3], 50 mmol/L KCl), 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each deoxynucleoside triphosphate, and 0.2 mmol/L of each primer. One microliter of genomic DNA was added to each reaction. The following parameters were used for amplification: denaturation at 95°C for 5 minutes, 30 subsequent cycles of amplification, each consisting of 1 minute at 95°C, 1 minute at 50°C, and 30 seconds at 72°C, with a final extension at 72°C for 7 minutes. PCR products were analyzed by gel electrophoresis on a 1% agarose gel, and the amplicon size was evaluated by comparing it with a 1 kb ladder (Invitrogen). PCR products were purified by using the QIAquick PCR purification kit (Qiagen). Sequencing, with forward and reverse primers, was performed on the ABI 377 DNA sequencer with Big Dye chemistry (Applied Biosystems), according to the manufacturer's instructions.

PCR for quantitative DNA analysis of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was performed. The respective genes chosen for bacterial detection were the *S. pneumoniae ply* gene [32], *H. influenzae* 16S rRNA gene [33] and the *M. catarrhalis ompJ* gene [34].

Virology

Samples were analyzed by multiplex PCR as previously described [35]. Briefly, upon thawing, nucleic acids were extracted from each sample, using the MagNA Pure LC System and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science) according to manufacturer's instructions. A multiplex real-time PCR assay containing 15 different viral pathogens was used. This assay was designed for the detection of specific viral genomes belonging to influenzavirus (IV) type A and B, coronavirus (CoV) 229E and OC43, human bocavirus (hBoV), enterovirus (EV), adenovirus (AdV), parechovirus (PeV), parainfluenzavirus (PIV) types 1-4, human metapneumovirus (hMPV), rhinovirus (RV) and respiratory syncytial virus (RSV). An internal control consisting of phocine

herpesvirus (PhPV, IC DNA control) and equine arthritis virus (EAV, IC RNA control) was included in the assay. RNA was reverse transcribed to cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) in a 50 µl reaction mix containing 20 µl of nucleic acid isolate and random hexamers as primers, according to the manufacturer's instructions. PCRs were performed on the LightCycler 480 instrument using LightCycler 480 Probes Master Mix (Roche Diagnostics). Validated primer/probe-mixes were purchased from Diagenode and used according to the manufacturer's instructions. Cycling conditions were 95°C for 5 minutes, followed by 50 cycles of 95°C for 15 seconds and 55°C for 15 seconds and 72°C for 20 seconds. The amount of virus was recorded semi-quantitatively based on the Ct value (cycle threshold value).

Statistical analysis

Statistical analysis was performed using the Statistical Package of Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA). The chi-square test or Mann-Whitney *U*-test was used when appropriate to calculate the statistical differences between patient baseline characteristics. The chi-square test was used in the analysis of categorical data obtained from bacterial and viral culture and PCR.

Results

Bacterial patterns in the middle ear do not discriminate between rAOM and COME

From April 2008 to July 2009, 177 children were enrolled in this study. Selected demographic and clinical characteristics of the cohort are shown in Table 1. Recurrent AOM was mostly diagnosed at a younger age, while the prevalence of COME increased by age (rAOM 75% during the first year of life vs. 17% at 5 years of age; COME 25% during the first year of life vs. 83% at 5 years of age). A middle ear fluid specimen was collected from 72% of the children (n=127), and there was a significant difference in the presence of middle ear fluid and OM diagnosis (rAOM n=27 (59%) vs. COME n=99 (76%), P=0.03). Children suffering rAOM presented with less ENT-related surgery in their medical history when compared to children suffering from COME, but received significantly more antibiotics <1 year prior to surgery.

In rAOM, no pneumococci were cultured from middle ear fluid, whereas *H. influenzae* and *M. catarrhalis* were detected in 23% and 4% of rAOM, respectively (Table 2).

Table 1. Characteristics of children suffering from rAOM and COME

	rAOM	COME	*P*-value
	n=46	*n*=131	
Male sex, *n* (%)	31/46 (67)	70/131 (53)	0.10
Mean age (years [SD])	3.065 [1.686]	3.974 [1.406]	**0.01**
Presence of middle ear fluid, *n* (%)	27/46 (59)	99/131 (76)	**0.03**
≥4 URTI in the preceding year, *n* (%)[1]	17/43 (40)	31/119 (26)	0.09
Antibiotics prescribed in the preceding year, *n* (%)	12/44 (27)	15/127 (12)	**0.02**
History of ENT surgery, *n* (%)	9/32 (28)	50/94 (53)	**0.01**
Asthma/wheezing, *n* (%)	3/44 (7)	8/121 (7)	0.96
Allergic rhinitis, *n* (%)	5/43 (12)	5/120 (4)	0.08
Eczema, *n* (%)	7/44 (16)	20/120 (17)	0.90
Birth weight, mean (g)	3374	3370	0.93
Breast-feeding <3 months, *n* (%)	29/42 (69)	69/120 (58)	0.19
Prevnar immunization, *n* (%)[2]	18/38 (47)	35/110 (32)	0.08
Tobacco smoke exposure, *n* (%)	16/42 (38)	34/118 (29)	0.26
Older siblings, *n* (%)	24/33 (73)	66/104 (63)	0.68
Day-care attendance, *n* (%)	22/43 (51)	57/118 (48)	0.75

Significant differences are shown in bold

[1]Upper respiratory tract infections

[2]The Prevnar vaccine was introduced in the Dutch National Immunization Program for children born after 01-04-2006

S. pneumoniae was observed in 5% of the COME specimens, *H. influenzae* in 19% and *M. catarrhalis* in 9%. Based on bacterial culture, the presence of multiple bacteria in middle ear fluid was found sporadically and only in patients diagnosed with COME. Co-infection by *S. pneumoniae* and *H. influenzae* was found in only a single patient (<1%), as was co-infection by *H. influenzae* and *M. catarrhalis* (<1%). The less frequently described otopathogens *S. aureus*, *S. pyogenes*, *H. parainfluenzae*, *P. aeruginosa* and *A. otitidis* were only cultured sporadically and therefore excluded from further analysis.

PCR detection of the bacterial species present in rAOM middle ear fluids generated 12%, 46% and 27% positive results for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* bacteria, respectively, compared to 6%, 31% and 25% in the middle ear fluids of children suffering from COME. With respect to co-culture, rAOM PCR analysis indicated co-infection with *S. pneumoniae* and *M. catarrhalis* in a single child (4%), co-infection of *H. influenzae* and *M. catarrhalis* in 2 children (8%), and PCR positivity for all 3 pathogens in a single child (4%). In children suffering from COME, bacterial

Table 2. Frequency of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in middle ear fluid and the nasopharynx of children suffering from rAOM and COME

	Bacterial culture middle ear fluid		Bacterial PCR middle ear fluid		Bacterial culture nasopharynx	
	rAOM *n*=26	COME *n*=97	rAOM *n*=26	COME *n*=100	rAOM *n*=46	COME *n*=130
No bacteria	19 (73)	67 (69)	9 (35)	48 (48)	5 (11)	14 (11)
S. pneumoniae	0 (0)	5 (5)	3 (12)	6 (6)	**16 (35)**	**78 (60)***
H. influenzae	6 (23)	18 (19)	12 (46)	31 (31)	30 (65)	89 (68)
M. catarrhalis	1 (4)	9 (9)	7 (27)	25 (25)	17 (37)	57 (44)

* *P*=0.003

co-infections with *S. pneumoniae* and *M. catarrhalis* were found in 3 children (3%), *M. catarrhalis* and *H. influenzae* in 5 children (5%), and *S. pneumoniae* and *H. influenzae* in 2 children (2%).

A comparative analysis relating the detection of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* by culture and PCR was performed on 121 middle ear samples. As expected, PCR detected bacterial DNA in a significant percentage of culture negative middle ear effusions: *S. pneumoniae* in 4.1% (NS), *H. influenzae* in 16.5% (*P*<0.001) and *M. catarrhalis* in 19% (*P*<0.001).

Finally, the presence of each specific pathogen, measured using conventional culture techniques or qualitative PCR, was not significantly different between the rAOM and COME groups. Additionally, quantitative PCR detected no significant difference in bacterial load between the two groups (data not shown).

Nasopharyngeal carriage with a single pathogen dominates in rAOM patients

H. influenzae was the most frequently detected bacterial pathogen isolated from the nasopharynx and equally present in both rAOM and COME groups (65% versus 68%, respectively). *M. catarrhalis* was detected in 37% and 44% of the rAOM and COME patients, respectively. Eleven percent of the children were non-carriers, a result that was again similar between both patient cohorts. Importantly however, a significant difference in *S. pneumoniae* colonization was observed between the groups, comprising 35% positivity in rAOM patients compared to 60% positivity in COME patients (*P*=0.003) (Table 2).

Table 3. Presence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, as either single or multiple species, in the nasopharynx of children suffering rAOM or COME

Nasopharynx, bacterial culture	rAOM *n*=46, (%)	COME *n*=130, (%)	*P*-value
no pathogens	5 (11)	14 (11)	0.99
1 pathogen	22 (48)	38 (29)	**0.02**
≥2 pathogens	19 (42)	78 (60)	**0.02**

Significant differences are shown in bold

A single pathogen was more frequently detected in the nasopharynx of children suffering from rAOM, compared to those suffering from COME (48 vs. 29%; *P*=0.02) (Table 3), with co-colonization by more than 2 pathogens being significantly higher in children suffering from COME (42 vs. 60%, *P*=0.02). Moreover, co-colonization of two or more pathogens was significantly associated with the presence of bacteria in middle ear fluid of patients suffering from COME (*P*=0.04).

Bacteria in the nasopharynx and middle ear are often genetically identical

The presence of *S. pneumoniae* or *H. influenzae* in middle ear fluid was positively correlated with the presence of either *S. pneumoniae* or *H. influenzae* in the nasopharynx (*S. pneumoniae P*=0.049, *H. influenzae P*=0.034). *M. catarrhalis* was found in the nasopharynx of 42% of the children, with simultaneous occurrence of *M. catarrhalis* in the middle ear in 8% of the children. This association was not significant.

Serotype analysis of the 5 *S. pneumoniae* isolates cultured from middle ear fluid revealed the following serotypes: 23A, 23F, 6B, 19A, 7F. In all cases, the corresponding serotype was also found in the nasopharynx. Statistically significant differences were not observed between 1) pneumococcal vaccine types (i.e. those present in Pneumococcal Conjugate Vaccine 7) and non-vaccine types (NVT: rAOM 71%; COME 66%), or 2) between typeable and non-typeable (NT) pneumococcal strains, when children suffering rAOM and COME were compared (NT: rAOM 14%; COME 3%).

Ninety-six percent of the *H. influenzae* isolates (*n*=23) cultured from the middle ear were non-typeable, compared to 95% non-typeable isolates cultured from the nasopharynx (*n*=113). Serotype E was detected in 4% of the middle ear fluid and 4% of the nasopharynx specimens. Serotype B was detected in 1 nasopharyngeal specimen (1%).

MLST analysis revealed that the majority of nasopharyngeal and middle ear fluid isolates obtained from the same child were genetically identical (*S. pneumoniae* 100%; *H. influenzae* 77%; *M. catarrhalis* 100%). Further, no clonal relationships were observed for any of the 3 bacterial pathogens, i.e. there was no evidence to suggest that isolates originated from a single 'founder' isolate spreading throughout the community. This diversity of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* genotypes probably reflects a genetically heterogeneous population structure for these bacterial pathogens within this region of The Netherlands.

Rhinovirus dominates in both rAOM and COME patients and contributes to bacterial colonization

Rhinovirus was the most frequently detected virus present in middle ear fluids in both patient groups (rAOM 44%; COME 45%), followed by enteroviruses (7% and 8%, respectively), coronavirus (7% and 2%, respectively), and parainfluenzaviruses (4% and 1% respectively) (Table 4). The presence of enteroviruses in middle ear fluid was associated with the presence of rhinovirus, as 8 out of the 9 enterovirus-infected middle ears were also infected with rhinovirus (88%, P=0.01). Nevertheless, in the vast majority of the samples only a single virus was detected.

Rhinoviruses were also the most frequently detected viruses in the nasopharynx, (rAOM 51%; COME 50%), followed by parainfluenza viruses 1-4 (both 11%), adenoviruses (11% and 5%, respectively) and RSV (0% and 6%, respectively) (Table 4). The frequency of the different virus infections was not significantly different between the two groups.

Table 4. Presence of viruses in middle ear fluid and the nasopharynx of children suffering rAOM or COME

	Middle ear fluid		Nasopharynx	
	rAOM	COME	rAOM	COME
	n=27 (23%)	*n*=91 (76%)	*n*=37 (25%)	*n*=109 (75%)
No viruses	11 (41)	42 (47)	9 (24)	38 (35)
Rhinovirus	12 (44)	41 (45)	19 (51)	54 (50)
Enterovirus	2 (7)	7 (8)	6 (16)	9 (8)
Parainfluenza virus 1-4	1 (4)	1 (1)	4 (11)	12 (11)
Others*	2 (7)	9 (10)	6 (16)	23 (21)

*AdV, CoV, IV type A and B, hMPV, PeV, hBoV, RSV

The presence of rhinovirus in the nasopharynx was significantly correlated with co-occurrence of this virus in the middle ear (P=0.02), though this was not true for enteroviruses or parainfluenzaviruses.

Interestingly, a significant association was observed for bacterial-viral co-occurrence in the nasopharynx, the presence of *S. pneumoniae* or *H. influenzae* being significantly associated with the presence of rhinoviruses (P=0.047 and P=0.02 respectively), and the presence of *M. catarrhalis* being significantly associated with the presence of enteroviruses (P=0.02). No significant difference in bacterial-viral co-colonization was detected between children with rAOM and COME.

Discussion

In this study, we investigated whether the clinical signs and symptoms specific for rAOM and COME could be differentiated microbiologically by the presence of bacteria and viruses in middle ear fluid. The results of our study show that in this region of The Netherlands, the predominant bacterial pathogen associated with middle ear fluids obtained from children suffering from rAOM and COME is *H. influenzae*, and that presence of *S. pneumoniae* (8%), *H. influenzae* (36%) or *M. catarrhalis* (25%) is not significantly different between rAOM or COME children. However, Coates *et al.* (2008) demonstrated that bacteria can reside inter- and even intracellularly within the middle ear mucosa, leading to persistent otitis media [18, 33, 36]. Since we analyzed middle ear fluid *per se* and not the middle ear mucosa in this study, our bacterial findings might represent an underestimation of the actual frequency of bacterial pathogens present in the middle ears of rAOM and COME children.

With respect to the nasopharynx, as with middle ear fluids, *H. influenzae* was the most frequent bacterial pathogen detected, though the role of nasopharyngeal carriage of the bacterial pathogens *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* on the risk for OM disease is not yet fully understood. Some studies have reported that increased bacterial carriage rates are associated with an increase in OM [19, 37], while other studies have failed to demonstrate a correlation [38]. Further, the colonization density [8], as well as early colonization of pathogens in the nasopharynx, is considered to increase the risk of OM [20, 39], and it is also suggested that carriage of newly acquired bacteria is associated with OM [40]. Further studies are required in order to determine whether any of these factors play a significant role in the development of disease in rAOM and COME children compared with healthy children.

We provide evidence that co-colonization with 2 or more pathogens in the nasopharynx is significantly associated with the presence of bacteria in the middle ear of patients suffering from COME. However, this effect might be partially explained by the subtle yet statistically significant age differences observed between the two patient groups in this cohort study.

Previous studies described nasopharyngeal colonization in relation to the bacteria found in OM, but did not emphasize on nasopharyngeal colonization with multiple pathogens [16, 41]. Others showed correlations between nasopharyngeal colonization of either *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* [42], or related nasopharyngeal colonization to clinical observations of AOM [26, 38]. Krishnamurthy *et al.* (2009) were the first who described a murine colonization model, which showed that increased incidence of pneumococcal OM was affected by polymicrobial colonization, and in particular *M. catarrhalis* [43].

Fifty-five per cent of the children in this study did not possess *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* in their middle ear fluids, and 11% of the children did not have any of these 3 pathogens in their nasopharynx. Hence, it appears that bacterial colonization of the nasopharynx is only a poor indicator of the presence of bacteria in the middle ear of children suffering from rAOM and COME. Although we found a positive association between *H. influenzae* in the nasopharynx and *H. influenzae* in the middle ear, as well as *S. pneumoniae* in the nasopharynx and middle ear, the actual numbers of bacteria present in the middle ear fluids were small, and these associations were only observed when the rAOM and COME study groups were combined.

Pathogen isolates were characterized using serotyping and/or MLST in order to investigate whether the pathogens present in the nasopharynx and middle ear were genetically identical within the same child (but genetically distinct between different children). Studies investigating the genetic relatedness of bacteria obtained from different locations of the upper respiratory tract, and within a single patient, tend to appear sporadically in the literature. Among the different studies described, various approaches were used to determine this genetic relatedness, i.e. ribotyping, pulsed-field gel electrophoresis and amplified fragment length polymorfism [44-47]. The bacterial pathogens that were detected in the middle ear fluids of our cohort were genetically identical to those isolates cultured from the nasopharynx, as compared by MLST, which is in line with previous publications [44-47]. Further, the *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* genotypes isolated from the total cohort of children represented a genetically heterogeneous population structure, i.e. the isolates were genetically highly diverse,

even though they all originated from a relatively restricted geographical region of The Netherlands.

The ability of upper respiratory tract viruses to pave the way for secondary bacterial infection in AOM has been described extensively in literature [27, 48-49]. Influenza A and *S. pneumoniae*, RSV and *H. influenzae*, and PIV and *M. catarrhalis* are just a few examples of viral-bacterial interaction investigated in both animal models and human studies [50]. Further, rhinoviruses have been implicated in the pathogenesis of COME [51]. However, whether the presence of upper respiratory viruses is a causative etiologic factor, or merely a remnant of previous infection, in rAOM and COME disease remains largely unknown. In the present study, a positive association for the co-occurrence of: 1) rhinoviruses and *S. pneumoniae*, 2) rhinoviruses and *H. influenzae*, and 3) enteroviruses and *M. catarrhalis* was observed in the nasopharynx, though no association between the co-occurrence of these viruses and bacteria was established in middle ear fluids. To our knowledge, we are the first group to analyze the co-occurrence of bacteria and viruses in middle ear fluids and its relationship to nasopharyngeal colonization.

In conclusion, a prospective cohort study was performed in Nijmegen, The Netherlands, to investigate the frequency of bacterial and viral pathogens in the middle ear and nasopharynx of children suffering from rAOM or COME. Results showed that the same pathogenic bacterial species and viruses are implicated in the pathogenesis of both rAOM and COME disease. Therefore, our findings do not support the assumption that COME is merely a consequence of persistent sterile inflammation in the middle ear.

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Chapter 7

Differential virulence gene expression of group A *Streptococcus* serotype M3 in response to co-culture with *Moraxella catarrhalis*

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Abstract

Group A *Streptococcus* (GAS) and *Moraxella catarrhalis* are important colonizers and opportunistic pathogens of the respiratory tract. However, the majority of knowledge gathered to date regarding the colonization and pathogenic potential of these two pathogens has been mainly derived from work involving single bacterial species. Importantly, evidence increasingly indicates the significance of polymicrobial infections in facilitating respiratory disease pathology.

In this respect, comparative co-culture experiments were set up in order to study the effect of *M. catarrhalis* co-cultivation on the global GAS serotype M3 gene expression profile. Co-culture generated a distinct GAS gene transcription profile, with a significantly increased (≥4-fold) gene expression for the GAS virulence genes hyaluronan synthase (*hasA*), streptococcal mitogenic exotoxin Z (*smeZ*) and IgG-endopeptidase (*ideS*). In contrast, significant decreased (≥4-fold) gene expression was observed with respect to genes involved in energy metabolism.

This study provides the first evidence to indicate that polymicrobial infections involving GAS and *M. catarrhalis* may affect the expression of GAS related virulence and biofilm genes, possibly leading to an increased pathogenic potential for GAS during polymicrobial infections compared to pure GAS infections alone.

Introduction

Streptococcus pyogenes, group A *Streptococcus*, is responsible for a wide range of infections including the relatively benign GAS-mediated pharyngitis, the life-threatening necrotizing fasciitis and streptococcal toxic shock syndrome. GAS is also an important cause of post-infectious sequelae such as post-streptococcal glomerulonephritis and rheumatic fever [1-4]. In contrast, *Moraxella catarrhalis* is an exclusive colonizer and opportunistic pathogen of the human respiratory tract, the bacterium being associated with both upper (e.g. otitis media (OM)) and lower (e.g exacerbations of chronic obstructive pulmonary disease (COPD)) respiratory tract infections. However, both GAS and *M. catarrhalis* share the same respiratory biological niche, particularly with respect to upper respiratory tract infections [5]. For example, although *Streptococcus pneumoniae*, *Haemophilus influenzae* and *M. catarrhalis* are considered the predominant organisms causing OM, studies have shown that GAS may also be included as one of the most frequent causative agents of OM, including in the development of OM complications such as acute mastoiditis [6-7]. However, the role of GAS in OM is often underappreciated due to the effectiveness of β-lactam antibiotics in eliminating this organism. Further, recent communications have indicated an increase in *S. pyogenes* related OM infections in Hungary between 2006 and 2008 (personal communication Dr. Éva Ban, Semmelweis University, Budapest, Hungary; manuscript in preparation).

Research has shown that co-colonization of bacterial respiratory pathogens leads to increased colonization and infection. For example, Xu *et al.* (2009) reported that *H. influenzae* could enhance the binding of *S. pyogenes* to epithelial cells [8]. Further, Armbruster *et al.* (2010) showed that co-colonization of *M. catarrhalis* and *H. influenzae* could influence biofilm formation and antibiotic resistance, while Verhaegh *et al.* (2010) showed that co-colonization of infants by *M. catarrhalis* and *H. influenzae* was significantly more likely than single species colonization alone [9-10]. In fact, Armbruster *et al.* (2010) showed that a quorum sensing signaling mechanism positively influences *M. catarrhalis* biofilm formation, a system utilized by many pathogenic bacterial species to coordinate their gene expression as a response to the local bacterial population density. Until recently, there was little association between GAS and biofilm formation reported in the literature. One of the first observations was reported by Akiyama *et al.* (2003), and a limited number of studies since have demonstrated GAS biofilm formation *in vitro* and *in vivo* [11]. Nowadays, there is a growing understanding of the importance of biofilm formation in GAS-related colonization and infection. It has been found that

many GAS genes required for biofilm formation are also important virulence factors. Finally, polymicrobial infections may also promote the survival of bacterial species via the phenomenon of 'indirect pathogenicity'. For example, *S. pneumoniae* is protected from the action of certain β-lactam antibiotics in the presence of BRO β-lactamase positive *M. catarrhalis* [12-13].

Due to the increasing evidence linking the importance of polymicrobial infections to the pathogenesis of respiratory disease, we sought to investigate the changes in global gene transcriptome patterns in GAS during GAS and *M. catarrhalis* co-culture. We utilized an established GAS microarray protocol to identify significantly up- and down-regulated GAS genes, including those that could have an influence on GAS pathogenicity during polymicrobial clinical disease.

Methods

Bacterial strains and growth conditions

The isolates used in this study were a serotype M3 GAS strain MGAS16655 (cultured from a patient with pharyngitis and closely related to the reference strain MGAS315) [14], and *M. catarrhalis* strain JMF150 (isolated from a child presenting with OM and provided by Texas Children's Hospital, Houston, United States). For co-culture experiments, both organisms were cultured in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37°C with 5% CO_2 aeration. Further, each strain was grown in triplicate so that triplicate experiments could be performed. In order to quantify viable GAS and *M. catarrhalis*, both as single cultures and in co-culture, bacteria were grown in THY broth, and enumerated (colony forming units, CFU/ml) and OD_{600} readings determined at serial time points.

RNA extraction from co-cultures was obtained from isolates in the exponential/early-stationary phase of growth after an incubation period of 2.5 hours, with control RNA being extracted from MGAS16655 grown under identical conditions as described above, but in pure culture. One milliliter of co-culture was added to volumes of RNA protect (Qiagen) and bacteria were incubated at room temperature for 5 minutes, then pelleted by centrifugation at 4000×g at 4°C for 10 minutes, and stored at -80°C until ready for RNA extraction.

RNA extraction

RNA was extracted from exponential/early-stationary phase bacterial cells using the QIAGEN RNeasy mini kit according to the manufacturer's instructions. RNA samples were treated with Ambion TURBO DNase to remove any contaminating DNA, and the quality of the RNA was analyzed using an Agilent 2100 bioanalyzer.

cDNA synthesis, fragmentation, 3' end-labeling and hybridization

cDNA synthesis, fragmentation, 3' end-labeling and hybridization was prepared from total RNA according to the GeneChip® expression analysis technical manual supplied by the manufacturer (Affymetrix Inc.), with slight modifications. Poly-A RNA controls were added to each RNA aliquot, and random primers to a final dilution of 25 ng/µl (Invitrogen) were annealed (10 minutes at 70°C, followed by 10 minutes at 25°C). First-strand cDNA synthesis was performed using 25 U/µl SuperScript III (Invitrogen) in the presence of 0.5 mM dNTPs, 0.5 U/µl SUPERase•In RNase inhibitor (Ambion), and 10 mM dithiothreitol (10 minutes at 25°C, followed by 60 minutes at 37°C, 60 minutes at 42°C and 10 minutes at 70°C). RNA was removed by hydrolysis in 1 N NaOH (30 minutes at 65°C), and neutralized with 1 N HCl before cDNA purification using the QIAquick 96 kit (Qiagen) according to the manufacturer's recommendations. For cDNA fragmentation, 6-10 µg of cDNA and 0.2 U/µg of DNase I (Roche) were used (5 minutes at 37°C, followed by 10 minutes at 98°C). The fragmented cDNA (averaging 50 to 200 bases) was 3' end-labeled using GeneChip® DNA labeling reagent (Affymetrix Inc.) (60 minutes at 37°C) according to the manufacturer's instructions. The fragmented and end-labeled cDNA was added to the microarray hybridization solution without further purification. End-labeled cDNA was added to a custom GAS Affymetrix microarray [15] and incubated at 45°C overnight (~16 hours). The slides were then washed using a Fluidics Station 450 (Affymetrix Inc.). Microarray slides were scanned using a GeneChip® Scanner 3000 (Affymetrix Inc.) and analyzed.

Microarray transcriptome expression analysis

Microarray expression studies were performed in triplicate using a custom-made Affymetrix GeneChip that contained 100% of the ORFs of the GAS reference strain MGAS315 as previously described [15-17]. Briefly, samples used for microarray analysis were collected from cultures in THY at 2.5 hours after co-culturing. Controls comprised RNA extracted from GAS grown in pure culture under identical conditions as co-culture experiments.

Genes that were statistically significant (two-sample *t*-test; $P<0.05$) and had a ≥4-fold change in expression were included in the analysis. As a final step, the complete genome of *M. catarrhalis* strain RH4 [18] was compared to the genome of GAS strain MGAS315 for regions of sequence similarity. If a gene was found to be similar in both species using a cut off of >95% similarity, then this gene was removed from the analyses due to possible problems with cross-hybridization of GAS and *M. catarrhalis* RNA transcripts.

Quantitative PCR analysis

Confirmatory quantitative gene transcript analysis was performed on 2 important GAS virulence associated genes, and the constitutively expressed control gene *pros*, using TaqMan quantitative real-time PCR (qRT-PCR) and a 7500 Fast Real-Time PCR System (Applied Biosystems) as previously described [15, 19-20]. Transcripts were compared to the internal reference gene *tufA* as previously described [21]. Sequence data for the respective TaqMan primers and probes are listed in Table 1. All TaqMan experiments were performed in quadruplicate.

Table 1. TaqMan quantitative real-time PCR primers and probes utilized in this study

Gene	Spy no. in strain MGAS315	5′ primer	3′ primer	TaqMan probe
hasA	SpyM3_1851	ACCGTTCCCTTGTCAATAAAGG	CGTCAGCGTCAGATCTTTCAAA	CGCCATGCTCAAGCGTGGGC
proS	SpyM3_1688	TGAATTTATCATGAAAGACGGCTATAGTTTC	AATAGCTTCGTAAGCTTGACGATAATC	TCGTAGGTCACATCTAAATCTTCATAGTTG
speB	SpyM3_1742	CGCACTAAACCCTTCAGCTCTT	ACAGCACTTTGGTAACCGTTGA	GCCTGCGCCGCCACCAGTA

Results

Characterization of serotype M3 GAS strain MGAS16655 and *M. catarrhalis* JMF150 growth as single cultures and in co-culture

Prior to characterizing the transcriptome of GAS, we studied the growth of strains MGAS16655 and JMF150 in THY medium in co-culture and as pure cultures. Both strains

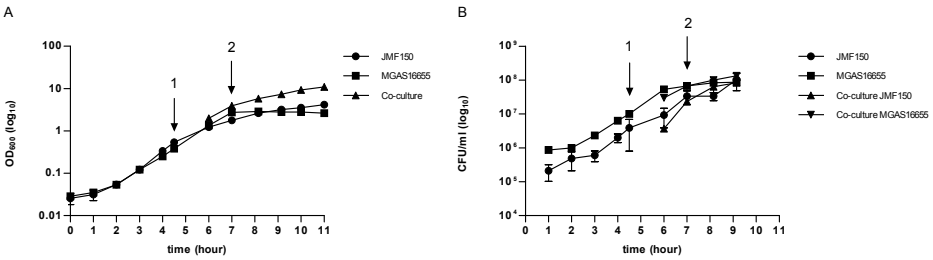

Figure 1. Characterization of growth curves for GAS serotype M3 MGAS16655 [■], *M. catarrhalis* isolate JMF150 [●], and co-culture [▲ or ▼]. Bacterial growth was measured over time in 3 independent experiments by determination of A) the OD_{600} and B) the CFU/ml, at hourly time points. The growth curve displayed represents the mean OD_{600} and CFU/ml values. Error bars indicate the standard error of the mean between individual experiments. Sampling time points for the start of co-culture [1], and for the harvesting of cells for RNA-isolation [2] are indicated by arrows.

grew rapidly in this medium, and the bacterial density reached ~10^8 CFUs/ml in the exponential/early-stationary phase (Figure 1). Further, the experiments showed that we were able to reproducibly correlate OD_{600} readings to CFU concentrations for both MGAS16655 and JMF150.

Changes in the GAS transcriptome induced by co-culture with *M. catarrhalis*

Microarray expression analysis was used to test the hypothesis that GAS gene transcript levels differed significantly during growth in co-culture with *M. catarrhalis* compared to GAS pure culture alone. Principal component analysis (PCA; Partek Genomics Suite) was performed and indicated that the two different growth conditions (co-culture versus single culture) generated distinct GAS gene transcription profiles, with multiple virulence and energy metabolism genes being differentially expressed upon co-culture compared to the constitutively expressed control gene *proS* (Table 2).

GAS transcription expression levels were affected by a factor of at least 4-fold or greater for a total of 207 genes (61 genes with increased expression and 147 genes with decreased expression), after growth in co-culture and relative to the growth of GAS alone. Further, BLAST searching of whole genome sequences revealed that only a small percentage of the *M. catarrhalis* RNA transcripts were predicted to cross-hybridize with the GAS MGAS315 gene chip. When these potentially cross-hybridizing genes were

Table 2. List of GAS serotype M3 MGAS16655 genes exhibiting the most significant difference in expression during co-culture with *M. catarrhalis* when compared to GAS serotype M3 culture alone

Spy no. in strain MGAS315	Fold change	Gene	Function
SpyM3_1851	181,321	*hasA*	Hyaluronan synthase
SpyM3_0616	34,675	*pyrF*	Orotidine phosphate decarboxylase
SpyM3_0617	29,003	*pyrE*	Orotate phosphoribosyltransferase
SpyM3_0558	26,203	*pyrR*	Uracil phosphoribosyltransferase / Pyrimidine operon regulatory protein PyrR
SpyM3_0559	24,299	*pyrP*	Uracil permease
SpyM3_1716	22,044	-	Streptococcal mitogenic exotoxin Z (SmeZ)
SpyM3_0132	20,776	-	hypothetical protein
SpyM3_1853	20,445	*hasC*	UTP-glucose-1-phosphate uridylyltransferase
SpyM3_0777	19,294	*radC*	DNA repair protein RadC
SpyM3_1543	16,992	-	Nicotinamidase
SpyM3_0618	15,182	*amiC*	6-aminohexanoate-cyclic-dimer hydrolase
SpyM3_0561	13,653	*carA*	Carbamoyl-phosphate synthase small chain
SpyM3_0583	12,703	-	Immunoglobulin G-endopeptidase (IdeS) / Secreted immunoglobulin binding protein (Sib38)
SpyM3_0560	12,479	*pyrB*	Aspartate carbamoyltransferase
SpyM3_0562	11,733	-	-
SpyM3_1652	-49,579	*salA*	Lantibiotic salivaricin A
SpyM3_1655	-51,635	*lacF*	PTS system, lactose-specific IIA component
SpyM3_1678	-53,819	*ulaA*	Transport protein SgaT, putative
SpyM3_1657	-54,741	*lacC.2*	Tagatose-6-phosphate kinase (EC 2.7.1.144) / 1-phosphofructokinase
SpyM3_1482	-58,292	*lacD.1*	Tagatose 1,6-diphosphate aldolase
SpyM3_1486	-78,036	-	PTS system, galactose-specific IIC component
SpyM3_1742	-79,421	*speB*	Strepotococcal cysteine protease (Streptopain) / Streptococcal pyrogenic exotoxin B (SpeB)
SpyM3_1484	-84,780	*lacB.1*	Galactose-6-phosphate isomerase, LacB subunit
SpyM3_1679	-93,265	-	PTS system IIB component
SpyM3_1656	-106,265	*lacD.2*	Tagatose 1,6-diphosphate aldolase
SpyM3_1659	-109,750	*lacA.2*	Galactose-6-phosphate isomerase, LacA subunit
SpyM3_1485	-125,047	*lacA.1*	Galactose-6-phosphate isomerase, LacA subunit
SpyM3_1751	-162,809	-	PTS system, cellobiose-specific IIB component
SpyM3_1680	-371,559	-	Transcription antiterminator, BglG family
SpyM3_1677	-431,501	-	Transaldolase

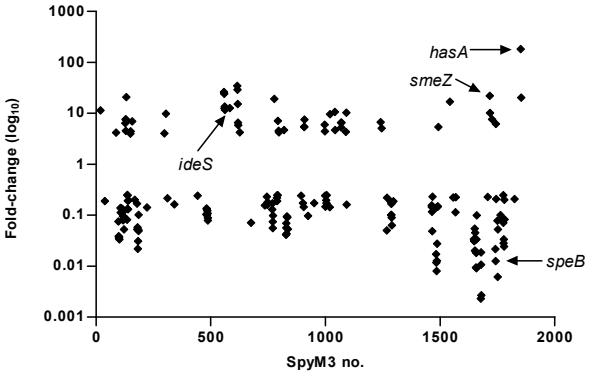

Figure 2. Genes showing significant changes in expression, using a 4-fold cut-off value, for GAS serotype M3 MGAS16655 when grown in co-culture with *M. catarrhalis*. Results were calculated relative to gene expression values obtained for GAS M3 MGAS16655 grown in pure culture alone. One hundred and ninety-two genes were either 4-fold increased (52 genes) or 4-fold decreased (140 genes) in expression, which corresponds to 10% of the MGAS315 genome. The *hasA*, *ideS*, *smeZ* and *speB* genes have been associated with GAS virulence.

subtracted from the list of statistically significant, ≥4-fold, GAS expression transcripts, a total of 192 genes (207 minus 15 genes) remained. The final list comprised 52 genes with significantly increased expression and 140 genes with significantly decreased expression (Figure 2).

Significant down-regulation was observed for many genes of the functional subcategories as shown in Figure 3 (73% of the genes whose expression was modified were down-regulated in co-culture relative to single culture), for example genes involved in signal transduction were all down-regulated. In contrast, genes encoding proteins involved in purine/pyrimidine metabolism were generally up-regulated. A key finding was that a ~180-fold increase and ~80-fold decrease in the expression of *hasA* and *speB* was observed, along with a significant decrease in the expression of many genes involved in carbohydrate utilization. Further, gene expression in co-culture resulted in significant differences in several GAS two-component systems and response regulatory systems (Table 3), compared to the results observed for GAS pure culture alone.

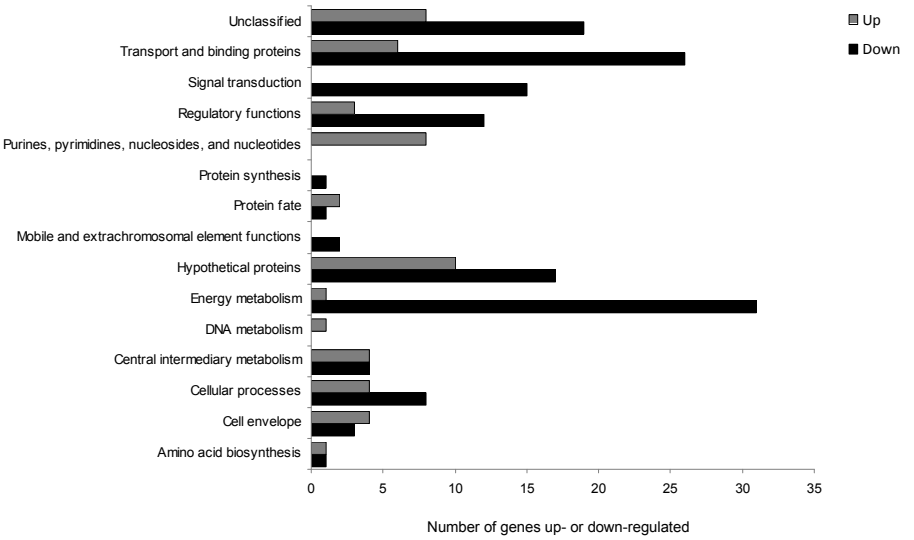

Figure 3. Number of genes up- or down-regulated after GAS co-culture with *M. catarrhalis*. Genes were classified into 13 main functional categories. Genes associated with energy metabolism comprised the most frequent down-regulated genes.

Table 3. Significant differences observed in GAS response regulatory (RR) systems for GAS serotype M3 MGAS16655 and *M. catarrhalis* JM150 grown in co-culture and relative to GAS serotype M3 MGAS16655 grown as a pure culture

Colonization					Persistence				
Spy no. in strain MGAS315	RR	Gene	Kreikemeyer *et al*. (2003)	This study (fold change)	Spy no. in strain MGAS315	RR	Gene	Kreikemeyer *et al.* (2003)	This study (fold change)
SpyM3_1227	Mga	*emm*	+	+1.7	SpyM3_0480	RALPs	*sagA*	-	-2.8
SpyM3_1726	Mga	*scpA*	+	+7.6	SpyM3_0097	RALPs	*nra*	-	-3.9
SpyM3_1630	Mga	*fba*	+	+1.7	SpyM3_1728	RALPs	*mga*	-	+1.6
SpyM3_0097	Mga	*nra*	+	-3.9	SpyM3_1301	RALPs	*speA*	-	+2.5
SpyM3_1728	Mga	*mga*	+	+1.6	SpyM3_1742	RALPs	*speB*	-	-79

Validation of DNA microarray data

TaqMan quantitative real-time reverse transcription PCR analysis confirmed the validity of the DNA microarray results for 2 representative and significantly up- or down-regulated virulence genes, and the constitutively expressed control gene *proS* (Table 4).

Table 4. Fold-change in gene expression observed for 2 virulence-associated genes (and a control *proS*) during co-culture versus pure culture, using a GAS MGAS16655 microarray and gene specific TaqMan assays

Gene	Spy no. in strain MGAS315	Fold-change co-culture (MGAS16655 Array)	Fold-change co-culture (MGAS16655 TaqMan)
hasA	SpyM3_1851	181,3212	472,4987
proS	SpyM3_1688	-1,9155	-2,2436
speB	SpyM3_1742	-79,4210	-75116,0372

Discussion

The primary goal of this study was to investigate the effect of *M. catarrhalis* co-culture on global GAS serotype M3 gene expression, compared to GAS serotype M3 growth in pure culture alone. In this comparative co-culture study, the expression of 192 GAS genes were found to be significantly affected by at least a 4-fold difference as compared to growth of GAS serotype M3 alone. Several of the most significantly up-regulated genes during co-culture were known virulence genes (hyaluronan synthase, streptococcal mitogenic exotoxin Z and IgG-endopeptidase), as well as genes involved in purine and pyrimidine metabolism. In contrast, in general, the genes most significantly down-regulated upon co-culture were genes involved in energy metabolism.

The hyaluronic acid capsule (hyaluronan synthase gene *hasA*) is a virulence factor of GAS, playing a role in resistance to phagocytosis, bacterial aggregation, biofilm maturation, resistance to host defense peptides and neutrophil extracellular killing [2, 22]. GAS also produces a number of highly potent exotoxins that act as superantigens. The streptococcal mitogenic exotoxin Z (SmeZ) is the most potent bacterial superantigen so far discovered and enhances the cascade of pro-inflammatory events that follow invasive streptococcal infection [23-24]. GAS also secretes a highly effective IgG-endopeptidase (IdeS) that inhibits phagocytic killing by cleavage of specific IgG [25-27]. These virulence genes were all up-regulated during co-culture, indicating that the presence of *M. catarrhalis* could lead to an increase in the pathogenic potential of GAS (with concomitant clinical implications) compared to GAS infection alone.

Biofilm formation may be considered a developmental process that is characterized by the expression and regulation of distinct structural and regulatory genes during the steps that are required for cell-surface interactions (binding), maturation (sessile

phase) and for a return to the planktonic lifestyle [28]. Recently, biofilms have become a topic of interest in the study of GAS, as many GAS genes show a cell density-dependent pattern of regulation. Further, GAS genes required for the development of biofilms are also important virulence factors, being important for invasion and survival of GAS in the human host [29-34]. For example, in a study performed by Cho *et al.* (2005), the hyaluronic acid capsule was found to be essential in biofilm formation, and an increase in *speB* expression was observed in biofilm formation compared to planktonic culture [34]. However, these results were in contrast to the study of Roberts *et al.* (2010), who found that an increased activity of SpeB contributed to the loss of the biofilm phenotype [30]. From our own results, *speB* was down-regulated 80-fold, though confusingly, *hasA* showed a 180-fold increase in expression. These often contradictory findings between *hasA* and *speB* may possibly be explained by the fact that only a subset of GAS strains is able to form biofilms, and that the studies performed used different GAS serotypes [31-32]. However, the authors could find no information regarding the biofilm forming capacity of serotype M3 GAS, or previous research on the effect of *M. catarrhalis* on GAS gene expression and biofilm forming capacity.

With respect to GAS response regulators and regulatory networks, Kreikemeyer *et al.* (2003) indicated distinct patterns of gene regulation associated with particular global response regulators during colonization, persistence and spread of GAS [35]. Though our results are not conclusive, comparison of our data to the data presented by Kreikemeyer *et al.* (2003), appeared to indicate a transition from GAS colonization to persistence during co-culture, which would be consistent with a hypothesis involving biofilm formation. Moreover, our results were obtained by comparing co-culture GAS with pure culture GAS, this means that our results were corrected for any possible effect associated with the time of isolate sampling, i.e. sampling at the transition from exponential to early-stationary phase. Co-culture may therefore increase the GAS population of cells undergoing the transition from a colonizing to a persistence phase in a polymicrobial culture.

Our findings with respect to transcriptome expression are particularly relevant in the context of recent research detailing the effect of quorum sensing on the polymicrobial interactions of other respiratory bacterial pathogens. In particular, a recent study by Armbruster *et al.* (2010) showed that co-colonization of *M. catarrhalis* and *H. influenzae* (bacterial pathogens sharing the same respiratory niche as GAS) results in increased *M. catarrhalis* biofilm formation and resistance to antibiotics. This process was achieved via an autoinducer-2 (AI-2, *luxS*) quorum signaling system [9]. At the same time,

Verhaegh *et al.* (2010), showed that co-colonization of *M. catarrhalis* and *H. influenzae* was significantly more likely than single species colonization with either *M. catarrhalis* or *H. influenzae* alone, adding clinical epidemiological evidence to the laboratory-based findings [10]. In fact, many species of bacteria use quorum sensing systems to coordinate their gene expression and biofilm formation, dependent on the local bacterial cell density. A homolog of *luxS*, the genetic determinant for AI-2 production, has been identified in GAS, though a *luxS* homolog in *M. catarrhalis* has not been found. However, evidence from whole genome sequencing does suggest that *M. catarrhalis* possesses an AI-1 *luxR* sensing system, but no quorum signaling system [36]. Currently, a role for quorum sensing systems in facilitating the observed GAS - *M. catarrhalis* polymicrobial interaction is still open to question, not least due to the fact that less 'targeted' mechanisms could be responsible for the change in GAS transcriptome profile during co-culture, including competition for nutrients.

The 15 genes that showed the most significant decrease in expression during co-culture were mainly involved in energy metabolism, with an emphasis on galactose metabolism. Nutrient availability is a signal by which pathogens sense their external environment and to which they respond through regulated production of various virulence factors. Studies have shown that co-cultures compete for nutrients and that interactions between co-cultured strains also involve species-specific pH limits for growth and differential utilization of growth substrates [37-38]. The success of GAS, which thrives in diverse host niches, depends on the acquisition of nutrients from very different sources and GAS virulence gene expression is highly responsive to carbohydrate source and availability; however, the pathways linking metabolism and virulence remain poorly understood [15, 39]. The authors observed trends in the metabolism-related transcriptional response of GAS during co-culture with *M. catarrhalis*, with the majority of the most significant differences occurring in the down-regulation of genes involved in carbohydrate utilization. This finding could be associated with a GAS transcriptional response to depletion of carbohydrate (and possible other nutrients) within the environment, thereby helping GAS to conserve its 'energy reserves', and ultimately facilitating entry into a sessile 'state of rest' (associated with biofilm formation) [40]. However, it should be noted that *M. catarrhalis* is biochemically asaccharolytic, meaning that it is unable to metabolize glucose or other carbohydrates [41]. Therefore, the true relationship between *M. catarrhalis* and GAS co-culture, carbohydrate depletion, and the down-regulation of genes involved in carbohydrate utilization remains to be further elucidated.

Conclusion

The transcriptome profile of GAS is markedly altered in response to co-culture with *M. catarrhalis*, with genes involved in virulence (up-regulated) and carbohydrate utilization (down-regulated) being especially affected. There also appeared to be an increase in GAS transition from a colonizing to a persistence phase. This study is a first step in understanding the mechanisms that help facilitate colonization and disease during polymicrobial infections with GAS and *M. catarrhalis*. The results may be useful in determining the prognosis of infection, with respect to polymicrobial versus pure culture, as well as identifying new pathologies in GAS-*M. catarrhalis* polymicrobial morbidity.

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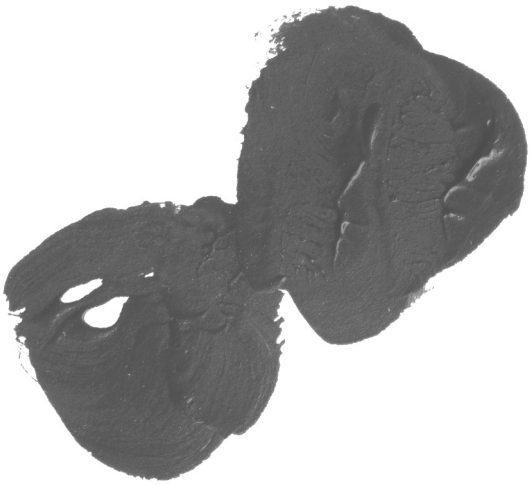
Humoral immune response



Chapter 8

Temporal development of the humoral immune response to surface antigens of *Moraxella catarrhalis* in young infants

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Abstract

The primary *Moraxella catarrhalis*-specific humoral immune response, and its association with nasopharyngeal colonization, was studied in a cohort of infants from birth to 2 years of age.

Results indicated that the levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time, with IgM and IgA levels to all 9 OMPs being relatively low throughout the study period. In contrast, the level of antigen-specific IgG was significantly higher for $Hag^{385-863}$, $MID^{764-913}$, $MID^{902-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ OMPs in cord blood compared to 6 months of age ($P \leq 0.001$). This was a consequence of maternal transmission of antigen-specific IgG to newborn babies, possibly indicating a future role for these three surface antigens in the development of an effective humoral immune response to *M. catarrhalis*. Further, genetic variation in immunologically relevant regions of vaccine candidate genes was observed, indicating that the *M. catarrhalis* OMPs UspA1, UspA2 and Hag/MID are likely to possess major epitopes for immune evasion.

Finally, at 2 years of age, the levels of antigen-specific IgG still remained far below that obtained from cord blood samples, indicating that the immune response to *M. catarrhalis* has not matured at 2 years of age. We provide evidence that a humoral antibody response to OMPs UspA1, UspA2 and Hag/MID may play a role in the immune response to community acquired *M. catarrhalis* colonization events.

Introduction

Moraxella catarrhalis is an aerobic, Gram-negative diplococcal commensal of the respiratory tract. Although healthy children are frequently colonized with this bacterium, it is also able to cause disease, being especially associated with otitis media (OM), as well as exacerbations of chronic obstructive pulmonary disease (COPD) in adults.

Studies have shown that the bacterium colonizes the nasopharynx soon after birth and that the carriage rate of *M. catarrhalis* in healthy children may differ per geographical region, season and year of isolation [1]. For example, in a German study (November 1991 to April 1992), 9% of children attending day-care centers ranging in age from 4 months to 3 years old were colonized with *M. catarrhalis* [2]. In a Japanese study conducted in 1999, children aged 1 month to 5 years attending day-care centers, 35% were found to be colonized [3]. In The Netherlands, a comparative study of 1.5 to 14 month old children born between February 2003 and August 2005 indicated a carriage rate for children ranging from 11.8% at the age of 1.5 months to 29.9% at the age of 6 months and 29.7% at the age of 14 months [4]. In general, despite local geographical variation, infants tend to become colonized with *M. catarrhalis* at a very early age, resulting in a nasopharyngeal colonization peak for *M. catarrhalis* at 2 years of age [5].

Bacterial adherence to the respiratory mucosa is an essential step towards colonization of the human respiratory tract epithelium, and research has indicated that the most important adhesins responsible for the attachment of *M. catarrhalis* to host cells include the outer membrane proteins (OMPs) UspA1, UspA2 and Hag/MID, though several other surface-exposed outer membrane proteins have been described that may also play a role in the process. Further, with respect to *M. catarrhalis*, it has been shown that colonization of the human respiratory tract epithelium results in an increased risk of disease, specifically OM disease (both chronic and acute) in children [6, 7]. Therefore, it is reasonable to expect that an effective immune response raised against UspA1, UspA2 and Hag/MID, for example via vaccination, will have a significant effect on colonization and disease.

OM is one of the major childhood diseases that necessitate visits to general practitioners [8]. In 2004, the American Academy of Pediatrics (AAP) published new guidelines that addressed the diagnosis and treatment of acute otitis media (AOM), largely because the treatment of AOM is not always appropriate, and the long-term overuse of antibiotics increases the risk of the development of antimicrobial resistance. The AAP guidelines recommended the use of observation as a potential strategy for the treatment of AOM, although global rates of antibiotic prescription for AOM still vary greatly [9-11].

An alternative strategy to the use of antibiotics in the treatment of OM disease is vaccination [12]. However, there is currently no licensed vaccine available against *M. catarrhalis*, and none of the antigens so far described (which may serve as potential vaccine candidates) have progressed to clinical trials. The challenge in identifying potential vaccine candidates for *M. catarrhalis* lies in identifying antigens that are able to generate an appropriate immune response that prevents the process leading from colonization to infection. It is known that healthy adults possess naturally acquired serum antibodies directed against several *M. catarrhalis* OMPs, apparently via the acquisition and elimination of many different *M. catarrhalis* strains [13]. Further, changes in antibody response are observed in adults suffering from *M. catarrhalis*-mediated COPD disease [14].

Several new *M. catarrhalis* OMP vaccine candidates have been described in the literature, and previous studies have indicated that a multivalent vaccine comprising a combination of epitopes of these *M. catarrhalis* OMP vaccine candidates should form the basis of a vaccine to prevent *M. catarrhalis*-mediated colonization and disease [13, 15, 16].

However, relatively little is known about the humoral immune response to these vaccine candidates, especially within the first few years of life. The present study was performed to determine the humoral immune response to currently available *M. catarrhalis* OMPs in healthy Dutch children from birth to 2 years of age. Further, the relationship between *M. catarrhalis* colonization and immune response was also investigated.

Materials and methods

Study cohort

This study was embedded in the Generation R Study, a population-based prospective cohort study, designed to identify early environmental and genetic causes of normal and abnormal growth, development and health from fetal life until young adulthood [17]. This study was performed in a randomly selected subgroup of Dutch children whose parents are ethnically homogeneous (two parents and four grandparents born in The Netherlands), in order to exclude possible confounding factors associated with ethnicity.

In total, 57 infants who were born between February 2003 and August 2005 were included in this study. Three or 4 serial serum samples were collected from each infant for inclusion in the study. The collection totalled 177 samples, comprising 54 (31%) cord blood samples, 32 (18%) samples obtained at 6 months, 46 (26%) samples obtained

at 14 months, and 45 (25%) samples obtained at 24 months of age. The bacterial colonization status was determined by taking nasopharyngeal swabs at the ages of 1.5, 6, 14 and 24 months of age, with swabs being taken at the same time as serum samples. Swabs were obtained from 40 (70%), 49 (86%), 50 (88%) and 48 (84%) infants at 1.5, 6, 14 and 24 months of age, respectively. The colonization status was determined using standard *M. catarrhalis* culture and detection techniques [1].

Moraxella catarrhalis antigens

The previously described *M. catarrhalis* recombinant proteins UspA1$^{557\text{-}704}$ (aa 557–704 of UspA1), UspA2$^{165\text{-}318}$, MID$^{764\text{-}913}$, MID$^{902\text{-}1200}$, Hag$^{385\text{-}863}$, MhaC, McaP$^{51\text{-}333}$, orf 238 and orf 296 were used in this study [13, 18-22]. These recombinant proteins represent the majority of the *M. catarrhalis* immunogenic proteins discovered to date.

Antigen coupling

Recombinant proteins were coupled to SeroMAP™ beads, which are carboxylated beads that are developed for serological applications. The coupling procedure was performed as detailed by Verkaik *et al.* [23]. Briefly, 5.0×10^6 microspheres were resuspended in 100 mmol/L monobasic sodium phosphate (pH 6.2) buffer. For activation of the carboxyl groups on the surface of the beads, 10 µl of 50 mg/ml of *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino) ethanesulfonic acid, pH 5.0 (Sigma-Aldrich) in which 25 µg of protein was added. The final concentration of microspheres was adjusted to 4000 beads/µl with blocking-storage buffer (PBS-BN; PBS, 1% bovine serum albumin, and 0.05% sodium azide [pH 7.4]). The microspheres were protected from light and stored at 4°C until required. All centrifugation steps were performed at 12,000 g for 2 minutes at room temperature (RT).

Uncoupled beads were used as a negative control, and to determine non-specific binding. If minor non-specific binding was observed, then the median fluorescence intensity (MFI) values obtained from this non-specific binding was subtracted from the antigen-specific results.

Multiplex *M. catarrhalis* antibody assay

The multiplex procedure was performed as described elsewhere [23]. Briefly, after validation of the assay (where human pooled serum (HPS) MFI multiplex assay val-

ues were compared to corresponding HPS MFI singleplex assay values), the different antigen-coupled microspheres were mixed to a working concentration of 4000 beads per color per well. Serum samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG and IgA and 1:50 for measurement of IgM. Fifty microliters per diluted sample was incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 minutes at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with PBS-BN that was aspirated by vacuum manifold, and the microspheres were resuspended in 50 µl of PBS-BN. In separate wells, 50 µl of a 1:100 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human IgG and IgA and 50 µl of a 1:50 dilution of RPE-conjugated donkey anti-human IgM (Jackson Immuno Research) were added. The plate was incubated for 35 minutes at room temperature and washed. The microspheres were resuspended in 100 µl of PBS-BN. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed in duplicate, and the fluorescence intensity values, reflecting quantitative antibody levels, were averaged. The coefficient of variation of these values was then calculated for each serum sample and averaged per protein and antibody isotype.

Vaccine gene carriage

M. catarrhalis isolates were grown from glycerol stocks at 37°C overnight on blood agar plates. DNA was extracted using the MagNA Pure LC System (Roche Applied Science). PCR was performed to detect the major identified *M. catarrhalis* vaccine candidates *uspA1*, *uspA2* and *hag/mid* genes. Primer pairs were used to detect the *uspA1* (RTF1-8 5'-cgttatgcactaaaagagcaggtc and RTB1-8 5'-gcatctgaccagcttagaccaatc) and *uspA2* (RTF2-10 5'-gcatctgcggataccaagtttg and RTB2-10 5'-ttgagccatagccaccaagtgc) genes according to the protocol of Meier *et al.* [24]. For the detection of the *hag/mid* gene, the primers McatHag-2 (5'-gtcagcatgtatcatttttttaagg) and McatHagR4 (5'-tgagcggtaaatggtt-taagtg) were used [16].

Further, PCR was performed to detect *ompJ* types (a surrogate genetic lineage marker), and 16S rRNA types as previously described by Hays *et al.* and Verhaegh *et al.* [16, 25].

To identify the 16S rRNA types of individual *M. catarrhalis* isolates, 16S rRNA PCR products were digested using the enzymes *Fsp*BI (10 U) and *Hha*I (10 U) according to Verhaegh *et al.* [16].

Isolate genotyping and vaccine-related gene diversity

M. catarrhalis genotyping was performed using pulsed-field gel electrophoresis (PFGE) as detailed by Verduin *et al.* [26]. Briefly, *M. catarrhalis* plug digestions were performed using *Spe*I at 20 U/reaction and an electrophoresis protocol comprising a 1st block with a constant voltage of 6 V cm^{-1}, a pulse time from 3.5 to 25 seconds during the first 12 hours, followed by a 2nd block of 8 hours where the pulse time increased linearly from 1 to 5 seconds. All PFGE patterns were analyzed using BioNumerics (Applied Maths), with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set to 1.5%. PFGE products between 48.5 and 339.5 kb were included in the band matching analysis.

The genetic diversity of the top three vaccine candidate genes (*uspA1*, *uspA2* and *hag*/*mid*) was assessed by aligning available NCBI gene sequences (http://www.ncbi.nlm.nih.gov/guide/) of *M. catarrhalis uspA1*, *uspA2* and *hag/mid* genes (Sci Ed Central for Windows 95, v1.2), in order to determine any variation present within *M. catarrhalis* OMP regions of interest [19, 20, 22].

Statistical analysis

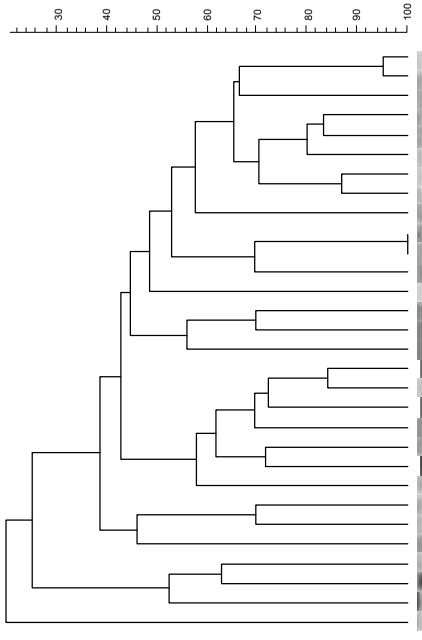
Statistical analyses were performed using SPSS PASW version 17. The Wilcoxon signed-rank test was used to compare the anti-*Moraxella* antibody levels between different age groups. The Mann-Whitney *U*-test was used to compare differences in antibody levels between colonized and non-colonized children. A *P*-value of ≤0.05 was considered to be statistically significant.

Results

Isolate genotyping, vaccine gene carriage and vaccine-related gene diversity

A high degree of genotypic heterogeneity in *M. catarrhalis* isolates colonizing children in the focus cohort was maintained over the entire study period, with no association found between genotype and any of the antigen-specific MFI values (Figure 1).

Ninety-seven percent (29/30) of the consecutive *M. catarrhalis* isolates were found to be positive for *uspA1*, with 90% (27/30) positive for *uspA2*, and 87% (26/30) positive for *hag/mid* gene carriage. In total, 87% (26/30) of the *M. catarrhalis* isolates



Age	Month	Year	Hag$^{385-863}$	MID$^{764-913}$	MID$^{902-1200}$	UspA1$^{557-704}$	UspA2$^{165-318}$
14	2	2006	168	1350	21	5112	3988
6	9	2004	693	1067	3	1587	629
6	1	2005	48	94	0	194	421
24	6	2005	76	1394	38	413	1563
6	8	2005	653	138	0	228	101
14	5	2006	77	607	8	263	170
24	1	2006	39	366	48	1196	368
14	2	2006	0	3189	1070	1399	2782
6	11	2005	175	294	0	83	148
14	12	2004	62	1568	118	5612	224
14	12	2004	10	7382	311	971	1740
14	12	2004	183	10073	488	484	10939
14	4	2006	247	3631	279	1411	252
6	12	2004	10	402	0	871	1098
24	7	2006	81	571	13	*	9773
14	6	2006	28	978	533	2481	322
24	1	2006	137	3518	1044	6207	4949
24	5	2006	415	6240	270	3003	3695
24	2	2006	77	1178	0	3838	7379
14	5	2006	98	673	51	397	409
24	11	2005	419	3041	62	3468	2467
6	12	2005	21	157	22	172	188
24	4	2007	142	1568	175	1953	2532
6	10	2004	183	1310	38	243	257
14	10	2004	489	981	0	459	963
6	2	2005	428	3937	0	476	995
24	6	2005	360	4461	322	1883	3082
6	8	2005	71	668	171	1319	2210
24	8	2005	125	4658	779	1664	8099
24	3	2007	3	1424	0	988	516

Figure 1. Relationship between consecutively isolated *M. catarrhalis* genotypes (isolated at 6, 14 and 24 months of age), and concurrent MFI values. No relationship between MFI value and genetic relatedness was observed for these isolates. Age nasopharyngeal swabs were taken: 6, 6 months; 14, 14 months; 24, 24 months, after birth. The month and year of isolate culture from the nasopharynx of children is also shown.

were categorized into 16S type lineage 1 (16S type 1; sero-resistant lineage), with the remaining 13% belonging to the 16S type lineage 2 (16S type 2 and 3; sero-sensitive lineage).

Of the 16 available NCBI complete gene sequences available for UspA1, when translated into protein, three submissions showed a deletion in the CEACAM-binding region, located in the vaccine region of UspA1$^{557-704}$. Alignment of the 15 available UspA2 protein sequence data revealed a high degree of variability within the vaccine region UspA2$^{165-318}$, including deletions and insertions when compared to the reference strain *M. catarrhalis* ATCC25238. In parallel, alignment of the 8 available Hag/MID protein sequence data for the vaccine candidate Hag$^{385-863}$ revealed a high degree of variability within the vaccine region, including deletions and insertions when compared to the reference strain *M. catarrhalis* O35E. For the recombinant proteins MID$^{764-913}$ and MID$^{902-1200}$, alignment of the 8 NCBI Hag/MID protein sequences revealed a high degree of gene variability within the MID$^{764-913}$ protein, including insertions and deletions, while for the IgD-binding protein MID$^{902-1200}$, 97% similarity was observed for the vaccine region between the two proteins sequences that differed the most in the initial alignment.

Dynamics of the anti-*Moraxella* antibody response

The changes measured in anti-*M. catarrhalis* IgG, IgA and IgM during the first 2 years of life are shown in Figure 2. The levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time. The level of antigen-specific IgG in cord blood (maternal antibody) was significantly higher for Hag$^{385-863}$, MID$^{764-913}$, MID$^{902-1200}$, UspA1$^{557-704}$ and UspA2$^{165-318}$ than at 6 months of age ($P \leq 0.001$), presumably due to passive immunization by maternally acquired IgG antibodies *in utero*. Such passive immunity typically remains until approximately 6 months after birth [27].

IgG levels against MID$^{764-913}$, MID$^{902-1200}$, UspA1$^{557-704}$ and UspA2$^{165-318}$ rose significantly between 6 months to 2 years of age. IgG levels to *M. catarrhalis* OMPs Hag$^{385-863}$, McaP$^{51-333}$, MhaC, orf238 and orf296 remained relatively low and did not significantly increase over the 6 month to 2 year time period.

IgM and IgA levels to all 9 OMPs were relatively low throughout the study period. However, IgM levels to the OMPs MhaC, MID$^{764-913}$, MID$^{902-1200}$, UspA1$^{557-704}$ and UspA2$^{165-318}$, and IgA levels to Hag$^{385-863}$, MID$^{764-913}$, MID$^{902-1200}$, UspA1$^{557-704}$ and UspA2$^{165-318}$ increased significantly ($P \leq 0.05$) over the 6 month to 2 year time period. Finally, not every infant developed an antigen-specific IgG, IgA or IgM response to all of the proteins tested in the first 2 years of life.

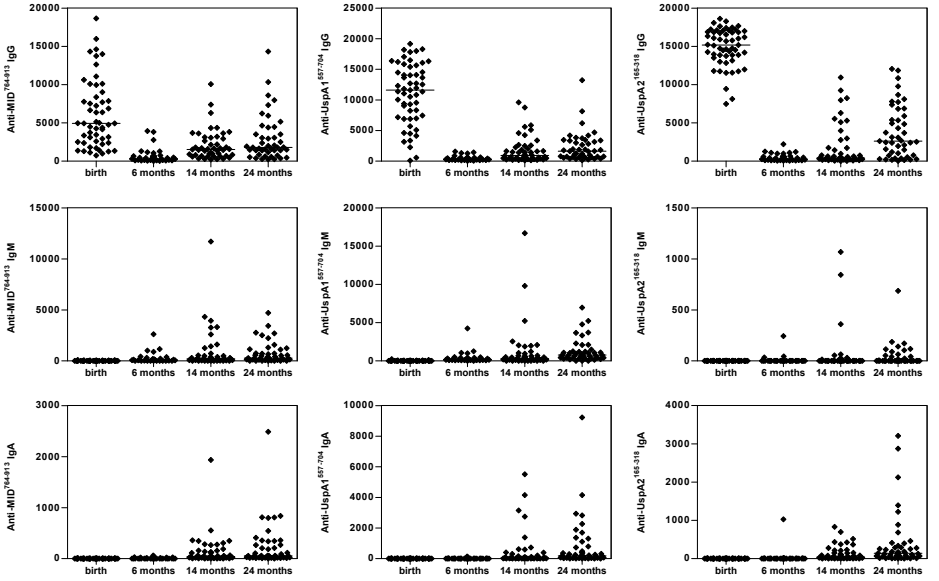


Figure 2. Levels of IgG, IgM and IgA directed against *M. catarrhalis* immunoglobulin D-binding protein (MID) and ubiquitous surface proteins A1 (UspA1) and A2 (UspA2) in 57 children at birth, 6 months, 14 months and 24 months. Antibody levels are reflected by MFI values. Each dot represents a serum sample. Median values are indicated by horizontal line.

Relationship between colonization and anti-*M. catarrhalis* antibody levels

In order to relate colonization status to changes in anti-*M. catarrhalis* antibody levels (which would provide an estimation of the efficacy of the immune response in preventing *M. catarrhalis* colonization), results were utilized from sera and nasopharyngeal colonization data of children at 6, 14 and 24 months of age where concurrent sera and nasopharyngeal swab data were available. Children were divided into colonized or non-colonized at each time period and their IgG levels to Hag$^{385-863}$, MID$^{764-913}$, MID$^{902-1200}$, UspA1$^{557-704}$ and UspA2$^{165-318}$ plotted.

In total, 9 (33%), 10 (24%) and 11 (29%) of the children were found to be colonized with *M. catarrhalis* at the time of sampling at 6, 14 and 24 months, respectively. There was no significant difference in IgG levels for all antigens between colonized and non-colonized children, except for MID$^{902-1200}$ at 24 months of age (P=0.04) (Figure 3). Further, the increase in IgG antibody response did not result in a decrease in the percentage of infants nasopharyngeal colonized by *M. catarrhalis*, although antigen-

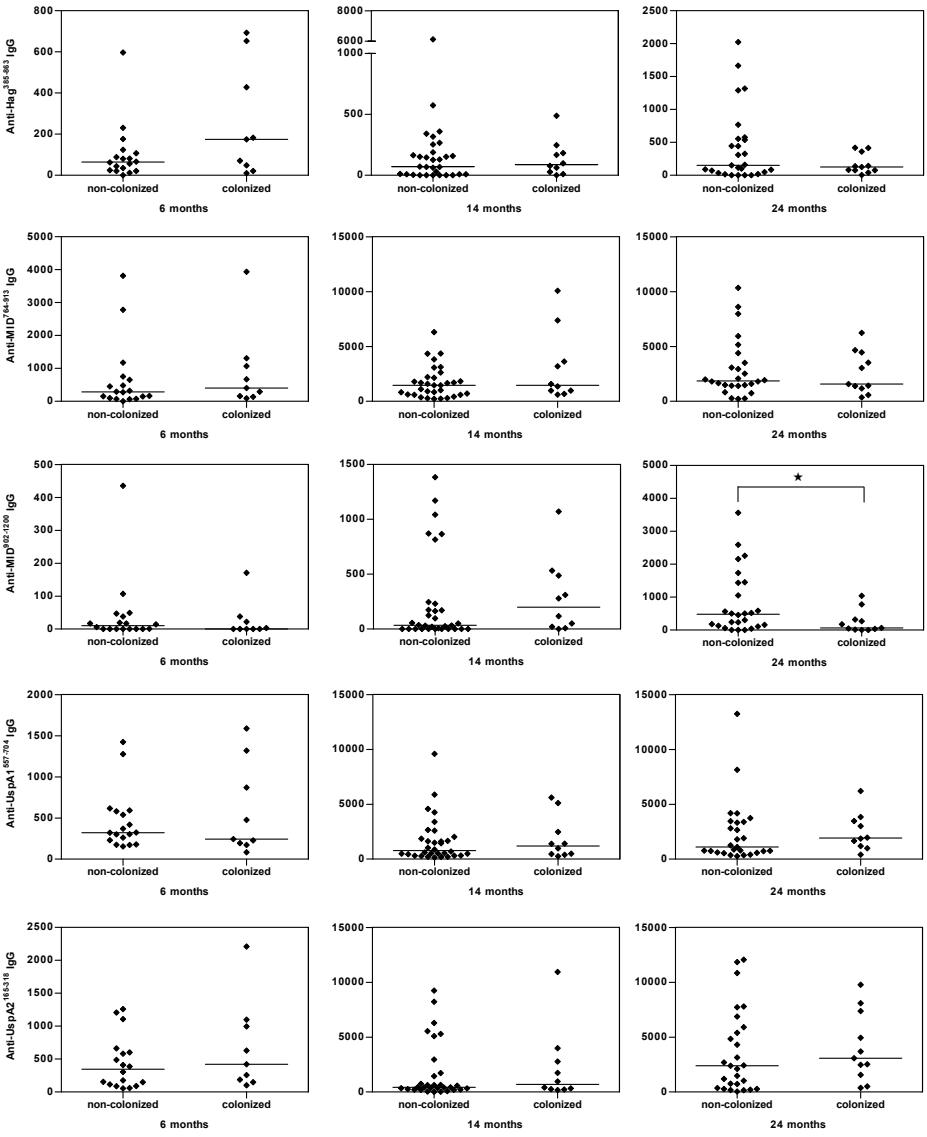


Figure 3. Relationship between *M. catarrhalis* colonization and anti-Hag/Mid, UspA1 and UspA2 IgG levels at 6, 14 and 24 months of age, as reflected by MFI values. Median values are indicated by horizontal line. A significant difference between non-colonized and colonized children was observed for MID$^{902-1200}$ at 24 months of age (* P=0.04).

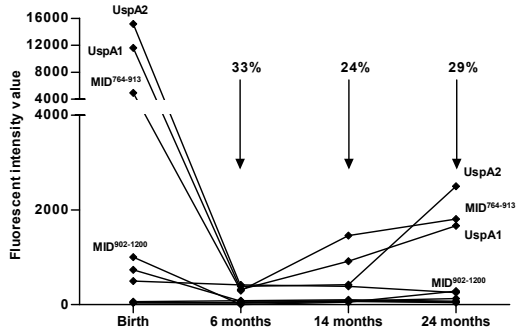


Figure 4. Relationship between *M. catarrhalis* colonization and anti-*M. catarrhalis* IgG levels at 6, 14 and 24 months of age, as reflected by MFI values.

specific IgG levels significantly increased for $MID^{764-913}$, $MID^{902-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ between 6 months and 2 years of age (Figure 4).

Discussion

The research performed in this publication represents the most extensive study of the infant immune response to potential vaccine candidates of *M. catarrhalis* performed to date, utilizing 9 different *M. catarrhalis* OMPs in a cohort of 57 healthy children followed from birth until 2 years of age. Further, the study was performed using multiplexed Luminex's xMAP technology that proved to be a rapid method for research into humoral immune response changes during *M. catarrhalis* colonization.

In our study, the level of antigen-specific IgG to *M. catarrhalis* antigens in cord blood was significantly higher compared to the anti-*M. catarrhalis* IgG level at 6 months, most likely due to the presence of maternally derived IgG antibodies that were transferred to the fetus through the placenta. The passage of antibodies between mother and baby, via the umbilical cord, gives rise to 'passive immunity', which generally tends to confer humoral protection against infection until approximately 6 months after birth [27]. During this 6 month period, passively acquired antibodies disappear and are replaced by antibodies generated by the infants' own 'actively acquired' humoral immune response. This actively acquired immune response may be generated by successive rounds of colonization and/or infection by pathogens, leading to the development of a host-specific immune response and eventual pathogen clearance. In this respect, Ejlertsen *et*

al. (1994) showed a significant fall in antibody concentration during the first 3 months of life, and a steady low level was maintained in the age group from 3 to 10 months, similar to the results obtained in this study [28]. Further, from the age of 1 year, the immune response of the children in the study of Ejlertsen *et al.* (1994) and Tan *et al.* (2006) increased slowly to reach maternal levels at the age of 10 years and in healthy adults, and though only sampling children up to 2 years of age, our study also showed increases in IgG antibody response for the antigens $MID^{764-913}$, $MID^{902-1200}$, $UspA1^{557-704}$ and $UspA2^{165-318}$ between years 1 and 2. The peak level of *M. catarrhalis* colonization (2 years of age) and where children have a high susceptibility to *M. catarrhalis* infections coincides with the age where the levels of *M. catarrhalis* IgG antibodies are low [5, 28]. This suggests an association between the lack of a host-specific protective humoral immunity towards *M. catarrhalis* and colonization.

Although *M. catarrhalis* is considered to be a major mucosal pathogen of the human respiratory tract, the salivary immunoglobulin A (IgA) response towards OMPs of *M. catarrhalis* remained relatively low throughout the study period. Two forms of IgA can be distinguished based upon their location (serum IgA and secretory IgA). In its secretory form, IgA is the main immunoglobulin found in mucous secretions, including respiratory epithelium. Studies have shown that human salivary IgA response is directed consistently against a small number of major OMPs in healthy adults and adults suffering from COPD. It is also found in small amounts in blood [29]. This may explain the relatively low levels of IgA found in this study, as serum antigen-specific IgA levels were measured and not secretory IgA [30, 31].

The IgM levels to all 9 OMPs were also relatively low throughout the study period. IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure. In contrast to IgG, IgM (and also IgA) antibodies do not pass across the human placenta.

Though an antibody response was generated against our *M. catarrhalis* OMP vaccine candidates in our focus cohort group during the first 2 years of life, the relatively constant level of *M. catarrhalis* nasopharyngeal colonization observed within the cohort suggests that the antibody response measured did not provide significant protection against *M. catarrhalis* nasopharyngeal colonization up to 2 years of age, with the exception of antigen $MID^{902-1200}$. Non-colonized children showed significantly higher IgG levels for $MID^{902-1200}$ compared to colonized children at 24 months of age. This result provides preliminary evidence that antibodies raised against $MID^{902-1200}$ could offer protection against *M. catarrhalis* colonization. If indeed confirmed, then a $MID^{902-1200}$

vaccine may possibly be used to boost immunity levels at or before 2 years of age, in order to provide protection against *M. catarrhalis* colonization, and hence disease. However, further research is required to investigate this hypothesis.

The factors influencing *M. catarrhalis* colonization and elimination are not yet fully understood, though genetic variation and adhesion to mucosal receptors appear to play an important role in colonization dynamics [32]. For example, several studies have shown that children acquire and eliminate a number of different strains throughout the first 2 years of life by the ability of evading the host immune system, caused by phase variation and antigenic variation [33]. Under 'immune pressure', antigenic variation due to sequence changes in virulence genes may provide a selective advantage for bacterial isolates expressing novel sequence variants. Alternatively, mutations may generate phase variable gene expression, switching off genes that are recognized by the immune system. Specifically, *M. catarrhalis* OMPs UspA1, UspA2 and Hag/MID are known to undergo phase variation, with antigenic variation reported in the target region of monoclonal antibody (MAb) 17C7 (a conserved UspA1 and UspA2 binding site) [34-39]. The relatively constant level of *M. catarrhalis* nasopharyngeal colonization observed within the cohort could also be related to host factors, for example relatively low levels of antibody at 2 years of age, or lack of effective antibody neutralizing activity [28, 40]. In any case, our genotyping results indicated that circulating *M. catarrhalis* isolates cultured during the study period were non-clonal in nature, a factor that may need to be taken into consideration upon further investigation of the efficacy of the OMP isolates utilized in this study.

Further research is required in order to determine whether increased IgG levels against the OMPs UspA1, UspA2, and Hag/MID (induced for example via vaccination) would significantly reduce the incidence of *M. catarrhalis* colonization and infection in infants up to 2 years of age (and in later life). In this respect, further studies are being planned at 5 years of age.

The introduction of a vaccination strategy against *M. catarrhalis* (either in children and/or in adults) is still a topic for debate, though the continuing high prevalence of OM disease in children and the rising prevalence of COPD in adults means that *M. catarrhalis*-associated disease continues to increase in global significance. Further, the introduction of successful vaccines against respiratory bacterial pathogens that occupy the same niche as *M. catarrhalis*, e.g. *S. pneumoniae* and *H. influenzae*, could facilitate a concomitant increase in *M. catarrhalis* colonization and infection. Though further research is required, our results indicate that at 2 years of age, the antibody response

to *M. catarrhalis* is still developing, and is largely based on an IgG isotype of antibodies raised against 3 major OMPs (i.e. UspA1, UspA2 and Hag/MID). We also provide preliminary evidence to suggest that antibodies directed against Hag/MID may be effective in preventing *M. catarrhalis* colonization, though natural variation in amino acid sequences of this protein may act to limit the potential of vaccines created to generate an immune response against Hag/MID.

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Transparency declaration

The Generation R Study is conducted by the Erasmus MC, Rotterdam, in close collaboration with the School of Law and Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service Rotterdam area, the Rotterdam Homecare Foundation and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond (STAR), Rotterdam. The first phase of the Generation R Study was made possible by financial support from the Erasmus MC, Rotterdam, the Erasmus University Rotterdam and The Netherlands Organization for Health Research and Development (ZonMw). Additionally, an unrestricted grant from the Europe Container Terminals (ECT) Rotterdam funded this project. Finally, this work was supported by a grant from the European Union, FP6 Project OMVac 037653.

All authors declare that they have no conflicts of interest.

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Chapter 9

Comparative analysis of the humoral immune response to *Moraxella catarrhalis* and *Streptococcus pneumoniae* surface antigens in children suffering from recurrent acute otitis media (rAOM) and chronic otitis media with effusion (COME)

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In preparation

Abstract

A prospective clinical cohort study was established to investigate the humoral immune responses against bacterial surface proteins in children suffering from rAOM and COME disease. Specifically, the humoral immune responses against putative vaccine candidates of *M. catarrhalis* and *S. pneumoniae* were determined in serum and MEF and their association with COME and rAOM disease was investigated. The association between humoral immune response and nasopharyngeal colonization was also studied.

Results indicated that the levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variation, with significantly higher serum IgG levels observed for the pneumococcal proteins PLY and PsaA in children suffering from COME compared to rAOM (P=0.029 and P=0.039). In MEF, significantly higher IgG and IgA levels were observed for children suffering from COME compared to rAOM for the pneumococcal proteins sp0376 and PpmA (P=0.044 and P=0.046). Day-care attendance was a risk factor for *M. catarrhalis* colonization in COME, and *M. catarrhalis* non-colonized children suffering from COME showed significantly higher IgG levels against the *M. catarrhalis* antigen $MID^{764-913}$ when compared to COME colonized children (P=0.027). Further, OM *S. pneumoniae* negative children suffering from COME showed significantly higher MEF IgG and IgA levels to the pneumococcal proteins BVH-3 and IgA-1 compared to OM *S. pneumoniae* positive children (P=0.046 and P=0.022). A strong correlation was observed between antigen-specific serum and MEF IgG levels.

Based on our preliminary findings, the pneumococcal BVH-3 and IgA-1 proteins and *M. catarrhalis* Hag/MID antigen should be further investigated as possible vaccine candidates in COME children.

Introduction

Otitis media (OM) is an important upper respiratory tract disease of early childhood and the primary reason for children to visit a physician. The disease has a considerable negative impact on the quality of life during childhood and causes much concern to parents. OM encompasses a spectrum of conditions, including acute otitis media (AOM) and otitis media with effusion (OME), with approximately 80% of children experiencing an episode of AOM by the age of 3 years. Up to one-third of children will have experienced recurrent infections, with many of these episodes being facilitated by a bacterial infection [1-2]. In fact, bacteria may be isolated from the middle ear fluid (MEF) of approximately 80% of children with AOM, and 30% of chronic middle ear effusions obtained from children presenting with OME [3]. In many countries, OM is a common reason to prescribe antibiotics, or to undergo surgery for the placement of grommets [4-5], resulting in a significant burden on healthcare systems and being a key contributor to antibiotic resistance [6]. This means that the direct costs associated with OM are substantial [7], and that the prevention of OM disease via alternative methods such as vaccination offer a promising approach to reduce the burden of OM disease and its economic consequences.

Streptococcus pneumoniae is reported to be the predominant bacterial species cultured in AOM disease, followed by *Haemophilus influenzae* and *Moraxella catarrhalis*. However, *H. influenzae* tends to predominate in OME disease, followed to a lesser extent by *S. pneumoniae* and *M. catarrhalis* [8-10]. These rankings are subject to some debate, as there are still issues relating to which bacterial species actually play the most significant role in middle ear infections [9, 11], though all of these common OM-related bacterial species may be cultured from the nasopharynx of children during OM episodes, either as single pathogens or as co-cultures [12]. Normal healthy children are only occasionally colonized with these pathogens, and after they become colonized, they rapidly eliminate the pathogens. In contrast, children suffering from OM are colonized earlier, more frequently and for longer periods. In fact, the pattern of nasopharyngeal colonization is an important determinant for OM disease [13-14]. Research has also indicated that, as well as bacterial species, the adaptive and native immune system, Eustachian-tube dysfunction, viral load, and genetic and environmental factors are all involved in the pathogenesis of OM [15-20].

The recent recognition of *M. catarrhalis* as an important human pathogen has stimulated active investigation into the molecular mechanisms of pathogenesis of *M.*

catarrhalis. An essential step in colonization and infection is bacterial adherence to the respiratory tract mucosa of the human respiratory tract epithelium. A growing number of adhesins have been identified in *M. catarrhalis* and most of these proteins are highly conserved, immunogenic and express epitopes on the bacterial surface. This means that they may be suitable as potential *M. catarrhalis* vaccine candidates [21]. However, relatively little is known regarding the development of the humoral immune response to these potential vaccine candidates in children. As yet, no licensed vaccine has been marketed against *M. catarrhalis*, and to date, none of the putative vaccine candidates so far described in the literature have actually progressed to clinical trials.

Currently, several pneumococcal surface proteins and combinations have been suggested as putative vaccine candidates [22-24]. These proteins could serve, either alone or in combination with the pneumococcal conjugate vaccines, as more effective vaccines than these currently available, providing a broad coverage against most pneumococcal serotypes. However, similar to the situation faced by vaccine candidates of *M. catarrhalis*, relatively little is known about the development of the humoral immune response to *S. pneumoniae* protein-based vaccine candidates within children.

With this study, we provide insights into the anti-*M. catarrhalis* and anti-pneumococcal humoral immune response in a cohort comprising Dutch children exhibiting recurrent and chronic episodes of OM. Studying their immune response will allow us to distinguish the bacterial factors that are expressed *in vivo* and which may be involved in the pathogenesis of recurrent acute otitis media (rAOM) and chronic otitis media with effusion (COME). This may lead to a better understanding into the pathogenesis of OM disease. Furthermore, the relationship between the immune response and the presence of *M. catarrhalis* and *S. pneumoniae* in the nasopharynx and in MEF was also investigated.

Materials and methods

Study cohort

This study was performed as part of a prospective clinical cohort study (Stol *et al.*, manuscript in preparation) set up at Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands, to determine the immune response to putative vaccine candidates of *M. catarrhalis* and *S. pneumoniae* in children suffering OM disease.

Patients were enrolled in two hospitals in Nijmegen, The Netherlands, from April 1st 2008 to July 1st 2009, their ages ranging from birth up to 5 years of age and suffering

from rAOM or COME for whom tympanostomy tube insertion was indicated. Recurrent OM was defined as 3 or more episodes of AOM in the last 6 months or 4 episodes in the last 12 months. The COME patient population consisted of children who experienced a period of persistent OM lasting longer than 3 months. Signs, symptoms, audiometry and tympanometry were documented prior to surgery by an ear, nose and throat (ENT) specialist in order to confirm OM diagnosis.

Patient characteristics and risk factors were identified using a questionnaire. Permission was obtained from the Committee on Research Involving Human Subjects in January 2008 (CMO 2007/239, international trial register number: NCT00847756).

Clinical materials and bacterial culture

Middle ear fluid and a nasopharyngeal swab were collected during surgery. Nasopharyngeal swabs were cultured according to standard laboratory procedures in order to determine the presence of *M. catarrhalis* and *S. pneumoniae*. Quantitative real-time PCR (Q-PCR) was performed in order to determine the presence of *M. catarrhalis* and *S. pneumoniae* in MEF (Stol *et al.*, manuscript in preparation).

Moraxella catarrhalis antigens

The previously described *M. catarrhalis* recombinant proteins used in this study comprised: ubiquitous surface proteins A (UspA1$^{557-704}$ (aa 557–704 of UspA1) and UspA2$^{165-318}$), *Moraxella* immunoglobulin D-binding proteins (MID$^{764-913}$ and MID$^{902-120}$), human erythrocyte agglutinin (Hag$^{385-863}$), *M. catarrhalis* hemagglutinin-like proteins (MhaB and MhaC), *M. catarrhalis* adherence protein (McaP$^{51-333}$), and the hypothetical proteins orf 238 and orf 296 [25-29].

Streptococcus pneumonia antigens

The previously described *S. pneumoniae* recombinant proteins used in this study comprised: choline binding protein A (PspC/CbpA), α-enolase (Eno), hyaluronidase (Hyl), immunoglobulin A1 (IgA-1) protease, neuraminidase (NanA), pneumolysin (PLY), a double mutant of pneumolysin (PdbD), putative protease maturation protein A (PpmA), pneumococcal surface adhesion A (PsaA), pneumococcal surface protein A (PspA), the pneumococcal histidine triad (Pht) proteins (SP1003 (PhtD) and BVH-3 (PhtE)), streptococcal lipoprotein rotamase A (SlrA), *S. pneumoniae* proteins (SP proteins) SP0189 (hypothetical protein), SP0376 (response regulator, intracellular location), SP1651 (thiol peroxidase, intracellular location), and Pilus A [24, 30].

Multiplex *M. catarrhalis* and *S. pneumoniae* antibody assay

Recombinant proteins were coupled to SeroMAP™ beads, carboxylated beads that are developed for serological applications, as detailed by Verkaik *et al.* [31]. Uncoupled beads were used as a negative control and to determine non-specific binding. If non-specific binding was observed, then the median fluorescent intensity (MFI) values obtained from this non-specific binding was subtracted from the antigen-specific results.

The Luminex multiplex procedure was performed as described previously [31]. Briefly, after validation of the assay (achieved by comparison of human pooled serum (HPS) MFI values obtained using the multiplex assay with HPS MFI values obtained using the singleplex assay), the different antigen-coupled microspheres were mixed to a working concentration of 4000 beads per color per well. Serum samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG. Fifty microliters per diluted sample were incubated with the microspheres in a 96-well filter microtiter plate (Millipore) for 35 minutes at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with assay buffer (PBS-BN) and aspirated using a vacuum manifold. The microspheres were resuspended in 50 µl of assay buffer, and 50 µl of a 1:200 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-human IgG (Jackson Immuno Research) was added in separate wells. The plate was incubated for 35 minutes at room temperature and washed. The microspheres were resuspended in 100 µl of assay buffer. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed in duplicate, and the MFI values, reflecting quantitative antibody levels, were averaged. The coefficient of variation was calculated for each serum sample and averaged per protein and antibody isotype. The procedure for MEF was identical to that outlined above, except that IgA and IgM were also measured. Briefly, MEF samples were diluted 1:100 in PBS-BN for measurement of antigen-specific IgG and 1:50 for measurement of IgA and IgM. Fifty microliters of a 1:100 dilution of RPE-conjugated AffiniPure goat anti-human IgG and IgA and 50 µl of a 1:200 dilution of RPE-conjugated donkey anti-human IgM were then added. For assay validation, MFI values obtained from pooled MEF (PMEF) using the multiplex assay were compared to MFI values obtained from PMEF obtained using the singleplex assay.

Statistical analysis

Statistical analyses were performed using SPSS PASW Statistics version 17. The Mann-Whitney *U*-test was used to compare anti-*Moraxella* and anti-pneumococcal immunoglobulin (Ig) levels between children diagnosed with rAOM and COME and to compare differences in Ig levels between colonized and non-colonized children. We also analyzed whether the presence of siblings and day-care attendance influenced *M. catarrhalis* and *S. pneumoniae* colonization using Fisher's exact test. Correlations between antigen-specific IgG in serum and MEF were assessed using Spearman's correlation coefficient. A *P*-value of ≤0.05 was considered to be statistically significant.

Results

Dynamics of the anti-*Moraxella* and anti-pneumococcal antibody response in children with recurrent and chronic otitis media disease

A total of 156 children were included in the analysis of the antibody response to *M. catarrhalis* and *S. pneumoniae* proteins in serum. Forty-two children were diagnosed with rAOM, whereas 114 children were diagnosed with COME. No significant difference in anti-*M. catarrhalis* serum MFI values (anti-*M. catarrhalis* IgG levels) was observed between the rAOM or COME groups for all antigens tested, though significantly higher IgG levels were observed for the pneumococcal proteins PLY and PsaA in children suffering from COME compared to children with rAOM (P=0.029 and P=0.039) (Figure 1).

A total of 121 children were included in the analysis of the antibody response to *M. catarrhalis* and *S. pneumoniae* proteins in MEF, with 25 children and 96 children diagnosed with rAOM and COME, respectively. No significant difference in *M. catarrhalis* and pneumococcal MEF IgG levels were found for all proteins tested, except for the pneumococcal protein sp0376 (P=0.044). Additionally, there was no significant difference in IgA and IgM levels in MEF, with the exception of anti-PpmA IgA (P=0.046) (Figure 1).

Effect of the presence of siblings and day-care attendance on nasopharyngeal colonization

Fisher's exact test was used to determine whether the presence of siblings and day-care attendance influenced *M. catarrhalis* and *S. pneumoniae* nasopharyngeal colonization

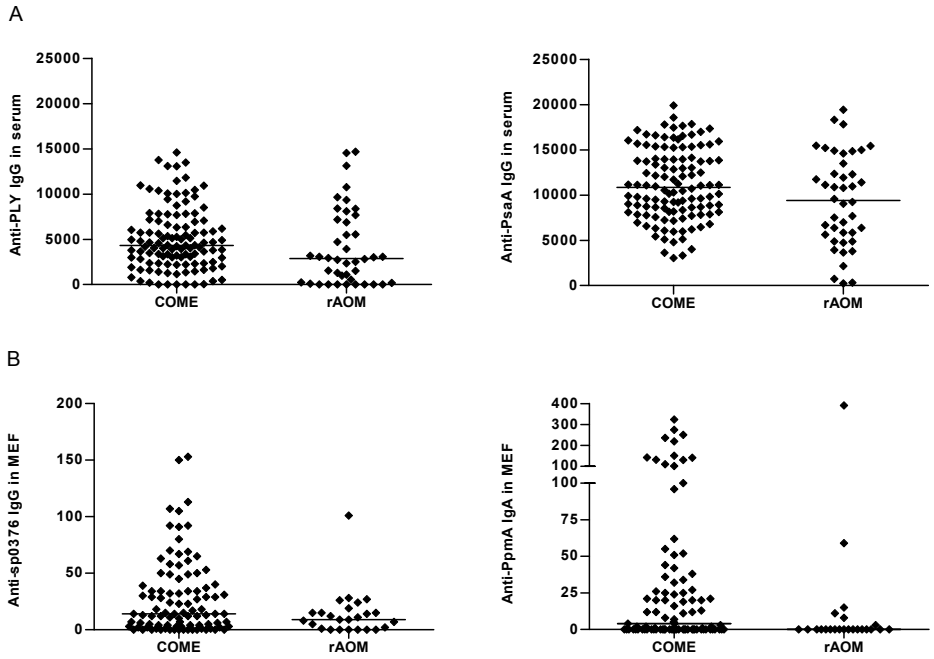

Figure 1. Relationship between disease state and antibody response in children suffering from chronic otitis media disease, as reflected by MFI values. A. Serum: children suffering from COME possessed significantly higher levels of serum IgG against the pneumococcal antigens PLY and PsaA. B. MEF: Significantly higher levels of IgG were observed against sp0376 in MEF, as well as significantly higher levels of IgA to PpmA.

(Stol *et al.*, manuscript in preparation). With respect to *M. catarrhalis*, no statistically significant difference was found between children with siblings or with no siblings, and *M. catarrhalis* colonization (P=0.35), though significant results were observed for day-care attendance (P=0.05). With respect to *S. pneumoniae*, no statistically significant difference was observed between children with siblings or with no siblings, (P=0.15), or day-care attendance and *S. pneumoniae* colonization (P=0.20).

Relationship between colonization and anti-*Moraxella* and anti-pneumococcal antibody levels in children with recurrent and chronic otitis media disease

A total of 156 children were included in the analysis of the antibody response to *M. catarrhalis* and *S. pneumoniae* proteins in serum. Forty-two children were diagnosed

with rAOM, whereas 114 children were diagnosed with COME. *M. catarrhalis* non-colonized children suffering from COME showed significantly higher IgG levels against the *M. catarrhalis* antigen MID$^{764\text{-}913}$ compared to COME colonized children (P=0.027). No significant difference was observed for *S. pneumoniae* colonization and serum IgG levels against the pneumococcal antigens (Figure 2).

A total of 117 children were included in the analysis of the antibody response to *M. catarrhalis* and *S. pneumoniae* proteins in MEF, including 23 children suffering from rAOM and 94 children suffering from COME. Similar to the antibody response in serum, no significant difference in IgG, IgA and IgM levels in MEF was observed for all *M. catarrhalis* and *S. pneumoniae* antigens between OM *M. catarrhalis* and *S. pneumoniae* positive and OM *M. catarrhalis* and *S. pneumoniae* negative children suffering from

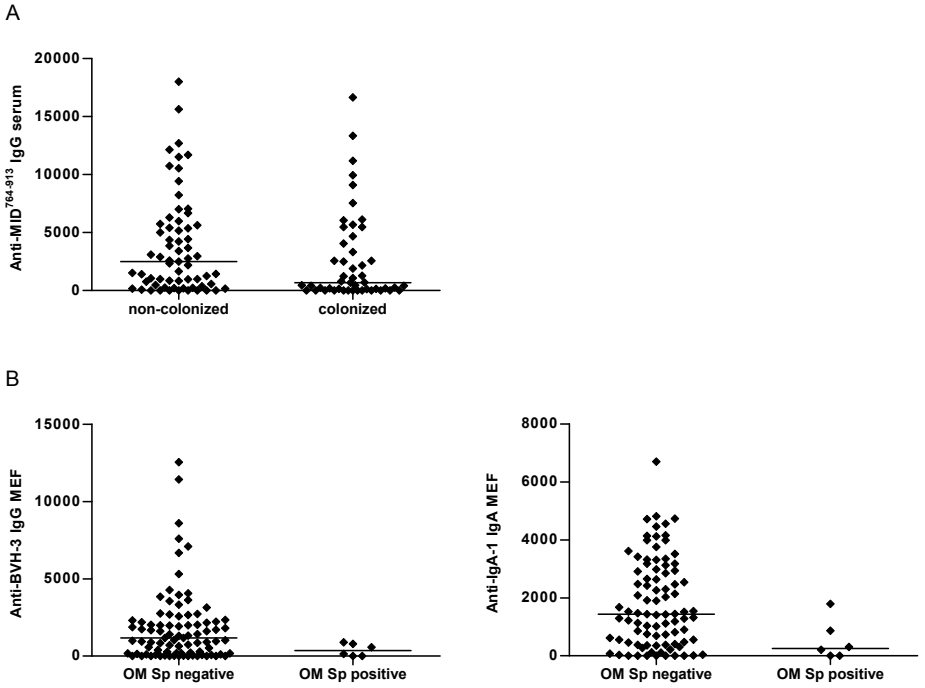

Figure 2. Relationship between COME colonization and anti-*Moraxella* and anti-pneumococcal IgG levels in serum and MEF, as reflected by MFI values. A. Non-colonized children suffering from COME showed significantly higher serum IgG levels against the *M. catarrhalis* antigen MID$^{764\text{-}913}$. B. OM *S. pneumoniae* negative children suffering from COME showed significantly higher MEF IgG and IgA levels against the *S. pneumoniae* antigens BVH-3 and IgA-1, respectively. OM, otitis media; Sp, *Streptococcus pneumoniae*.

either rAOM or COME. However, OM *S. pneumoniae* negative children suffering from COME showed significant higher IgG and IgA levels against the pneumococcal proteins BVH-3 and IgA-1 compared to OM *S. pneumoniae* positive children, respectively (P=0.046 and P=0.022) (Figure 2).

Correlation between anti-*M. catarrhalis* and anti-pneumococcal IgG levels in serum and MEF

To determine the correlation between the levels of anti-*Moraxella* and anti-pneumococcal IgG in serum compared to MEF, samples from 111 children who donated both blood and MEF at tympanostomy surgery were included. The mean IgG levels (reflected by MFI values) in these samples were calculated for each protein (Figure 3). IgG levels in serum showed a strong correlation to IgG levels in MEF for both *M. catarrhalis* (R^2 = 0.97) as well as *S. pneumoniae* (R^2 = 0.89).

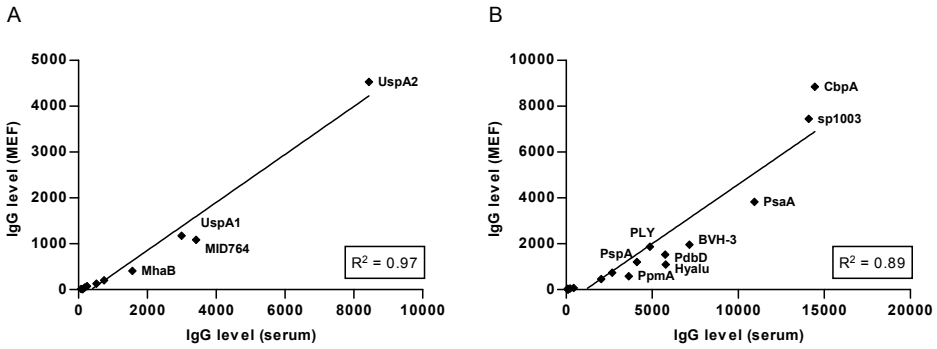

Figure 3. Correlation between IgG levels in serum and MEF. Mean IgG levels in serum and MEF were used to calculate the correlation statistics for each respective *M. catarrhalis* (A) or *S. pneumoniae* (B) protein.

Discussion

This study was performed as part of a prospective cohort study to determine the immune response to the major vaccine candidate outer membrane proteins of *M. catarrhalis* and protein vaccine candidates of *S. pneumoniae* in children suffering from rAOM and COME.

Children are frequently colonized with *M. catarrhalis* at an early age and frequent colonization with *M. catarrhalis* has been reported to increase the risk of OM [13].

Studies have shown that the presence of siblings and day-care attendance are important risk factors for early and frequent colonization by *M. catarrhalis* and *S. pneumoniae* in the first years of life [16, 18, 32]. In this study, the presence of siblings and attending day-care did not result in an increase in *M. catarrhalis* and *S. pneumoniae* colonization within this cohort of children, with the exception of *M. catarrhalis* colonization and day-care attendance. Previous studies in healthy children have however indicated that both the number of siblings and day-care attendance are associated with significant nasopharyngeal colonization by both of these respiratory pathogens [16, 18]. The apparent difference observed between the current and previous studies could be explained by the fact that previous publications published data on healthy children, whilst the current study has been performed in a group of children exhibiting recurrent and chronic episodes of OM. Children suffering from OM tend to be colonized earlier, more frequently, and for longer periods of time compared to healthy children [33], meaning that these populations may be unsuitable for comparative association statistics involving nasopharyngeal colonization events.

The adaptive and native immune system are also important factors in the pathogenesis of OM [17]. The high incidence and high rate of 'spontaneous' recovery from OM suggests that recovery is a natural phenomenon and part of the gradual maturation of the child's immune system. However, a defective or immature antibody response to pathogens causing OM may explain the increased susceptibility of some children to OM [34]. With respect to the adaptive immune system, significantly higher serum IgG levels were observed for the pneumococcal proteins PLY and PsaA in children suffering from COME, compared to children with rAOM. This observation may be explained by a previous observation indicating significantly higher numbers of *S. pneumoniae* in the nasopharynx of children with COME compared to children with rAOM (Stol *et al.*, manuscript in preparation). Additionally, significantly higher serum IgG levels to the putative *M. catarrhalis* vaccine candidate antigen $MID^{764-913}$ was observed in COME *M. catarrhalis* non-colonized versus COME *M. catarrhalis* colonized children. A recent study of healthy children by Verhaegh *et al.* (manuscript submitted) indicated that the Hag/MID outer membrane protein may be involved in protecting healthy children against *M. catarrhalis* nasopharyngeal colonization, as significantly higher IgG levels against $MID^{902-1200}$ were observed in healthy *M. catarrhalis* non-colonized versus healthy *M. catarrhalis* colonized children. However, though both $MID^{764-913}$ and $MID^{902-1200}$ are derived from the same outer membrane protein (Hag/MID), there was no significant difference in IgG levels against the $MID^{764-913}$ antigen observed in healthy children. This observation may be

related to the fact that in the study of Verhaegh *et al*., healthy children were followed up to a maximum of 2 years of age, while the median age of the current rAOM/COME group was 4 years of age (range 10 months – 6 years). The immune response to pathogens develops rapidly during the first few years of life, as children become exposed to an increasing number of microbial species and strains. The difference in Hag/MID IgG levels observed between healthy and rAOM/COME children could therefore be related to 'maturation' of the humoral immune response against Hag/MID, as the children become exposed to multiple strains of *M. catarrhalis* between birth and 4 years of age. With respect to clinical applications, it has been found that *M. catarrhalis* DNA is detected in a larger proportion of cases of OME than of AOM, and involves bacterial biofilm formation on the middle-ear [21, 35]. The significantly higher IgG levels to the *M. catarrhalis* antigen $MID^{764\text{-}913}$ observed in non-colonized children suffering from COME may indicate that the Hag/MID protein should be further investigated as a potential vaccine antigen to reduce *M. catarrhalis* biofilm formation and hence *M. catarrhalis*-mediated COME disease.

Local production of secretory antibodies is another immunological defense mechanism of the upper respiratory tract. Secretory IgA inhibits pathogen adherence and reduces nasopharyngeal bacterial colonization, and children with recurrent OM might lack secretory IgA [36]. The MFI levels of antigen-specific IgA showed extensive inter-individual variability over time, with IgA levels to all *M. catarrhalis* and *S. pneumoniae* OMPs being relatively low throughout the study period. However, OM *S. pneumoniae* negative children suffering from COME showed significant higher MEF IgA levels for the pneumococcal IgA-1 protease protein compared to OM *S. pneumoniae* positive children. Many mucosal pathogens, including *S. pneumoniae*, express an IgA-1 protease that may circumvent the protective effects of IgA, facilitating bacterial adhesion and mucosal surface persistence [37]. In this study, we provide some preliminary evidence to indicate that antibodies against IgA-1 may have a protective effect in COME children. Interestingly, OM *S. pneumoniae* negative children suffering from COME showed significantly higher MEF IgG levels against the *S. pneumoniae* antigen BVH-3. This finding may also suggest that this protein has a protective effect in children suffering from COME, and indeed, in previous studies, the BVH-3 protein has been shown to be capable of eliciting protective immunity against pneumococcal disease [38-39].

Finally, a strong correlation was observed between IgG antibody levels in serum and in MEF, for both *M. catarrhalis* and pneumococcal proteins. This is an interesting clinical finding, as it indicates that serum antibody levels might be predictive of local antibody production.

Conclusion

Both rAOM and COME children generate antibodies (IgG, IgA and IgM) against a variety of novel *M. catarrhalis* outer membrane and *S. pneumoniae* proteins. Further, significant differences in antibody levels (reflected by MFI values) were observed between 'pathogen colonized' and 'pathogen non-colonized' children suffering from COME. Based on our preliminary findings, the *Moraxella* Hag/MID and pneumococcal BVH-3 and IgA-1 proteins may be further investigated as possible vaccine candidates in COME children.

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General discussion

Chapter 10

Summarizing discussion

Moraxella catarrhalis colonization and infection

Moraxella catarrhalis is part of the normal bacterial flora of the human respiratory tract. It was only relatively recently acknowledged as a true bacterial pathogen, rather than simply being a 'colonizing commensal' (**Chapter 2**). In general, the organism is mostly associated with upper respiratory tract infections in children (including otitis media (OM) and sinusitis) and lower respiratory tract infections in adults (e.g. exacerbations of chronic obstructive pulmonary disease (COPD)). *M. catarrhalis* is also associated with more serious sporadic cases including meningitis, urinary tract infection and conjunctivitis [1, 2].

Although colonization by itself may not always result in the onset of symptoms, it is the first step towards invasive disease, as well as being a source of horizontal spread of *M. catarrhalis* in the community [3]. Frequent (re-)colonization with *M. catarrhalis* has been reported to increase the risk of OM disease. With this in mind, the risk factors associated with *M. catarrhalis* colonization have been studied in different countries and age groups, in which crowding and contact with children (including siblings) were identified (**Chapter 3**). Interestingly, the same risk factors are also associated with colonization by *Streptococcus pneumoniae* and *Haemophilus influenzae* [4].

In **Chapter 4**, we showed a trend towards a regular 6-monthly *M. catarrhalis* colonization cycle in a cohort of healthy children living in Rotterdam (the Generation R study), with no pronounced seasonal colonization peak. This observation tends to contrast with other studies reporting a seasonal influence on *M. catarrhalis* colonization. We hypothesized that colonization with *M. catarrhalis* occurs via regular cycles of colonization and clearance, with the apparent increase in *M. catarrhalis* detection during the winter months probably being a consequence of increased viral respiratory infections, resulting in increased opportunity for secondary bacterial infection by bacterial pathogens [5]. Essentially, a respiratory viral infection may cause i) prolonged dysfunction of the Eustachian tube, ii) congestion of the respiratory mucosa, and iii) alteration in middle ear pressure, all of which tend to lead to the accumulation of fluid within the middle ear. This abnormal pathology may then facilitate invasion, persistence and proliferation of nasopharyngeal-colonizing microbial pathogens into the middle ear [6].

Polymicrobial infections and biofilms involving *M. catarrhalis*

There is growing evidence indicating a role for polymicrobial infection and biofilm formation in the development of disease [7, 8]. In this respect, *M. catarrhalis* is no exception,

although there remains some disagreement regarding the exact role of *M. catarrhalis* in polymicrobial disease [9]. Recently, Armbruster *et al.* (2010) showed that co-infection with *M. catarrhalis* and *H. influenzae* promotes increased biofilm formation and thereby resistance to antibiotics, while also hindering host clearance of the bacteria. Further, it was shown that this phenomenon occurred as a result of quorum sensing signaling via an AI-2 messenger [10]. Interestingly, at the same time we published data from a healthy cohort of children (the Generation R Study) indicating that co-colonization by *M. catarrhalis* and *H. influenzae* was significantly more likely than colonization by either bacterium alone (**Chapter 4**). Additionally, in **Chapter 6**, we showed that co-colonization in the nasopharynx with two or more pathogens is associated with the presence of bacteria in the middle ear of patients suffering from COME. Taken together, these publications indicate the importance of the polymicrobial interaction between *M. catarrhalis* and *H. influenzae* in facilitating *M. catarrhalis* colonization. Further research is necessary in order to determine the direct effects of this increased colonization on (the severity of) resultant disease.

Nowadays, there is also a growing understanding of the importance of biofilm formation in group A *Streptococcus* (GAS)-related colonization and disease [11-13]. While the published literature mainly reports on *S. pneumoniae* and *H. influenzae* as being associated with *M. catarrhalis* polymicrobial infections, the interactions between *M. catarrhalis* and GAS are poorly understood [14]. This is important with respect to the increasing number of GAS-associated OM infections in certain countries of Europe. The incidence of *S. pyogenes*-mediated OM disease reported in the literature is low, and even rare in the West. However, recent communications have indicated an increase in *S. pyogenes* related OM infections in Hungary between 2006 and 2008 (personal communication, Dr. Éva Ban, Semmelweiss University, Budapest, Hungary).

Therefore, in **Chapter 7**, initial studies were performed to investigate the effect of *M. catarrhalis*-GAS co-culture on GAS gene expression, with particular emphasis on (GAS) virulence gene expression. We provided evidence that *M. catarrhalis* may positively affect the expression of GAS genes related to virulence and biofilm formation. Theoretically, this could lead to increased colonization and pathogenicity of GAS during *M. catarrhalis*-GAS polymicrobial infections. Further research is necessary in order to confirm this interesting finding.

Current treatment of *M. catarrhalis*-associated disease

The recognition of its pathogenic status means that the detection of *M. catarrhalis* in clinical specimens is now a relevant topic in the diagnosis and management of disease. It is especially relevant with respect to OM-related disease, a disease that is currently associated with the highest prevalence of *M. catarrhalis* infections. Acute, recurrent and chronic forms of OM are the most common reasons to prescribe antibiotics or to undergo surgery for the placement of grommets in many countries [15, 16]. This results in a significant burden on healthcare systems and national economies, as parents take time off from work to visit their general practitioner [17, 18]. The American Academy of Pediatrics (AAP) published guidelines in 2004 that address the diagnosis and treatment of AOM. The diagnosis of AOM is complicated by a lack of understanding of the correlation between clinical signs and symptoms and responsible pathogens. Consequently, antibiotic treatment of AOM is not always appropriate, and the long-term overuse of antibiotics in AOM reduces the effectiveness of treatment and places children at an increased risk of antibiotic-resistant infections [16]. Although the AAP guidelines recommended the use of watchful waiting as a potential strategy for the treatment of AOM, actual rates of antibiotic prescription for AOM still vary to a great extent internationally [17, 19, 20]. Differences in regional antibiotics prescribing practices may lead to an increase in the prevalence of antibiotic resistant organisms. For example, in a recent publication, Khan *et al.* (2010) showed a significant difference in *bro* gene (β-lactamase resistance) prevalence in non-clonal global *M. catarrhalis* isolates recovered from children and adults. Consistently higher β-lactam minimum inhibitory concentrations were found for isolates from the Far East [21]. These differences are most likely associated with differences in antibiotic prescribing practices between adults and children and between the Far East and other areas of the world. Research has also indicated that the presence of β-lactamase positive *M. catarrhalis* may actually protect some other bacterial species against β-lactam antibiotics [22].

It has been calculated that COPD will become the 5th most prevalent disease in the world by 2020, and *M. catarrhalis* causes an estimated 2-4 million exacerbations of COPD in adults annually in the United States [23, 24]. If treatment solely relies on the use of antimicrobial chemotherapy, then the prevalence of antibiotics resistance (not only within *M. catarrhalis* but also in other non-targeted bacterial species) is likely to increase. One method to decrease the reliance on antimicrobial treatment in *M. catarrhalis*-mediated disease would be via the development and implementation of a

vaccine against *M. catarrhalis*. This may both help in reducing the burden of antibiotics resistance as well as the medical and economic consequences associated with *M. catarrhalis* colonization and infection.

Vaccine development

In spite of the technical challenges associated with the development of vaccines that target respiratory bacterial pathogens, vaccines have been successfully developed for both *S. pneumoniae* and *H. influenzae*. A 7-valent vaccine against *S. pneumoniae* (PCV-7), which targets the 7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, became available in the United States in April 2000 and is recommended by the AAP for universal use in children 23 months of age and younger to prevent pneumococcal infections [25]. In March 2001, the same vaccine was marketed in The Netherlands for use in infants and young children. The aim for the use of this vaccine was to prevent invasive pneumococcal disease (e.g. meningitis and pneumonia) at this age. The introduction of PCV-7 initially led to a noticeable reduction in the incidence of *S. pneumoniae* vaccine targeted strains in the etiology of AOM [20]. However, subsequent studies have shown an increase in the carriage of non-vaccine targeted serotypes and a consequent increase in non-vaccine serotype-associated invasive disease. This increase in invasive disease could reduce or even negate the benefits initially obtained through vaccination [26, 27], and possibly lead to an increase in antibiotic resistant *S. pneumoniae* serotype 19A infections [28].

With respect to *H. influenzae*, a vaccine for diphtheria, tetanus, pertussis (a-cellular), poliomyelitis and *H. influenzae* type b (DKTP-Hib) was introduced in The Netherlands in 2006. Before introduction of the vaccine, ten percent of all AOM-infections were caused by *H. influenzae* type b. Currently, mainly non-typeable (NT) *H. influenzae* is isolated from AOM-infections. DKTP-Hib is not effective against NT *H. influenzae* and, to date, no vaccine against NT *H. influenzae* has been developed, although *H. influenzae* protein D has been used as an active carrier protein for pneumococcal vaccines [29].

In 1996, a collaborative surveillance network was established in Europe to monitor the impact of routine *H. influenzae* type b (Hib) vaccination on the epidemiology of invasive *H. influenzae* disease. The data collected between 1996 and 2006 showed that the incidence of invasive non-type b *H. influenzae* was higher than the incidence of Hib and was also associated with a higher rate of case fatality [30]. Although there is little direct information on the effect of Hib vaccination on the prevalence of OM, it seems likely that the serotype replacement effect observed for non-type b invasive disease will also be observed for OM-related diseases.

In contrast to *S. pneumoniae* and *H. influenzae*, there is currently no licensed vaccine available against *M. catarrhalis*, and, to date, none of the putative vaccine candidates described so far have progressed into clinical trials (several outer membrane proteins (OMPs) and lipooligosaccharides (LOS) are described in the literature as potential vaccine candidates). The lack of i) correlates of protection and ii) a suitable animal model to study *M. catarrhalis*-mediated disease (especially OM disease), are seriously hindering the development of an effective vaccine against *M. catarrhalis* [31]. A mouse pulmonary clearance model, which measures clearance of bacteria from the lungs following bacterial challenge directly into the airways, has been useful for assessing putative vaccine antigens [32-35]. However, this model system does not simulate human respiratory tract infection, as *M. catarrhalis* is cleared within 24 hours from the middle ear of mice. Although mouse, and also chinchilla, models of OM have yielded important observations in the case of *S. pneumoniae* [36] and *H. influenzae* [37, 38], they have not been useful in studying *M. catarrhalis* [39].

Current advances in *M. catarrhalis* vaccine development

Bacterial adherence to the respiratory mucosa is an essential step towards colonization of the human respiratory tract epithelium. Adhesins responsible for the attachment of *M. catarrhalis* to host cells include i) the family of ubiquitous surface proteins A (UspAs), ii) *M. catarrhalis* immunoglobulin D (IgD) binding protein (MID), also referred to as the hemagglutinin (Hag), iii) *M. catarrhalis* adherence protein (McaP), iv) OMPCD, *M. catarrhalis* hemagglutinin-like proteins (Mha), v) surface-exposed structures such as pili, and vi) LOS structures [40-49]. Other putative vaccine candidates for *M. catarrhalis* are involved in nutrient acquisition and virulence and include i) the *M. catarrhalis* surface proteins (Msp) Msp22 and Msp75 [50], ii) outer membrane porin M35 [51], iii) *M. catarrhalis* outer membrane protein B (CopB) [52], iv) transferrin binding proteins A and B (TbpA and TbpB) [53], v) OMPE [54], vi) lactoferrin binding proteins A and B (LbpA and LbpB) [55] and vii) oligopeptide permease protein A (OppA) [32]. Most of these proteins and LOS are highly conserved, immunogenic and express epitopes on the bacterial surface, which means that they may be suitable as potential *M. catarrhalis* vaccine candidates. The challenge in identifying potential vaccine candidates for an OM vaccine lies in identifying antigens that are present on the surface of all strains, antigenically conserved among strains, and expressed during infection of the human host. In **Chapter 5**, we found that isolates from children and adults are genetically diverse and

that differences exist in the incidence and expression of outer membrane-associated virulence genes between child and adult-associated isolates within a global context. From these results, it would appear that an ideal *M. catarrhalis* vaccine may have to be based on a multivalent vaccine that contains a combination of immunogenic epitopes from multiple vaccine candidates. A multivalent vaccine may offer better protection against *M. catarrhalis*-mediated disease in both children and adults.

As a first step in validating putative vaccine candidate antigens, the antigens need to be proven to be immunogenic and capable of inducing a protective immune response. Unfortunately, relatively little is known about the development of the humoral immune response to *M. catarrhalis* vaccine candidates in either healthy or ill individuals. In this respect, the studies presented in **Chapters 8** and **9** were established in order to investigate whether specific antibodies against currently identified outer membrane protein vaccine candidates of *M. catarrhalis* (and additionally *S. pneumoniae*, **Chapter 9** only) were detectable in the sera of healthy and sick (chronic otitis media with effusion and recurrent acute otitis media) cohorts of children. We were also interested in determining whether antibody levels could be related to colonization or non-colonization prevalences. Our results indicated that at 2 years of age, the antibody response to *M. catarrhalis* is still developing, and is largely based on IgG isotypes of antibodies raised against 3 major OMPs: UspA1, UspA2 and Hag/MID. Further, we provided preliminary evidence that the Hag/MID antigen may be a good vaccine candidate for further investigation in *M. catarrhalis*-mediated disease. Specifically, this vaccine antigen may be useful in helping to reduce *M. catarrhalis* colonization and disease in children suffering from chronic and recurrent OM infections. Natural variation in the amino acid sequence of this protein may however limit the potential of a vaccine based solely on this outer membrane component.

Novel *M. catarrhalis* prevention and treatment options

Currently, watchful waiting and/or the prescription of antibiotics are the most favored options for the treatment of *M. catarrhalis*-associated disease. Treatment with antibiotics seems effective, but the chances of the development of antibiotic resistance in *M. catarrhalis* (or non-targeted bacteria) remain high. However, a vaccine against *M. catarrhalis* is not currently available, nor likely to be so within the next 5 years.

Targeting bacterial communication could be a novel treatment methodology, as biofilm formation is facilitated by bacterial communication, being controlled in part through cell density-dependent quorum signaling networks [56, 57]. Many bacteria rely on

quorum signaling molecules to control the expression of virulence and regulatory genes, which ultimately influence the establishment and progress of disease. If a method could be found to block quorum signaling systems and molecules, then the processes leading to development of biofilm formation and disease may also be blocked.

The study by Armbruster *et al.* (2010) indicates that a quorum signaling molecule can be an ideal target for disrupting polymicrobial signaling and pathogenicity [10]. This disruption could occur via the development of i) anti-AI-2 molecule antibodies, ii) enzymes that cleave the AI-2 molecule, or iii) an AI-2 molecule mimic that blocks AI-2 receptor sites without activating quorum sensing systems [58, 59]. Unfortunately, the interspecies targeting of autoinducer molecules may result in undesirable implications, as disruption of AI-2 signaling may promote virulence or virulence-related phenotypes in *Staphylococcus aureus* and *S. pyogenes*. Further, targeting of AI-2 has a potential impact on the normal microbiota, as AI-2 signaling may be critical for the establishment and maintenance of a normal microbiota and in the defense against pathogens [60].

Future perspectives

OM is an important children's disease that can be caused by *M. catarrhalis*. This organism also appears to play a significant role in exacerbations of COPD. A vaccine directed against *M. catarrhalis* should help reduce the prevalence of its major infections, as well as less prevalent diseases such as sinusitis and pneumonia. This will impact on patient morbidity, and on the negative economic consequences that are associated with these diseases. Further, such a vaccine will provide an alternative to excessive antibiotic use, which often leads to the generation of antibiotic resistance.

Unfortunately however, the introduction of vaccines targeting *S. pneumoniae* and *H. influenzae* has tended to lead to the replacement of 'traditionally' virulent bacterial strains with 'non-traditional', but equally virulent, strains of the same species. The development of a multivalent vaccine consisting of several *M. catarrhalis* antigens should help reduce any 'serotype replacement' effect if or when it reaches the market. The cost-effectiveness of vaccination compared to current treatment methods for *M. catarrhalis*-mediated disease, as well as the ecological effects on healthy flora, also need to be considered.

Ultimately, the successful development and introduction of a *M. catarrhalis* vaccine will be largely dependent upon economic factors. A vaccine that is effective against *M. catarrhalis* and also against other respiratory bacterial and viral pathogens will have the highest chance of success.

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Chapter 11

Nederlandse samenvatting

Kolonisatie en infectie

Moraxella catarrhalis is een commensaal van de luchtwegen bij mensen, en is pas recentelijk erkend als pathogeen (**Hoofdstuk 2**). *M. catarrhalis* wordt voornamelijk geassocieerd met middenoorontsteking (otitis media; OM) bij jonge kinderen. OM is een veel voorkomende aandoening van de bovenste luchtwegen, en een belangrijke reden voor een bezoek aan de huisarts of het ziekenhuis. Deze aandoening kan ertoe leiden dat de kwaliteit van leven in de eerste levensjaren van kinderen aanzienlijk achteruit gaat, en baart ouders grote zorgen. Ongeveer 80% van de kinderen heeft rond het derde levensjaar een episode van OM doorgemaakt. Bij volwassenen komt *M. catarrhalis* voornamelijk voor in de onderste luchtwegen en wordt bij deze groep geassocieerd met het verergeren van de klachten van chronische obstructieve longziekten (chronic obstructive pulmonary disease; COPD).

Hoewel kolonisatie niet per direct hoeft te leiden tot ziekte, is het wel een eerste stap in deze richting, en bovendien een oorzaak van verspreiding van *M. catarrhalis* tussen personen. Studies hebben aangetoond dat frequente kolonisatie met *M. catarrhalis* bij jonge kinderen kan leiden tot OM. Het is daarom belangrijk te weten wat de risicofactoren zijn die geassocieerd worden met *M. catarrhalis* kolonisatie. In **Hoofdstuk 3** hebben we aangetoond dat crèche bezoek en het hebben van broers en/of zussen belangrijke risicofactoren zijn voor *M. catarrhalis* kolonisatie.

Polymicrobiële infecties

De afgelopen jaren is er meer en meer bewijs verzameld voor het feit dat de aanwezigheid van meerdere soorten bacteriën en het vormen van biofilms een belangrijke rol spelen in de ontwikkeling van ziekten (**Hoofdstuk 6**). Een biofilm is een laag van bacteriën vastgehecht aan een oppervlak, bijvoorbeeld het middenoor. De bacteriën die in een biofilm leven kunnen totaal andere eigenschappen krijgen dan vrijlevende micro-organismen van dezelfde soort. Er is recentelijk aangetoond dat co-infectie van *Haemophilus influenzae* met *M. catarrhalis* er voor zorgt dat deze bacteriën in biofilms beter bestand zijn tegen toxische stoffen zoals antibiotica. Celdichtheid-afhankelijke genexpressie, ook wel quorum signaling genoemd, is één van de factoren die daarvoor verantwoordelijk is. Dit is een interessante ontdekking, aangezien wij met onze studie in **Hoofdstuk 4** hebben aangetoond dat co-kolonisatie van *M. catarrhalis* en *H. influenzae* waarschijnlijker is dan kolonisatie van *M. catarrhalis* of *H. Influenzae* alleen. Ondanks dat er meer onderzoek nodig is, laten de studies zien dat de interactie tussen *M. catarrhalis* en *H. influenzae* een belangrijke rol speelt in de kolonisatie van *M. catarrhalis*.

Een ander organisme waarbij biofilms een belangrijke rol spelen in kolonisatie en infectie, is *Streptococcus pyogenes*, een β-hemolytische groep A streptokok (GAS). Hoewel *Streptococcus pneumoniae*, *H. influenzae* en *M. catarrhalis* in de literatuur voornamelijk worden beschreven als de belangrijkste veroorzakers van OM, kan GAS ook worden beschouwd als een 'echt' OM-pathogeen. In het Westen zijn GAS-geassocieerde OM-infecties zeldzaam. Niettemin is de incidentie van GAS-gerelateerde OM-infecties in sommige landen van de Europese Unie aan het stijgen. Een voorbeeld is Hongarije, waar tussen 2006 en 2008 een toename in GAS-gerelateerde OM infecties is waargenomen. Er is weinig bekend over de interactie tussen *M. catarrhalis* en GAS. Daarom is de studie in **Hoofdstuk 7** opgezet om het effect van *M. catarrhalis* op de genexpressie van GAS te bestuderen, en dan met name de genen die betrokken zijn bij virulentie. Hoewel er meer onderzoek nodig is om onze resultaten te bevestigen, is deze studie het eerste bewijs dat *M. catarrhalis* van invloed is op de genexpressie van GAS-genen die onder andere betrokken zijn bij virulentie en biofilm formatie. Deze invloed kan mogelijk leiden tot een verhoogde kans op kolonisatie en pathogeniteit van GAS tijdens polymicrobiële infecties.

Huidige behandelmethoden

Het erkennen van de pathogene status van *M. catarrhalis* heeft ertoe geleid dat het aantonen van *M. catarrhalis* in klinische specimens een relevant onderwerp is geworden bij de diagnose en behandeling van onder andere OM. In veel landen is het gebruikelijk om antibiotica voor te schrijven of om buisjes te plaatsen in de oren wanneer een kind lijdt aan OM. Deze behandelmethoden hebben echter een enorme impact op de gezondheidszorg en de landelijke economie.

De Amerikaanse academie voor kinderartsen (American Academy of Pediatrics; AAP) publiceerde in 2004 richtlijnen met betrekking tot de diagnose en behandeling van OM. Het vaststellen van de diagnose OM wordt bemoeilijkt door een onduidelijke samenhang tussen klinische symptomen en de daadwerkelijke veroorzakers van OM, met als gevolg een mogelijk onjuiste behandeling. De verschillende vormen van OM, maar ook COPD bij volwassenen, zijn in veel landen belangrijke redenen voor het voorschrijven van antibiotica. De hoeveelheid antibiotica die hierbij wordt voorgeschreven varieert sterk tussen verschillende landen. Veelvuldig gebruik van antibiotica reduceert de effectiviteit van de behandeling, en tevens lopen kinderen een verhoogd risico om geïnfecteerd te raken met antibioticumresistente micro-organismen. De richtlijnen van de AAP schrijven dan ook de 'watch and wait'-methode voor. Hierbij luidt het advies bij vermoedens van een OM-infectie eerst een paar dagen af te wachten en paracetamol te gebruiken.

Vaccinontwikkeling

Een veelbelovend alternatief voor het gebruik van antibiotica is vaccinatie. Preventie van OM door het introduceren en toepassen van bestaande en toekomstige vaccins lijkt een hoopgevende manier om de last van OM en de bijbehorende economische consequenties te verminderen, zowel voor het Westen als voor de ontwikkelingslanden.

Een 7-valent *S. pneumoniae* vaccin (pneumococcal conjugate vaccine 7; PCV-7) gericht tegen de serotypen 4, 6B, 9V, 14, 18C, 19F en 23F kwam in april 2000 in de Verenigde Staten op de markt. Dit vaccin is aanbevolen door de AAP voor gebruik bij kinderen van 23 maanden en jonger ter preventie van pneumokokkeninfecties. In maart 2001 werd hetzelfde vaccin in Nederland op de markt gebracht voor gebruik bij kleuters en jonge kinderen ter voorkoming van onder andere meningitis en longontsteking. De introductie van PCV-7 leidde ook tot een aanzienlijke reductie in de prevalentie van *S. pneumoniae* met betrekking tot OM. Hoewel het gebruik van PCV-7 in eerste instantie tot een afname van *S. pneumoniae* in de etiologie van OM heeft geleid, is er een toename in dragerschap van non-vaccin serotypen gesignaleerd. De opkomst van deze non-vaccin serotypen is zorgelijk, en kan in de toekomst zelfs het effect van vaccineren teniet doen.

In 2006 is in Nederland een vaccin voor difterie, tetanus, kinkhoest, polio en *H. influenzae* type b (DKTP-Hib) geïntroduceerd. Vóór de introductie van het vaccin werd 10% van de OM-infecties veroorzaakt door *H. influenzae* type b, terwijl op dit moment voornamelijk de niet-typeerbare *H. influenzae* (NTHi) bij een OM-infectie wordt geïsoleerd. Het Hib-vaccin is helaas niet effectief tegen de niet-typeerbare variant van *H. influenzae*. Er is tot nu toe geen vaccin ontwikkeld tegen NTHi, hoewel het *H. influenzae* eiwit D als dragereiwit wordt gebruikt voor pneumokokken vaccins.

Tot op heden is er nog geen vaccin op de markt gebracht tegen *M. catarrhalis*. In wetenschappelijke publicaties zijn verschillende membraanoppervlakeiwitten en lipopolysacchariden als vaccinkandidaten geïdentificeerd. Geen enkele van de tot dusver beschreven vaccinkandidaten is echter in klinische studies getest. De uitdaging in het vinden van vaccinkandidaten ligt in het feit dat potentiële antigenen aanwezig dienen te zijn op het membraanoppervlak van alle *M. catarrhalis* stammen, deze geconserveerd dienen te zijn tussen *M. catarrhalis* stammen, en zij bovendien tot expressie moeten komen gedurende infectie. In **Hoofdstuk 5** hebben we aangetoond dat er genetische verschillen bestaan tussen stammen geïsoleerd bij kinderen en volwassenen, waarbij er een verschil is in aanwezigheid en expressie van Hag/MID, een potentiële vaccinkandidaat. Een ideaal *M. catarrhalis* vaccin dient daarom opgebouwd te zijn uit immunogene epitopen van meerdere *M. catarrhalis* vaccinkandidaten. Een dergelijk zogenaamd multi-

valent vaccin biedt daardoor een brede bescherming tegen *M. catarrhalis* gerelateerde ziekten in zowel kinderen als volwassenen.

Een eerste stap in het valideren van vaccinkandidaten is aan te tonen dat ze in staat zijn tot het opwekken van een immuunrespons die uiteindelijk beschermend werkt. Er is tot op heden weinig bekend over de ontwikkeling van de humorale immuun respons tegen *M. catarrhalis* vaccinkandidaten in gezonde en zieke personen. De studies in **Hoofdstuk 8** en **9** zijn opgezet om te bepalen of antilichamen tegen *M. catarrhalis* (en *S. pneumoniae*, **Hoofdstuk 9**) vaccinkandidaten aanwezig zijn in het bloed van gezonde en zieke kinderen. We hebben ook bestudeerd of de aanwezigheid van deze antilichamen van invloed was op *M. catarrhalis* kolonisatie. Uit deze studies blijkt dat op tweejarige leeftijd de immuunrespons gericht tegen *M. catarrhalis* nog in ontwikkeling is, en voornamelijk bestaat uit IgG antilichamen opgewekt tegen de oppervlakte membraaneiwitten UspA1, UspA2 en Hag/MID. We hebben ook aangetoond dat Hag/MID een mogelijke vaccinkandidaat is, welke een potentiële rol kan spelen in het reduceren van *M. catarrhalis* kolonisatie en infectie.

Nieuwe preventie- en behandelmethoden

Op dit moment zijn de de 'watch and wait'-methode en het gebruik van antibiotica de meest voorkomende vormen van behandeling tegen *M. catarrhalis*-geassocieerde ziekten. We hebben al eerder kunnen lezen dat het gebruik van antibiotica voor een toename zorgt in het vóórkomen van resistente micro-organismen. Daarnaast is het niet waarschijnlijk dat binnen nu en 5 jaar een vaccin tegen *M. catarrhalis* op de markt komt. Een alternatieve behandelmethode is daarom gewenst. Het aanpakken van de communicatie tussen bacteriën zou een goede optie kunnen zijn ter preventie en behandeling van *M. catarrhalis* infectie. Veel bacteriën vertrouwen op quorum signaling moleculen om de expressie van regulatoire en virulentiegenen te reguleren. Met het blokkeren van deze communicatie kan de pathogeniciteit van de betrokken micro-organismen afnemen.

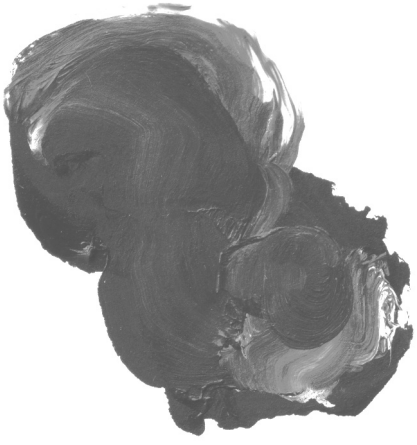
Toekomstperspectief

OM is een belangrijke aandoening bij kinderen die in de meeste gevallen wordt veroorzaakt door een selecte groep bacteriële pathogenen, waaronder *M. catarrhalis*. Behalve in OM speelt *M. catarrhalis* ook een belangrijke rol in de klachten van COPD. Vaccinatie is mogelijk een goed alternatief voor het excessieve gebruik van antibiotica. Een vaccin gericht tegen *M. catarrhalis* moet in staat zijn tot het terugdringen van het aantal *M. catarrhalis* gerelateerde ziekten, en de last verlichten die deze ziekten veroorzaken.

Helaas heeft de introductie van de huidige vaccins geleid tot vervanging van 'traditioneel virulente' bacteriële stammen met 'niet-traditionele, maar wel virulente' stammen van dezelfde, maar soms ook van andere species in relatie tot OM. Er wordt gezocht naar een vaccin dat een brede bescherming biedt tegen zowel virulente als niet-virulente stammen van dezelfde bacterie (om bijvoorbeeld serotype-'replacement' te voorkomen). Daarnaast is er meer onderzoek gewenst in de ontwikkeling van multivalente vaccins, die effectief zijn tegen zowel bacteriële als virale pathogenen.

Andere aspecten die van belang zijn voor een succesvolle introductie van een mogelijk vaccin zijn de leeftijd waarop kinderen gevaccineerd dienen te worden; of de verworven immuniteit na vaccinatie beschermend werkt op latere leeftijd; en of de kosten van vaccineren lager zijn dan de kosten die nu gemoeid zijn met pijnstilling, antibioticumgebruik en het werkverzuim van ouders.

Appendices



Dankwoord

En dan het dankwoord. Het laatst geschreven stuk van mijn boekje, maar zeker niet het stuk waar het minst over is nagedacht. Met het schrijven van dit dankwoord is het laatste puzzelstukje gelegd en valt alles op zijn plek.

Ik heb de afgelopen 4 jaar hulp gehad van veel mensen, en bij deze daarom ook dank aan een ieder die op welke manier dan ook heeft bijgedragen aan dit proefschrift!
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Curriculum vitae

Suzanne Johanna Christina Verhaegh was born on April 23rd, 1978 in Tegelen, The Netherlands. In 1995, she finished her secondary school education (HAVO) at Blariacum College in Blerick, and began further education at the Gilde Opleidingen (Middelbaar Beroepsonderwijs), studying 'Medical Laboratory Techniques'. During this time she specialized in microbiology, performing research on the plant pathogenic fungi *Fusarium oxysporum* and *Mycosphaerella graminicola* at the Plant Research International (formerly IPO-DLO), Wageningen, The Netherlands. After graduating in 1998, she continued her education (Hoger Beroepsonderwijs) at the Larenstein International Agricultural College, taking a course in 'Laboratory Science' with a specialization in microbiology. As part of this course, Suzanne completed an internship entitled 'Interactions between *Acanthamoeba polyphaga* and the intracellular pathogen *Listeria monocytogenes*' at the Department of Biological Sciences, University of Warwick, Coventry, United Kingdom, under the supervision of Prof. Wellington. Further, she also performed research on 'The effect of antidiabetic components on the glucose uptake in L6 muscle cells' at Numico Research BV, Wageningen, The Netherlands, under the supervision of Dr. Nieuwenhuizen. In July 2001 she graduated and in that same year, started an MSc course in 'Biology' at the Wageningen University where she completed two MSc theses. The first thesis entitled 'Assessment and evaluation of the effect of two probiotic strains on fecal microbiota of patients with inflammatory bowel disease' was performed in the Laboratory of Microbiology, Wageningen, The Netherlands, under the supervision of Dr. Ben-Amor. A second traineeship was performed at the Department of Civil and Environmental Engineering, University of Washington, Seattle, United States, under the supervision of Prof. Stahl, where she analyzed gastrointestinal community shifts in pigs in response to dietary fiber. In January 2005 she graduated from the MSc course 'Biology' and from October 2005 until December 2006 worked as a research technician at the Department of Human Genetics at the Leiden University Medical Center. In January 2007, she continued practicing science as a PhD-student at the Department of Medical Microbiology and Infectious Diseases of the Erasmus MC, under the supervision of Dr. Hays and Prof. van Belkum. The majority of this research was performed as part of the European Union FP6 project 'Development of a prophylactic vaccine and diagnostic markers to prevent and diagnose otitis media (OMVac)', and the results obtained form

the majority of the publications presented in this thesis. As well as doing research, Suzanne participated in several courses, established by the Molecular Medicine Postgraduate School. Further, the results of her research were presented at several national and international scientific symposia and conferences, including the Dutch Scientific Spring Meeting (NVMM), the 1st International Workshop on *Haemophilus influenzae* and *Moraxella catarrhalis* (HinMax), and the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID). During her PhD studies, Suzanne also obtained funding to conduct research on a short traineeship at The Methodist Hospital Research Institute, Houston, Texas, United States, under the supervision of Dr. Musser, for which she received both an ESCMID travel grant for training in a foreign institution and a Ter Meulen Fund grant, from the Royal Netherlands Academy of Arts and Sciences. Initial studies were performed to study the effect of *Moraxella catarrhalis* on group A *Streptococcus* gene expression, particularly with respect to GAS virulence gene expression. Suzanne is currently continuing her research in the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, The Netherlands.

List of publications

Verhaegh SJC, Snippe ML, Levy F, Verbrugh HA, Jaddoe VWV, Hofman A, Moll HA, van Belkum A, Hays JP. Colonization of healthy children by *Moraxella catarrhalis* is characterized by genotype heterogeneity, virulence gene diversity and co-colonization with *Haemophilus influenzae*. Microbiology. 2011. 157(Pt 1):169-78.

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Verhaegh SJC and Hays JP. Chapter 80 *Moraxella*. In: Liu D (ed), Molecular Detection of Human Bacterial Pathogens. 2011. Taylor & Francis CRC Press, Boca Raton, FL, USA. In press.

Verhaegh SJC, de Vogel CP, Riesbeck K, Lafontaine ER, Murphy TF, Verbrugh HA, Jaddoe VWV, Hofman A, Moll HA, van Belkum A, Hays JP. Temporal development of the humoral immune response to surface antigens of *Moraxella catarrhalis* in young infants. Submitted for publication.

Verhaegh SJC, Flores AR, van Belkum A, Musser JM, Hays JP. Differential virulence gene expression of group A *Streptococcus* serotype M3 in response to co-culture with *Moraxella catarrhalis*. Manuscript in preparation.

Stol K*, **Verhaegh SJC***, Hays JP, Warris A, Hermans PWM. Microbial analysis does not facilitate the differentiation of childhood recurrent acute otitis media (rAOM) from chronic otitis media with effusion (COME). Manuscript in preparation.

Verhaegh SJC*, Stol K*, de Vogel CP, Belkum A, Hermans PWM, Hays JP. Comparative analysis of the humoral immune response to *Moraxella catarrhalis* and *Streptococcus pneumoniae* surface antigens in children suffering from recurrent acute otitis media (rAOM) and chronic otitis media with effusion (COME). Manuscript in preparation.

Smidt M*, Bättig P*, **Verhaegh SJC**, Oleksiewicz MB, Niebisch A, Hanner M, Schlick P, Noiges B, Schüler W, Lundberg U, Nagy E, Meinke AL, Hays JP, Selak S, Henriques-Normark B. Identification of novel *Moraxella* vaccine candidates by antigenome technology. Manuscript in preparation.

*These authors contributed equally to the work.

PhD Portfolio

Name PhD student:	Suzanne J.C. Verhaegh
Erasmus MC department:	Medical Microbiology and Infectious Diseases
PhD period:	January 2007 - June 2011
Research school:	Erasmus Postgraduate School Molecular Medicine
Promotor:	Prof.dr.dr. Alex van Belkum
Copromotor:	Dr. John P. Hays

PhD training

General academic skills	**Year**	**ECTS**
▪ Academic Writing in English for PhD Students	2008	2
In-depth courses Postgraduate School Molecular Medicine		
▪ Molecular Diagnostics	2007	1
▪ Bioinformatic Analysis, Tools and Services	2008	0.3
▪ Phylogeny & Genetics in Microbiology and Virology	2008	0.7
▪ Molecular Microbiology of Infectious Diseases	2008	1
▪ Biomedical Research Techniques	2008	1.6
▪ Basic Data Analysis on Gene Expression Arrays	2008	1.2
▪ Molecular Medicine	2010	0.7
▪ Research Management for PhD Students and Postdocs	2010	1
▪ Photoshop and Illustrator CS4	2010	0.3
▪ Writing Successful Grant Proposals	2011	0.5
Seminars and workshops		
▪ Departmental Journal Clubs	2007-2010	3
▪ Departmental Research Meetings	2007-2010	2
▪ PhD Day Erasmus MC	2008-2009	0.7

National and international conferences

▪ Scientific Spring Meeting NVMM (oral presentation)	2007	2
▪ Molecular Medicine Day (poster presentation)	2008	1
▪ Scientific Spring Meeting NVMM (poster presentation)	2008	2
▪ HinMax, Rotterdam, The Netherlands (oral presentation)	2008	2
▪ Scientific Spring Meeting NVMM (poster presentation)	2009	2
▪ 19th ECCMID, Helsinki, Finland (oral presentation)	2009	2
▪ Molecular Medicine Day (poster presentation)	2010	1
▪ Scientific Spring Meeting NVMM (oral presentation)	2010	2
▪ 20th ECCMID, Vienna, Austria (poster presentation)	2010	2
▪ Scientific Spring Meeting NVMM (poster presentation)	2011	2

Scientific meetings

▪ OMVac consortium meetings	2006-2010	5
▪ Departmental Research days (oral presentations)	2008-2010	3

Other activities

▪ PhD committee Postgraduate School Molecular Medicine	2008-2011	4
▪ Organization Departmental Research days	2008-2010	4
▪ Organization Get out of your Lab Days Postgraduate School Molecular Medicine	2009	2

Research experience abroad

- The Methodist Hospital Research Institute, Houston, Texas, United States. Laboratory training in global gene expression analysis using microarrays. Supervision: Dr. Anthony F. Flores and Dr. James M. Musser. June - August 2010

Grants

- ESCMID travel grant for training in a foreign institution
- Ter Meulen Fund grant, the Royal Netherlands Academy of Arts and Sciences

Teaching

Supervision of students

▪ Supervision of Bachelor of Science students	2007-2009	8
▪ Supervision of medical students 'Vaardigheidsonderwijs Infectieziekten'	2007-2011	4