

Studies into the Genetic Diversity and Complement
Resistance Phenotype of *Moraxella catarrhalis*.

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**Studies into the Genetic Diversity and
Complement Resistance Phenotype of
Moraxella catarrhalis.**

Onderzoek naar de genetische diversiteit en
het complement resistente fenotype van *Moraxella catarrhalis*

THESIS

to obtain the degree of Doctor from the Erasmus University Rotterdam

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“Earth Is The Cradle Of Humanity
But One Cannot Remain In The Cradle Forever”

Konstantin Tsiolkovsky 1895

To friends and relations (past, present and future)

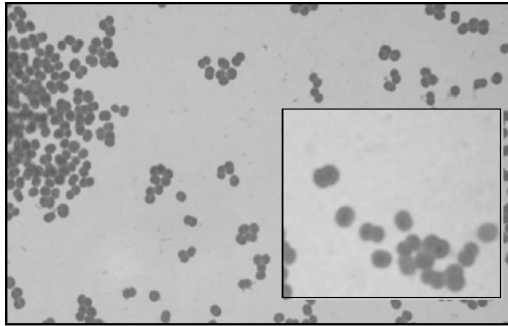
**Studies into the Genetic Diversity and Complement Resistance
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General Introduction



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The Genus Moraxella

The *Moraxella* genus currently contains at least 14 different species, including *M. catarrhalis*, *M. bovis*, *M. lacunata*, *M. osloensis*, *M. nonliquefaciens*, *M. atlantae*, *M. lincolni*, *M. ovis*, *M. caviae*, *M. canis*, *M. equi*, *M. cuniculi*, *M. caprae* and *M. boevertii*, which colonize both humans and animals. Four of these species, namely *M. catarrhalis*, *M. caviae*, *M. ovis*, and *M. cuniculi*, may be separated into a separate subgenus, the *Branbamella* subgenus (i.e. *M. (B.) catarrhalis*, *Moraxella (Branbamella) catarrhalis*). Using this system, the remaining species are included in the subgenus *Moraxella* (i.e. *M. (M.) lacunata*, *Moraxella (Moraxella) lacunata*). This division is based largely on morphological characteristics, in that subgenus *Moraxella (Branbamella)* spp. tend to exhibit coccoid morphology, whilst subgenus *Moraxella (Moraxella)* spp exhibit rod-like morphology. However, many publications tend to omit the (B). / (M). or (Branbamella) / (Moraxella) subgenus designation. Also, the *Moraxella* genus itself is under constant revision, with recent taxonomic restructuring placing the bacterial species formerly known as *Moraxella phenylpyruvica* in the genus *Psychrobacter* as *Psychrobacter phenylpyruvica* and *Moraxella urethralis* in the *Oligella* genus as *Oligella urethralis*. Common characteristics of the *Moraxella* genus include: a lack of colony pigmentation; Gram-negative staining coccoid/bacillus morphology with a tendency to resist decolorisation; positive with oxidase reagent and tetra-/dimethyl-*p*-phenylenediamine; and a GC content of between 40 and 47.5 mol%. Optimum growth conditions are achieved on blood agar plates under aerobic conditions at a temperature of approximately 33-37°C. In general, distinguishing between the different *Moraxella* species tends to be difficult, not least because of the asaccharolytic nature of the genus, though some publications have indicated that 16S rRNA sequence polymorphisms may be a useful adjunct to biochemical testing (12, 15, 19). The majority of *Moraxella* spp. are susceptible to penicillin-derived antibiotics, including cephalosporins, tetracyclines, quinolones and aminoglycosides. This contrasts with *M. catarrhalis*, where an alarming increase in the number of penicillin resistant isolates has been observed in the last 30 years (now approximating 90-95% of all isolations). This distinction in antibiotic sensitivity is most probably related to the fact that *M. catarrhalis* produces beta-lactamases enzymes, a phenomenon apparently rare in other *Moraxella* spp. (though at least one publication has indicated otherwise with respect to *M. nonliquefaciens* (11)).

Moraxella catarrhalis

The bacterial species known as *Moraxella catarrhalis* (previously referred to as *Micrococcus catarrhalis*, *Neisseria catarrhalis*, *Branhamella catarrhalis* and *Moraxella (Branhamella) catarrhalis*) was originally thought to be a common human commensal bacterium with little pathogenic potential, a characteristic currently shared by most other species within the *Moraxella* genus. However, growing evidence collected over the last 30 years has proven the clinical relevance of this organism in facilitating disease in both adults and children. The main question now remaining is the mechanism(s) by which the organism switches from being a harmless commensal to a facilitator of pathogenic disease.

Taxonomy

Moraxella catarrhalis has undergone several name changes since its first discovery in 1896 (13). Originally entitled *Micrococcus catarrhalis*, the organism became more familiar as *Neisseria catarrhalis* in the 1950's, largely due to its Gram-negative, diplococoid morphology and rapid oxidase positive reaction (characteristics associated with *Neisseria* species). In 1963 however, Berger showed that this genus may actually contain two distinct species (*Neisseria cinerea* and *Neisseria catarrhalis*), which could be separated by their biochemical ability to reduce nitrate and nitrite and to utilise tributyrin (3). With the introduction of DNA technology, further phylogenetic studies indicated a lack of chromosomal DNA homology, between the "true" *Neisseria spp.* and *N. catarrhalis*, so much so, that in 1970 the species was renamed *Branhamella catarrhalis* and relocated to the new genus of *Branhamella* to honour the famous microbiologist Sarah E. Branham who had previously conducted research on *Neisseria* (8). Fourteen years later, Bovre suggested that *B. catarrhalis* be renamed *Moraxella (Branhamella) catarrhalis* and be re-assigned to the genus *Moraxella*, not least because of the distinct physiological and genetic relatedness between *Branhamella catarrhalis*, and members of the *Moraxella* genus (5). In 1991, Catlin (6) suggested the formation of a new family (the *Branhamaceae*), which would accommodate both *Branhamella* and *Moraxella* genera and take into account the differences in morphology (cocci versus rods) and pathogenicity (important mucosal pathogens versus rare causes of disease). However, this suggestion has not been adopted by taxonomists, not least because 16S ribosomal RNA sequence analysis and genetic hybridisation studies tend to favour the current classification of *M.*

catarrhalis (12, 19). Currently, the *Moraxella* genus contains both rod-shaped (genus *Moraxella*, subgenus *Moraxella*) and coccoid (genus *Moraxella*, subgenus *Branhamella*) bacteria exhibiting similar genetic relatedness, though taxonomic revision of the genus is a continuing process. Indeed, there is growing evidence to suggest that the current *M. catarrhalis* genus may actually represent two different sub-species or even distinct species *per se* (4, 22).

Isolation and Identification

Optimum growth conditions for *M. catarrhalis* include a temperature of 35-37°C in an atmosphere containing 3%-7% CO₂, though the organism is also capable of growing at a larger range of temperatures (20-42°C) and in ambient air. Pure cultures of *M. catarrhalis* may be grown on standard laboratory media including Mueller Hinton agar, Columbia blood agar, and brain heart infusion or tryptic soy digest broths, though the addition of supplements e.g. boiled blood plus vitamin / amino acid enrichment (chocolate agar) may result in an enhancement of growth (9). A defined medium for *M. catarrhalis* culture was described by Juni et al in 1986 (17). Unfortunately however, the growth of *M. catarrhalis* from clinical specimens may be somewhat complicated by the presence of the normal bacterial flora, and in particular the presence of non-pathogenic *Neisseria*. For this reason, selective agars may best be used for the isolation of *M. catarrhalis* from clinical specimens such as sputum etc. Several types of selective media have been described for *M. catarrhalis* with a common feature being the inclusion of trimethoprim, vancomycin and an antifungal agent, with the further addition of acetazolamide (a synthetic sulphonamide) having been reported to enhance recovery (21). Of interest, it has been reported that *M. catarrhalis* isolated on modified New York City (MNYC) medium containing antibiotics selective for pathogenic *Neisseria*, may possess a greater virulence potential than isolates which do not grow on this medium (10). *M. catarrhalis* does not grow on Modified Thayer-Martin (MTM) medium. For long-term storage (several years) at -80°C, STGG (skimmed milk, tryptone, glucose, glycerol) medium has been reported to yield encouraging results (18).

M. catarrhalis colonies tend to be large, grey or non-pigmented, smooth, opaque and convex in nature and may be readily pushed intact over the surface of agar using a sterile loop. In a typical Gram film, the organism appears as a Gram-negative diplococcus with flattened sides, though physical appearance by itself is not enough to separate the species from related contaminating *Neisseria* spp. The identification of *M.*

catarrhalis to the species level involves biochemical testing with the production of oxidase, expression of a DNase, lack of acid production from glucose, sucrose, lactose, maltose and fructose, reduction of nitrate and nitrite, and the hydrolysis of tributyrin being important, though none of these tests are 100% specific or sensitive by themselves (7). The use of DNA technology, and in particular PCR, has allowed the direct detection of *M. catarrhalis* from clinical isolates (middle ear effusions, nasopharyngeal secretions etc) without the prior need for culture, with the validity of PCR detection having been proved in animal models (1, 20). Indeed, results show a superior sensitivity for PCR (down to six or seven genome equivalents over more traditional culturing methods (20)). Recent research has also shown that PCR may be used to follow quantitative changes in *M. catarrhalis* numbers in nasopharyngeal secretions in “real-time”, perhaps offering a sensitive and reliable means of following disease progress and/or treatment regimens in the future (14). However, perhaps the most useful aspect of PCR with regard to clinical diagnosis, is the ability of multiplex PCR protocols to detect several pathogens in a single reaction mix, including the bacterial pathogens *M. catarrhalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. Such multiplex PCRs have already been validated in animal models (2) and have been shown to be a reliable diagnostic tool allowing screening of middle ear effusions for several pathogens (including *Alloiococcus otitidis*) within a single reaction tube and within a single working day (16).

AIMS AND OUTLINE OF THE THESIS

The aim of this thesis was to help define the contribution of *Moraxella catarrhalis* genetic diversity on the ability of the bacterium to colonise and cause infection in the human host, as well as to investigate novel genes/mechanisms associated with isolates exhibiting the complement resistance phenotype.

In this respect, **Chapters 1** and **2** provide a detailed description of our current knowledge regarding the *Moraxella* genus in general and the *Moraxella catarrhalis* species in particular. The subsequent nine chapters are then divided into two main themes, relating to *M. catarrhalis* genotypes/genotypic changes (**Chapters 3 – 6**), and the

identification and characterisation of novel genes/mechanisms associated with the complement phenotype within this species (**Chapters 7 – 10**).

Chapter 3 investigates the genetic diversity of *M. catarrhalis* within a population of hospitalised children, and the effect of genotypic changes on concomitant disease. **Chapter 4** explores possible changes in *M. catarrhalis* genotypes and complement phenotype after vaccination with a *Streptococcus pneumoniae* vaccine (an organism that shares the same biological niche as *M. catarrhalis*). The following chapter (**Chapter 5**) represents the most extensive investigation into any group of *M. catarrhalis* isolates to date, using intensive whole-cell genotyping and protein profiling techniques to study complement phenotype-specific markers within this species. Such markers could be useful in developing screening techniques to distinguish between lineages of *M. catarrhalis* exhibiting differential virulence traits. **Chapter 6** studies genotypic and complement phenotype variation in *M. catarrhalis* with respect to otitis media prone and otitis media non-prone children. In the same chapter, low frequency intra-genomic variation in the UspA1 and UspA2 outer membrane proteins (previously associated with conferring complement resistance) are described.

With respect to genes/mechanisms associated with the complement phenotype, **Chapter 7** details research undertaken into the identification and characterisation of a novel major outer membrane protein of *M. catarrhalis* (OMP J) that exists in two major forms. Moreover, these forms are significantly associated with particular genetic lineages as well as the complement phenotype. The effect of knocking out the *ompJ2* gene on the pulmonary clearance of *M. catarrhalis* in a mouse model of infection is also investigated. In **Chapter 8**, studies are performed into the role of OMP J in the immune response to *M. catarrhalis* using sera collected from children suffering from acute otitis media (AOM). The effect of passive immunisation, using anti-OMP J antibodies, on the clearance of *M. catarrhalis* bacteria from the lungs of mice is also described. **Chapter 9** investigates the role of the mannose-binding lectin (MBL) pathway of complement activation on *M. catarrhalis* isolates cultured from children who suffered from recurrent acute otitis media episodes. This chapter provides information regarding the role of the MBL pathway on *M. catarrhalis* survival upon exposure to human serum. In **Chapter 10**, a search is performed for novel *M. catarrhalis* plasmids which may be useful as cloning and expression vectors for putative *M. catarrhalis* virulence genes (including complement resistance-associated genes). The chapter details the identification, isolation and characterisation of a small 3.5kb plasmid named pEMCJH04.

Finally, **Chapter 11** gives a summary of the research presented within this thesis and discusses the findings with respect to *M. catarrhalis* genotypic lineage and complement resistance phenotype.

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Moraxella catarrhalis:
**Virulence, Genetic Variation, Immune Response
and Putative Vaccines**

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ASSOCIATED DISEASES

Although once being considered a harmless commensal, it is now recognised that *Moraxella catarrhalis* is an important pathogen of humans, associated with upper and lower respiratory tract infections in both children and adults (140). In particular, *M. catarrhalis* tends to be associated with upper respiratory tract infections (including acute otitis media and sinusitis) in children, but lower respiratory tract infections in adults (usually with pre-existing pulmonary disease). Occasionally, the bacterium is reported as being the causative agent of certain non-respiratory tract associated infections, including: bacteremia (4, 70, 71, 126), endocarditis (187, 229), conjunctivitis/ keratitis (1, 155, 257), and tracheitis (83). The organism has very occasionally been associated with meningitis (63), ventriculitis (60, 215), ophthalmia neonatorum (197), acute mastoiditis (151) and septic arthritis (2, 191). Interestingly, many reports have indicated the seasonal incidence of *M. catarrhalis* infection, with the winter and spring months showing the greatest rates of isolation (202, 218, 258). Moreover, this seasonality is not a prominent feature of other commonly isolated respiratory tract-associated pathogens e.g. *Streptococcus pneumoniae* and *Haemophilus influenzae* (64, 68, 143), and there is some speculation that seasonal viral respiratory tract infection (e.g. respiratory syncytial virus (9, 78) but not adenoviruses (12)) may be a pre-disposing factor in seasonal *M. catarrhalis* infection. Another theory involves bacterial-bacterial competition involving *Corynebacterium spp.* which form part of the normal human throat microflora (213).

From a clinicians' perspective, it is also important to note that *M. catarrhalis* may be isolated as part of a mixed population of pathogens. For example, research has indicated that isolation of *M. catarrhalis* from sputa is accompanied with the isolation of other bacterial pathogens on approximately 40-50% of occasions (the most common being *S. pneumoniae* and *H. influenzae*) (110, 188, 202). In such cases, it may be clinically important to define the role of *M. catarrhalis* in the disease process, particularly with respect to antibiotic therapy, i.e. should antibiotic therapy be targeted against the co-isolated organism only, *M. catarrhalis* only, or should a broad spectrum antibacterial agent be used.

Childhood Infections

Otitis media - acute otitis media (AOM) is the most frequent infection associated with *M. catarrhalis* in children (86, 88, 92, 93), and comprises inflammation of the middle ear accompanied by a liquid effusion (145, 164). Approximately 50% of children will have experienced at least one episode of AOM by their first birthday, and this proportion rises to 70% by 3 years of age (144, 235, 236, 240), representing a tremendous disease burden for this age group and necessitating the widespread use of antibiotics (55, 200, 201). *M. catarrhalis* has been recognised as a specific pathogen associated with AOM for over 70 years (111), being the third most common bacterium isolated from this condition (behind *H. influenzae* and *S. pneumoniae*) (58, 102, 193). During routine visits, Faden et al (1994) (89), observed that otitis media prone children were colonised in 44.4% of occasions compared to only 16.7% of occasions for otitis non-prone children, suggesting a link between increased *M. catarrhalis* colonisation and the occurrence of otitis media. Of note, the actual rate of *M. catarrhalis* associated AOM may be underestimated, as tympanocentesis and culturing of middle ear effusions is not routinely performed. The use of new PCR based methodologies also provides evidence for the presence of *M. catarrhalis* in culture negative middle ear effusions from children suffering chronic otitis media (203). Also of note, the development and use of vaccines against *H. influenzae* and *S. pneumoniae* could facilitate a future increase in the percentage of AOM episodes attributable to *M. catarrhalis*. Historically, there appears to have been a marked increase in the number of reported *M. catarrhalis* isolations from middle ear effusions of children suffering from AOM since the 1980s (now approximating 15-20%), an increase accompanied by the appearance of beta-lactamase producing strains which now account for approximately 90-95% of all isolates (24, 147, 158, 194). Further, there is some suggestion that *M. catarrhalis* may facilitate the spread of beta-lactamase genes to other bacterial species during mixed bacterial infections (137). It is perhaps poor consolation that the actual numbers of bacteria isolated from middle ear effusions, and the severity of symptoms during AOM episodes, both appear to be lower when *M. catarrhalis* is isolated rather than *S. pneumoniae* or *H. influenzae* (87). Recent treatment recommendations for the treatment of acute otitis media were presented by Hoberman et al (2002) (118).

Sinusitis – is another very common infection in childhood, which has been estimated to account for approximately 5-10% of upper respiratory tract infections in this age group (249-253). Diagnosis may be difficult due to the presence of fairly non-

specific symptoms which are similar to allergy and upper respiratory tract viral disease (62). Clinically, sinusitis may be divided into two groups, the acute group (where symptoms persist for 10 to 30 days), and the subacute group (where symptoms persist for 30 to 120 days). In both cases, *M. catarrhalis* accounts for approximately 5-10% of bacterial infections, with *S. pneumoniae* and *H. influenzae* being more commonly isolated pathogens (35, 46, 94). However, *M. catarrhalis* does grow poorly in environments containing reduced oxygen concentrations (37).

Lower respiratory tract infections (LRTI) in children - the role of *M. catarrhalis* in the aetiology of LRTI in children seems to be clear cut, the organism having been isolated in pure culture from tracheal aspirates obtained from neonates, infants and children with pneumonia (18, 20, 34, 109). Interestingly, *M. catarrhalis* appears to be increasingly isolated from LRTI originating in hospitalised children, with Chong (1997) (51) indicating an increase in lower respiratory tract infections in hospitalised children from 11.4% in 1988 to 34.7% in 1995, a result possibly due to the increasing penicillin resistance observed in *M. catarrhalis* isolates over this period.

Adult infections

Bronchitis and pneumonia - though not a common cause of lower respiratory tract infection in healthy adults, *M. catarrhalis* may present a particular problem for adults with pre-existing pulmonary disease and in the elderly. In particular, bacterial exacerbations of chronic obstructive pulmonary disease (COPD) are often attributable to *S. pneumoniae*, non-typeable *H. influenzae* and *M. catarrhalis* infection in that order (10, 34, 81, 166, 174, 188, 202, 224). Bacterial pneumonias attributable to *M. catarrhalis* tend to be relatively mild, with the presence of patchy non-cavitary infiltrates on chest X-ray (259). Particularly susceptible to *M. catarrhalis* pneumonia are patients with end-stage pulmonary or malignant disease (14, 110), and many of these (>70%) are, or have been, smokers (68). *M. catarrhalis* pneumonia may occasionally be complicated by bacteremia (57, 126, 237).

Klingman et al (1995) (146) indicated that a proportion (approximately 20%), of *M. catarrhalis* mediated bronchiectasis patients maybe chronically colonised with up to four different strains of *M. catarrhalis*, though a direct relationship between the exacerbation of bronchiectasis and *M. catarrhalis* isolation was not proven.

Laryngitis - *M. catarrhalis* appears to be the most frequent bacterial pathogen associated with laryngitis in adults (220-222), though there remains some debate as to

whether the organism actually infects or simply colonises adults presenting with laryngitis (119). Further, the pathogenic potential of *M. catarrhalis* in laryngitic disease has been suggested by the fact that laryngitis has been induced in an animal model (128), and that patients with laryngitis are more likely to harbour isolates presenting with a complement resistant phenotype (119).

BACTERIAL TYPING AND EPIDEMIOLOGY

Traditionally, typing of *M. catarrhalis* isolates (for determining the relatedness of isolates during nosocomial transmission etc), has been performed using a series of non-molecular techniques, which have included: susceptibility to human serum mediated bacteriolysis, trypsin sensitivity/agglutination profiles against human group O erythrocytes (227), iso-electric focussing of beta-lactamase proteins (186), serological typing of lipopolysaccharide (241, 242), electrophoretic profiling of outer membrane proteins (15, 178), autoaggregation properties (127), and a few other miscellaneous techniques (65, 198, 199). However, advances in DNA technology now allows comparative whole genomic testing to be performed. The simplest of these techniques involves the use of restriction endonuclease enzymes to specifically digest the genomic DNA of isolates, followed by gel electrophoresis to separate the individual fragments (52, 142, 195). *Hae* III, *Hind* III, *Pst* I, *Cla* I and *Spe* I are particularly useful enzymes in this respect. Particularly successful and popular at present is the use of macro-restriction analysis (pulsed field gel electrophoresis or PFGE) which has been used to type *M. catarrhalis* isolates, particularly in the nosocomial situation (112, 159, 160, 248, 261). Interestingly, the use of several genomic techniques, including strain specific DNA probes (16, 254), automated ribotyping, and amplification fragment length polymorphism (AFLP) analysis (30, 247), have all indicated the division of *M. catarrhalis* isolates into 2 distinct phylogenetic clusters (or possibly sub-species). Moreover, *M. catarrhalis* isolates expressing various virulence traits (complement resistance, increased adherence to epithelial cells) have been found to be statistically associated with one of these two clusters (30, 247). Such genomic typing studies have also shown the genetically diverse nature of *M. catarrhalis* isolates, with successive clonal expansions (often linked to geographical location) apparently occurring (82, 159). Inter-species horizontal transfer of genes between *M. catarrhalis* and other species that inhabit the same niche also appears possible (31, 154).

Carriage and Spread

M. catarrhalis is an organism able to colonise the human respiratory tract without causing disease, though there exists a striking difference between the carriage rates of *M. catarrhalis* in children and in adults. In children, the carriage rate may be as high as 75% (89, 101, 241, 243), whilst in adults the carriage rate is very low at around 1 - 3% (65, 76). This inverse relationship between rate of colonisation and age has been known for approximately 100 years (11), and is still present today, though an adequate explanation for this phenomenon still remains elusive. Based on evidence provided by Meier and Stutzmann Meier (2003) (171, 231), one possible explanation for this phenomenon is that colonisation is dependent upon the development of strain specific mucosal immunoglobulin A (IgA), with specific IgA production being perhaps more important than specific IgG production (77). In this scenario, multiple colonisation and infection with different isolates of *M. catarrhalis* gradually increases the repertoire of specific neutralising IgA (or IgG) antibodies, such that the number of novel *M. catarrhalis* isolates able to colonise a particular individual decreases with time. Alternatively, otitis prone children may fail to produce a broadly protective antibody response (86), or could possibly have (sub-clinical) deficiencies in immune modulatory genes. Whatever the explanation, there appears to be a distinct correlation between the frequency of colonisation and the development of disease in children (38, 89, 92, 206, 241), a correlation which may also hold true for adults (146, 177).

There is as yet some debate regarding the mechanism of spread of *M. catarrhalis*, with possible mechanisms including person-to-person transmission or spread via environmental contamination (40, 125, 166). Both nursery schools and day care centres may be important reservoirs for strain exchange in children (161, 205, 261), and intra-familial spread has been observed (89). Outbreaks of nosocomial infection due to *M. catarrhalis* have been regularly documented (Figure 4) (19, 59, 66, 109, 160, 196, 211). However, the degree of (nosocomial) spread may be somewhat underestimated due to the fact that *M. catarrhalis* colonisation may not always lead to the appearance of overt disease (125).

Antibiotic Susceptibility

M. catarrhalis exhibits an almost universal resistance to penicillin related antibiotics, with several recent studies indicating that world-wide, 90-100% of *M.*

catarrhalis isolates produce beta-lactamase (3, 132, 136, 157, 173, 255, 264). This is a striking statistic when one considers that before 1970 few isolates produced beta-lactamase enzymes (44, 256). In fact, the increase in beta-lactamase producing isolates has been so rapid that in most countries it appears that a “saturation point” is close to being reached, with the increase in incidence of beta-lactamases positive *M. catarrhalis* strains now levelling off at this very high level (133, 226, 264). Research into *M. catarrhalis* beta-lactamase production has shown that 3 different isotype groups may be identified, BRO-1, BRO-2 and BRO-3 (54, 79, 149). However, by far the most common types are BRO-1 and BRO-2, being found in approximately 94% and 5% of beta-lactamase producers respectively (45). There is some suggestion that BRO-1 is gradually replacing BRO-2 over time (31, 85, 212) and that BRO-1 producing strains are less susceptible to penicillins than BRO-2 producing isolates (124). Both BRO-1 and BRO-2 enzymes appear to be constitutively expressed, chromosomally or plasmid encoded proteins, which differ by a single amino acid (32, 79, 80, 138). Interestingly, there is some evidence to suggest that the BRO beta-lactamases of *M. catarrhalis* were originally derived from a non-*Moraxella* species, this evidence includes: the lower G + C content of the BRO encoding (*bla*) genes (31% compared to an average of 41%), and the fact that the BRO-type beta-lactamases are lipidated in *M. catarrhalis* (suggesting a Gram-positive origin for the gene) (29, 256). A simple restriction digestion methodology to distinguish between the BRO-1 and BRO-2 beta-lactamase types has been published (72). Of clinical relevance, isolates harbouring the BRO-1 encoding gene tend to have higher transcription rates (and hence higher MICs) than BRO-2 harbouring isolates (137, 165). However, both BRO-1 and BRO-2 enzymes are inactivated by beta-lactamase inhibitors (36, 98, 189). In any case, the choice of treatment should ideally be based upon the type of disease and condition of the patient (e.g. simple or complicated COPD) (184). It is however perhaps prudent for clinicians to assume that all isolates of *M. catarrhalis* are resistant to penicillin, ampicillin, amoxicillin, piperacillin and cephalothin (69, 137, 152). Also important for clinicians to consider is the fact that the genes for BRO-type enzymes may be easily transferred by conjugation between different species (139, 256), a phenomenon which may be important in the treatment of mixed infections, and may even result in treatment failure (120, 194, 228). That said however, there is some evidence to suggest that beta-lactamase-producing *Moraxella catarrhalis* may actually prevent the emergence of penicillin-resistant *Streptococcus pneumoniae* in children with recurrent acute otitis media (131).

M. catarrhalis isolates may also show some inherent resistance to trimethoprim, with a recent study of 76 clinical isolates from Saudi Arabia indicating that 14.5% of isolates were resistant to this antibiotic (137). Further, a recent survey showed that approximately 10.5% of *M. catarrhalis* isolates in Latin America (130) and all isolates from an Indian study (232) were highly resistant to combination therapy using trimethoprim and sulphamethoxazole. In general however, *M. catarrhalis* isolates are consistently susceptible to most non-beta-lactamase antibiotics including, chloramphenicol, erythromycin, ciprofloxacin and gentamicin (17, 116, 117). Also, fluoroquinolones remain very active against *M. catarrhalis* (67, 84). New antibiotics for use against lower respiratory pathogens (including *M. catarrhalis*) are under constant development and testing (33, 134).

THE IMMUNE RESPONSE

The immune response to bacterial pathogens is a highly complex process involving both non-specific and specific mechanisms.

The Non-Specific Immune Response

Non-specific mechanisms include the presence of mucus and surfactant at pulmonary surfaces, mucociliary action, alveolar macrophage activity and complement-mediated lysis (238). Onofrio (1981) (192), published data looking at the removal of several bacterial species from the lungs of mice, and in contrast to non-typeable *H. influenzae* and *Streptococcus spp.*, found that *M. catarrhalis* was cleared relatively slowly from the lungs with a correspondingly significant increase (400x) in the numbers of granulocytes. Further, the release of "neutrophil defensins" (peptides exhibiting broad-spectrum antimicrobial activity and released by neutrophils as part of the inflammatory process), have been shown to actually stimulate adherence of *M. catarrhalis* (107) (a process conceivably involving the phase variable expression of bacterial adherence genes). Finally, it has recently been reported that *M. catarrhalis* induces mast cell activation and nuclear factor kappa B-dependent cytokine (IL-6 and MCP-1) synthesis after direct contact with mast cells (with neither bacterial lipopolysaccharide or bacterial supernatants inducing cytokine secretion) (148). The role of complement and

complement resistance in the non-specific immune response to *M. catarrhalis* mediated infections is discussed later.

The Specific Immune Response

Several studies have demonstrated the presence of bactericidal antibodies in *M. catarrhalis* associated infections, including: AOM (90, 91, 150, 162), lower respiratory tract infections (23, 47, 53), pneumonia (100) and exacerbations of COPD (13). Using whole-cell *M. catarrhalis* bacteria as coating antigen and an ELISA assay format, Goldblatt et al (1990) (105) showed that in the majority of cases, normal human sera tested positive for the binding of IgG1, IgG2 and IgG3 sub-classes but not IgG4. Further, the IgG3 sub-class of antibody may be particularly important in *M. catarrhalis* infection (208) as IgG3 specific antibodies are low or undetectable in children under 4 years of age (48, 106). These findings may explain the differences in colonisation rates observed between children and adults (11, 89). However, studies have indicated that host Gm allotype (i.e. genetic markers present on IgG molecules) may influence the IgG subclass composition of specific antibody responses to *M. catarrhalis* outer membrane proteins (42), possibly via a complex interaction between B-cells, T-cells, HLA type and Gm-linked V region antibody expression (104). If the IgG3 sub-class is particularly important and allotype variation results in differences in IgG3 expression in certain individuals, then this could explain why some children suffer from chronic *M. catarrhalis* infections (e.g. chronic otitis media)

At mucosal surfaces IgA antibody responses predominate and specific IgA has been detected in middle ear effusions of children with otitis media (90, 141, 233) and in the saliva of healthy adult volunteers (171). Stutzmann Meier (2003) (231) indicated that *M. catarrhalis* colonisation in early infancy was associated with a consistent IgA immune response directed against the outer membrane proteins. However, it has been shown that the development of (mucosal) IgA antibody may not necessarily correspond to the development of (systemic) IgG antibody (216, 230). Of note, Mandrell et al (1992) (156) and Troncoso et al (2004) (239) identified cross-reactive antigens between *M. catarrhalis*, *N. meningitidis* and *N. lactamica*, and showed the presence of cross-reactive antibodies in both healthy individuals and patients convalescing from meningococcal meningitis.

SERUM AND COMPLEMENT RESISTANCE

The complement system is a complex series of proteins found in serum which are part of the host defense mechanism against microbial pathogens (43, 153). It is known that the ability of Gram-negative bacteria to withstand the effects of complement is an important virulence factor (39, 214), and many *M. catarrhalis* disease causing isolates are apparently resistant to the effects of complement in human serum (121, 122, 135). Several studies have shown the importance of complement resistance to *M. catarrhalis* pathogenicity (37, 176, 227), with isolates from the upper respiratory tract of children or healthy adults tending to be complement sensitive, whilst isolates from children and adults with lower respiratory tract infections tending to be complement resistant (122, 244). There appears to be no relationship between beta-lactamase production (another virulence factor) and complement resistance in *M. catarrhalis* (223), possibly reflecting the plasmid borne nature of beta-lactamase production or the multifactorial nature of complement resistance. Our knowledge regarding complement resistance mechanisms in *M. catarrhalis* is as yet limited. Prellner et al (1980) (204), provided evidence for high level binding of C1q complement component to *M. catarrhalis*, a phenomenon which would render the organism sensitive to the anti-bacterial activity of complement. Research by Verduin et al (1994) (245), showed that a least one resistance mechanism involves inhibition of formation of the membrane attack complex of complement, involving the binding of vitronectin (a natural inhibitor of complement found in serum) to the UspA2 protein present on the surface of *M. catarrhalis* (169, 246). An *M. catarrhalis* mutant lacking the UspA2 gene was found to be sensitive to complement-mediated killing, whilst the parent isolate was resistant (6). Further, UspA2 protein shares similarity with the *H. ducreyi* DsrA (*ducreyi* serum resistance protein A), which has also been shown to be involved in serum resistance (56). A *copB* gene knockout *M. catarrhalis* mutant was used by Helminen et al (1993) (114) to show that reduced expression of the CopB (or OMP B2) iron acquisition protein, decreased serum resistance and survival *in vivo*. Confusingly however, Furano et al (99), recently indicated that constitutive expression of iron-regulated proteins (via inactivation of the ferric iron uptake regulator gene *fur*) actually increases the susceptibility of *M. catarrhalis* to the bactericidal activity of normal human sera, and suggested that this most probably occurs due to the up-regulated expression of (outer membrane) proteins increasing the presence of multiple antigenic targets for human antibody binding and hence complement activation. One further study of *M.*

catarrhalis outer membrane protein expression indicated that OMP E (a putative fatty acid transporter) may be involved in serum sensitivity (179). In 2000, Zaleski et al (263) implicated the *M. catarrhalis* LOS in complement resistance, as inactivation of the *galE* gene which encodes a UDP-glucose-4-epimerase (involved in the biosynthesis of LOS) resulted in enhanced serum susceptibility of the mutant. Interestingly, similarities in outer membrane proteins and LOS between *M. catarrhalis* and *Neisseria* spp. (e.g. *N. gonorrhoea* and *N. meningitidis*), may provide further clues as to the mechanisms by which complement resistance is mediated in *M. catarrhalis*.

Finally, several studies have indicated that complement resistant and complement sensitive isolate of *M. catarrhalis* may actually represent two different sub-species (30, 247).

PUTATIVE VACCINE CANDIDATES

Currently, the efforts of many researchers in the *M. catarrhalis* field is geared towards the identification and development of specific vaccine candidates (reviewed in (167, 168, 170)) with particular emphasis being placed on 1) the lipooligosaccharide/lipopolysaccharide (LOS/LPS) and 2) various highly expressed outer membrane proteins (178).

Several studies have indicated that an antibody response to the LPS of *M. catarrhalis* is generated and that this response is not serotype specific but directed against common epitopes present on the LPS (95, 129, 190, 209), though confusingly there is some evidence to suggest that the antibody response (in rabbits) is indeed serotype specific (207). This LPS antibody response appears to be particularly directed against the oligosaccharide region of the LPS (73-75). The inoculation of non-viable *M. catarrhalis* into the middle ear bullae of guinea pigs also induces middle ear inflammation and mucoperiosteal histopathology, a type of inflammatory picture most probably mediated by bacterial outer membrane components (including the LOS) (219). Based on this data, research by several groups has indicated that the LPS/LOS may be a promising vaccine candidate so long as its inherent toxicity is removed and it is linked to a protein conjugate to improve immunogenicity (108, 123, 129, 234). To complicate matters, there are at least 3 serotypes of LPS, each exhibiting some form of cross-reactivity (242), possibly dependant on differences in the lengths of the immunodominant oligosaccharides (75). However, no relationship between serotype and

severity of infection have been noted, implying that all three of these common LOS serotypes (types A, B and C) should ideally be included in a future LPS/LOS based vaccine.

Most research regarding the development of a vaccine against *M. catarrhalis* has concentrated on the identification and investigation of several major outer membrane proteins (OMPs), which currently fall into roughly 2 distinct groups. One group includes OMPs associated with bacterial adherence, including; UspA1/2 (7, 48, 49, 115), OMP CD (182, 183, 260) and MID (96, 97), whilst the other group includes OMPs associated with iron and fatty acid (nutrient) acquisition, LbpA, LbpB, TbpA, TbpB/B1/74kDa protein (25, 27, 28, 41, 50, 163), CopB (8, 114, 225) and OMP E (21, 179).

The UspA proteins (UspA1 and UspA2) are closely related proteins which share some conserved, semi-conserved and repeat sequence domains (7, 61, 112). Both proteins share a conserved epitope that binds the monoclonal antibody 17C7 (mab17C7), which could be useful in the progress towards a *M. catarrhalis* vaccine (172). Moreover, both proteins appear to elicit (protective) antibodies in humans (48, 216, 217, 231), mice (115, 169) and guinea pigs (49). OMP CD is a protein which has been shown to specifically bind human middle ear and nasal mucins but not salivary mucin (210), and exhibits a high degree of sequence conservation (181, 260). The protein has also been shown to be a target for both the mucosal and systemic immune response (183). However, the degree of sequence conservation in OMP CD could indicate that this protein does not actually come under immuno-selective pressure *in vivo*, and may therefore not actually be a good vaccine candidate in humans. A recent study has also indicated that a 200kDa IgD binding protein (MID) is also an adhesin (96) able to induce human B-lymphocyte (but not T-lymphocyte) activation (103). This protein also appears to be conserved and is apparently present in most *M. catarrhalis* isolates (175). However, the usefulness of this MID protein as a future vaccine candidate has yet to be determined.

Studies have shown that *M. catarrhalis* expresses two lactoferrin receptors (LbpA and LbpB) (26) and two transferrin receptors (TbpA and TbpB*/OMP B1) (185). Yu (1999) (262) showed that of these four iron acquisition proteins only the LbpB and TbpB proteins show strong reactivity with acute- and convalescent- phase sera from patients with *M. catarrhalis* associated pulmonary infections. Moreover, there appeared to be considerable antigenic heterogeneity in reactivity to the TbpBs and LbpB proteins isolated from different *M. catarrhalis* isolates. CopB (also known as OMP

B2) is an iron-repressible 81kDa outer membrane protein which is largely conserved between *M. catarrhalis* isolates with discrete regions of moderate heterogeneity (225). A copB binding monoclonal antibody (Mab10F3) was shown to enhance the pulmonary clearance of *M. catarrhalis* in a mouse model and was able to bind to 70% of *M. catarrhalis* isolates tested (5, 114). Moreover, significant rises in anti-copB antibody titres were observed in patients with *M. catarrhalis* infections (113, 162, 171). OMP E exhibits weak homology to the fatty acid transporter FadL of *E. coli* (21). The 47kDa protein appears to be highly conserved among *M. catarrhalis* isolates (180), and a majority of patients with chronic bronchitis were shown to have IgA antibodies to OMP E in their sputum, (though surprisingly none of ten adults with *M. catarrhalis* lower respiratory tract infections demonstrated a clear-cut rise in OMP E antibody titer) (22). Further, two *M. catarrhalis* gene knockout mutants lacking OMP E were shown to be more readily killed by normal human serum compared to the isogenic parent strains (179).

*N.B. Several groups have independently worked with the TbpB protein, such that two other names exist for TbpB, namely B1 (41) and 74kDA protein (50).

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Chapter 3

Changes in genetic types and population dynamics of *Moraxella catarrhalis* in hospitalized children are not associated with an exacerbation of existing disease.

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ABSTRACT

Pulsed field gel electrophoresis typing was performed on a retrospective set of 129 *Moraxella catarrhalis* isolates obtained over a 20 month period from 70 children admitted to, or presenting at, the Erasmus University Medical Center, Rotterdam, The Netherlands. The mean age of the children (at the end of the study) was 2.5 years with an age range of between 6 months to 15 years. 51 different *M. catarrhalis* types were isolated from the hospitalised children, with 31% (22/70) being infected with two particularly prevalent *M. catarrhalis* types. These two prevalent types also exhibited different protein profiles. 72% (16/22) of the children infected with these two predominant types had spent at least one week on two paediatric intensive care wards. No exacerbation of existing disease or new disease was observed in children for whom *M. catarrhalis* type changes occurred.

INTRODUCTION

Moraxella catarrhalis is often found as a commensal bacterium of humans, though the species has also been recognized as a major respiratory pathogen of both children and adults (Verduin et al., 2002). The organism is most often associated with the respiratory disease states of : acute and chronic otitis media (Faden, 1995; Faden et al., 1997), sinusitis (Finegold et al., 2002) and acute exacerbations of chronic obstructive pulmonary disease (COPD) (Pfaller et al., 2001; Sethi and Murphy, 2001). In common with several other respiratory pathogens, *M. catarrhalis* has the ability to adhere to bronchial epithelial cells, facilitate ciliotoxicity and ultimately damage pulmonary epithelia (van Alphen et al., 1995), all of which add to the pathogenic potential of this species.

Nosocomial pneumonia is the most frequently acquired infection in the intensive care unit (ICU) setting and the second most frequently acquired infection in hospitals overall (Lode et al., 2000). In ventilated children who contract nosocomial pneumonia, an attributable mortality of 27% may be observed (rising to 43% where *Pseudomonas* or *Acinetobacter* species are cultured) (Fagon et al., 1993). Reports of nosocomial infections due to *M. catarrhalis* have already been published (Cook et al., 1989; Denamur et al., 1989; Kasian et al., 1989; Morgan et al., 1992; Patterson et al., 1988; Richards et al., 1993), with Ikram et al. (1993) indicating that the average length

of stay in hospital is considerably longer for children colonized with *M. catarrhalis* compared to those not colonized and providing evidence that re-colonization with different *M. catarrhalis* types occurs. Recently, it has been observed that the isolation of a new strain of *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* is associated with a significantly increased risk of experiencing an exacerbation of chronic obstructive airways disease in adults (Sethi et al., 2002).

The aim of this study was to retrospectively assess *M. catarrhalis* population diversity/dynamics in hospitalized children and to determine if *M. catarrhalis* type changes resulted in exacerbation of existing disease within this particular study population.

MATERIALS AND METHODS

Study population. *M. catarrhalis* isolates were collected retrospectively from 70 children admitted to, or presenting at, the Erasmus Medical Center, Rotterdam, The Netherlands during the study period of July 2000 to March 2002. Sixty one of these seventy children had been admitted to diverse pediatric wards within the hospital (including neonatology, pediatric intensive care, children's surgery and children's health departments) for various periods of time. Nine children had attended outpatient clinics and had not been admitted to the Erasmus Medical Center between the beginning of the study (July 2000) and the date of isolation of the first positive culture. All of the children lived in geographically diverse regions within The Netherlands and suffered from unrelated disease states (e.g. tetralogy of Fallot, HIV positive, Down syndrome etc). The mean age of children from which isolates were obtained was 2.5 years with an age range of between 0.5 to 15 years.

***M. catarrhalis* specimens.** One hundred and twenty nine retrospectively cultured and stored *M. catarrhalis* isolates, (which had been isolated from routine swabs/swabs taken from children presenting with overt signs of disease over a 20 month study period) were collected ready for typing. During the time period of the study, 4 or more *M. catarrhalis* culture positives were available from 10% (7/70) of the children, with only a single positive culture being available from 76% (53/70) of the children. *M. catarrhalis* positive cultures came from 79 sputa, 38 bronchial aspirates, 9 bronchial lavages, 1 eye swab, 1 nose swab and 1 drain tip.

***M. catarrhalis* typing.** Pulsed field gel electrophoresis (PFGE) was performed as detailed by Verduin et al (Verduin et al., 2000). PFGE patterns were analyzed using GelCompar software (Applied Maths, Gent, Belgium) with band tolerance set at 2.5% after gel lanes had been normalized against a lambda DNA ladder. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous Laemmli buffer system (4% stacking gel and 11 % separating gel) and the Mini-PROTEAN® 3 Cell electrophoresis kit (Bio-Rad, USA). Eight *M. catarrhalis* isolates of PFGE type E or M (4 from each group) were chosen at random for SDS-PAGE analysis.

Clinical data. Clinical data was collected from patients' clinical records containing details of existing disease, dates and wards to which children were admitted, dates discharged from hospital, any treatment procedures (e.g. decanulation, extubation), any reports of exacerbation of disease (e.g. increased mucus, fever) and bacterial/viral investigations (e.g. bacterial culture, viral immunofluorescence).

RESULTS AND DISCUSSION

Diversity in *M. catarrhalis* types. During the twenty month study period (i.e. July 2000 to March 2002), a total of 51 different types of *M. catarrhalis* were isolated from seventy children admitted to, or presenting at the Erasmus Medical Center. Of these 51 types, 4 different types (8%) were each isolated from two distinct children, and two “predominant” types (types E and M) were isolated from fifteen (21%) and nine (13%) children respectively (Figure 1).

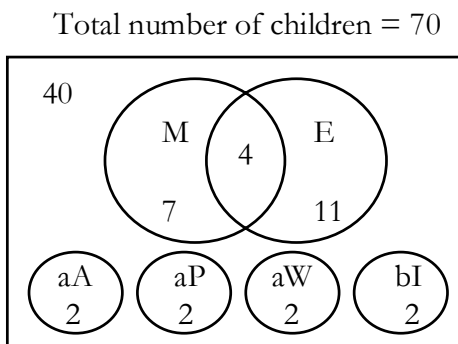


Figure 1. Venn diagram showing the number of different children colonized with particular *M. catarrhalis* pulsed field gel electrophoresis (PFGE) pattern types. Letters = PFGE types. Numbers = number of individual children infected with that particular PFGE type. 40 = number of children in which only a single PFGE type was isolated during the study period.

The dendrogram generated when these 51 different *M. catarrhalis* types were compared is shown in Figure 2; multiple isolates of the same type from the same child were removed in order to generate a more accurate view of the diversity of *M. catarrhalis* types present within the study population. A large number of individual *M. catarrhalis* PFGE types were observed within the study population of hospitalized children. This large number of types probably reflects the high degree of genetic diversity present within this species, a diversity which appears to be a feature of *M. catarrhalis* infection and colonization (Faden et al., 1994; Hays et al., 2003; Martinez et al., 1999) and which may present problems for vaccine design. However, despite this diversity, two distinct but closely related clusters of PFGE types (types E and M) could be observed within the dendrogram of isolates.

Upon SDS-PAGE analysis of 4 randomly chosen isolates from groups E and M (Figure 3), it was observed that individual isolates within the E and M clusters also shared similar protein profiles and that these protein profiles differed between the two E and M clusters. The presence/absence of an approximately 40 kDa protein was particularly noticeable. The inclusion of PFGE banding patterns obtained from four randomly selected complement sensitive and complement resistant isolates (acquired from Verduin et al., 2000) to the dendrogram shown in Figure 2., indicated no grouping of complement resistant or sensitive PFGE banding patterns to one or other of the clusters E / M, or to any particular region of the dendrogram (data not shown).

The hospital setting. Examination of *M. catarrhalis* type data and hospital location, indicated that diverse types could be found on all children's wards of the hospital. Moreover, though two dendrogram clusters of PFGE types could be observed, neither of these clusters was found to be 100% associated with children admitted to any particular ward. However, more specific analysis, showed an association between isolation of the predominant types E and M and admission of children for one week or more to two adjacent pediatric intensive care wards (Table 1.).

By comparing the number of children colonized with type M and admitted to pediatric intensive care wards compared with children colonized with type M and admitted to other wards, it was found that colonization by type M was statistically associated to the pediatric intensive care wards (2-tailed Fisher's Exact test $p < 0.01$), though colonization of children with type M was not absolutely dependent on a stay of

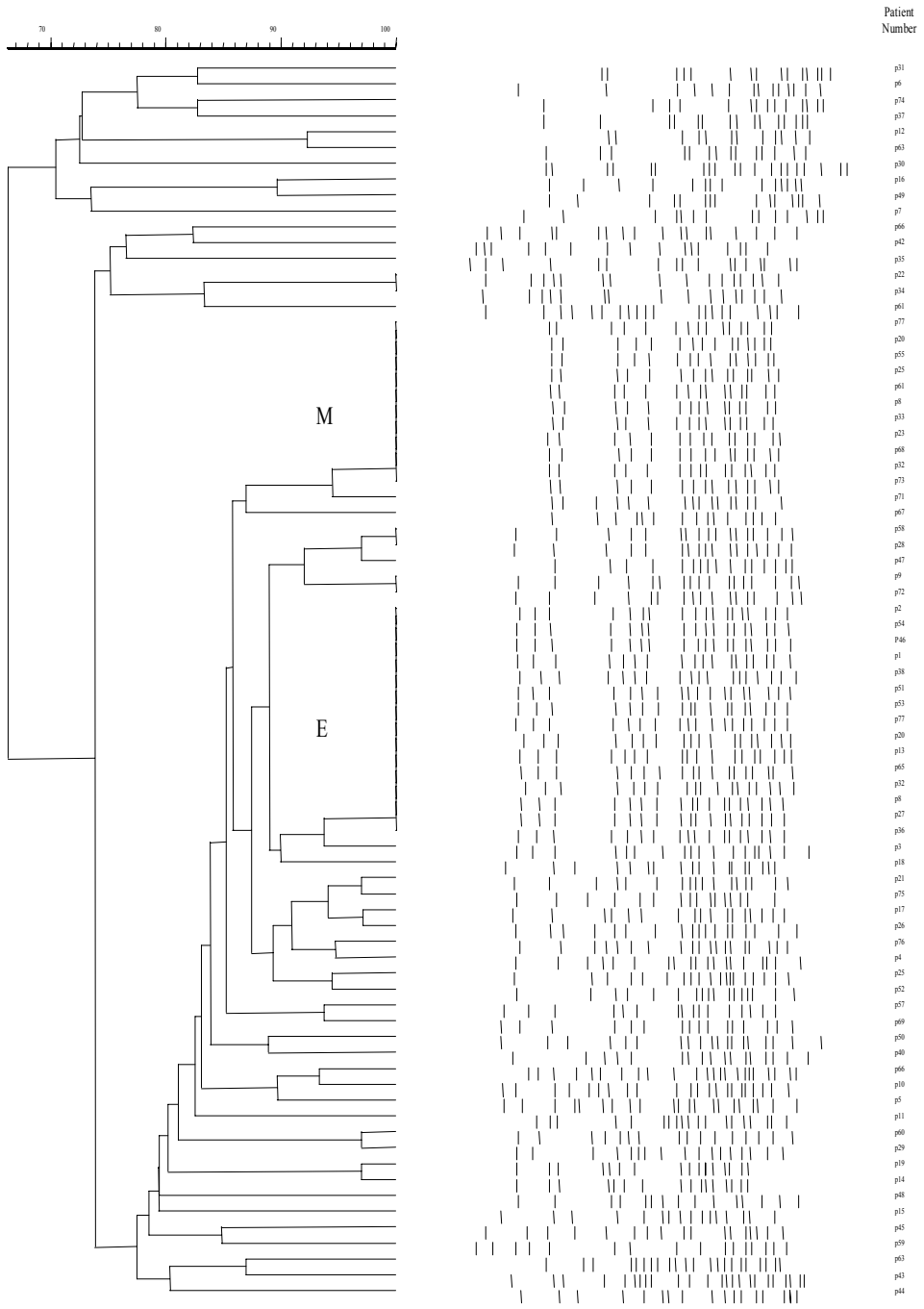


FIG. 2. Dendrogram showing pulsed field gel electrophoresis (PFGE) patterns of *M. catarrhalis* isolates obtained from children admitted to or attending a hospital in the Netherlands. PFGE was performed as detailed by Verduin *et al* 2000 and patterns were analyzed using GelCompar software (Applied Maths, Gent, Belgium) with band tolerance set at 2.5% after gel lanes had been normalized against a lambda DNA ladder. Letters E and M represent isolates with similar PFGE patterns which were subsequently grouped into *M. catarrhalis* types E and M.

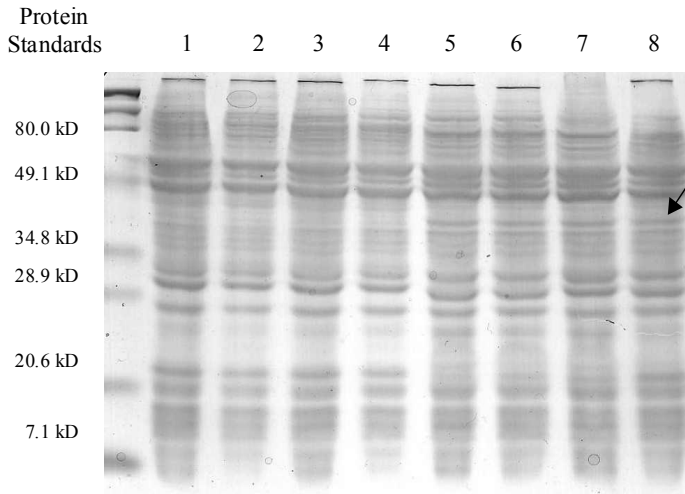


Figure 3. SDS-PAGE protein profiles obtained using four randomly chosen *M. catarrhalis* type M isolates (numbers 1- 4) and four randomly chosen *M. catarrhalis* type E isolates (numbers 5 - 8). Arrow = major difference in protein band profile observed between types E and M.

TABLE 1. *M. catarrhalis* pulsed field gel electrophoresis type and length of stay for 70 children admitted to or attending a Dutch hospital.

		PFGE Type			
		Time spent on wards other than pediatric intensive care		Time spent on two pediatric intensive care	
		< 1 week	> 1 week	< 1 week	> 1 week
E	B	E		C	E/M E/M
aP	aC	X		aU	aI E
aE	E	aM		Z	D bL
bG	aG	aL		aZ	E bK
K	aS	aW		bA	aW E
A	E	bI		aR	E/M E
Y	aQ	bI		aA	M M
P	W	bM		aF/M	bb/M
E	aX	I		E	bH/G
bE	aH	E		aA	M
H		aB		aT	M
		aN		J	E/M
		bJ		F	
		bC/aK			
		N			
		Ap			
		M			

greater than one week in pediatric intensive care (2-tailed Fisher's Exact test $p = 0.067$). Of the four other *M. catarrhalis* types isolated from more than one child (Figure 1.); type aA was associated with 2 children admitted to the pediatric intensive care wards (where types E and M were predominant), type bI was isolated from two children who were admitted to the same neonatology ward, and types aP and aW were isolated from children with no common link. It is possible that a larger study would indicate that other PFGE types are statistically associated with the pediatric intensive care setting, or that colonization of children with the same *M. catarrhalis* type is not only restricted to the pediatric intensive care setting. It should also be noted that there might have been a natural bias towards the isolation of common types within the two pediatric intensive care wards. This results from the larger number of swabs regularly taken from the children admitted to these two wards. However, this possible bias does not affect our results regarding the diversity and dynamics of *M. catarrhalis* types isolated from hospitalized children. We may have merely underestimated the extent of *M. catarrhalis* dynamic type changes within this environment.

***M. catarrhalis* type switching.** Based on the findings of Table 1., a timeline could be drawn for children admitted to the two pediatric intensive care wards and colonized with types E and M during the course of the study (Figure 4). Using this timeline, a dynamic relationship was observed between these two dominant types and an important time period (between February and April 2001), where type switching had occurred. The actual mechanism underlying this type switching is not fully understood, though three possible mechanisms are possible; 1) changes in the dominant type present within a population of organisms colonizing a single individual leads to isolation of different types from that individual over different time frames, 2) a new (potentially more virulent) type is introduced into the hospital from the outside community and colonizes hospitalized individuals/health care workers e.g. during the visiting hours, or 3) transfer of types occurs within the hospital environment *per se* e.g. from child to child or from health care worker to child. Interestingly, isolates from child p77 showed switching between types E and M within the same month on several occasions (Figure 4), possibly indicating that a mixed population of *M. catarrhalis* types may have colonized this particular child (repeated cycles of infection, involving clearance and re-infection with these two types seems unlikely as the child had an intact immune system). However, with regard to community and hospital spread of types, of thirty seven *M. catarrhalis* isolates from children who had not been admitted to the two

pediatric intensive care wards where types E and M were predominant, 14% (5/37) of children were colonized with *M. catarrhalis* type E, indicating that type E was circulating either within the community or within the whole of the hospital during the time-course of the study. In contrast, type M was only ever isolated from the pediatric intensive care wards, indicating that the spread of this particular type was most probably occurring within these wards themselves. With reference to this data and the timeline of Fig 4., it seems likely that child p77 may have been the index case for the spread of type M within these pediatric intensive care wards. Certainly, the previous transmission of viruses from child to child and/or child to health care worker in a neonatal and pediatric intensive care unit has been documented (Gagneur et al., 2002a; Gagneur et al., 2002b).

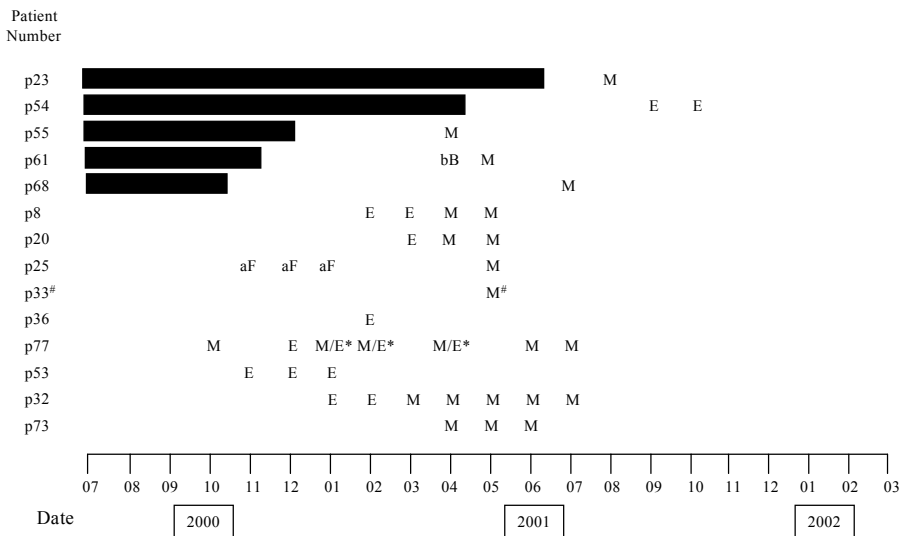


Figure 4. Timeline showing the dynamic population changes observed between *M. catarrhalis* PFGE types E and M in hospitalized children on two geographically linked pediatric intensive care wards. Filled rectangles = child not yet born. Letters = pulsed field electrophoresis type. p23 = child number 23. * = both PFGE types E and M were isolated during these months. # = this isolate was cultured from a child who had previously been admitted to pediatric intensive care but at the time of *M. catarrhalis* culture was present on an unrelated child health ward (none of the other children had been admitted to this particular child health ward).

Changes in *M. catarrhalis* type and clinical data. Analysis of clinical data, showed no obvious new treatment procedures taking place on the pediatric wards during the period of the study, and no other epidemic infectious agents (e.g. bacteria and viruses such as *Mycoplasma pneumoniae*, Parainfluenzaviruses etc) had been isolated from the wards. Also, no link was found between change in *M. catarrhalis* type and isolation in “pure” culture of *M. catarrhalis*, or change in *M. catarrhalis* type and isolation in combination with other bacterial pathogens (e.g. *H. influenzae*, *P. aeruginosa*, *S. aureus* etc). No change in antibiotic resistance profiles occurred after a change in *M. catarrhalis* type, with both types E and M being resistant to penicillin and ampicillin but sensitive to augmentin, ceftriaxon and tetracycline. During the 20 month time period of the study, several other infectious agents were isolated from one or more of the hospitalized children. These infectious agents included among others cytomegalovirus, parainfluenzavirus type 3, enterovirus, respiratory syncytial virus etc. Interestingly, the clinical data showed that one child (p29) had been diagnosed with pneumonia whilst admitted to one of the pediatric intensive care wards. This child had recently undergone surgery to repair patent ductus arteriosus of the heart and both *M. catarrhalis* and *S. aureus* were isolated from consecutive sputum samples. However, the *M. catarrhalis* cultured from this child was not PFGE type E or M (Figure 2.) and the child was discharged in good health approximately 3 weeks later. Adults with chronic obstructive pulmonary disease (COPD) have been previously shown to develop new serum IgG and new mucosal IgM to *M. catarrhalis* after exacerbations (Bakri et al., 2002). Moreover, a recent study has indicated an association between the isolation of a new strain of a particular bacterial species (including *M. catarrhalis*) and exacerbation of chronic obstructive pulmonary disease in adults (Sethi et al., 2002). However, our own studies of available clinical records, indicated no link between the acquisition of a new *M. catarrhalis* type and subsequent exacerbation of existing disease (or development of new disease) within hospitalized children.

These results indicate that dynamic nosocomial *M. catarrhalis* population changes occur in individual hospitalized children over time, and that these changes are set against an already genetically diverse background of *M. catarrhalis* types. In contrast to recent results, no relationship could be determined between change in *M. catarrhalis* type and exacerbation of disease. However, more detailed and rigorously controlled studies would be useful in assessing the exact effect of *M. catarrhalis* type changes on

child morbidity, as well as helping to elucidate the mechanism by which such population changes occur.

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Chapter 4

Pneumococcal vaccination does not affect the genetic diversity of *Moraxella catarrhalis* isolates in children.

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Eur J Clin Microbiol Infect Dis. 2004 Oct;23(10):801-3

Moraxella catarrhalis is an acknowledged pathogen of the respiratory tract in both adults and children [1], occupying a similar niche to that of *Streptococcus pneumoniae* (the “pneumococcus”) and *Haemophilus influenzae*. In a recent study, Veenhoven et al [2], showed that toddlers and older children (who had previously experienced episodes of acute otitis media (AOM)) when vaccinated with a conjugate pneumococcal vaccine followed by a polysaccharide pneumococcal vaccine, did not experience a reduction of AOM episodes. Further, isolations of *M. catarrhalis* did not differ between the two groups, but *Staphylococcus aureus* was isolated more often in the vaccinated group than in the control (hepatitis B or A) vaccinated group ($p = 0.02$). It was also noted that an “immediate and complete” replacement of *S. pneumoniae* vaccine serotypes by non-vaccine serotypes tended to occur within the vaccinated group. As bacterial interference of *M. catarrhalis* by competing *Streptococcal* species has been demonstrated *in vitro* [3], the aim of this study was to determine whether vaccination against *S. pneumoniae* (and hence its removal from the *M. catarrhalis* niche) had an effect in altering the genetic diversity of *M. catarrhalis* isolates compared to a control vaccinated group.

During the course of the study, children were followed for a total of 18 months, with routine microbiological and clinical investigation occurring 1, 7, 14, 20 and 26 months after vaccination as well as during any episode of AOM. Bacterial cultures were obtained from nasopharyngeal swabs at routine visits and also from middle ear fluids during episodes of AOM. In total, 41 *M. catarrhalis* isolates obtained from 13 children vaccinated with pneumococcal vaccine and 21 *M. catarrhalis* isolates obtained from 6 children vaccinated with a control (hepatitis A or B) vaccine were genotyped using pulsed field gel electrophoresis (PFGE), as described by Verduin et al (2000) [4]. The results of PFGE analysis are presented as a dendrogram in Fig. 1. As an indication of pathogenic potential, all isolates were also tested for the phenotypic expression of complement resistance using the “culture and spot” test [5].

PFGE analysis of the isolates indicated the presence of four clusters comprising a wide range of genotypes. The high degree of diversity within the *M. catarrhalis* species has already been documented in several publications [6, 7]. No pattern could be observed between PFGE cluster and vaccination status, or PFGE cluster and the isolation of other (co-colonizing) bacteria. *M. catarrhalis* PFGE types belonging to different clusters were found to occur within the same patient over time (e.g. patient 1001), this observation also holding true for PFGE types associated with episodes of AOM (e.g. patient 4025).

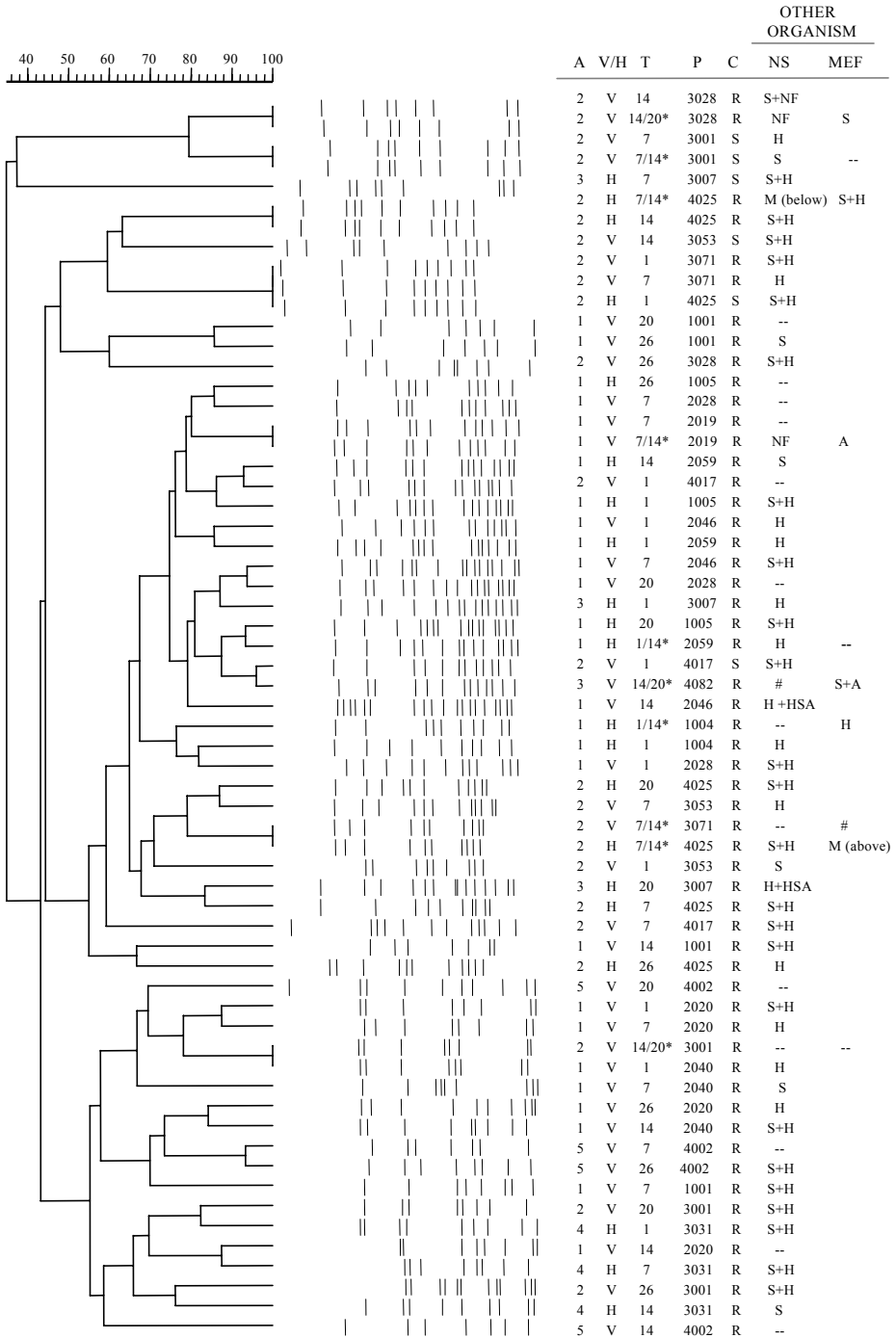


Fig. 1 Dendrogram showing relatedness of *M. catarrhalis* PFGE types isolated from an *S. pneumoniae* vaccine trial. Middle ear fluids were only collected during acute otitis media episodes. A = age after final vaccination. V/H = vaccinated *S. pneumoniae* / vaccinated Hepatitis A or B. T = time of routine planned visit after final vaccination, where 1, 7, 14, 20 and 26 = 1, 7, 14, 20, 26 months respectively. 7/14 etc = acute otitis media occurring between routine visits of 7 and 14 months etc. P = patient. C = complement phenotype, where R = resistant and S = sensitive. NS = nose swab. MEF = middle ear fluid/swab. * = culture from middle ear fluid after an acute otitis media episode. S = *Streptococcus pneumoniae*. H = *Haemophilus influenzae*. A = *Staphylococcus aureus*. NF = non-lactose fermenter. M = a second *M. catarrhalis* PFGE type isolated at the same time but from another site. # = *M. catarrhalis* of same PFGE type isolated from this site. -- = no organism isolated.

The vast majority of isolates 90% (56/62) were found to be resistant to the effect of complement in human serum, and there was no difference between the isolation of complement resistant and sensitive phenotypes between the two vaccinated groups (Fisher's Exact Test $p = 1$). The percentage of complement resistant *M. catarrhalis* isolated appears to be relatively high when compared to some studies involving healthy children (90% versus 30 -60% respectively [8]), though other studies have yielded similar results [9]. The percentage complement resistant isolates could have been influenced by the fact that the children enrolled on the study had previously experienced episodes of AOM disease, possibly resulting in an enhanced immune response (including complement mediated responses) against potential bacterial pathogens.

In this study, vaccination against *S. pneumoniae* in older children did not affect the diverse genetic nature of *M. catarrhalis* isolates cultured when compared to a control vaccinated group.

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Chapter 5

Whole-cell genomic analysis and one-dimensional protein expression profiling in the search for lineage-specific and virulence-associated markers of *Moraxella catarrhalis*.

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Abstract

Previous research has shown that a genetically distinct subpopulation/lineage of *Moraxella catarrhalis* exist which are associated with virulence traits. Therefore, the development of an assay, which could distinguish between different *Moraxella catarrhalis* lineages, would be advantageous in identifying isolates with an increased virulence potential. In this study, both intensive whole-cell genomic analysis and one-dimensional protein expression profiling was performed on 25 complement resistant and 23 complement sensitive isolates of *Moraxella catarrhalis* in order to identify markers associated with both genetic lineage and complement phenotype (a model for virulence potential). High-throughput amplified fragment length polymorphism (htAFLP) analysis generated 2,273 fragment length polymorphisms, with an average of 862 polymorphisms per isolate, revealing the presence of two phylogenetic lineages and 40 lineage-specific markers. However, no complement phenotype-specific polymorphisms were found, though 361 polymorphisms (16%) were found to be significantly associated with complement phenotype (Fisher's exact test $P < 0.05$). Simple one-dimensional SDS-PAGE analysis revealed the presence of 6 major protein expression profile markers significantly associated with either the complement resistant or sensitive phenotype (Fisher's exact test $p < 0.01$). Subsequent sequencing and MALDI-TOF analysis of a number of genetic and protein markers revealed that most markers originated in housekeeping genes. However, genetic markers associated with a putative competence protein and major outer membrane protein CopB, as well as the differential expression of an outer membrane hemagglutinin (Hag) were observed.

1. Introduction

Moraxella catarrhalis is a commensal of the human respiratory tract, which is also recognized as a pathogen of both children and adults. Carriage rates differ widely between children and adult populations, being approximately 77.5% and 2% respectively [1,2]. In children, the organism has been associated with acute otitis media [3], sinusitis [4], and infrequently pneumonia [5] and bacteremia [6]. Isolates appear to spread in children via nursery schools and day care centers [7], though respiratory tract colonization by a new *M. catarrhalis* genotype does not necessarily result in the production of overt disease [8]. In adults, the organism has been associated with

exacerbations of chronic obstructive airways disease (COPD) / bronchitis [9,10], and pneumonia [11].

Previous studies using a variety of different DNA typing techniques, including PFGE/RAPD [12], and single-adapter AFLP (sAFLP) / probe-generated RFLP (pRFLP) / 16S rRNA sequencing [13], have suggested that genetic lineages or sub-populations of *M. catarrhalis* isolates exist [14], and that these lineages exhibit different virulence potentials. One particularly important virulence factor is that of complement resistance [15-18], with evidence suggesting that the mechanism of resistance may be facilitated via inhibition of membrane attack complex (MAC) formation, possibly by the binding of the complement inhibitor vitronectin to particular forms of the bacterial ubiquitous surface protein A2 (UspA2) [19-23]. However, this is not the only mechanism that has been described, as the iron acquisition protein CopB [24,25], a 50kDa outer membrane protein OmpE [26], a lipooligosaccharide P(k) epitope [27], as well as a ferric uptake regulator (*fur*) [28], have also been implicated in facilitating complement resistance.

In this study, detailed whole-cell genomic analysis (high-throughput amplification fragment length polymorphism), and one-dimensional protein expression profiling was performed, in an attempt to identify lineage and complement phenotype-specific markers which could be useful in the development of assays able to distinguish between different virulent *M. catarrhalis* lineages, especially those exhibiting an increased pathogenic potential.

2. Materials and methods

2.1. Bacterial isolates

Twenty five complement resistant and 23 complement sensitive isolates of *M. catarrhalis* were chosen from the collection of isolates within the Department of Microbiology and Infectious Diseases at the Erasmus MC, Rotterdam, The Netherlands (Table 1). These isolates had been previously characterized using several genotyping techniques by Verduin et al [12]. The Dutch isolates used were considered representative of the *M. catarrhalis* isolates circulating within The Netherlands during the time period 1989 - 1997.

Table 1
M. catarrhalis isolates used in htAFLP analysis

Isolate	Origin	Complement Phenotype	Isolate	Origin	Complement Phenotype
1.9	NN	Sensitive	F3.57	HN	Sensitive
1.12	NN	Resistant	F4.64K	HN	Sensitive
1.24	NN	Resistant	F4.64N	HN	Sensitive
1.38	NN	Resistant	F5.82	HN	Resistant
1.39	NN	Sensitive	F6.90	HN	Resistant
3.9	NN	Resistant	F6.92	HN	Resistant
3.14	NN	Sensitive	F6.93	HN	Sensitive
3.18	NN	Sensitive	38935	FN	Sensitive
3.21	NN	Sensitive	55183	FN	Sensitive
3.24	NN	Sensitive	60832	FN	Sensitive
4.16	NN	Resistant	69982	FN	Sensitive
4.22	NN	Sensitive	129822	FN	Resistant
5.12	NN	Sensitive	131472	FN	Resistant
6.2	NN	Sensitive	H2	UN	Resistant
6.12	NN	Sensitive	H12	UN	Resistant
7.2	NN	Sensitive	97/951	RN	Resistant
7.10	NN	Sensitive	97/0233	RN	Resistant
7.13	NN	Resistant	25240	ATCC	Resistant
8.3	NN	Sensitive	B22	A	Resistant
F1.3	HN	Resistant	D14	A	Resistant
F1.11	HN	Sensitive	F17	A	Resistant
F2.42	HN	Sensitive	Q01	A	Resistant
F2.44	HN	Resistant	R02	A	Resistant
F3.46	HN	Resistant	V02	A	Resistant

NN = isolates from children at a primary school, Nieuwegein, The Netherlands (1989). HN = isolates from children at a primary school, Heerenveen, The Netherlands (1993). FN = clinical isolates from adult lower respiratory tract infections, Friesland, The Netherlands (1993). UN = positive blood cultures from children, Utrecht, The Netherlands (1989). RN = positive blood cultures from children, Rotterdam, The Netherlands (1997). ATCC = Isolate from the American Type Culture Collection. A = Ghanaian isolates (1995)

2.2. Complement resistance testing

The complement resistant or sensitive phenotype of the 48 *M. catarrhalis* isolates used in this study had been previously determined using the “culture-and-spot” test by Verduin et al [29]. This is a rapid and simple test for determining the complement resistance phenotype of *M. catarrhalis*, which exhibits a statistically significant concordance with the serum bactericidal assay ($P < 0.0001$). For the purposes of this publication any isolate determined as expressing the intermediate complement resistant phenotype using the “culture and spot” test (isolates F5.82, 1.24 and F6.92) was regarded as complement resistant.

2.3. Whole-cell genomic htAFLP and phylogenetic analysis

High-throughput AFLP (htAFLP) analysis was performed at Keygene N.V. (Wageningen, The Netherlands), using primers labeled with radioactive phosphorous-33 as previously described [30]. Genomic DNA was digested using the restriction enzymes *MboI* and *MseI*, heat inactivated, and *MseI* and *MboI* adapters ligated to the restriction products. htAFLP amplification was subsequently performed using i) an *MboI* primer (5'-GTAGACTGCGTACCGATC-3') incorporating an extra selective nucleotide at its 3'-end (labeled with radioactive phosphorous-33) and ii) an *MseI* primer (5'-GACGATGAGTCCIGAGTAA-3') incorporating 2 extra selective nucleotides at its 3'-end (both unlabelled). In total, 21 primer combinations were used in the htAFLP analysis of the 48 *M. catarrhalis* isolates tested (Table 2). Analysis of htAFLP gel fingerprints was performed using 1µl of each htAFLP reaction, which was loaded onto a 5% denaturing polyacrylamide sequencing gel along with a radioactively-labeled internal size marker. Fragments were separated by electrophoresis for 2 hours at constant power (110 W). After electrophoresis, gels were fixed for 30 min in 10% acetic acid, dried on glass plates and exposed to Fuji phosphor image plates for 16 hours. Fingerprint patterns were visualized using a Fuji BAS-2000 phosphor image plate scanner and the scanned images were analyzed using AFLP-QuantarPro software (Keygene Products N.V.). Any fragment found to be present in 100% of *M. catarrhalis* isolates (after exclusion of any ambiguous results) was discarded from the total set of fragments generated and not therefore used for comparative analysis. Comparative

phylogenetic analysis of the data was performed using two-dimensional hierarchical clustering with a complete linkage algorithm and the Tanimoto similarity metric (OmniVizR version 3.6 software; OmniViz Inc., USA). Similarity was based on the number of positive attributes that two records have in common and the results were ordered by dendrogram.

Table 2
3'-end discriminating bases added to
htAFLP linker primers

		<i>Mbo</i> I Primer			
		+A	+C	+G	+T
<i>M</i>	+ AA		+		
	+ AC	+			+
	+ AG				+
	+ AT		+	+	
<i>P</i>	+ CA	+			
	+ CT	+			+
	+ GA	+			+
	+ GT	+			+
<i>r</i>	+ TA		+	+	
	+ TC	+			+
	+ TG	+			+
	+ TT		+	+	

2.4. htAFLP polymorphic fragment sequencing

A PCR sequencing methodology was used to identify DNA sequences associated with particular htAFLP polymorphic fragments. Essentially, a small piece of the relevant polymorphic fragment was picked out of the htAFLP gel and added directly to a PCR sequencing reaction mix containing the relevant htAFLP linker primer. PCR sequencing was performed using dye terminators on an ABI 3700 capillary sequencer. The sequence data from each polymorphic fragment was then compared to the unannotated *M. catarrhalis* genome sequence available at GenBank, accession

numbers AX067426-AX067466 (comprising 41 contigs ranging in size from 429bp to 261,300bp in length), in order to identify homologous open reading frame (ORF) sequences. The corresponding homologous ORF was then translated into protein and the resultant protein sequence used to search publicly available protein sequence databases to identify the nature of the original gene.

2.5. One-dimensional SDS-PAGE protein expression profiling

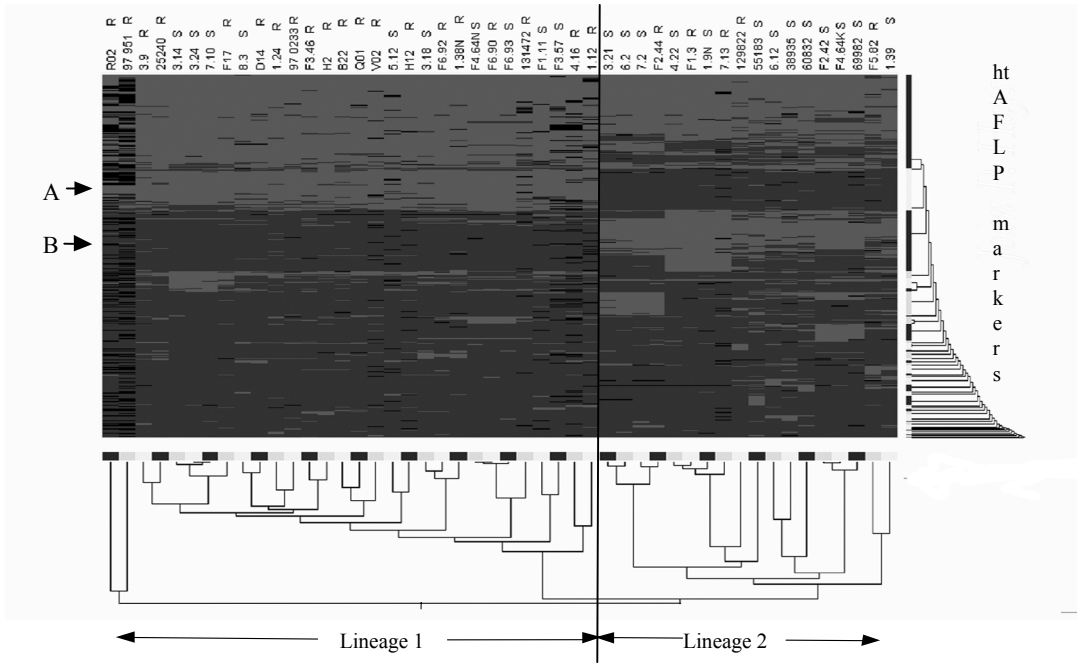
One-dimensional (1D) SDS-PAGE was performed using standard techniques, with analysis being performed on all 48 *M. catarrhalis* isolates after growth under identical conditions (overnight aerobic growth on Mueller Hinton agar at 37°C). Differential protein markers were then identified by comparing the patterns of protein expression between the complement resistant and complement sensitive isolates. A differential protein expression marker was identified as a series of protein bands of approximately the same molecular mass which were significantly associated with either the complement resistant or complement sensitive phenotype (Fisher's Exact Test $p < 0.01$). Once differential protein expression markers had been identified, representative bands for each marker were picked from the 1D SDS-PAGE gel and internal polypeptide fragment sequences identified using MALDI-TOF mass spectrometry. These fragments were then compared to a translated (all 6 possible reading frames) proteomic map of the unannotated *M. catarrhalis* genome (available at GenBank accession numbers AX067426-AX067466), in order to identify the component protein(s) present within each band/marker.

3. Results

3.1. Whole-cell genomic htAFLP analysis

In total, 41,364 fragments were obtained using 21 htAFLP primer combinations on this set of 48 *M. catarrhalis* isolates, revealing the presence of 2,273 fragment length polymorphisms. Comparative phylogenetic analysis of the data indicated the presence of two distinct phylogenetic lineages, exhibiting approximately 5% difference in their branch depth (Fig. 1, Top). These two lineages were differentiated by approximately 250 and 242 lineage-defining polymorphisms

Sub-population Lineages



Complement Phenotype

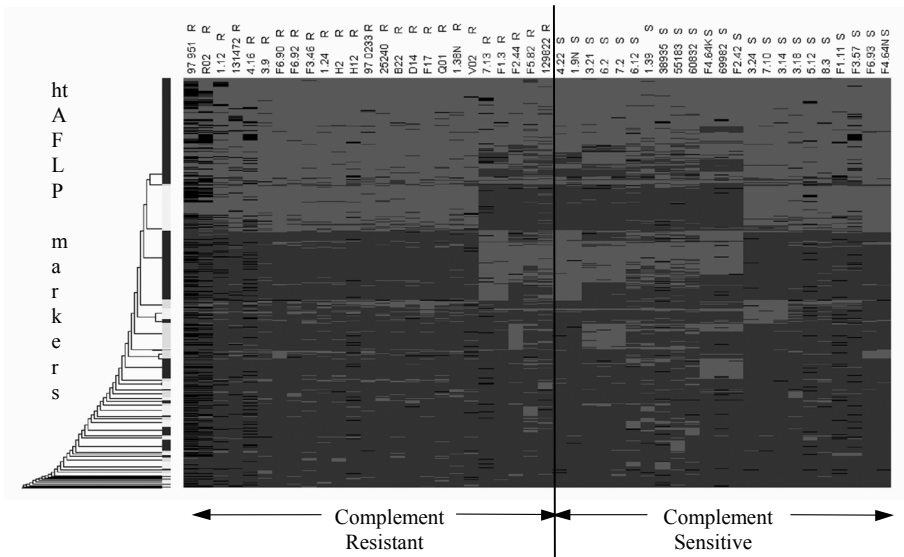


Fig. 1. Phylogenetic analysis of a set of 48 *M. catarrhalis* isolates using htAFLP polymorphism data (Top), and subsequent rearrangement into complement phenotype (Bottom). Vertical text = *M. catarrhalis* isolate designations, where R = complement resistant, and S = complement sensitive phenotype. Light gray areas represent the presence of a particular htAFLP polymorphism, whilst dark gray areas represent the absence of a particular htAFLP polymorphism. Black areas represent regions where the presence or absence of htAFLP polymorphic data could not be reliably determined. A and B = subpopulation associated polymorphisms.

respectively (A and B in Fig. 1), with 40 (1.8%) of the htAFLP polymorphisms found to be 100% specific for one or other of the two lineages identified. Additionally, there was a significant correlation between complement phenotype and the two phylogenetic lineages (Fisher's exact test $P = 0.016$, Pearson $r = 0.38$ (0.10 – 0.60), $P = 0.008$). Unfortunately however, no complement phenotype-specific htAFLP polymorphisms were found (Fig. 1. Bottom), though 361 polymorphisms (16%) were found which were statistically associated with complement phenotype, including 172 polymorphisms associated with complement resistance and 189 with complement sensitivity (Fisher's exact test $P < 0.05$).

3.2 htAFLP polymorphic fragment sequencing

Using the results obtained from the htAFLP analysis, an arbitrary selection of 13 lineage-specific and 17 complement phenotype-associated polymorphic markers were chosen and sequenced (Table 3). Of the lineage-specific markers, 11 mapped to putative housekeeping genes, whilst 2 mapped to putative virulence associated genes. The majority of the complement phenotype-associated markers were mapped to housekeeping genes, though polymorphic marker F3121.511<N was found to reside within the *copB* gene, a gene associated with iron acquisition [31], as well as being a target for antibodies involved in pulmonary clearance [32] and being implicated in altering serum resistance in vivo [24].

The sequence data for these htAFLP fragments are available from GenBank, accession numbers AY771621 – AY771641 and AY944723 – AY944731.

Table 3
htAFLP polymorphic fragments associated with *M. catarrhalis* lineage and complement phenotype

htAFLP Fragment	Size (bp)	Lineage	Putative gene description	Predicted function of identified ORF	Accession number
<i>100% specific for genetic lineage</i>					
A / AC F-134.585<N*	109	1	Probable carboxyl-terminal protease	Protein metabolism	AY771621
A / AC F-379.208<N*	353	1	N-acetylglutamate synthase	Protein metabolism	AY771622
A / CA F-166.034<N*	145	1	Phosphatase domain containing protein	Unknown	AY771623
A / CA F-174.909<N*	151	1	Lipid A disaccharide synthase (lpxB)	Biosynthesis of lipopolysaccharide	AY771624
A / CA F-177.244<N*	163	1	Elongation factor G	Protein metabolism	AY771625
A / CA F-266.454<N*	245	1	DnaJ domain containing protein	Molecular chaperone	AY771626
A / GA F-189.403<N*	160	1	Signal transduction histidine kinase	Signal transduction	AY771629
A / GA F-256.710<N*	228	1	Colicin tolerance gene (<i>tolB</i>) [#]	Colicin A sensitivity/resistance	AY771631
A / GA F-375.414<N*	352	1	Ferredoxin-NADP+ reductase	Protect against oxidative stress	AY771632
A / TC F-227.958<N*	201	1	Lipoprotein precursor (<i>nlpD</i>)	Cell wall formation	AY771634
A / AC F-239.018<N**	217	2	Flavodoxin reductase	Protects against oxygen radicals	AY771638
A / CA F-173.831<N**	144	2	Putative competence factor [#]	Bacterial competence	AY771639
A / CT F-301.112<N**	280	2	Hypothetical protein	Unknown	AY771640
<i>Associated with the complement resistant phenotype (P < 0.05)</i>					
A / CA F-315.261<N	287		Putative monooxygenase	Energy production + conversion	AY771627
A / GA F-112.386<N	86		Mg-dependent DNase	DNase	AY771628
A / GA F-217.923<N	135		Response regulator (<i>gacA</i>)	Transcription/response regulator	AY771630
A / CT F-321.511<N	296		Major outer membrane protein CopB	Iron acquisition / Serum resistance	AY944723
A / TC F-360.194<N	260		Adenylosuccinate synthetase	Purine ribonucleotide synthesis	AY771635
A / TC F-217.777<N	193		Macrophage infectivity potentiator protein - Acyl-coA dehydrogenase (intergenic)	Macrophage infection - Energy production	AY771633
T / TG F-536.245<N	501		Homoserine dehydrogenase	Protein metabolism	AY944724
C / TA F-194.337<N	163		16S rRNA processing protein (RimM)	Maturation of 30S rRNA	AY944725
C / TA F-224.476<N	174		Membrane fusion protein - Cation/multidrug efflux pump (intergenic)	Membrane fusion / Efflux pump	AY771636
G / TA F-391.095<N	363		ParA ATPase	Partitioning of bacterial plasmids	AY771637
G / AT F-191.261<N	121		Leucyl, phenylalanyl-tRNA-protein transferase	Protein metabolism	AY944726
<i>Associated with the complement sensitive phenotype (P < 0.05)</i>					
A / AC F-316.046<N	289		Succinate-semi-aldehyde dehydrogenase	Energy production + conversion	AY944727
A / TC F-318.602<N	303		Dihydroliipoamide acetyltransferase	Energy production + conversion	AY771641
T / AG F-168.547<N	142		Pyridoxamine 5-phosphate oxidase	Co-enzyme metabolism	AY944728
C / AT F-214.578<N	189		Guanosine-3', 5'- bis (diphosphate)	Purine metabolism	AY944729
G / AT F-142.047<N	115		Replication helicase	DNA unwinding	AY944730
G / AT F-310.917<N	285		Promoter for Acetaldehyde dehydrogenase II	Energy production + conversion	AY944731

* = also associated with the complement resistant phenotype ($P < 0.05$). ** = also associated with the complement sensitive phenotype ($P < 0.05$). # = putative virulence gene

3.3. 1D SDS-PAGE protein expression profiling

One-dimensional SDS-PAGE protein analysis of isolates grown under identical conditions revealed six major protein expression markers significantly associated with either the complement resistant or complement sensitive phenotype (Fisher's Exact Test $p < 0.01$). These markers are shown in Fig. 2. MALDI-TOF mass spectrometric analysis of representative bands from each of these protein expression markers indicated the possible identities of the differentially expressed proteins associated with each marker (Table 4). A simple PCR screening method indicated that at least one gene (*ompCD*) was present in all 48 isolates tested despite its differential expression (data not shown).

Table 4

Composition of 6 differential protein expression markers associated with complement phenotype in 48 *Moraxella catarrhalis* isolates.

Protein marker	Marker identity	Entrez accession no.
X**	ATP-dependent helicase / RNA polymerase Beta subunit / DNA topoisomerase I	giI37526066IrefINP_929410 giI46914940IembICAG21715.1 giI33152456IrefINP_873809.1
A*	Outer membrane haemagglutinin (Hag)	giI22000942IgbIAAL78285.1
B*	Omp CD precursor	giI481874IpirIS39866
D**	Hypothetical bacterial protein	giI41690179IrefIZP_00146711
E*/F**	28kDa outer membrane lipoprotein or ABC-type metal transporter	giI16272562IrefINP_438778.1 giI46133096IrefIZP_00156582.2

* / ** = significant at $P < 0.001$ for complement resistant or complement sensitive phenotype respectively. N.B. Protein markers E and F could not be distinguished by MALDI-TOF.

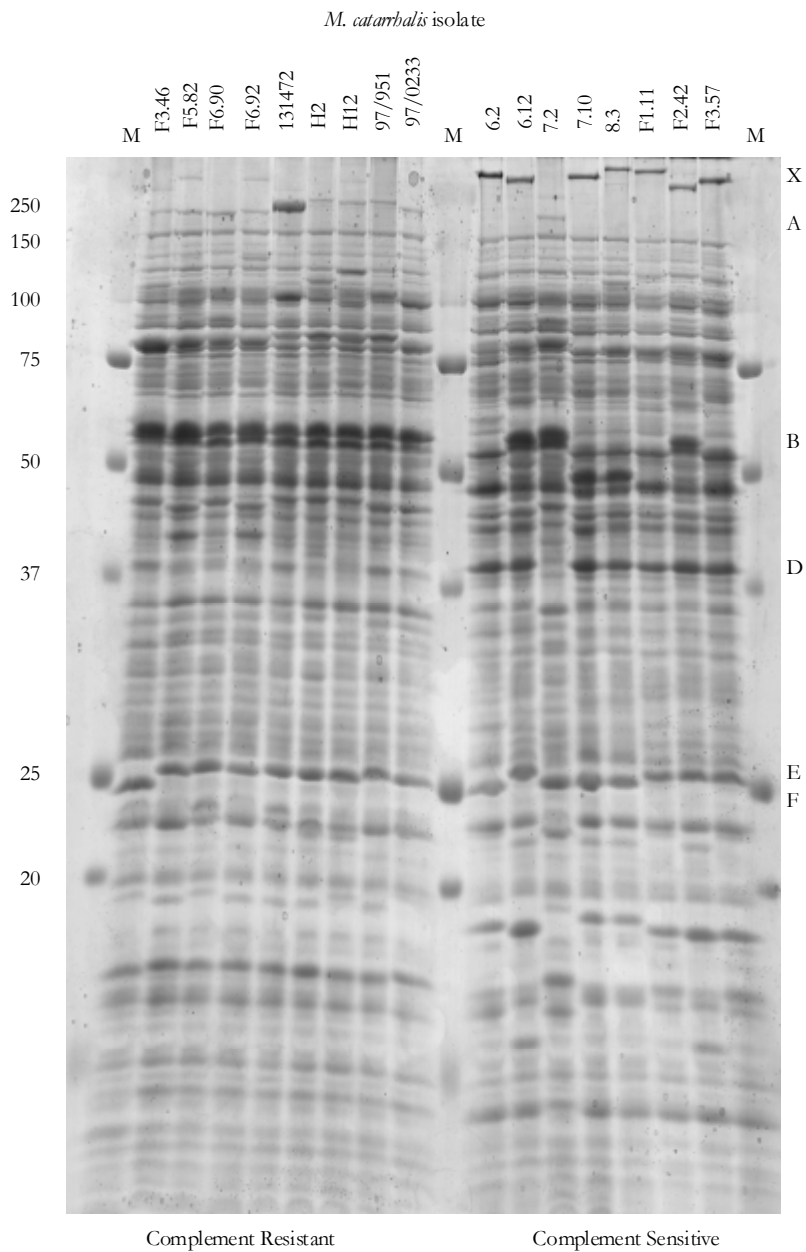


Fig. 2. Representative one-dimensional SDS-PAGE gel showing differential protein expression markers (labeled X, A, B, D, E and F) obtained from 48 *M. catarrhalis* isolates grown overnight under identical (nutrient replete) conditions. M = molecular weight marker in kDa.

4. Discussion

The expression of virulence traits within the *M. catarrhalis* species has previously been associated with distinct sub-populations/lineages of isolates. Therefore, the identification of lineage and/or virulence related markers would be a useful first step in the development of a “universal” assay which could be used to distinguish between isolates belonging to different *M. catarrhalis* lineages, and hence identify isolates exhibiting an increased virulence potential. Such assays could be useful in the clinical situation e.g. in influencing the decision over whether to use antibiotic therapy or not.

To achieve this aim, the most intensive analysis to date of a cohort of *M. catarrhalis* isolates was performed, utilizing both whole-cell genomic polymorphism analysis (high throughput AFLP), and one-dimensional protein expression profiling, to identify possible “universal” lineage-specific and complement phenotype-associated (virulence-associated) markers for this species. The wide potential of the AFLP technique in the study of evolutionary / epidemiological aspects of taxonomy in general [33,34], and bacterial taxonomy in particular [35-37], has already been established. Indeed, the AFLP technique has several advantages over other genomic typing techniques in that it may be applied to generate genetic fingerprints from organisms without prior knowledge of genomic DNA sequences, and that mutations of only a single base pair may be detected. In this study, an intensive high-throughput AFLP strategy was utilized, revealing the presence of 40 lineage-specific genetic markers and 361 genetic markers significantly associated with the complement phenotype. The subsequent identification of the genetic origin of a number of these markers, now allows for a more targeted approach to be adopted (involving the testing of a larger number of geographically diverse *M. catarrhalis* isolates), in order to determine the “universal” nature of these lineage-specific and complement-associated markers within this species. Further, the fact that most genetic markers were found to reside in putative housekeeping genes, as opposed to genes under immune selection pressure, means that they are likely to remain conserved within individual genetic lineages.

One-dimensional SDS-PAGE gel electrophoresis of outer membrane protein extracts has previously been used to compare *M. catarrhalis* isolates [38] and identify possible vaccine candidates within this species [39,40]. However, the identification of different protein expression markers between isolates exhibiting differential virulence characteristics has been limited [41]. In this study, one-dimensional SDS-PAGE analysis identified 6 major protein expression markers (within the approximately 50 possible protein markers visible upon each SDS-PAGE gel), which were significantly associated with either the complement resistant or complement sensitive phenotype for 48 *M. catarrhalis* isolates. MALDI-TOF analysis indicated the component protein(s) present within these 6 expression markers as including two virulence associated proteins (a hemagglutinin [42,43] and an Omp CD precursor protein [44,45]). As with the htAFLP results, these findings should be considered as forming the basis for a more targeted protein expression profile approach, using a greater number of geographically diverse *M. catarrhalis* isolates and possibly two-dimensional SDS-PAGE, to further screen and validate our results. If such markers are to be used as potential virulence markers, then the actual number of component proteins within each individual differential protein expression marker is not as important as the reproducibility of the marker per se. With respect to the mechanisms possibly generating differential protein expression profiles, these may include: 1) the presence/absence of a particular gene between isolates, 2) differences in gene or promoter sequences which influence protein expression, 3) the lack of recognition of promoter sequences in horizontally transmitted genes between isolates, 4) differential receptor responses to identical growth conditions and 5) differential post-translational processing. Indeed, both horizontal transmission and phase variable expression of genes in *M. catarrhalis* have been described [46,47].

Finally, though the complement resistance phenotype is a virulence trait for *M. catarrhalis*, complement resistance is not always a feature of isolates found within virulence-associated clusters [12,13]. Also, though the *uspA2* gene has been shown to be involved in the expression of the complement resistant phenotype [20,21], it is not the only mechanism currently associated with this phenotype and the presence of the *uspA2* gene does not necessarily lead to the expression of complement resistance per se [13,20]. Therefore, assays that concentrate purely on the presence/absence of the *uspA2* gene, or are based solely on the complement phenotype per se, may not accurately indicate the true virulence potential of a particular *M. catarrhalis* isolate. By combining

lineage-specific and complement phenotype-associated markers, the ability to specifically identify virulent *M. catarrhalis* isolates should be increased.

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Chapter 6

Total genome polymorphism and low frequency of intra-genomic variation in the *uspA1* and *uspA2* genes of *Moraxella catarrhalis* in otitis prone and non-prone children up to 2 years of age. Consequences for vaccine design?

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ABSTRACT

Intra-genomic variation in the *uspA1* and *uspA2* genes of *Moraxella catarrhalis* was studied using pulsed field gel electrophoresis (PFGE) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. From a set of 91 *Moraxella catarrhalis* isolates, 19 pairs of PFGE identical isolates were found. 5 pairs originated from otitis non-prone children, 11 pairs from otitis prone children and for 3 pairs, one of the pair originated from an otitis prone and the other from an otitis non-prone child. No particular *M. catarrhalis* isolate was associated with either the otitis prone or non-prone children. 1 of these 19 pairs of isolates was found to exhibit both *uspA1* and *uspA2* intra-genomic variation, whilst another pair exhibited *uspA2* intra-genomic variation only. Sequence data obtained from these variants showed that PCR-RFLP pattern differences reflected actual changes in predicted amino-acid composition and that minor amino-acid changes in a 23 base pair “NINNIY” repeat region (a conserved UspA1 and UspA2 binding site for the neutralizing antibody mAb17C7) occurred. Variation in the *uspA2* 5’ non-coding “AGAT” repeat region was also observed. These results may have implications for future *M. catarrhalis* vaccines comprising UspA1 or UspA2 components.

1. Introduction

Moraxella catarrhalis is a common commensal of the human upper respiratory tract, which has been associated with a number of disease states [1-3]. The organism has been found to be the third most common cause of acute otitis media in children [4,5] and has also been implicated in chronic otitis media [6]. In adults, *M. catarrhalis* has been associated with both acute and chronic bronchitis [7,8].

The outer membrane proteins of *M. catarrhalis* express major antigenic determinants [9], with different isolates apparently sharing similar protein profiles [10]. Two important examples of these outer membrane proteins are the ubiquitous surface proteins A1 and A2 (UspA1 and UspA2) which share similar overall morphology (comprising an N-terminal head, a left-handed coiled coil segment and a C-terminal membrane anchor region [11]) as well as several conserved amino acid repeat motifs (often present in multiple copies) [12,13]. The UspA1 protein appears to play a role in cellular attachment [13,14], whilst the UspA2 protein appears to be mainly involved in

complement resistance [15]. Most infants have maternally derived serum IgG antibodies to these UspA1 and UspA2 proteins at 2 months of age and adults have significantly higher IgG titers than children. Antibodies to UspA1 and UspA2 cross-react and are associated with bactericidal activity [16] and both UspA1 and UspA2 are currently being considered as possible vaccine candidates for *M. catarrhalis* [17]. Indeed, a recent publication has indicated that a common epitope to mAb17C7 (a murine *M. catarrhalis* bactericidal IgG₁ antibody [18]) was present in 97% of *M. catarrhalis* isolates from young children. [19].

In this study, a set of 91 previously characterized *M. catarrhalis* isolates (obtained from children followed longitudinally up to 2 years of age) were investigated using pulsed-field gel electrophoresis (PFGE), PCR - restriction fragment length polymorphism (PCR-RFLP) and PCR sequencing, in order to determine the presence or absence of *uspA1* and/or *uspA2* intra-genomic variation. In order to further assess *uspA1* and *uspA2* polymorphisms, changes in the “NINNIY” repeat sequence by partial sequencing of the *uspA1* genes and variations in the “AGAT” 5’ non-coding repeat region of the *uspA2* genes were investigated for intra-genomic variants. Complement resistance phenotypes for all specimens were also obtained.

2. Materials and Methods

2.1. Bacterial isolates.

The bacterial isolates comprised 91 *M. catarrhalis* isolates longitudinally collected from 18 different children in the first 2 years of life [4]. Nasopharyngeal swabs were taken at 1-6, 8, 10, 12, 15, 18, 21 and 24 months, with tympanometry and pneumatic otoscopy also being performed. *M. catarrhalis* isolates were classified using the initials of the patient followed by a sequential number (dependant on whether the isolate was the first, second, third etc isolate from that particular child).

2.2. Pulsed field gel electrophoresis (PFGE).

Pulsed field gel electrophoresis was performed as detailed by Verduin *et al* [20]. PFGE patterns were analyzed using GelCompar software (Applied Maths, Gent, Belgium). For the definition of sub-types, up to three small band-size differences were tolerated when compared to the reference pattern.

2.3. *UspA1* and *uspA2* PCR-RFLP.

uspA1 and *uspA2* genes were PCR amplified using standard techniques from isolates exhibiting identical PFGE patterns using primer pairs USPA1t1/USPA1t2 and USPA2t1/USPA2 [13] respectively (both primer pairs include non-coding regions). After PCR amplification, restriction digestion of 15µl of PCR product was performed using 10U of *Hae* III or 10U of *Alu* I (New England Biolabs Inc., MA, USA) according to the manufacturer's instructions. The *uspA1* and *uspA2* genes from isolates with identical PFGE patterns but variant PCR-RFLP patterns were re-amplified using primer pairs *uspA1start*/RTB1-8 and P8959/RTB2-10 respectively (genomic primers) and re-digested with *Alu* I and *Hae* III. Primer details are shown in Figure 1.

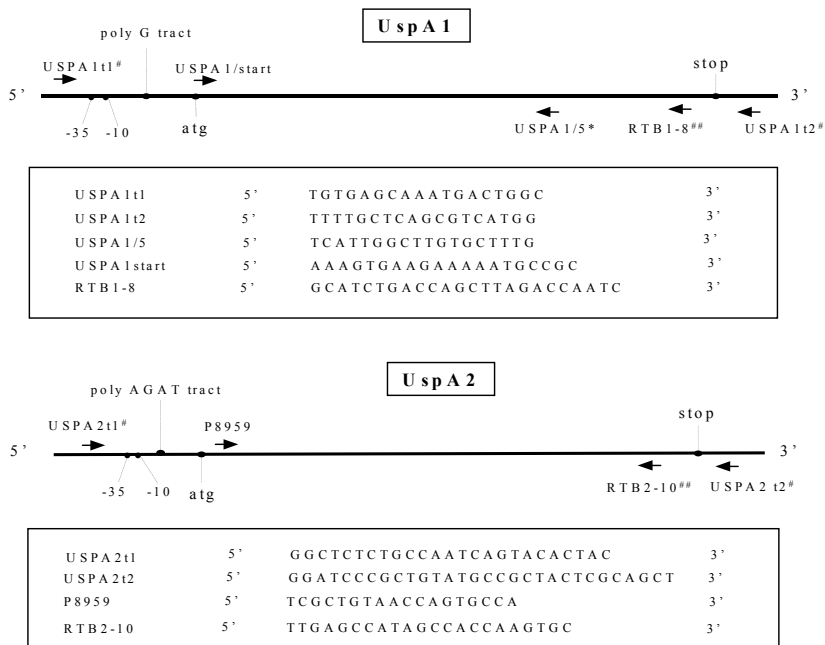


Figure 1. Schematic representation of the positions of *uspA1* and *uspA2* PCR and sequencing primers. * = sequencing primer used for “NINNIY” repeat. # = Primer sequences obtained from Aebi et al, [13]. ## = Primer sequences obtained from Stutzmann Meier et al, [19]. atg = start codon. -35, -10 = RNA polymerase binding sites. Poly G tract / poly AGAT tract = short sequence repeats.

2.4. *UspA1* and *uspA2* sequencing reactions.

In order to study variation in the “NINNIY” repeat sequence (a conserved UspA1 and UspA2 epitope recognized by monoclonal antibody mAb17C7); partial sequencing of the *uspA1* gene of intra-genomic variants 3JB-2, 4LK-2, 16CK-2 and 16CK-3 was performed using primer USPA1/5. The *uspA2* 5' non-coding “AGAT” repeat regions of these intra-genomic variants were also compared using sequence data derived from primer USPA2t1 (see Fig. 1. for primer sequences).

2.5. Complement resistance assay.

Complement resistance was determined using the “culture and spot” method [21,22]. Basically, 100 µl of a 1.5×10^8 suspension of *M. catarrhalis* was spread on a blood agar plate and allowed to dry. Next, 50 µl of 50% human pooled serum and 50 µl of heat treated (56°C for 30 minutes) 50% human pooled serum was spotted onto the lawn of bacteria and the plates incubated at 37°C overnight. The phenotype of the isolate was then determined. Complement resistant, intermediate and sensitive phenotypes were graded as follows (i.e. ≤ 50 colonies = sensitive; normal growth = resistant; > 50 colonies = intermediate).

3. Results

3.1. Pulsed field gel electrophoresis (PFGE).

62 different PFGE band patterns were observed in this particular set of 91 *M. catarrhalis* isolates (Fig. 2.), with 19 identical patterns attributable to 2 or more *M. catarrhalis* isolates. Of these 19 pairs of identical isolates, 5 pairs originated from otitis non-prone children (e.g. isolates MG2-1 and MG2-2), 11 pairs from otitis prone children (e.g. isolates EE11-3 and EE11-4), and 3 pairs where one isolate originated from otitis prone and the other isolate from otitis non-prone children (e.g. isolates CA9-3 and BW18-6). No particular PFGE pattern was associated with either otitis prone or otitis non-prone children. At least one major change in PFGE pattern was observed in every child with three or more *M. catarrhalis* culture positive specimens. The dendrogram obtained for this set of *M. catarrhalis* isolates indicated a division into two major clusters. In one cluster, the two complement sensitive and four complement intermediate isolates were grouped, whilst in the other cluster, the remaining sixty eight complement resistant and seventeen intermediate isolates were found.

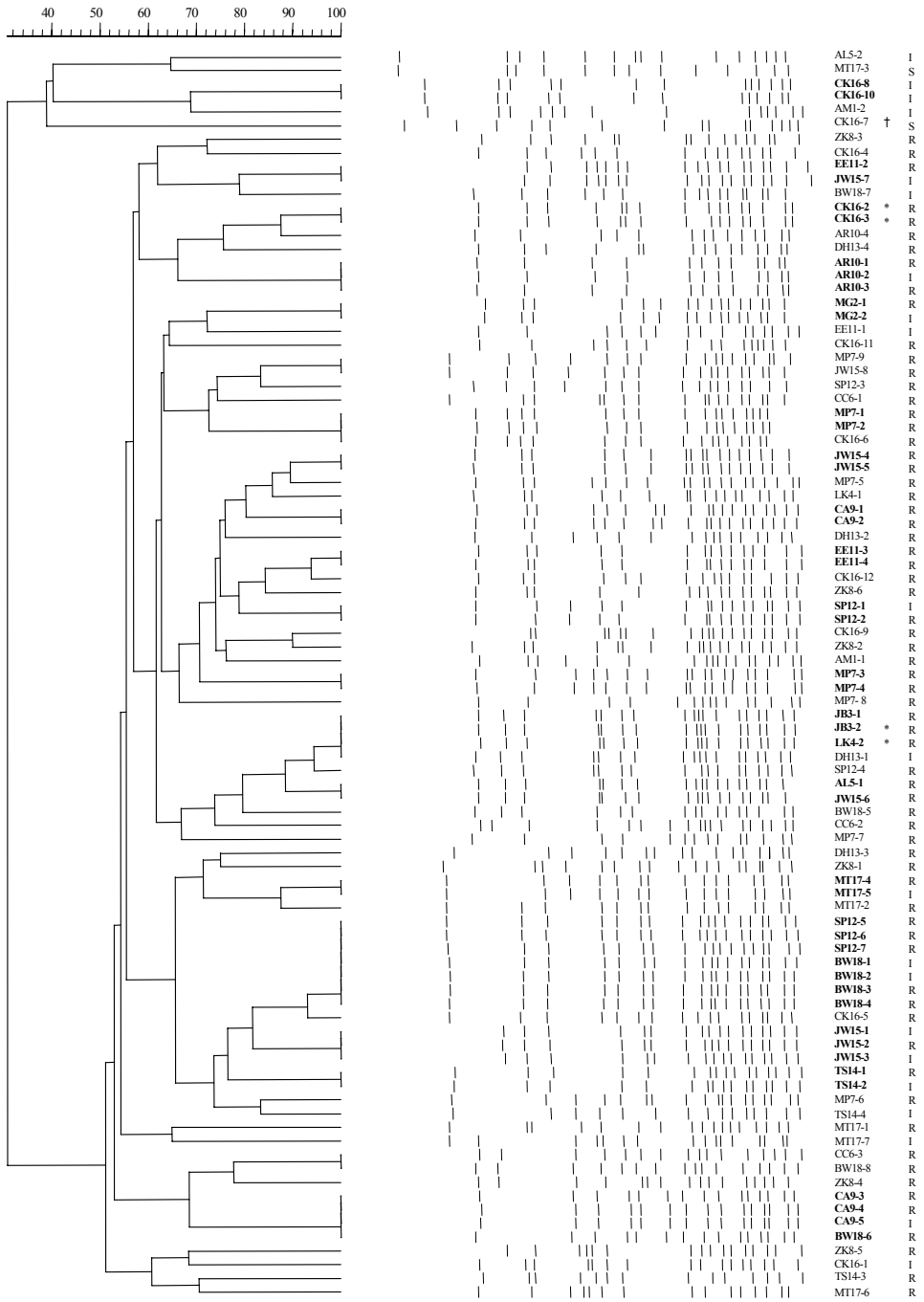


Figure 2. Dendrogram showing the relatedness of *Moraxella catarrhalis* isolates on the basis of PFGE patterns. Band tolerance was set at 1.5% with bands normalized against reference isolate CK16-7†. Bold type = comparable PFGE patterns. * = *uspA1* and/or *uspA2* intra-genomic variants. S = complement sensitive, I = intermediate resistant, R = resistant. Children MP7, ZK8, CA9, AR10, EE11, SP12, DH13, TS14 and JW15 were otitis prone.

3.2. *UspA1* and *uspA2* PCR-RFLP.

UspA1 and/or *uspA2* intra-genomic variation (different *uspA1* and/or *uspA2* PCR-RFLP types in isolates exhibiting the same PFGE pattern) was observed in 2 pairs or 4% (4/91) of *M. catarrhalis* isolates (Figs. 2. and 3.), infecting 17% (3/18) of the children followed (children 3JB, 4LK and 16CK). Intra-genomic variation was observed using both gene-only encoding primer pairs as well as for primers which also amplified portions of the 5' and 3' non-coding regions. Both pairs of intra-genomic isolates originated from otitis non-prone children, with 1 pair (3JB-2 and 4LK-2) exhibiting both *uspA1* and *uspA2* intra-genomic variation and the other pair (16CK-2 and 16CK-3) exhibiting *uspA2* intra-genomic variation only (Fig. 3.)

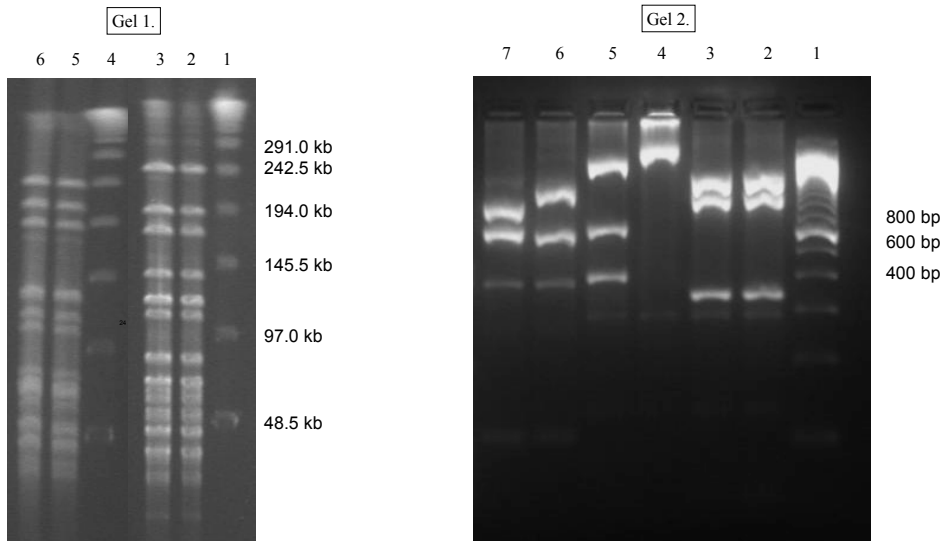


Figure. 3. Intra-genomic variation in *M. catarrhalis*, as determined by pulsed field gel electrophoresis (PFGE) pattern similarity after *Spe* I digestion of genomic DNA (Gel 1.) and subsequent PCR-RFLP analysis of *uspA1* and *uspA2* genes using *Hae* III (Gel 2.). PFGE patterns (Gel 1) : lane 1 = lambda ladder, lane 2 = 16CK-2, lane 3 = 16CK-3, lane 4 = lambda ladder, lane 5 = 3JB-2 and lane 6 = 4LK-2. PCR-RFLP patterns (Gel 2): lane 1 = 600bp molecular weight marker, lane 2 = 16CK-2 *uspA1*, lane 3 = 16CK-3 *uspA1*, lane 4 = 3JK-2 *uspA1*, lane 5 = 4LK-2 *uspA1*, lane 6 = 16CK-2 *uspA2* and lane 7 = 16CK-3 *uspA2*.

3.3 *UspA1* and *uspA2* sequencing.

Partial *uspA1* gene sequencing and translation revealed that the “NINNIY” epitope within these intra-genomic variants contained only minor amino-acid changes (Fig. 4.). Moreover, comparison of the sequenced regions against the previously published *UspA1* sequences for *M. catarrhalis* isolates O35E, O12E and O46E indicated that the partial *UspA1* amino-acid sequence of isolate 4LK-2 had some homology to the previously published isolate O46E *UspA1*. In contrast, this same intra-genomic variant isolate, when isolated from child 3JB, showed homology to the previously published isolate O35E. Comparisons of the *uspA2* 5' non-coding regions also indicated polymorphism, in that the number of “AGAT” repeats was highly variable (data not shown), with 11 “AGAT” repeats for isolate 16CK-2, 14 for isolate 16CK-3 and 20 for isolate 4LK-2. The “AGAT” repeat sequence was absent in the intra-genomic variant isolated from child 3JB (3JB-2), this absence being explained by homology to the previously published TTA37 *uspA2*H hybrid sequence (which combines a N-terminal half *UspA1* protein with a C-terminal half *UspA2* protein and therefore does not contain a 5' non-coding “AGAT” repeat sequence) [15].

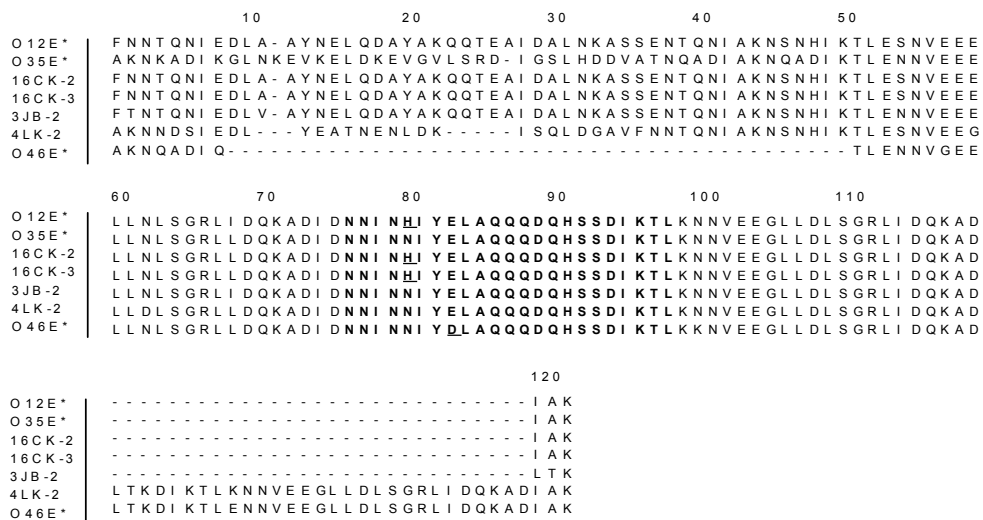


Figure 4. Translated *UspA1* gene sequence alignments (MegAlign 4.0 ©, DNASTAR Inc., Madison, USA) obtained using duplicate *USPA1/5* primer sequencing reactions. * = Partial *UspA1* amino-acid sequences obtained from the National Library of Medicine “PubMed” website (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>). Bold = “NINNIY” conserved repeat sequence to which neutralizing antibody Mab17C7 binds. Underlined = amino acid changes in “NINNIY” sequence.

3.4. Complement resistance assay.

75% of isolates (68/91) were found to be fully complement resistant, 23% (21/91) complement intermediate resistant and 2% (2/91) complement sensitive. 43% (9/21) of the complement intermediate isolates were isolated from otitis prone children compared to 59% (40/68) of the complement resistant isolates (Fig. 2).

4. Discussion

A high degree of isolate variability within *M. catarrhalis* isolates has previously been reported [20,23-26]. However, the finding of low frequency intra-genomic variation in the *uspA1* and/or *uspA2* genes of 4% of isolates within this study adds further to the extent of genomic variation present within this species.

The distribution of PFGE patterns within this set of isolates appeared to be random in nature, with no particular PFGE pattern being associated with the otitis prone or otitis non-prone children. This random distribution of PFGE patterns was also observed for “first acquisition” isolates within the two groups. These observations indicate that re-colonization and re-infection was a frequent event in this cohort of children and that genetic factors within otitis prone children themselves probably predisposes them to repeated *M. catarrhalis* infection, as opposed to pathogenic factors associated with any particular *M. catarrhalis* isolate per se.

Despite the random distribution of PFGE patterns between otitis prone and non-prone children, two distinct clusters of PFGE patterns could be observed, namely between complement sensitive and complement resistant isolates. Moreover, though only two complement sensitive isolates were found within this set of isolates, these results (when added to those previously published by [27] and [20]), further the suggestion of clonality in complement resistant *M. catarrhalis* isolates [28]. It therefore appears that complement resistant *M. catarrhalis* isolates either originated more recently than complement sensitive isolates or perhaps, that the complement resistance phenotype in some way constrains genetic diversity within this species.

Complement resistance in *M. catarrhalis* has been previously associated with disease causing isolates [29]. In relation to other studies [19,29-33], the percentage of complement resistant isolates from this set of USA isolates (98%) was high. This could be attributable to the young age of the patients from which the isolates were cultured. During the first two years of life, infants have relatively naïve immune systems and are

likely to come into contact with many novel infectious agents possibly leading to more frequent activation of the complement system in this age group. In this scenario it is likely that complement resistant *M. catarrhalis* isolates would have a distinct advantage in establishing infection.

The UspA1 and UspA2 proteins have been put forward as possible vaccine candidates [19,34,35,36,37], with encouraging results being published regarding UspA immunized mice [17,38], as well as passive immunization with UspA reactive antibody [18]. Indeed, previous research has indicated that conserved identical repeat regions within both the UspA1 and UspA2 proteins could contain important neutralizing epitopes (e.g. the “NINNIY” repeat and monoclonal antibody mAb17C7 [12,13,18,19,35]). Interestingly, partial *uspA1* gene sequencing results for the “NINNIY” repeat sequence in the intra-genomic variants found within this set of isolates, indicated that displacement of this region could occur in identical isolates (3JB-2 and 4LK-2). However, it should be noted that alignment of these “NINNIY” sequences with previously published UspA1 protein sequences, indicated that 3JB-2 and 4LK-2 UspA1 proteins were related to two different isolates (O35E and O46E respectively). This raises the possibility that either *uspA1* (partial) gene transfer occurs between identical isolates, or that PFGE is not a reliable method for the determination of isolate identities in *M. catarrhalis*.

Variation in the *uspA2* 5' non-coding “AGAT” repeat sequence may possibly be linked to phase variation in expression of the UspA2 protein, in that variation in a poly “G” tract region (occupying an analogous position in the 5' non-coding region of the *M. catarrhalis uspA1* gene) has previously been linked to variation in expression of the *uspA1* gene [39]. Such phase variable expression of the *uspA2* gene could be important as a survival mechanism for *M. catarrhalis*. In this scenario, the up-regulation of UspA2 expression would be advantageous in complement rich environments, whilst down-regulation would be advantageous against an immune response which has generated a particularly effective UspA2 bactericidal antibody. However, further work is required in order to confirm or refute this theory.

This is the first report of intra-genomic variation within the *M. catarrhalis uspA1* and *uspA2* genes. These results indicate that diversity within the *M. catarrhalis* species is greater than originally reported, (though occurring at a relatively low frequency), and that minor changes in conserved epitopes may occur in identical strains. Previously published reports have indicated that the presence of these conserved epitopes within the UspA1 and UspA2 proteins could be useful in the design of a future *M. catarrhalis*

vaccine. However, two important questions remain to be answered about the potency of these conserved epitopes with respect to human disease. Firstly, why do repeat *M. catarrhalis* infections and/or re-infections occur if conserved epitopes act as sites for the production of bactericidal antibody? Secondly, if conserved epitopes provide protection against the host immune system, what process is driving *M. catarrhalis* variation and *uspA1* / *uspA2* intra-genomic variation? Maybe the mouse model (on which previous neutralizing antibody studies have been based) does not accurately represent in vivo *M. catarrhalis* infection in humans.

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Chapter 7

**Identification and characterization of a novel outer membrane
protein (OMP J) of *Moraxella catarrhalis*
that exists in two major forms.**

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ABSTRACT

Moraxella catarrhalis is a common commensal of the human respiratory tract, which has been associated with a number of disease states, including acute otitis media in children and exacerbations of chronic obstructive pulmonary disease in adults. During studies to investigate the outer membrane proteins of this bacterium, two novel major proteins of approximately 19kDa and 16kDa (named OMP J1 and OMP J2 respectively) were identified. Further analysis indicated that these two proteins possessed almost identical gene sequences apart from two insertion/deletion events in predicted external loops present within the putative barrel-like structure of the proteins. Development of a PCR screening strategy found a 100% (96/96) incidence for the gene encoding the OMP J1 and OMP J2 proteins within a set of geographically diverse *M. catarrhalis* isolates, as well as a significant association of OMP J1 / OMP J2 with both genetic lineage and complement phenotype (Fisher's Exact Test $P < 0.01$). Experiments using two $\Delta ompJ2$ mutants (one complement resistant and the other complement sensitive), indicated that both were less easily cleared from the lungs of mice compared to their isogenic wild-type counterparts, with a significant difference in bacterial clearance being observed for the complement resistant isolate but not its isogenic $\Delta ompJ2$ mutant (unpaired Student's t-test $P < 0.001$ and $P = 0.32$). In this publication, the authors characterize a novel outer membrane protein of *Moraxella catarrhalis* which exists in two variant forms associated with particular genetic lineages, and which are suggested to contribute to bacterial clearance from the lungs.

INTRODUCTION

The Gram-negative bacterium *Moraxella catarrhalis* is a common commensal of the human upper respiratory tract which has been associated with a number of disease states, including acute otitis media in children (9, 14) and both acute and chronic bronchitis in adults (16, 32). Nosocomial outbreaks of this pathogen have also been reported (10, 34), as well as cases of near fatal pneumonia (11). The morbidity burden of *M. catarrhalis* is particularly noticeable in young children suffering from recurrent otitis media episodes (13) and in adults presenting with chronic obstructive pulmonary disease (COPD) (35).

One particularly important virulence trait of *M. catarrhalis* is serum resistance (21), with several outer membrane proteins (OMPs) being implicated in the expression of this particular phenotype. Of particular importance is the UspA2 protein, a vitronectin binding protein whose N-terminal half may confer complement resistance in certain isolates (1, 33, 41). Other OMPs associated with virulence include the iron acquisition protein CopB (20), a hemagglutinin (28), and the lipooligosaccharide (44). Interestingly, there is increasing evidence to suggest that particular virulence traits are associated with distinct sub-populations of *M. catarrhalis* (8, 12, 42).

Several OMPs of *M. catarrhalis* have been shown to elicit an antibody response in humans and have therefore been suggested as potential vaccine candidates, these include the immunoglobulin D-binding protein (MID) (15), and the major heat-modifiable protein Omp CD (31, 43). However, an ideal vaccine candidate has not yet been described.

In this article, a novel outer membrane protein of *M. catarrhalis* that exists in two major forms (OMP J1 and OMP J2) is described and characterized. Sequence variation of the two forms and their relationship with both genetic lineage and complement phenotype are discussed. Preliminary investigations into the role of the protein were performed by comparing the clearance of two OMP J2 knockout ($\Delta ompJ2$) mutants against their isogenic wild-type counterparts in a mouse pulmonary model of infection

MATERIALS AND METHODS

Bacterial isolates. In total, a group of 96 *M. catarrhalis* isolates were utilized in this study, comprising 35 isolates from The Netherlands (1989 – 1997), 6 from Ghana (1995), and 55 from the USA (1991 – 1994, kindly supplied by Dr. H. Faden, Department of Pediatrics, Children's Hospital of Buffalo, New York). All of the isolates were cultured from children on Columbia blood agar, apart from 6 of the Dutch isolates, which were cultured from adults.

Identification of OMP J. One-dimensional SDS-PAGE analysis was performed using standard techniques with 100 μ g protein loaded per gel lane. Membrane fractions of *M. catarrhalis* were isolated by ultrasonic treatment and extraction in 1% sarcosyl according to the methodology previously described by Klingman and Murphy (24). Tandem mass spectrometry was performed on trypsin digests of 19kDa and 16kDa

proteins (OMP J1 and OMP J2 respectively) to identify amino acid sequences which were compared to an *in silico* translation (all 6 reading frames) of the unannotated *M. catarrhalis* genome sequence available at GenBank (accession numbers AX067426-AX067466, comprising 41 contigs ranging in size from 429bp to 261,300bp in length). Identification of the relevant protein and gene sequence allowed PCR screening and sequencing primers to be designed.

PCR screening and sequencing of *ompJ1* / *ompJ2* genes. PCR screening of *M. catarrhalis* isolates for *ompJ* and its genetic variants was performed using standard PCR techniques and primer pair 19kDres.f and 19kDres.r (primer details for all primers used are given in Table 1). Expected PCR product sizes were approximately 363bp (*ompJ1*) and 333bp (*ompJ2*) in size. PCR sequencing of the *ompJ* gene from 14 *M. catarrhalis* isolates (representative of the total group of 96 isolates used in this study), was performed using PCR sequencing primers 19kDseqf, 19kDseqf2, 19kDseqr and 19kDseqr2.

TABLE 1. List of primers used

Primer	Sequence
19kDres.f	5'-CTAACGCTGCCATCAGCTAT-3'
19kDres.r	5'-GTTGCATTACGGCTGGTAAC-3'
19kDseqf	5'-CGTCATTTGGAACAAGACAGTT-3'
19kDseqf2	5'-TGAGTGCTAACGCTGCCATC-3'
19kDseqr	5'-AAAGATAATTTATCATGATGGCTTA-3'
19kDseqr2	5'-CAACACCAACACCGCCTGCT-3'
19kDKO1f.Bam	5'-GACTGGG GATCCGCGTATCACCGCCAATCACT-3'
19kDKO1r.Pst	5'-GACTGCTGCAGCGCCAATAACGAAGAAGCTG-3'
19kDKO2r.Bam	5'-GACTGGG GATCCTCACATCATGCGTGGACT-3'
19kDKO2f.Pst	5'- GACTGCTGCAGGCGTTGAAGCAAGCTACA-3'
19kDaKO.ctrlf	5'-GCACAGCAAGCGTAGTTATG-3'
19kDaKO.ctrlr	5'-TGCGTTAAGCCTTGGTGATG-3'
K _{an} R1	5'-CTCGTGAAGAAGGTGTTGC-3'
K _{an} R2	5'-ATAAATGGGCTCGCGATAATGTC-3

Details of the 14 representative isolates chosen for *ompJ* sequencing are shown in Table 2. PFGE genotype / complement phenotypes were obtained by reference to Verduin et al. and Hays et al. (18, 42).

TABLE 2. *M. catarrhalis* isolates chosen for *ompJ* gene sequencing.

Isolate	Origin	Complement Phenotype
1.24	The Netherlands (1989)	RESISTANT
1.39	The Netherlands (1989)	SENSITIVE
3.14	The Netherlands (1989)	SENSITIVE
3.18	The Netherlands (1989)	SENSITIVE
3.9	The Netherlands (1989)	RESISTANT
F3.57	The Netherlands (1993)	SENSITIVE
H12	The Netherlands (1989)	RESISTANT
97/0233	The Netherlands (1997)	RESISTANT
25240	ATTC reference isolate	RESISTANT
V02	Ghana, Africa (1995)	RESISTANT
CK16-7	New York, USA (1990-92)	SENSITIVE
EE11-2	New York, USA (1990-92)	RESISTANT
MT17-3	New York, USA (1990-92)	SENSITIVE
MT17-6	New York, USA (1990-92)	RESISTANT

1.24 - F3.57 = isolates from healthy (colonized) children. H12 - 97/0233 = positive blood culture isolates from children. CK16-7 - MT17-3 = isolates from children prone to acute otitis media

Preparation of $\Delta ompJ$ gene knockout isolates. The regions immediately flanking the *ompJ* gene were amplified using primer pair 19kDKO1f.Bam / 19kDKO1r.Pst (700bp upstream fragment), and primer pair 19kDKO2r.Bam / 19kDKO2f (500bp downstream fragment). Both fragments were then digested with *Pst I*, ligated, and the 1200bp product (minus an internal 410bp fragment of *ompJ*), re-amplified using primers 19kDKO1f.Bam and 19kDKO2r.Bam. This PCR product was digested with *BamH I*, ligated into plasmid pGEM-7zf(+) (Promega Corporation), and used to transform One Shot[®] TOP10 *E. coli* cells (Invitrogen). Plasmids containing the insert were selected by PCR analysis of white, ampicillin resistant *E. coli* colonies. After extraction, the plasmid was digested using *Pst I*, and an internal kanamycin resistance gene cassette (obtained by *Pst I* digestion of plasmid pUC4K (Amersham Pharmacia Biotech)) ligated into the *ompJ* *Pst I* site. The construct was used to transform One Shot[®] TOP10 *E. coli* with selection on Mueller Hinton (MH) agar containing 5 μ g/ml kanamycin. *M. catarrhalis* isolates were subsequently naturally transformed with PCR amplification products (primer pair 19kDKO1f.Bam / 19kDKO2r.Bam and pGEM-

7zf(+) $\Delta ompJ \Delta kanR$ as template) and selected on Mueller Hinton (MH) agar containing 5 μ g/ml kanamycin. The presence of the $\Delta ompJ$ knockout construct in the chromosome of kanamycin resistant *M. catarrhalis* colonies was confirmed by PCR (primers 19kDaKO.ctrlf, 19kDaKO.ctrlr, KanR1 and KanR2), as well as the absence of OMP J protein expression (established using 1D SDS-PAGE analysis of outer membrane protein extracts). $\Delta ompJ2$ knockout constructs were prepared in *M. catarrhalis* isolates 3.9 and 3.18.

Pulmonary clearance study. In order to determine the effect of OMP J on pulmonary clearance in a mouse model, 2 *M. catarrhalis ompJ2* containing gene knockout mutants (3.9 $\Delta ompJ2$ and 3.18 $\Delta ompJ2$) were constructed, and compared to the isogenic wild-type isolate in a mouse pulmonary clearance study. The mouse pulmonary clearance protocol was based upon that published by Forsgren et al (15) and Unhanand et al (39). Basically, *M. catarrhalis* isolates were grown overnight at 37°C on either MH agar (wild-type isogenic isolates) or MH agar incorporating 5 μ g/ml kanamycin ($\Delta ompJ2$ mutants), and then grown to mid-log phase in MH broth. A 50 μ l volume containing 1 x 10⁸ CFU of each isolate was inoculated intra-nasally into the lungs of anaesthetized BALB/c mice which were sacrificed at 1/2 hour and 3 hours post-infection. Colony counts for surviving *M. catarrhalis* were performed on MH agar after overnight incubation at 37°C. Five mice were sacrificed for each isolate tested (total = 2 x 20 mice). Percentage survival values 3 hours post-inoculation were calculated by taking the average CFU count of each isolate at time point 1/2 hour as 100%. Statistical analysis was performed using a two-tailed unpaired t-test comparing the difference in lung log₁₀ CFU/ml survivors at times 1/2 and 3 hours after nasal inoculation. Further analysis involved generating growth curves for both wild-type and isogenic $\Delta ompJ2$ mutants in order to verify that no significant difference in growth rates existed. The animal studies described in this publication were performed in accordance with the ethical and legal requirements of the Erasmus MC, Rotterdam, and under approval of the Animal Studies Ethics Committee of the same institution.

Serum bactericidal testing. Serum bactericidal survival for *M. catarrhalis* isolates 3.9, 3.18 and their isogenic $\Delta ompJ2$ mutants was based on the protocol described by Attia et al (1). Briefly, bacterial cultures were grown to mid-log phase (approximately 5 x 10⁸ CFU/ml) in Mueller Hinton (MH) broth and diluted 1/1000 in Veronal-buffered saline containing 0.1% (wt/vol) gelatin. Twenty microliters of this

bacterial suspension was added to 160µl Veronal buffered saline/gelatin and 20µl of human pooled serum (or 20µl heat inactivated human pooled serum previously incubated at 56°C for 30 minutes) added. Ten microliter aliquots of each reaction mix were plated onto MH agar after 0 and 30 minutes of incubation at 37°C. Four independent experiments were performed per bacterial isolate, using human pooled serum (HPS) obtained from 8 healthy adult volunteers. Percentage survival values were calculated by comparing the means of results at zero minutes and 30 minutes of exposure to HPS from four independent experiments. Statistical differences between wild type and $\Delta ompJ2$ isolates were calculated using log₁₀ CFU/ml survival values after 30 minutes of HPS exposure and a two-tailed Student's unpaired *t* test (after first ensuring that there was no significant difference in CFU/ml results after zero minutes of exposure to HPS).

RESULTS

Identification of OMP J. One-dimensional SDS-PAGE analysis of outer membrane protein extracts from 48 out of the 96 *M. catarrhalis* isolates used in this study, revealed the presence of two putative proteins (of approximately 19kDa and 16kDa) and an association between protein and complement resistant or sensitive phenotype (Fig. 1). Subsequent tandem mass spectroscopy analysis indicated that the two proteins consisted mainly of identical polypeptide sequences, indicating that both proteins were in fact two variants of the same protein. These sequences were found in only one translated ORF within the whole *M. catarrhalis* proteome, allowing the protein and its genomic location to be identified. Using this data, the surrounding ORFs of *ompJ* were found to include proteins involved in the recognition and processing of DNA lesions (*uvrC*), glycolate metabolism (*pgp*), glutamyl-tRNA synthetase (*gluRS*), and in the suppression of a DnaK-like heat shock protein (data not shown).

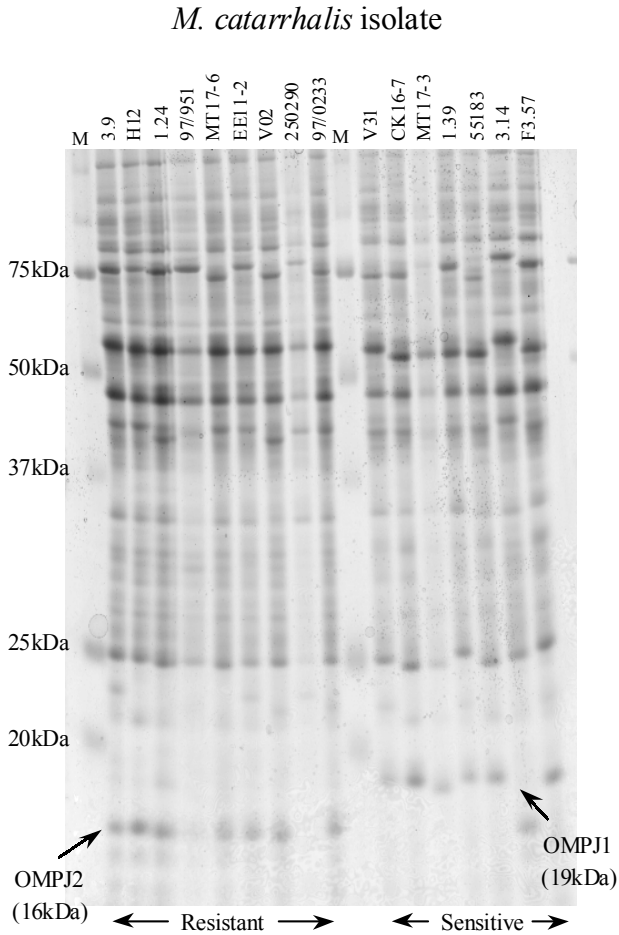


Fig 1. One-dimensional SDS-PAGE gel of outer membrane proteins extracted from several complement resistant and complement sensitive isolates of *M. catarrhalis*. M = SDS-PAGE standards. Filled arrows indicate positions of the two major forms of the OMP J protein. Resistant = complement resistant phenotype, Sensitive = complement sensitive phenotype

Sequencing and PCR screening of *ompJ* genes. Sequence analysis of the *ompJ* gene from 14 geographically distinct *M. catarrhalis* isolates indicated that two distinct phylogenetic lineages existed (GenBank, accession numbers DQ008974-DQ008986 and DQ105644), with 90% identity (over 579bp) between the two most divergent

sequences (Fig. 2). This clustering occurred independent of geographical origin of the isolates. Translation of the 14 gene sequences showed 3 insertion/deletion events which were common to each cluster, occurring at amino-acids positions 69-71,82-97, 138-144 and 151-153 of the isolate F3.57 sequence. Sequence comparisons of OMP J1 with known protein sequences indicated that the protein was similar to a hypothetical protein found in the closely related *Psychrobacter spp.*, (GenBank accession number gi|52853456|ref|ZP_00145674.2|), but contained little sequence similarity with other known proteins. Secondary structure analysis revealed the putative position of α -helix and β -sheet regions within the OMP J sequence, providing indications that OMP J may form a beta-barrel-type structure with considerable structural similarity to a superfamily of proteins which include the Omp21 protein from *Comamonas (Deftia) acidovorans* (3, 4), *Neisseria* opacity associated protein (Opa) (17), its homologue *Neisseria* surface protein A (NspA) (40), and *Enterobacter cloacae* outer membrane protein X (OmpX) (37, 38). The position of two of the major insertion/deletion events occurred in a putative external loop (loop 2) of the predicted barrel-like structure.

PCR screening primers for *ompJ* were designed using conserved sequences found in both *ompJ1* and *ompJ2* genes and yielded positive PCR products in 100% (96/96) of *M. catarrhalis* isolates tested (Fig. 3). Of these, 97% (72/74) of complement resistant isolates generated shorter PCR products of approximately 333bp (*ompJ2*-like), whilst 74% (14/22) of complement sensitive isolates generated larger PCR products of approximately 363bp (*ompJ1*-like). This distribution of *ompJ* gene to complement phenotype was found to be highly significant (Fisher's Exact Test $P < 0.001$). Importantly, there also appeared to be a significant correlation between the two major forms of *ompJ* gene and genetic lineage (as determined by PFGE). In particular, by cross-referencing the results obtained with 41 isolates previously genotyped by Verduin et al (42), it was observed that 22/28 isolates from a short branched lineage harbored the *ompJ2* gene and 9/13 isolates from a longer branched lineage harbored the larger *ompJ1* gene. Further cross-referencing of *ompJ* PCR data to 55 American isolates previously genotyped by Hays et al (18), indicated that 50/50 isolates from a short branched lineage harbored the *ompJ2*-like gene and 4/5 isolates from a longer branched lineage harbored the larger *ompJ1*-like gene (data not shown). These results were highly significant (Fisher's Exact Test $P = 0.005$ and $P < 0.0001$ respectively).

	10	20	30	$\beta 1$	40	L1	50	
Majority	M K T L K T L L A V S A S S L L A M S A N A A I S Y G N S A D A Q P Y V G A K I G Q V D A K Q I N G							
3.18								
MT 17.6								
1.24								
EE 11.2								
3.14								
3.9								
V02								
97.0233								
H12								
25240								
1.39								
CK 16.7								
MT 17.3						V		
F3.57						V		
	$\beta 2$	60	T1	70	$\beta 3$	80	L2 90	100
Majority	K N T A V G I Y A G N F D Q N F G V E A E F V G S D A K E F N A G V S - - - - - P V K							
3.18								
MT 17.6							M	
1.24							M	
EE 11.2							M	
3.14							M	
3.9							M	
V02							M	
97.0233							T	
H12								
25240							M	
1.39				A		D V T	P A V - G T T P A V V L	
CK 16.7				A	V	D V T	P A V - G T T P A V V L	
MT 17.3				A	V	D V T	P A V V G V S P A V V L	
F3.57				A		D V T	P A V V G V S P A V V L	
	$\beta 4$	110	T2	120	$\beta 5$	130	140	L3 150
Majority	G D V K S F G A Y G T Y R N F I N T P F Y A K G K L G I A K T K V D V T S R N A T T - Y S N K S D							
3.18								
MT 17.6								
1.24								
EE 11.2								
3.14								
3.9								
V02								
97.0233								
H12								
25240								
1.39							G	A S
CK 16.7							G	A S
MT 17.3							G	K A S
F3.57							G	K A S
	$\beta 6$	160	T3	$\beta 7$	170	L4 180	$\beta 8$	190
Majority	K T S L A G G V G V G F K P L A N V G V E A S V N V L S E D A N A I S L G A H L A F							
3.18								
MT 17.6								
1.24								
EE 11.2								
3.14								
3.9								
V02								
97.0233								
H12								
25240								
1.39	E	N						
CK 16.7	E	N						
MT 17.3	E							
F3.57	E							

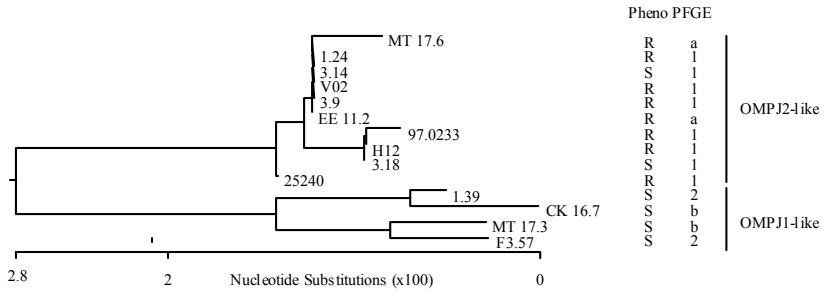


Fig. 2. (Top) OMP J amino-acid sequences of 14 *M. catarrhalis* isolates. 25240, 3.14, 3.9, 97/0233, H12, 1.24, 1.39, F3.57 = Dutch isolates. EE 11.2, CK 16.7, MT 17.3, MT17-6 = American isolates. V02 = Ghanaian isolate. Dotted line above sequence = signal peptide; filled line above sequence = β -strands numbered β 1 - β 8; L1 - L4 = surface exposed loops; T1 - T3 = periplasmic turns; boxed residues = residues contributing to aromatic girdles; residues in bold type = residues in β -strands facing the lipids.

(Bottom) Dendrogram of the nucleotide sequences (DICE coefficient with UPGMA). Pheno = complement phenotype where R = resistant S = sensitive. PFGE = genotypic lineages as previously determined by Verduin et al, 1994 (1 or 2), and Hays et al, 2003 (a and b).

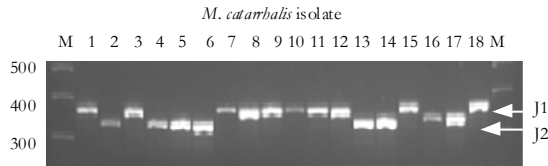


Fig. 3. Gel showing *ompJ* PCR screening results obtained for 18 Dutch *M. catarrhalis* isolates. M = molecular weight marker (bp). 1 - 18 = Dutch *M. catarrhalis* isolate F1.11/ F1.3 / 8.3 / 7.13 / 7.10 / 7.2 / 6.12 / 6.2 / 5.12 / 4.22 / 4.16 / 3.24 / 3.21 / 3.18 / 3.14/ 1.38 / 1.12 / 1.9 respectively. Note differences in size between *ompJ*1-like (363bp) and *ompJ*2-like (333bp) genes.

PCR screening results did not reveal the presence of multiple *ompJ* PCR products within individual isolates. Further, sequence searching of the only (unannotated) *M. catarrhalis* whole genome sequence publicly available (GenBank accession numbers AX067426-AX067466), revealed the presence of only a single copy of the *ompJ* gene (*ompJ2*) within this isolate.

Δ ompJ knockouts and pulmonary clearance studies. Attempts at creating Δ *ompJ2* gene knockouts in *M. catarrhalis* was successful for the complement resistant isolate 3.9 and complement sensitive isolate 3.18 (Fig. 4), with further studies indicating that knocking out these *ompJ2* gene did not affect the expression of other outer membrane proteins (Fig. 5).

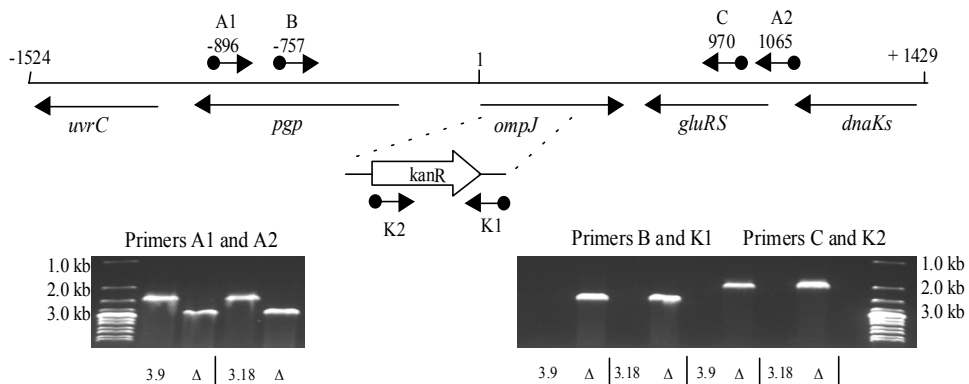


Diagram and PCR gels showing insertion of kanamycin resistance cassette into *ompJ* genes of *M. catarrhalis* isolates 3.9 and 3.18. Primers: A1 = 19kDaKO.ctrlf, A2 = 19kDaKO.ctrlf; B = 19kDKO1f.Bam; C = 19kDKO2R.Bam; K1 = KanR1 and K2 = KanR2. 3.9 and 3.18 = isolates 3.9 and 3.18 respectively. Δ = the respective isogenic Δ *ompJ* knockout mutant. Numbers indicate the position of the 5'-end of primers with respect to the 5'-end of the *ompJ* gene as obtained from the unannotated *M. catarrhalis* genome sequence available at GenBank (accession numbers AX067426-AX067466). The kanamycin resistance cassette inserted into the *ompJ* gene is 1240bp in length. Open reading frames in the vicinity of the *ompJ* gene include: *uvrC* = excinuclease ABC subunit C (gi|46141700|ref|ZP_00147050.2|); *pgp* = predicted phosphoglycolate phosphatase (gi|52853459|ref|ZP_00145679.2|); *ompJ* = hypothetical *Psychrobacter* protein Psyc03002166 (gi|52853456|ref|ZP_00145674.2|); *gluRS* = glutamyl- and glutaminyI-tRNA synthetase (gi|41690542|ref|ZP_00147074.1|); *dnaKs* = gene for DnaK suppressor protein (gi|41690541|ref|ZP_00147073.1|).

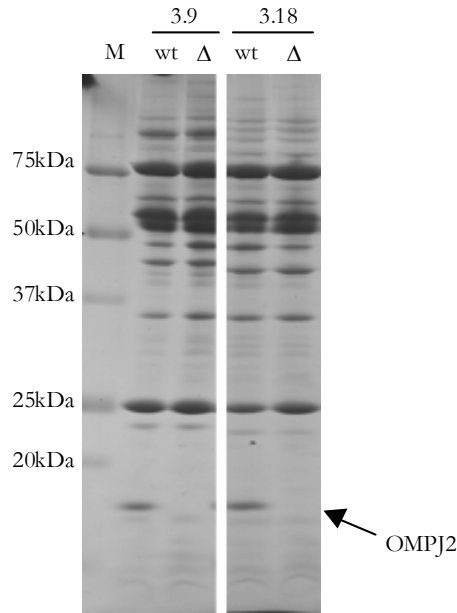


Fig. 5. Composite one-dimensional SDS-PAGE gel showing outer membrane protein profiles of *M. catarrhalis* isolates 3.9 and 3.18 along with their respective isogenic $\Delta ompJ$ mutants.

The average *M. catarrhalis* survival in a pulmonary mouse model 3 hours after inoculation was measured as 23%, 78%, 80% and 92% for isolates 3.9, 3.9 $\Delta ompJ2$, 3.18 and 3.18 $\Delta ompJ2$ respectively (Fig. 6). Statistical analysis using a two-tailed unpaired Student's t-test indicated a statistically significant decrease in \log_{10} CFU/ml bacterial survivors after 3 hours for isolate 3.9 ($P < 0.001$), but not for its isogenic $\Delta ompJ2$ mutant ($P = 0.32$), isolate 3.18 ($P = 0.25$) or its isogenic $\Delta ompJ2$ mutant ($P = 0.49$). Moreover, both $\Delta ompJ2$ mutants survived in greater numbers than their respective wild-type parents. Growth curve comparisons showed no difference in exponential growth rate between the $\Delta ompJ2$ mutants and their respective isogenic isolates, though it was noted that the final concentration of $\Delta ompJ2$ mutant cells was somewhat lower in the plateau phase of the growth cycle (Fig. 7).

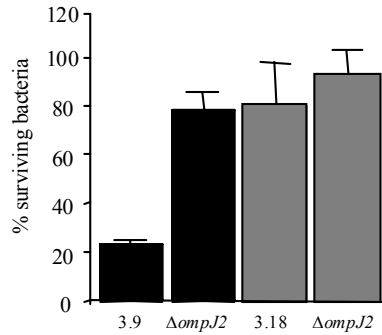


Fig. 6. Graph showing percentage pulmonary survival in a mouse challenge study for two wild-type isolates of *M. catarrhalis* and two isogenic $\Delta ompJ2$ gene knockout mutants. 3.9 and 3.18 = wild-type isolates 3.9 (complement resistant) and 3.18 (complement sensitive) respectively. $\Delta ompJ2$ = respective isogenic *ompJ2* gene knockout mutant. Percentage survival was determined by comparing CFU/ml values 3 hours post-inoculation with CFU/ml values $\frac{1}{2}$ hour post-inoculation. Data are representative of 5 independent experiments. Error bars indicate SEM using percentage data.

Serum bactericidal testing. Serum bactericidal survival results for *M. catarrhalis* isolates 3.9, 3.18 and their respective isogenic $\Delta ompJ2$ mutants are shown in Figure 8. No significant difference was observed between wild-type isolate 3.9 and its $\Delta ompJ2$ mutant in either HPS or heat-inactivated HPS ($P = 0.44$ and 0.16 respectively), though percentage survival values were reduced for the 3.9 $\Delta ompJ2$ knockout isolate in both HPS and inactivated HPS. The complement sensitive isolate 3.18 actually showed an increase in survival in both HPS and heat-inactivated HPS. However, the significance of any conclusions that could be drawn were limited by the detection limit of the methodology used (zero colonies were recorded after incubation in HPS).

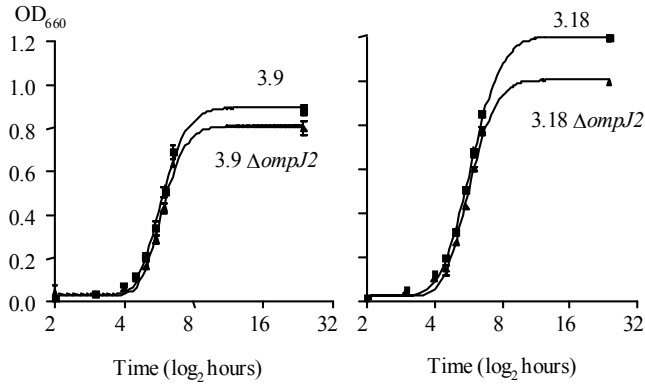


Fig. 7. Growth curves for two *Moraxella catarrhalis* wild-type isolates (3.9 and 3.18) and isogenic $\Delta ompJ2$ gene mutants (3.9 $\Delta ompJ2$ and 3.18 $\Delta ompJ2$).

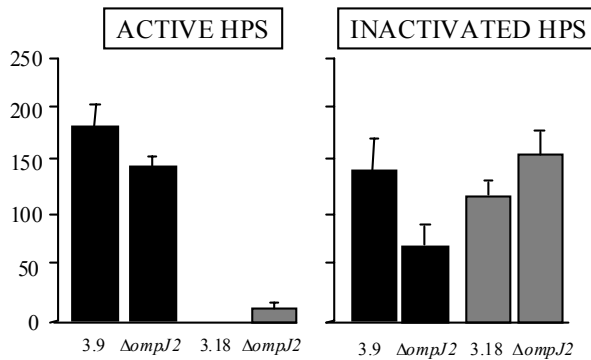


Fig. 8. Effect of 10% (vol/vol) human pooled serum (HPS) and 10% heat-inactivated HPS on the percentage survival of *M. catarrhalis* isolates 3.9 (complement resistant) and 3.18 (complement sensitive) compared to their respective $\Delta ompJ2$ gene mutants. Colony counts were performed at time zero and 30 minutes after addition of serum, with percentage survival being calculated relative to time zero. Standard error bars are calculated from the means of 4 independent experiments.

DISCUSSION

Early studies on the outer membrane proteins of *M. catarrhalis* identified and characterized eight major proteins within this species, ranging from 98kDa to 21kDa and named OMP A to OMP H respectively (29). Several of these OMPs have now been characterized and include proteins involved in iron acquisition e.g. CopB (7), LbpB/A (6) and TbpB (36), fatty acid uptake (5), and adhesion (22, 23, 26). A role for some of these proteins in *M. catarrhalis* virulence and pathogenicity has been suggested, including the UspA2 (1, 41) and OMP E (30) proteins, which appear to facilitate serum resistance. Other experiments have shown that a CopB binding monoclonal antibody (Mab10F3) could enhance the clearance of *M. catarrhalis* in a mouse pulmonary model, binding to 70% of *M. catarrhalis* isolates tested (19). Further, the finding that adults develop new serum IgG and mucosal IgA to bacterial surface epitopes after exacerbations of COPD, shows the importance of the humoral immune response to *M. catarrhalis* mediated infection (2).

In this publication, 1D SDS-PAGE analysis of outer membrane protein extracts from several isolates of *M. catarrhalis*, revealed the presence of a small and novel major outer membrane protein (OMP J), which was found to exist in two major forms, with molecular weights of approximately 19kDa and 16kDa (OMP J1 and OMP J2 respectively). Sequence analysis and database searching indicated a limited homology between the OMP J protein and *ompJ* gene with other known protein and gene sequences, with the possible exception being a hypothetical protein found in the closely related *Psychrobacter sp.* However, secondary structure prediction for OMP J indicated that the protein might possess a barrel-like tertiary structure, which taken in context with the presence of a signal sequence, suggests that OMP J may be an integral membrane protein. Indeed, the sequence/structure results suggest that OMP J belongs to a superfamily of proteins that include the OPA (opacity) family of proteins of *Neisseria species*, a family of proteins which mediate bacterial adherence to epithelial cells by interacting with (for example) the receptors for the human carcinoembryonic antigen cell adhesion molecule (CEACAM) on human polymorphonuclear phagocytes. Other members of this superfamily include the *Neisseria* surface protein A (NspA), a highly conserved protein of unknown function, but which is a promising vaccine candidate against both *Neisseria meningitidis* and *Neisseria gonorrhoeae* (27, 40). Structurally, the major difference between the two forms of OMP J seems to reside in the deletion

of 12 amino acids forming part of a putative loop 2 region, though the consequences of this deletion with respect to the function and antigenic properties of the two proteins has yet to be determined.

PCR screening of isolates suggested that only a single copy of the *ompJ* gene is present in the *M. catarrhalis* species and that it may be found in 100% of isolates, indicating a significant role for OMP J in the *M. catarrhalis* life-cycle. No clear indication of the likely function of OMP J was obtained by inspecting neighboring ORFs, which appeared to comprise a mix of putative housekeeping genes involved in various metabolic and DNA repair activities. Of note however, the direction of transcription of the *ompJ* gene lies in the opposite orientation to neighboring ORFs.

A statistically significant association between the two major forms of OMP J, genetic lineage, and complement phenotype, was observed in diverse geographical isolates. However, serum resistance experiments using two $\Delta ompJ2$ mutants did not indicate a significant role for OMP J2 in facilitating complement resistance. It seems likely that the association between OMP J1 and OMP J2 with complement phenotype is simply a consequence of their association with different genetic lineages, lineages previously associated with the differential expression of virulence traits (8). In fact, most evidence implicates the UspA2 outer membrane protein as the major contributor to the complement resistance phenotype within this species (1,41).

Previous investigations have shown that alteration in the expression of outer membrane proteins and LOS in *M. catarrhalis* may significantly impact on the in-vivo clearance of isogenic mutants in a mouse pulmonary model of infection (25). Studies investigating the role of *ompJ2* in the clearance of *M. catarrhalis* from the lungs of mice showed that the absence of OMP J2 resulted in a reduction in bacterial clearance from the lungs, suggesting that OMP J2 may actually be a target for the immune system.

In this publication, the authors identified and characterized a novel outer membrane protein (OMP J) of *M. catarrhalis* which appears to be present in two major, lineage specific, forms. Further, the *ompJ* gene appears to be universally present within the species and may play a role in immune-mediated bacterial clearance from the lungs.

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Chapter 8

Investigations into the immune response and pulmonary clearance after passive immunization using the novel outer membrane proteins OMP J1 and OMP J2 of *Moraxella catarrhalis*.

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In Preparation

ABSTRACT

A novel major outer membrane protein that exists in 2 major forms, named OMP J1 and OMP J2, has recently been described in *Moraxella catarrhalis*, a bacterium associated with nosocomial infections and respiratory tract disease in both children and adults. In order to investigate the role of OMP J1 and OMP J2 in the immune response against this bacterial pathogen, the respective proteins were cloned and used in an ELISA protocol to investigate the total serum IgG response in groups of children suffering from acute otitis media, as well as children/young adults with no acute otitis media disease. Additionally, passive immunization studies were performed in a mouse pulmonary model of infection using anti-OMP J1 and anti-OMP J2 rabbit hyperimmune sera. ELISA results indicated that the total serum IgG response remained constant during the first two years of life, and that no significant difference in total serum IgG could be found between acute and convalescent sera within groups of children suffering from acute otitis media. However, a significant difference was observed in anti-OMP J1 and anti-OMP J2 total IgG reactivity between children and young adults. Passive immunization using hyperimmune serum raised against OMP J1 resulted in an almost significant reduction in the percentage bacterial survival of an OMP J1 expressing *M. catarrhalis* isolate in the lungs of mice ($P = 0.06$). These results suggest that OMP J1 and OMP J2 are immunogenic in humans and that antibodies against these proteins help facilitate bacterial clearance from the lungs of mice.

INTRODUCTION

Over the last few decades, the nature of the bacterium *Moraxella catarrhalis* has been redefined from a commensal to an organism with pathogenic potential in its own right (5, 29). It is currently known that the bacterium may be associated with a variety of respiratory tract infections in both adults and children, including exacerbations of COPD (25) and acute otitis media (27). Further, nasopharyngeal colonization is very common in early childhood and high rates of colonization are associated with an increased risk of otitis media disease (7).

Several studies have indicated the role of the immune response, and in particular the antibody response, to *M. catarrhalis* infection (6, 9, 18), with a highly significant correlation having been observed between the level of specific anti-*M. catarrhalis* IgG3

and certain heavy chain IgG (Gm) phenotypes in patients with chronic/recurrent sinusitis (12). Further, the development of new serum IgG raised during *M. catarrhalis* mediated exacerbations of COPD has been reported (1).

Several research groups are currently in the process of identifying and developing an effective vaccine against *M. catarrhalis* (20), with particular emphasis having been placed on a range of outer membrane proteins (OMPs), as well as the lipooligosaccharide/lipopolysaccharide (LOS/LPS) as potential candidates. The *M. catarrhalis* OMPs identified as putative vaccine candidates comprise proteins associated with bacterial pathogenicity and ion/nutrient acquisition. These include the immunoglobulin D-binding protein (MID) (10), the major heat-modifiable protein OMP CD (30), lactoferrin/transferrin receptors (3, 31), the hemin utilization protein (HumA) (11), OMP E (involved in fatty acid transport) (22) and the ubiquitous surface proteins A1 and A2 (UspA1 and UspA2), (2), though the usefulness of UspA2 has recently been questioned (21). *M. catarrhalis* LOS linked to immunogenic outer membrane proteins has been shown to be a promising multi-component vaccine combination (13, 15, 16).

Recently, a novel major outer membrane protein of *M. catarrhalis* that exists in a 19kDa (OMP J1) and a 16kDa (OMP J2) form, has been described, with each form being significantly associated with particular *M. catarrhalis* genetic lineages (14). In order to assess the immune response to OMP J and its possible potential as a putative vaccine candidate, the immune response to two recombinant OMP J1 and OMP J2 proteins was investigated. To this end, the *ompJ* genes from *M. catarrhalis* isolates V31 (*ompJ1*) and 3.9 (*ompJ2*) were cloned and expressed in *Escherichia coli* BL21 (DE3) cells and used in an ELISA to determine the total serum IgG response to OMP J1 and OMP J2 proteins in (Finnish) children presenting with acute otitis media (AOM), and in (Dutch) children/young adults presenting with non-AOM related respiratory disease (i.e. allergies). The AOM sera were used as a model for human *M. catarrhalis* infection, with the sera from patients suffering respiratory allergies serving as a control group. Recombinant OMP J1 and OMP J2 proteins were also used to generate hyperimmune sera in rabbits for use in mouse passive immunization experiments assessing the role of anti-OMP J antibodies in mediating *M. catarrhalis* clearance.

MATERIALS AND METHODS

Expression of OMP J proteins and polyclonal antibody production. Cloning and expression of OMP J1 and OMP J2 was achieved by PCR cloning the *ompJ1* gene from *M. catarrhalis* isolate V31 and the *ompJ2* gene from *M. catarrhalis* isolate 3.9 into the cloning and expression vector pET-11c (New England Biolabs Inc) using His-tagged primer 19kD.XP.Nhe (5'-ACGTGCTAGCCATCACCATCACCATCACGCCATCAGCTATGGCAAATTCT-3') and primer 19kD.XP.Bam (5'-ATCGGGATCCTTAGAAA GCCAAATGAGCGCC-3'), followed by transformation into BL21(DE3) *E. coli* cells. After sequencing of the cloned inserts to ensure that they contained the correct gene sequence, protein expression was facilitated by induction of phage T7 RNA polymerase via the addition of IPTG to log phase cells grown in LB medium. After sonification, the relevant protein was purified by nickel-column absorption / elution, followed by dialysis to remove the imidazole. Finally, the protein was re-solubilized in 8M urea ready for immunization into rabbits (performed by Eurogentec S.A., Belgium). To renature the OMP J proteins, a variation of the method described by Oomen et al (23) and Vandeputte-Rutten et al (28) was used. Basically, the denatured protein was diluted 5 times in 20mM ethanolamine (pH 10-11) containing 1% (wt/vol) detergent SB14, ensuring that the pH of the ethanolamine solution was 1.5 –2 times above the calculated isoelectric point of the OMP J proteins. The protein solution (approximately 1mg/1ml) was then incubated overnight at room temperature, with 100µg aliquots being used to immunize rabbits. Final bleeding occurred 3 months after initial vaccination. The reactivity of OMP J1 and OMP J2 hyperimmune sera was tested by Western blotting a 1 in 30,000 dilution of serum against the outer membrane protein extracts of *M. catarrhalis* isolates CK16-7 and V31 (which express OMP J1), and isolates V02, 97/951 and 97/0233 (which express OMP J2).

OMP J ELISA. An in house OMP J ELISA protocol was developed using cloned, and renatured OMP J1 and OMP J2 proteins coated onto the surface of 96-well microtitre plates at a concentration of approximately 0.15µg/well. Plates were blocked using a solution containing PBS/0.05% Tween 20 (vol/vol) and 1% BSA (wt/vol) with sera to be tested being diluted 1/250 in PBS prior to use. Secondary total anti-human IgG antibody (Sigma A1543) was used at a 1/20000 dilution with 30 minutes allowed for color development. All sera were tested in duplicate and 4 negative PBS controls

were included per ELISA plate. Optical density (OD) readings were measured at a wavelength of 405nm.

Three different serum panels were tested using the OMP J1/OMP J2 ELISA, comprising: 1) a panel of 117 acute and convalescent sera obtained from Finnish children presenting with an episode of acute otitis media (AOM) with effusion (part of the *Finnish Otitis Media (FinOM) Cohort* study (24)), with an age range of 2 – 24 months (mean = 11 months); 2) a panel of 16 sera obtained from Dutch children with no otitis media disease but presenting at the Erasmus MC with a variety of respiratory ailments for which clinical chemistry analysis of serum had been requested (age range 8 –29 months, mean = 16 months); and 3) a similar panel of 68 sera obtained from Dutch children/young adults with no otitis media disease but presenting at the Erasmus MC with a variety of respiratory ailments (age range 3 – 18 years , mean = 7.9 years). Convalescent sera obtained from the Finnish children were taken 2-4 weeks (14 – 42 days) after initial diagnosis of AOM (acute phase serum), with middle ear fluid for bacterial culture being collected after myringotomy. Further, based on the AOM effusion culture results, the Finnish AOM sera could be subdivided into 3 subgroups comprising 22 children presenting with an *M. catarrhalis* culture positive AOM effusion, 46 children with a bacterial positive (other than *M. catarrhalis*) AOM effusion, and 49 children presenting with AOM effusions from which no bacteria were cultured.

Passive immunization studies. Passive immunization experiments studied the effect of hyperimmune rabbit serum raised against OMP J1 and OMP J2 proteins on the ability of BALB/c mice to clear homologous *M. catarrhalis* isolates in a mouse pulmonary clearance model. In total, 40 BALB/c mice were used for passive immunization experiments, utilizing 5 mice per experimental group. Pre- and post-immunization groups were immunized with 200µl of pre-immunized and 200µl of post-immune rabbit serum respectively at 24 hours and 1 hour prior to intranasal inoculation of 50µl bacterial culture containing 1×10^8 CFU *M. catarrhalis*. Mice were sacrificed 3 hours after intra-nasal inoculation and CFU counts on lung homogenates performed. Percentage survival was calculated by comparing the percentage CFU survival of *M. catarrhalis* in the pre- and post-immunization groups compared to the average survival (normalized to 100%) of a group of 5 mice immunized with 200µl PBS and sacrificed at the initiation of the experiment ($t = 1/2$ hour post-nasal inoculation). This method was based on that previously published by Forsgren et al (10). All of the animal studies described in this publication were performed in accordance with the

ethical and legal requirements of the Erasmus MC, Rotterdam, and under approval of the Animal Studies Ethics Committee of the same institution.

RESULTS

Expression of OMP J proteins and polyclonal antibody production. The cloning and expression of recombinant OMP J1 (*M. catarrhalis* isolate V31) and OMP J2 proteins (*M. catarrhalis* isolate 3.9) using the pET11 plasmid system and *E. coli* BL21(DE3) cells, yielded protein products of approximately 19kDa and 16kDa molecular weight respectively. The identity of the putative OMP J protein products was verified using MALDI-TOF and PCR sequencing of plasmids pET11c Ω ompJ1 (V31) and pET11c Ω ompJ2 (3.9). Hyperimmune serum raised against recombinant OMP J1 and OMP J2 proteins in rabbits were shown to specifically react against OMP J protein present in *M. catarrhalis* outer membrane extracts by Western blotting (Fig 1).

OMP J1 and OMP J2 ELISA. Results using an *in house* developed ELISA indicated that both anti-OMP J1 and anti-OMP J2 antibodies were present in all of the serum panels tested. With respect to age, the total IgG response to both OMP J1 and OMP J2 proteins remained constant for all children suffering AOM episodes between 2 and 24 months, and showed a steady increase with age for the non-AOM 3–18 year group (Fig. 2a). This increase in OMP J1 and OMP J2 IgG reactivity with age meant that a statistically significant difference was observed in ELISA results between AOM and non-AOM sera for the 3–18 year group (Fig. 2b).

Analysis of sera obtained from children suffering AOM episodes as a single group indicated no significant difference in anti-OMP J1 or anti-OMP J2 total IgG between acute and convalescent sera ($P = 0.27$ and $P = 0.50$, respectively). However, convalescent sera were found to exhibit an increased ELISA reactivity against OMP J1, but a decreased reactivity against OMP J2. This outcome was consistent (but not statistically significant) when AOM sera were sub-divided into 3 groups according to the organism isolated from their respective middle ear effusions (Fig. 2c). Further comparison of these 3 AOM sub-groups with each other yielded no significant differences in OMP J1 and OMP J2 ELISA reactivity (data not shown).

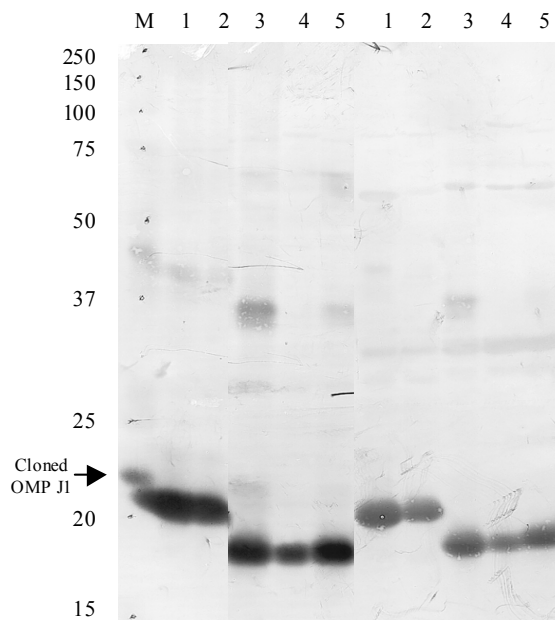


FIG. 1. Western blot showing reactivity of rabbit OMP J1 and OMP J2 hyperimmune sera against outer membrane protein extracts from a selection of *M. catarrhalis* isolates. M = Molecular weight marker with cloned OMP J1 protein. 1, 2 = *M. catarrhalis* isolates CK16-7 and V31 (OMP J1). 3, 4, 5 = *M. catarrhalis* isolates V02, 97/951 and 97/0233 (OMP J2).

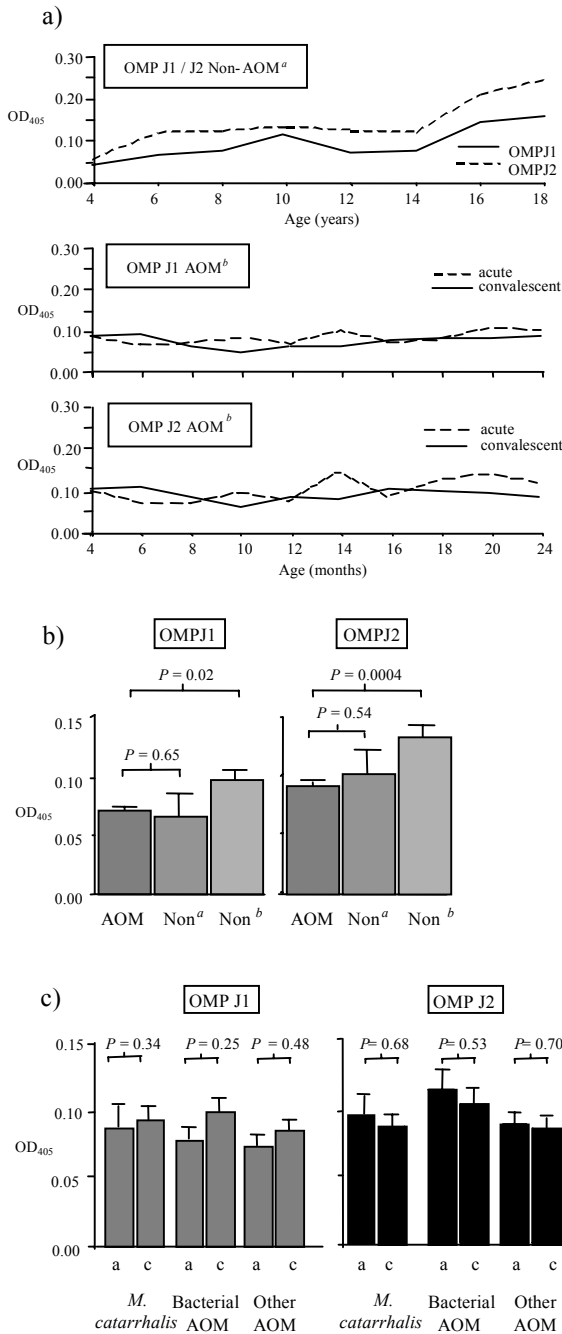


FIG. 2. a) ELISA results showing total serum IgG reactivity to *M. catarrhalis* OMP J1 and OMP J2 proteins in different population groups and ages. ^a Sera obtained from 69 Dutch children/young adults (3 - 18 years) with no AOM disease. ^b Sera obtained from 117 Finnish children (2 - 24 months) during and after an acute otitis media with effusion episode.

b) Statistical differences in total serum IgG response to *M. catarrhalis* OMP J1 and OMP J2 proteins in the convalescent phase serum of children with AOM compared to 2 age groups (single serum only) with no AOM disease. ^a AOM group age range 2 - 24 months. ^b Non-AOM group age range 8 - 29 months. ^c Non-AOM group age range 3 - 18 years. P = statistical significance of mean total serum IgG reactivity calculated using the unpaired Student's *t* test.

c) Total IgG immune response of children suffering from an acute otitis media episode with effusion. OMP J1 and OMP J2 = *M. catarrhalis* OMP J1 (isolate V31) and OMP J2 (isolate 3.9) proteins cloned and expressed in *E. coli* BL21(DE3) cells. a = serum taken during an acute AOM episode presenting with effusion; c = convalescent serum taken approximately 4 weeks (range 14-42 days) after acute AOM episode. *M. catarrhalis* = *M. catarrhalis* grown from middle ear effusion; Bacterial AOM = bacterial species other than *M. catarrhalis* grown from middle ear effusion; Other AOM = No bacteria grown from middle ear effusion; No AOM = control children not presenting with AOM symptoms. P values were calculated using the Student's paired *t*-test. Sera were diluted 1/250 prior to use.

Passive immunization studies. Passive immunization studies using rabbit hyperimmune serum raised against OMP J1 and OMP J2 proteins showed a trend towards a reduction in *M. catarrhalis* survival in the lungs of mice immunized with the corresponding OMP J hyperimmune serum (Fig. 3). Indeed, this reduction in survival was found to be almost statistically significant in mice passively immunized with OMP J1 hyperimmune serum and challenged with the OMP J1 expressing *M. catarrhalis* isolate V31 (2-tailed unpaired Student's *t* test, $P = 0.06$). However, no significant difference in percentage *M. catarrhalis* survival was observed in mice passively immunized with OMP J1 hyperimmune serum and challenged with OMP J2 expressing *M. catarrhalis* isolate 3.9, or in mice passively immunized with OMP J2 hyperimmune serum.

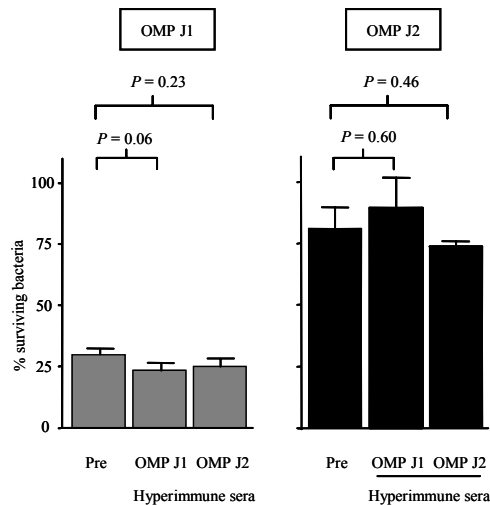


FIG. 3. Effect of passive immunization using hyper-immune rabbit serum raised against cloned OMP J1 and OMP J2 proteins (obtained from *M. catarrhalis* isolates V31 and 3.9 respectively) on the percent survival of *M. catarrhalis* V31 and 3.9 in the lungs of BALB/c mice. Mice were immunized with 200 μ l of the respective pre-immune (Pre) or hyperimmune (OMP J1 or OMP J2) serum at time periods 24 hours and 1 hour (booster) prior to nasal inoculation of 1×10^8 CFU bacteria. Mice were sacrificed 3 hours after nasal inoculation. Surviving bacteria % = percentage surviving *M. catarrhalis* bacteria compared to the number of bacteria detected at initiation of the experiment. Five mice were used per study group. Error bars indicate SEM of the mean. P values were calculated using a 2-tailed unpaired Student's *t* test.

DISCUSSION

Serological studies using recombinant OMP J proteins showed that hyperimmune sera raised against recombinant OMP J1 and OMP J2 proteins recognized both OMP J1 and OMP J2, indicating that the deletion of a small 12 amino-acid segment in loop 2 of OMP J2 did not seriously affect total IgG production against the OMP J2 protein. However, the presence of this additional 12 amino-acid segment in OMP J1 would be expected to provide extra epitopes against which OMP J1-specific (and possibly neutralizing) antibodies could be targeted. Further, the deletion of the loop 2 segment could in theory result in an alternative OMP J2 conformation, possibly leading to the shielding or exposing of important neutralizing epitopes.

When related to age, the reactivity of serum IgG against both OMP J1 and OMP J2 proteins remained constant during the first 2 years of life for both AOM and non-AOM groups, but steadily increased from 4–18 years of age (in the non-AOM group tested). Further, a significant difference between mean total IgG reactivity between the AOM and 3-18 year non-AOM group, but not the AOM and 8-39 month non-AOM group, was observed. This observation could be a consequence of an increased exposure to *M. catarrhalis* over time, resulting in an increase in isolate-specific anti-OMP J IgG titers, possibly contributing to a decrease in *M. catarrhalis* colonization with age.

The fact that no significant difference was observed in total serum IgG reactivity between acute and convalescent sera in children with AOM, indicates that anti-OMP J1 and anti-OMP J2 IgG antibodies are not important in the etiology of AOM disease, even though OMP J1 and OMP J2 are indeed immunogenic. Further, Mathers et al (19) reported that during AOM episodes, a rise in specific anti-*M. catarrhalis* OMP IgG was detectable in 83% of children 8 months of age or older, and also in 17% of younger children. Therefore, if OMP J were important in *M. catarrhalis* mediated AOM infections, then a rise in anti-OMP J IgG would have been expected to occur. However, there are indications to suggest that the antibody response to *M. catarrhalis* otitis media infection in children may (occasionally) be limited to the middle ear, with systemic antibody failing to be developed in a uniform manner (8). Also, our AOM groups may not have been large enough to detect changes in acute and convalescent sera. Interestingly, a consistent increase in anti-OMP J1 total IgG antibody was observed between acute and convalescent sera, whilst the same sera showed a consistent decrease in anti-OMP J2 total IgG antibody. In this context, recent

research has suggested that bacterial colonization of the nasopharynx does not necessarily lead to an increase in specific mucosal IgA antibodies, with for example *M. catarrhalis* anti-OMP E antibody actually showing a decline in response to colonization (4). Finally, the OMP J1 and OMP J2 total serum IgG response appeared to be similar in all AOM subgroups, including those subgroups where no evidence for *M. catarrhalis* mediated AOM was found. These results could indicate that OMP J cross-reactive antibodies might be found in human serum. Indeed, mouse anti-*Neisseria meningitidis* and anti-*Neisseria lactamica* sera have been shown to recognize *M. catarrhalis* 77, 62 and 32 kDa outer membrane antigens (26), indicating a possible interaction between human anti-*Neisseria* hyperimmune sera and *M. catarrhalis* proteins.

Serum levels of specific IgG and IgA have previously been shown to be correlated with pulmonary clearance of *M. catarrhalis* in a mouse model of infection (17). Therefore, we used a similar model to investigate the effect of passive immunization using OMP J1 and OMP J2 hyperimmune sera on the pulmonary clearance of *M. catarrhalis* in mice. These studies yielded interesting results, in that an almost significant decrease in *M. catarrhalis* survival was observed in mice passively immunized with OMP J1 hyperimmune serum and challenged with an OMP J1 expressing isolate, but not in mice passively immunized with OMP J2 hyperimmune serum and challenged with an OMP J2 expressing isolate. These results suggest that the “intact” loop 2 region of OMP J1 may play a role in the immune response against *M. catarrhalis* infection, with the truncated loop 2 of OMP J2 expressing isolates providing some protection against killing for OMP J2 expressing isolates. This finding could impact on any future plans to utilize the OMP J proteins as potential vaccine candidates, not least because previous research has indicated that the OMP J2 gene is significantly associated with *M. catarrhalis* lineages exhibiting an increased virulence potential (14).

In this article, investigations were performed into the immune response against the recently described *M. catarrhalis* outer membrane proteins OMP J1 and OMP J2. The ultimate goal of the research was to indicate whether OMP J could be useful as a potential vaccine candidate against *M. catarrhalis*, with the results suggesting a complex interaction between the host and these 2 forms of OMP J protein. In particular, the protein appears not to play a major role in AOM mediated disease, but alternatively may play a role in bacterial clearance from the lungs. Further research is necessary to clarify the exact role of the OMP J protein in the immune response against *M. catarrhalis*.

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Chapter 9

***Moraxella catarrhalis* is only a weak activator of the mannose-binding lectin (MBL) pathway of complement activation.**

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Alex van Belkum, Saskia. Kuipers.

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Abstract

A hemolytic bystander assay was used to assess the functional serum mannose-binding lectin (MBL) activating capacity of 5 isolates of *Moraxella catarrhalis* obtained from children who suffered recurrent acute otitis media episodes. Results showed that this organism is only a poor activator of the lectin pathway of complement activation, with subsequent consequences for the etiology of otitis media by this organism.

1. Introduction

Moraxella catarrhalis is a commensal bacterium associated with both the colonization and infection of adults and children. In particular, the bacterium is most often associated with respiratory tract infections, including acute and chronic otitis media and acute exacerbations of chronic obstructive pulmonary disease (COPD) [1]. In terms of the immunological response to *M. catarrhalis*, research has indicated that colonization/infection leads to the generation of opsonic antibodies against the relevant isolate [2], and that complement resistant isolates (which possess an increased virulence potential) possess the ability to bind vitronectin [3] and/or complement component C4b-binding protein [4]. However, there have as yet been no publications into the possible role of the mannose-binding lectin (MBL) arm of the complement system in the immune response to *M. catarrhalis*.

Mannose-binding lectin (MBL) is a high-molecular-weight protein, which is present in blood plasma at low concentrations (1.7 µg/ml), and which forms the initial component of the lectin pathway of the complement system. The protein acts by binding to specific sugar residues (i.e. mannose, N-acetylglucosamine, and fucose) present on the surface of microorganisms, leading to the subsequent activation of the (“innate”) MBL arm of the complement cascade system. In man, MBL deficiencies are quite common, with MBL gene polymorphisms leading to differential MBL expression levels, which in turn have been associated with susceptibility to several different types of infection [5]. In this brief report, a functional MBL assay was used to characterize *M. catarrhalis* as a weak or potent activator of the MBL arm of the complement system.

2. Materials and Methods

2.1 Bacterial isolates

Five isolates of *M. catarrhalis* were tested for MBL activating capacity, all obtained from children who suffered recurrent acute otitis media episodes. Three isolates (isolates 2040-1, 3001-14/20 and 4017-7) had been previously determined to be complement resistant and 2 isolates complement sensitive (isolates 3053-14 and 3007-7) via a simple “spot and drop” test [6,7].

2.2 Functional MBL assay

The functional MBL assay used to measure *M. catarrhalis*-induced MBL activation in this article was based upon that previously published by Kuipers et al [8], which makes use of microorganism-induced MBL activation in a dilution series of pooled human serum, followed by subsequent C5b6-mediated bystander hemolysis of chicken erythrocytes in the presence of a standardized concentration of MBL-deficient human serum. In this assay, the degree of MBL activation of microorganisms is related to the MBL-activating capacity of a standard isolate of the yeast *Saccharomyces cerevisiae* (in order to eliminate inter-experimental variation), using pooled human serum containing a known MBL concentration. With regard to *M. catarrhalis* testing, twelve different dilutions of the 5 *M. catarrhalis* isolates to be tested (previously grown on Columbia blood agar and resuspended to an OD₆₆₀ of 1.0, equivalent to approximately 1.5 x 10⁸ CFU/ml), or 3 x 10⁵ *S. cerevisiae* cells, were prepared in veronal buffered saline containing Ca²⁺ and Mg²⁺ (VSB⁺⁺). A checkerboard titration was then performed against dilutions of the standard pool of human serum. After incubation, the degree of bystander erythrocyte lysis was translated into the number of active sites per erythrocyte (Z value) using the equation of Borsos and Rapp [9]. As a control, pre-incubation of the standard human pooled serum with 2.5 mg/well mannose (which binds and blocks MBL binding), followed by repetition of the MBL activation experiments, was performed in order to indicate whether the bystander hemolysis attributable to *M. catarrhalis* was a consequence of MBL-specific activation. Activation of the alternative pathway of the complement system was excluded by testing in ethylene-glycol bis-amino-tetraacetic acid – veronal buffered saline [10]. The relative

MBL activation of *M. catarrhalis* was read off at a Z value of 0.2 (calculated as: $Z_{M. catarrhalis} / Z_{S. cerevisiae}$) and *M. catarrhalis*-mediated MBL activation ranked alongside other pathogenic bacteria using previously published data [8]. *S. cerevisiae* was used as the standard reference as this was the first reported microorganism to be associated with MBL [11], and the yeast cell wall of this organism is rich in mannan.

3. Results and discussion

The MBL activating capacity (as a measure of the level of bystander hemolysis of chicken erythrocytes) for the 5 *M. catarrhalis* isolates tested is presented in Fig. 1., with no difference being observed in MBL activating capacity between the complement resistant and complement sensitive isolates. A comparison of the *M. catarrhalis* results with bacterial species previously determined to be weak/potent activators of MBL is shown in Table 1. Experiments using mannose as a competitor for MBL activation showed that pre-incubation with mannose had a negative effect on the ability of *M. catarrhalis* to activate MBL (Fig. 1), indicating that *M. catarrhalis* MBL activating activity in the functional assay (though weak) was indeed a specific phenomenon.

These results indicate that *M. catarrhalis* is only a weak activator of the MBL arm of the complement system. In theory, this lack of MBL activation could be advantageous to the organism in allowing it to remain “hidden” from the MBL arm of the complement system (i.e. protected from lectin pathway-induced complement activation). In this scenario, serum MBL levels would be unlikely to influence *M. catarrhalis* colonization/infection. However, it should be noted that heavily mannosylated immunoglobulin A (IgA) is also found at tissue surfaces [12], and an interaction between IgA binding and MBL activation in *M. catarrhalis* mediated infection cannot be discounted at present.

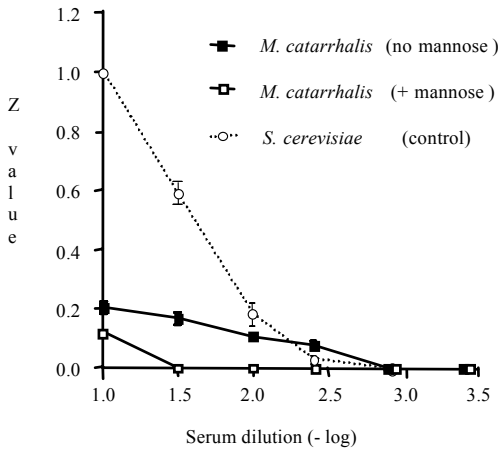


Fig. 1. Composite graph showing average MBL activation by *M. catarrhalis* (with and without the addition of competing mannose) and the yeast *Saccharomyces cerevisiae* (used as a standard MBL activating control organism to which organisms are compared in order to eliminate inter-experiment variation). Z value = mean number of active sites per chicken erythrocyte determined by the functional bystander assay. Experiments were performed in triplicate. Note the reduction in MBL activating capacity (Z value) of *M. catarrhalis* after pre-incubation of standard MBL containing human pooled serum with mannose (indicating the specificity of *M. catarrhalis* MBL activation). The 5 *M. catarrhalis* isolates used were all Dutch isolates, identification numbers 2040-1, 3001-14/20, 4017-7, 3053-14 and 3007-7.

Table 1. Comparison of potent and weak MBL activating microbial species (relative to the standard organism *S. cerevisiae*) as determined using a functional bystander hemolysis assay.

Microbe	Z _{microbe (OD660 = 1.0)}	1/Z _{S. cerevisiae (control)} ^a
P O T E N T	<i>Neisseria meningitidis</i> <i>Neisseria gonorrhoeae</i> <i>Salmonella typhimurium</i> <i>Mycobacterium bovis</i> <i>Pseudomonas aeruginosa</i>	* ^b * * * *
W E A K	<i>Candida albicans</i> ATCC 14053 <i>Yersinia enterocolitica</i> <i>Micrococcus lysodeikticus</i> <i>Helicobacter pylori</i> <i>E. coli</i> ATCC 29522 <i>M. catarrhalis</i> <i>Enterococcus faecalis</i>	0.80 0.77 0.62 0.54 0.49 0.21 No detectable activity

^a $Z_{\text{microbe (OD 1.0)}} / Z_{\text{S. cerevisiae (control)}} = \text{ratio}$ used to rank microorganism MBL activating capacity with respect to the standard reference organism *S. cerevisiae*, where $Z = -\ln(1 - \text{fraction erythrocytes lysed})$ a measure of the mean number of MBL active sites per chicken erythrocyte calculated using the equation of Borsos and Rapp (1963). ^b = The MBL activating capacity of these organisms exceed the maximum limit of this test at an OD₆₆₀ of 1.0.

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Immunology, UMCU – Wilhelmina Children’s Hospital, Utrecht, The Netherlands) and Dr. R. Veenhoven (Department of Pediatrics, Spaarne Hospital, Haarlem, The Netherlands) for kindly supplying the bacterial isolates used in this study.

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Chapter 10

A novel plasmid (pEMCJH03) isolated from *Moraxella catarrhalis* possibly useful as a cloning and expression vector within this species?

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Henri Verbrugh, and Alex van Belkum.

Plasmid 53 (2005) 263-268

ABSTRACT

A preliminary screening study of 6 *Moraxella catarrhalis* isolates from primary school children in the Netherlands identified a small 3.5kb plasmid (pEMCJH03), containing four open reading frames, which encoded three mobilizing and one replicase proteins. Insertion of a kanamycin containing transposon (yielding pEMCJH04) allowed selection and isolation of the plasmid in *E. coli*. Natural transformation of pEMCJH04 was successful in 25% (3/12) of non-isogenic isolates, with no link between (lack of) transformability and genetic lineage or (lack of) transformability and complement phenotype, though the transformation efficiency was found to be rather low at approximately 615 CFU/ μ g (range = 60 – 1040 CFU/ μ g). This is only the second publication detailing a plasmid isolated from this important respiratory pathogen, and the ability to clone and express foreign proteins in *M. catarrhalis* using pEMCJH04 could help in the development of a vaccine expression vector, as well as providing a useful tool for studying promoter activity and in complementation studies of gene knockout mutants.

Moraxella catarrhalis is a common commensal of the human upper respiratory tract which has been associated with a number of disease states including acute otitis media in children (Catlin, 1990; Faden *et al.*, 1994a; Faden *et al.*, 1994b) and both acute and chronic bronchitis in adults (Hager *et al.*, 1987; Nicotra *et al.*, 1986). Nosocomial spread and outbreaks of this pathogen have been reported (Cook *et al.*, 1989; Patterson *et al.*, 1988) as well as cases of near fatal pneumonia (Dyson *et al.*, 1990). Moreover, there exists a very high prevalence of β -lactamase production within the species (Thornsberry *et al.*, 2002). Vaccines against *M. catarrhalis* are currently being developed (Chen *et al.*, 1996; McMichael, 2000), though a suitable universal cloning vector for the expression of *M. catarrhalis* proteins within this species and other genera has yet to be fully described and developed. Several studies have investigated the carriage of plasmids in non-*Moraxella catarrhalis* species including *M. bovis* (McDonald and Pugh, 1986), and environmental *Moraxella* species (Kawasaki *et al.*, 1992; Rani *et al.*, 1996; Vasudevan and Paulraj, 1994), though only one plasmid has been fully characterized and sequenced within the *M. catarrhalis* species (plasmid pLQ510). This 12kb plasmid pLQ510 (Beaulieu *et al.*, 1988; Liu and Hansen, 1999) has previously been suggested as a possible cloning and expression vector, though the size of the plasmid (even without the presence of inserted genes) could reduce its ability to be efficiently transformed. Here, we report on

the finding of a small 3.5 kb plasmid in *M. catarrhalis*, which could possibly fulfill such a role.

Initial plasmid screening efforts utilized 6 *M. catarrhalis* isolates (3 colonizing isolates originally isolated in 1989 from primary school children in Nieuwegein, The Netherlands, and 3 colonizing isolates originally isolated in 1993 from primary school children in Heerenveen, The Netherlands). The isolates were retrieved from -80°C storage and grown overnight (approximately 16 hours) on 5% sheep blood agar at 37°C until semi-confluent, whereby 1/4 plate of growth was removed and embedded in agarose blocks according to established pulsed field gel electrophoresis (PFGE) protocols (Vu-Thien *et al.*, 1999), but without the addition of a restriction enzyme. PFGE was performed using a CHEF Mapper (BioRad) with a ramping protocol of 6 V/cm constant voltage at 15°C , and a pulse time increased from 2 to 10 seconds for 10 hours. After electrophoresis, the presence of any extrachromosomal plasmid DNA was detected using ethidium bromide staining. PFGE testing indicated the presence of at least 3 different extra-chromosomal elements within the range of isolates tested (Fig. 1), ranging in size from approximately 5kb to 60kb in size. At this point, the smallest plasmid (pEMCJH03), was isolated from its host (*M. catarrhalis* isolate 6.12) using the SV Wizard plus minipreps system (Promega), and recovered by insertion of a kanamycin encoding transposon using the EZ::TNTM <KAN-2> insertion kit (Epicentre[®]). The resultant pEMCJH03 plasmid containing the EZ::TNTM <KAN-2> transposon (plasmid pEMCJH04) was rescued in chemically competent *E. coli* TOP10 cells : F-*mcrA* Δ (*mrr-bsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-len*)7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG* (Invitrogen BV), followed by the selection of kanamycin resistant clones on Mueller Hinton agar containing 5 $\mu\text{g}/\text{ml}$ kanamycin (MH5Kan). A primer walking strategy was then undertaken to sequence both strands of the plasmid, using the primers provided with the EZ::TNTM <KAN-2> insertion kit as the initial pair of sequence primers.

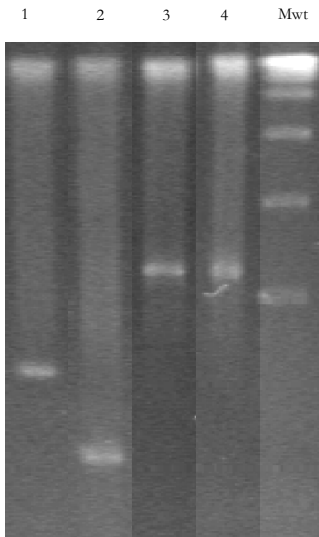


Fig. 1. Composite picture of extra-chromosomal elements observed in 4 *M. catarrhalis* isolates recovered from children living in The Netherlands during the years 1989 and 1993 using native pulsed field gel electrophoresis. 1 - 4 = *M. catarrhalis* isolates 3.21, 5.12N, 6.12K, F6.92 and F5.82 respectively. Mwt = lambda ladder molecular weight marker (kbp).

M. catarrhalis isolates to be naturally transformed were grown overnight at 37°C on Mueller Hinton (MH) agar. The following day, approximately 50ng of plasmid pEMCJH04 was spotted onto an area of confluent *M. catarrhalis* growth. The drops were then allowed to dry and the isolates returned to the 37°C incubator for 2 hours, after which time the *M. catarrhalis* cells within the “drop zone” were resuspended in 100ul water and plated onto both MH5kan agar (99µl) and MH agar (1µl positive growth control). After a further incubation step at 37°C for up to 48 hours, the number of kanamycin resistant colonies found on the MH5 plates were counted.

Sequencing of plasmid pEMCJH04 revealed that pEMCJH03 (the original plasmid without transposon insert) comprised 3,510 base pairs with a GC content of 44%, a GC content higher than that of the *M. catarrhalis* genome (41.7%) as well as the previously published plasmid pLQ510 (37.9%). Translation of the DNA sequence followed by database “BLAST” searching (<http://www.ncbi.nlm.nih.gov/BLAST/>) showed that the plasmid contained four open reading frames (ORFs), with three ORFs sharing conserved domain architecture to the mobilization (*mob*) genes of other bacterial species, and the fourth ORF comprising a putative replicase gene (replicase initiator protein). The plasmid contained no pilus synthesis (transfer or *tra*) genes. A schematic representation of plasmid pEMCJH03 and pEMCJH04 (pEMCJH03ΩEZ::TNTM<KAN-2>) is shown in Fig. 2.

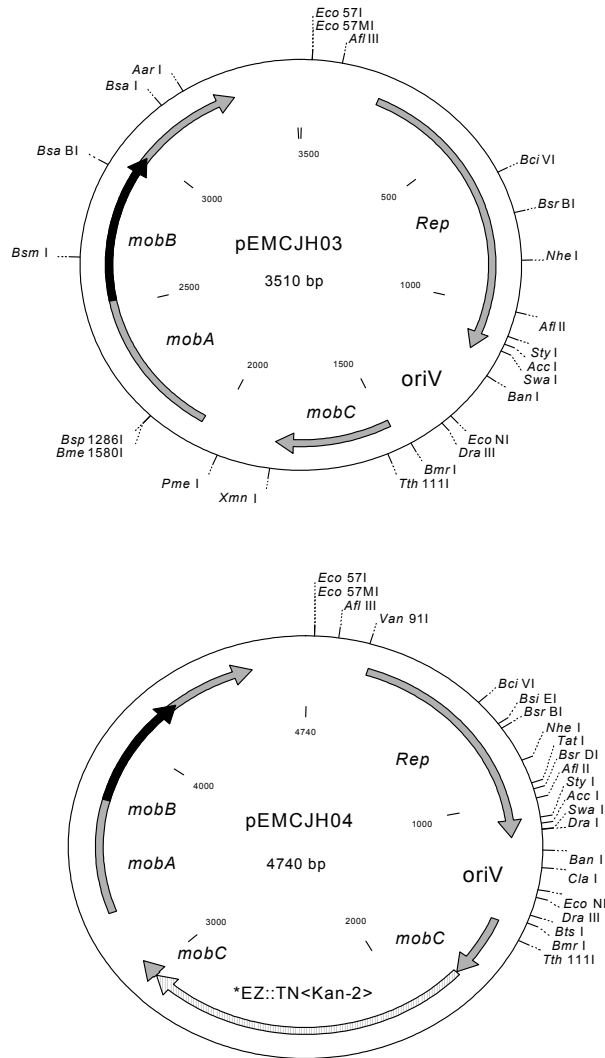


Fig. 2. Diagrammatic representation of the 3.5kb mobilizable plasmid pEMCJH03 and the EZ::TN<Kan2> transposon containing pEMCJH04. *mobA*, *mobB*, *mobC* = genes for putative mobilization proteins A, B and C respectively. *Rep* = gene for putative replicase protein. *oriV* = putative origin of replication. *oriT* = putative origin of transfer. * = transposon randomly inserted into *mobC* gene. The nucleotide sequences for pEMCJH03 and pEMCJH04 (pEMCJH03ΩEZ::TN<Kan-2>) are available at GenBank, (Accession Numbers [AY167745](#) and [AY453632](#), respectively).

pEMCJH03 ORF1 encoded a protein comprising 420 amino acids whose N-terminal half was similar to the mobilization protein MbeA of *Salmonella enteritidis* ([gi|19526579|gb|AAL87013.1|](#)) and the mobilization MobA protein of *E. coli* ([gi|78698|pir||S04789](#)), exhibiting identities of 27% and 28% over the total lengths of the translated proteins respectively. At the C-terminal end of the protein was a highly polar region comprising 8 glutamine, 2 arginine and 2 (non-polar) alanine amino acids. Functionally, MobA proteins act by binding and nicking double stranded DNA at the origin of transfer (*oriT*) site ready for DNA transfer, and are responsible for re-ligating the cleaved DNA strands once transfer of DNA is achieved. However, no *oriT* consensus sequence could be found which would place pEMCJH03 in either the P-type (YATCCTG/Y), Q-type (TAANWGCG/CCCT) or F-type (TGCGNNNGTNT/RNNNC) *oriT*-relaxase systems (LANKA AND WILLIAMS, 1995). ORF2 encoded a putative MobB protein, with 28% identity to the mobilization B protein of plasmid ColA ([gi|9507288|ref|NP_040389.1|](#)). It has been demonstrated that MobB proteins may interact with MobA proteins (in a region distinct from the nicking-ligating region of MobA), thereby assisting the relaxosome unit (Perwez and Meyer, 1999). ORF3 comprised 113 amino acids with homology to the mobC protein of *Pasteurella multocida* ([gi|10955979|ref|NP_054474.1|](#)) and *Proteus vulgaris* ([gi|11992063|gb|AAG42421.1|](#)), with identities of 42% and 38% respectively. MobC acts together with MobA and MobB proteins to form the relaxosome unit, with previous research indicating that lack of the MobC protein may lead to a 50-fold decrease in plasmid conjugal mobilization in some plasmids (Zhang and Meyer, 1997). The final ORF of pEMCJH03, encoded an *E. coli* like replication initiator protein similar to the replication initiator (Rep) proteins of theta-replicating plasmids pColE2 ([gi|78570|pir||S04455](#)) and pColE3 ([gi|78571|pir||S04456](#)), with protein identities of 41% and 39% respectively.

A 339bp region of DNA situated immediately downstream of the putative *Rep* gene and upstream to the putative *mobC* gene (Fig. 3), contained 2 sets of inverted repeats as well as 2 sets of direct repeat sequences. The direct repeat regions showed some homology to the Rep-binding sequence TGAGACCAGATAA-GCCT-TATCAGATAAGAGCGCC of the theta-replicating ColE2-type plasmids (del Solar et al., 1998), as well as homology to a conserved AAGCCTAAATCAGATAACAGCCG sequence (Expect value 0.085), associated with 2 partially mobilizable plasmids from the closely related *Acinetobacter* species (GenBank accession numbers:

[gi|6706107|emb|AJ251538.1|ALW251538](#),
[gi|6706102|emb|AJ250011.1|ALW250011](#)
and [gi|6706018|emb|AJ251518.1|ACA251518](#)).

These 2 sets of direct repeats also formed part of 2 larger direct repeat sequences which may be possible iteron-like sequences. A putative initiator host factor (IHF) binding sequence 5' – **TACAAGTGATTGTGAAGTTTTC**CAAGAA – 3' (with some similarity to the *E. coli* IHF consensus sequence 5' – **T.PyAA...PuTTGa.T.A.PuTT...pYAAcT**A – 3' of Craig and Nash 1984 (often cited as “YAANNNTTGATW” where Y = C/T and W = A/T)), was found upstream of the putative *mobA* gene and included the extreme 3'-end of the putative *mobC* gene. Neither the putative *oriV* region nor the putative IHF region resembled the corresponding replication region of the previously published *M. catarrhalis* plasmid pLQ510.

Natural transformation of plasmid pEMCJH04 was successful for 25% (3/12) of non-isogenic *M. catarrhalis*, though the transformation efficiency was found to be particularly low at approximately 615 CFU/μg (range = 60 – 1040 CFU/μg). In our experience, the agar plate method used for natural transformation is the most convenient and reliable method for naturally transforming multiple isolates of *M. catarrhalis*, though it is possible that other transformation methods, using well characterized isolates, may yield a greater transformation efficiency. However, *M. catarrhalis* has a strong tendency to form aggregates in broth culture, and *M. catarrhalis* competence has been associated with the expression of type 4 (MePhe) pili (a putative virulence trait) in certain isolates (Marrs and Weir, 1990; Luke *et al.*, 2004), a trait not investigated in our transformation experiments. However, the isolates used for transformation in this experiment, were chosen to represent: a) the two genetic lineages present within this species (Bootsma *et al.*, 2000), and b) isolates expressing either the complement resistant and complement sensitive phenotypes (an acknowledged virulence trait of *M. catarrhalis*) (Verduin *et al.*, 2000). Results however, indicated no significant difference with regard to transformability with pEMCJH04 and isolates from the two genetic lineages (2/7 from the clonal lineage transformable, 1/5 from the non-clonal lineage transformable; Fisher's exact test $p = 1$), or transformability and isolates

exhibiting the complement resistant or sensitive phenotype (2/6 complement resistant transformable, 1/6 complement sensitive transformable; Fisher's exact test $p = 0.6$).

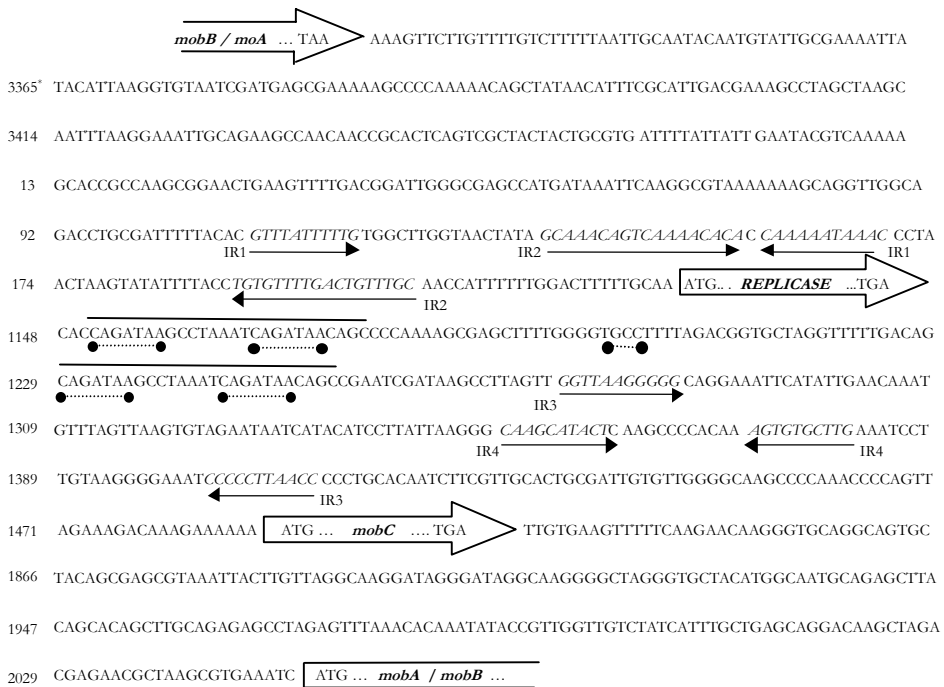


Fig. 3. Putative *oriV* region of plasmid pEMCJH03. Solid line with arrows = inverted repeat. IR1 = inverted repeat number 1. Solid line with filled circular ends = putative Rep-binding site similar to ColE2-type plasmid. Solid line above sequence = direct repeat (putative iteron sequence). Full stops = diagrammatic representation of the intervening locations of the *mob* and *Rep* genes. * = sequence numbering according to the pEMCJH03 sequence deposited at GenBank (accession number [AY167745](#)).

As a final note, the presence of restriction-modification systems (Seib *et al.*, 2002), and sequence mutations affecting the replication and/or promoter regions of *M. catarrhalis* genes (Bootsma *et al.*, 1996), mean that more research on the transformation characteristics and stability of this plasmid in *M. catarrhalis* is necessary if it is indeed to be used as a cloning/protein expression vector in this species.

The complete DNA sequences for plasmid pEMCJH03 and pEMCJH04 (pEMCJH03 Ω EZ::TN[™]<KAN-2>) are available at GenBank, accession numbers [AY167745](#) and [AY453632](#) respectively.

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SUMMARY AND CONCLUDING REMARKS

NEDERLANDSE SAMENVATTING

The bacterial species known as *Moraxella catarrhalis* was previously thought to be a common human commensal bacterium, able to colonize the human respiratory tract, but exhibiting little pathogenic potential. However, evidence over the last few decades has brought about a change in our perception of this species, indicating the clinical relevance of this organism in facilitating disease in both children and adults. In particular, *M. catarrhalis* tends to be associated with upper respiratory tract infections in children (e.g. acute otitis media and sinusitis), but lower respiratory tract infections in adults (e.g. bronchitis, pneumonia, laryngitis, exacerbations of chronic pulmonary obstructive disease), with the species occasionally being reported in a selection of non-respiratory tract associated disease states, including; bacteremia, endocarditis, conjunctivitis/keratitis, meningitis, ventriculitis, ophthalmia neonatorum, acute mastoiditis and septic arthritis. There also appears to be a striking difference in the carriage rates of this organism between children and adults (carriage rates being as high as 75% in children but only 1 – 3% in adults), as well as a distinct seasonality of isolation (the winter and spring months showing the greatest rates of isolation). The possible mechanisms of spread of *M. catarrhalis*, includes person-to-person transmission (including intra-familial spread) or spread via environmental contamination, with both nursery schools and day care centers having been shown as to be important reservoirs for strain exchange. Further outbreaks of nosocomial infection due to *M. catarrhalis* have been regularly documented.

Since *M. catarrhalis* was acknowledged as a pathogen in human disease, research has focussed on several key aspects of this species, namely genetic diversity, pathogenic traits, the immune response and putative vaccine designs. A detailed description of the current state of knowledge regarding these key aspects of research is given in **Chapter 2**. This information forms the basis for the research published in this thesis.

Genetic Diversity in *Moraxella catarrhalis*

Many different methods have been utilized to study the relatedness of different *M. catarrhalis* isolates, including protein expression profiling (12), DNA-DNA hybridization (1, 3, 19), restriction fragment length polymorphism analysis (5, 8), single-adaptor amplified fragment length polymorphism analysis/16S rRNA sequencing (2), randomly amplified polymorphic DNA analysis (18), and pulsed field gel electrophoresis (11, 17). However, there is as yet no published and accepted “gold standard” for genotyping this

species, though unpublished results (Mark Achtman, personal communication) have indicated that for example multi-locus sequence typing may be useful as the gold-standard technique for the future.

In **Chapter 3**, pulsed field gel electrophoresis (PFGE) was used to study a collection of 129 *Moraxella catarrhalis* isolates obtained over a 20 month period from 70 children admitted to, or presenting at, the Erasmus MC, Rotterdam, The Netherlands. Using PFGE, 51 different *M. catarrhalis* genotypes were identified, with 31% (22/70) of children being infected with two particularly prevalent *M. catarrhalis* types, which also exhibited different protein profiles. Further, 72% (16/22) of the children colonized with these two predominant types had spent at least one week on two pediatric intensive care wards within the Erasmus MC. By producing a time-line of colonization events, it was shown that replacement of one genotype with another could occur within this environment, though no exacerbation of existing disease or new disease was observed in any of the children for whom *M. catarrhalis* genotype changes occurred. These results indicate that genotype “switching” of *M. catarrhalis* genotypes occurs in the nosocomial environment, possibly paralleling events occurring in other closed environments (e.g. day care centers, at home etc). Moreover, genotype switching did not lead to the appearance of overt clinical disease, which may indicate that the true colonization rate of *M. catarrhalis* in children may be somewhat underestimated. However, some publications have in fact associated *M. catarrhalis* with pneumonia and bronchitis in pediatric intensive care units (4, 6, 14-16), indicating that *M. catarrhalis* may indeed facilitate the onset of overt disease within these environments. Taken together with our results, it is possible that certain *M. catarrhalis* genotypes may not actually be associated with facilitating tangible disease. Alternatively, it is also conceivable that certain human “genotypes” may be resistant to *M. catarrhalis* induced disease.

Chapter 4 also used the PFGE genotyping technique, but this time to investigate whether vaccination against common serotypes of *S. pneumoniae* (an organism that occupies the same biological niche as *M. catarrhalis*) affects the genetic diversity of *M. catarrhalis* isolates colonizing children with a history of acute otitis media episodes. In total, 41 *M. catarrhalis* isolates obtained from 13 children vaccinated with pneumococcal vaccine and 21 *M. catarrhalis* isolates obtained from 6 children vaccinated with a control (hepatitis A or B) vaccine were genotyped, indicating the presence of four clusters comprising a wide variety of genotypes. Moreover, no pattern could be observed between PFGE cluster and vaccination status, or PFGE cluster and the isolation of

other (co-colonizing) bacteria. *M. catarrhalis* PFGE types belonging to different clusters were found to occur within the same patient over time as well as being associated with different episodes of acute otitis media in the same patient over time. Further, the vast majority of isolates 90% (56/62) were found to exhibit the complement resistant phenotype, though no difference between complement phenotypes was observed between the vaccinated and unvaccinated groups (Fisher's Exact Test $P = 1$). The results of the investigation indicate that vaccination against *S. pneumoniae* in older children does not affect the diverse genetic nature of *M. catarrhalis* isolates when compared to a control vaccinated group, implying that a vaccine effective against a wide range of genotypes of *M. catarrhalis* will be necessary, even when *S. pneumoniae* is removed from the biological niche.

Chapter 5 utilized high-throughput amplification fragment length polymorphism (htAFLP) and one-dimensional SDS-PAGE analysis to investigate the presence/absence of genetic markers within different *M. catarrhalis* genotypic lineages and phenotypes (with specific reference to the complement resistance virulence trait). This study represented the most powerful genotypic analysis of the *M. catarrhalis* species yet published, analyzing 2,273 fragment length polymorphisms obtained from a population comprising 48 different isolates. Moreover, despite the presence of 40 lineage-specific polymorphisms, not one complement phenotype-specific marker was found. One-dimensional SDS-PAGE analysis revealed the presence of 6 major protein expression markers significantly associated with complement phenotype. By combining both lineage-specific and complement-phenotype associated markers, the ability to specifically identify virulent *M. catarrhalis* isolates should be enhanced.

Chapter 6 used PFGE to investigate the genetic diversity of *M. catarrhalis* isolates recovered from a set of 91 *Moraxella catarrhalis* isolates, originating from otitis prone and non-prone children. Further, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was used to investigate the presence of intra-genomic variation in the ubiquitous surface proteins of the *uspA1* and *uspA2* genes (the UspA1 and UspA2 proteins being potential vaccine candidates). Genotyping results indicated that no particular *M. catarrhalis* genotype was associated with either the otitis prone or non-prone children, and that a genetically diverse range of isolates was present. Nineteen pairs of PFGE identical isolates were found within the 91 isolates, with PCR-RFLP analysis showing that 1 of these 19 pairs of isolates exhibited both *uspA1* and *uspA2* intra-genomic variation, whilst another pair exhibited *uspA2* intra-genomic variation only. Sequence data obtained from these variants showed that PCR-

RFLP pattern differences reflected actual changes in predicted amino-acid composition and that these differences were associated with minor amino-acid changes in a 23 base pair “NINNIY” repeat region (a conserved UspA1 and UspA2 binding site for the neutralizing antibody mAb17C7). Sequence variation in the *uspA2* 5’ non-coding “AGAT” repeat region was also observed. These results showed that intra-genomic variation of an important epitope of both the *uspA1* and *uspA2* genes occurs, and indicates possible phase variation in the number of “AGAT” repeats in the *uspA2* gene. These findings may have implications for any future *M. catarrhalis* vaccines comprising UspA1 and UspA2 components. Indeed, one recent publication has indicated that the UspA1 and UspA2 proteins are unlikely to be good vaccine candidates, as distinct populations of *M. catarrhalis* with a reduced or complete absence of UspA1 and UspA2 expression may be isolated (13).

Complement Resistance Phenotype of *Moraxella catarrhalis*

Novel genes/mechanisms associated with the expression of the complement resistant phenotype were explored in the following four chapters. In **Chapter 7**, research was undertaken into a novel outer membrane protein (OMP J) of *M. catarrhalis* that exists in two major forms (of approximately 19kDa and 16kDa molecular weight), with each form being linked to either the complement resistant or complement sensitive phenotype. Moreover, this newly described protein was present in 100% of bacterial isolates tested and appeared to play a role in pulmonary clearance (as determined using an *ompJ2* gene knockout in a mouse model of infection). Subsequent research undertaken in **Chapter 8**, showed that OMP J is immunogenic and that total serum anti-OMP J immunoglobulin G (IgG) increases with age. However, there was no significant difference in anti-OMP J IgG when the acute and convalescent serum of children suffering from acute otitis media (AOM) disease was investigated. Passive immunization studies did however indicate that anti-OMP J antibodies might help facilitate the clearance of *M. catarrhalis* from the lungs during pulmonary infections.

The mannose binding lectin pathway (MBL) comprises one of the “arms” of the complement system, a system that is involved in the innate immune response to microbial pathogens (7). The ability of *M. catarrhalis* to activate (i.e. be recognized by) the MBL system was investigated using a functional MBL assay in **Chapter 9** (10). Using this technique, it was found that *M. catarrhalis* is only a weak activator of the

MBL system, a property that may in fact protect the organism against recognition and attack by the (innate) immune system. This would in turn facilitate colonization and/or infection by this organism.

Finally, **Chapter 10** details the identification, sequencing and characterization of a possible plasmid shuttle vector for the introduction and expression of complement resistance associated genes and/or potential vaccine candidates in *M. catarrhalis*. Also, despite the small-scale nature of this study, a range of plasmid sizes was found, and some of these plasmids could potentially carry virulence factors, including antimicrobial resistance cassettes and/or complement resistance-associated genes (9).

Conclusions and Future Perspectives

It is now accepted that *M. catarrhalis* is both a colonizer and a pathogen of man on a world-wide scale, though colonization and/or changes in genotype do not necessarily lead to the production of overt clinical disease, even in seriously ill children. Further, preliminary evidence suggests that children are more likely to be colonized with *M. catarrhalis* isolates that belong to distinct “clonal” lineages, whereas isolates obtained from adults tend to be more genetically diverse.

Previous research has identified several virulence factors (adhesins, iron scavenging proteins etc) that may be useful in promoting *M. catarrhalis* colonization, as well as genes possibly involved in the onset of disease (e.g. genes facilitating complement resistance). However, caution should be shown when statistical correlation data is used to directly attribute virulence traits (e.g. the complement resistance phenotype) to particular genes, as any association between a gene and virulence may simply be a consequence of the fact that that particular gene is more frequently found in a particularly virulent (clonal) *M. catarrhalis* lineage. In such cases, phenotypic studies using isogenic knockout isolates are necessary in order to provide a definitive association.

Currently, much research is geared towards the identification and evaluation of potential vaccine candidates against *M. catarrhalis*, with several promising proteins having been described. However, variation in the expression of putative vaccine candidate proteins has been recently documented, and the immune response to these proteins may be somewhat variable e.g. the decrease in anti-OMPE secretory IgA observed over time after nasopharyngeal colonization of children (Cripps, AW *et al*, 5th

Extraordinary International Symposium on Recent Advances in Otitis Media, 2005). It appears therefore, that in the foreseeable future, antibiotic therapy rather than vaccination will remain the preferred choice of treatment for *M. catarrhalis*-mediated infections.

Several advances have also been made with regard to the etiology of *M. catarrhalis*-mediated disease per se, with growing evidence to suggest that biofilms play a significant role in for example, the etiology of acute otitis media in children. This finding has an important bearing on the detection of organisms and the use of antibiotics in such infections, as planktonic (fixed) bacteria are extremely difficult to culture and very resistant to antibiotic therapy (*Post, JC. Otitis media with effusion as biofilm disease. 5th Extraordinary International Symposium on Recent Advances in Otitis Media, 2005*). Further research on the concept of “bacterial plurality” and the particular role of *M. catarrhalis* (in co-operation with *S. pneumoniae* and *H. influenzae*) in the formation of biofilms is warranted.

Finally, two major questions regarding the epidemiology of *M. catarrhalis*-mediated infection exist. Firstly, will the increase in smoking rates observed in “Third World” and Asian countries lead to an increase in the significance of *M. catarrhalis*-mediated infection within these countries, for example via an increase in chronic obstructive pulmonary disease (COPD) and *M. catarrhalis*-mediated exacerbations thereof. Secondly, will the introduction of vaccines against *Streptococcus pneumoniae* and *Haemophilus influenzae* reduce the importance of these organisms in respiratory disease, whilst at the same time leaving a biological niche open for exploitation by *M. catarrhalis*, possibly increasing the significance of this organism in upper and lower respiratory tract disease.

NEDERLANDSE SAMENVATTING

Het bacteriële species *M. catarrhalis* werd vroeger als een algemeen humaan commensaal organisme beschouwd met het vermogen tot kolonisatie van de luchtwegen. In de afgelopen decennia is er toenemend bewijs verzameld voor de rol van dit organisme bij luchtweginfecties van volwassenen en kinderen. Ontstekingen van de bovenste luchtwegen (bijv. middenoorontsteking en bij- of voorhoofdsholte ontstekingen) komen voornamelijk voor bij kinderen, terwijl ontstekingen van de onderste luchtwegen (bijv. bronchitis, longontstekingen, strottenhoofdontsteking) voornamelijk bij volwassenen worden geconstateerd. Af en toe wordt *M. catarrhalis* ook bij niet-luchtweginfecties geïsoleerd, met name bloedbaaninfecties, hartklepinfecties, bindvliesontsteking, hersenvliesontsteking, ooginfecties bij kinderen en kraakbeenontstekingen. Er blijkt een opvallend verschil in dragerschap te zijn (tot 75% in kinderen en 1 – 3% in volwassenen). Bovendien is *M. catarrhalis* seizoen gebonden en wordt het meest frequent geïsoleerd in de winter en lente maanden. Verspreiding van *M. catarrhalis* gebeurt via persoonlijk contact (speeksel) of via de handen enz., waarbij kinderdagverblijven en peuterscholen als belangrijke bronnen van verspreiding van bacteriële stammen tussen kinderen worden beschouwd. Een aantal uitbraken van infecties binnen ziekenhuizen van *M. catarrhalis* zijn ook waargenomen en gepubliceerd.

Onderzoek naar *M. catarrhalis* als pathogeen is gericht op verscheidene relevante bacteriële eigenschappen, zoals genetische diversiteit, ziekte verwekkende eigenschappen, de immuun respons tegen infecties en het zoeken naar een vaccin tegen *M. catarrhalis*. Een uitgebreide beschrijving van deze relevante eigenschappen is in **Hoofdstuk 2** weergegeven.

Genetische diversiteit van *Moraxella catarrhalis*

Tot nu toe zijn er veel verschillende technieken voor het bestuderen van de diversiteit in *M. catarrhalis* ontwikkeld, inclusief *protein expression profiling* (12), *DNA-DNA hybridisation* (1, 3, 19), *restriction fragment length polymorphism analysis* (5, 8), *single-adaptor amplified fragment length polymorphism analysis/16S rRNA sequencing* (2), *randomly amplified polymorphic DNA analysis* (18), en *pulsed field gel electrophoresis* (11, 17). Helaas

bestaat er nog geen “gouden standaard” techniek voor het genetisch karakteriseren van deze species.

In **Hoofdstuk 3**, werd *pulsed field gel electrophoresis* (PFGE) gebruikt en een stammencollectie van 129 *M. catarrhalis* isolaten bestudeerd om te onderzoeken of genetische veranderingen van *M. catarrhalis* stammen geïsoleerd binnen de pediatrieafdelingen van een ziekenhuis had plaatsgevonden. Verder werd ook een mogelijke relatie tussen genotype verandering en het verergeren van ziekten onderzocht. PFGE resulteerde in 51 verschillende genotypen, waarbij 31% (22/70) van de kinderen werden gekoloniseerd met twee bijzondere *M. catarrhalis* PFGE typen. Bij het uitzetten van kolonisatie experimenten in een tijdslijn, bleek dat binnen de patiënt het genotype van de koloniesant wisselde in de ziekenhuis omgeving, maar deze typewisseling leidde niet tot een verergering van de bestaande ziekte of nieuwe ziekte bij de kinderen. Deze resultaten laten zien dat *M. catarrhalis* “genotype wisseling” in kinderen voorkomt, en dat de reële incidentie van *M. catarrhalis* kolonisatie in kinderen grotendeels onderschat worden.

De PFGE techniek werd ook in **Hoofdstuk 4** gebruikt om een mogelijke relatie tussen het effect van vaccinatie tegen *S. pneumoniae* serotypen en kolonisatie met bepaalde genotypen van *M. catarrhalis* te onderzoeken. In totaal zijn 41 *M. catarrhalis* isolaten afkomstig van 13 met *S. pneumoniae* gevaccineerde kinderen en 21 *M. catarrhalis* isolaten afkomstig van 6 met hepatitis A/B (controle) gevaccineerde kinderen getypeerd. Geconcludeerd mag worden dat geen relatie bestaat tussen *M. catarrhalis* genotype en *S. pneumoniae* vaccinatie. Vaccinatie tegen *S. pneumoniae* serotypen blijkt de diversiteit van *M. catarrhalis* genotypen niet te beïnvloeden

In **Hoofdstuk 5** werden de *high throughput-amplification fragment length polymorphism* (htAFLP) techniek gebruikt om merkers voor fenotypische veranderingen, gecodeerd in het DNA, op te sporen. Dit was vooral gericht op het zoeken naar merkers welke met specifieke genetische groeperingen en/of complement resistente eigenschappen waren verbonden. Ondanks de aanwezigheid van 2,273 polymorfe merkers en 40 groepsspecifieke merkers, word er geen enkele merker gevonden welke specifiek is voor het complement fenotype. Eén-dimensionale SDS-PAGE gel electroforese toonde de aanwezigheid van 6 eiwit expressie merkers aan, die significant bleken voor het complement fenotype. De combinatie van deze genetische- en eiwit-specifieke merkers, zouden de identificatie van virulente *M. catarrhalis* isolaten kunnen vereenvoudigen.

De variatie in 2 genen (*uspA1* en *uspA2*), welke coderen voor verschillende oppervlakte eiwitten van *M. catarrhalis*, werden in **Hoofdstuk 6** onderzocht. In het

bijzonder werden stammen afkomstig van middenoorontsteking-gevoelige en middenoorontsteking-ongevoelige kinderen gebruikt. Resultaten laten zien dat er geen specifiek genotype van *M. catarrhalis* bij een van beide groepen werd gevonden, en dat variatie in de basepaar volgorde van de *uspA1* en *uspA2* genen kan ontstaan. Deze variaties kunnen gevolgen hebben voor toekomstige vaccins.

Complement resistentie van *Moraxella catarrhalis*

Het complement systeem is een reeks eiwitten die aanwezig zijn in het bloed van de mens, welke helpt bij bescherming tegen microbiële infecties. Deze eiwitten kunnen op drie verschillende manieren geactiveerd worden. In de volgende 4 hoofdstukken zijn factoren die geassocieerd zijn met het complement resistente fenotype van *M. catarrhalis* bestudeerd. In **Hoofdstuk 7** werd een nieuw oppervlakte eiwit (OMP J) beschreven dat eveneens bij complement resistentie is betrokken. Hierbij komen 2 bepaalde vormen van dit eiwit voor en is er een significante associatie tussen eiwitvorm en complement resistentie. Verder leidt het uitschakelen van dit eiwit tot een toename in de overlevingskansen van *M. catarrhalis* bacteriën in de longen van muizen (een model voor humane luchtweg infectie). In een vervolg onderzoek (beschreven in **Hoofdstuk 8**) bleek OMP J immunogeen, waarbij een evenredig verband met een toename van anti-OMP J immunoglobuline G (IgG) spiegels in serum en de leeftijd, werd gevonden. Er werd geen significant verschil in anti-OMP J IgG spiegels in sera afkomstig van kinderen met acute middenoorontsteking aangetoond. Echter, passieve immunisatie studies laten zien dat antilichamen tegen OMP J dekolonisatie/eradicatie van *M. catarrhalis* bacteriën uit de longen bevordert.

Een specifieke wijze van complement activatie loopt via de aanwezigheid van mannose (een suiker) op microbiële celoppervlakken. Deze suikers activeren de mannose-bindende lectine (MBL) activeringsweg van het complement systeem. In **Hoofdstuk 9** werd een specifieke toetsingsmethode gebruikt om het effect van *M. catarrhalis* op de activering van MBL te bepalen. Uit onze resultaten blijkt dat *M. catarrhalis* een zwakke activator van het complement systeem is.

Tot slot werd in **Hoofdstuk 10** een onbekend DNA fragment (plasmide pEMCJH03), dat zou kunnen functioneren als een vector voor het invoeren van bacteriële genen binnen verschillende *M. catarrhalis* stammen, in kaart gebracht. Het

plasmide zou een belangrijk hulpmiddel bij het maken van een vaccin tegen *M. catarrhalis* kunnen zijn.

Conclusies en Toekomstperspectieven

Het is bekend dat *M. catarrhalis* een bacterie is die zich over de hele wereld heeft verspreid. Tot nu toe heeft onderzoek diverse belangrijke eigenschappen van dit organisme in kaart gebracht, zoals genen en eiwitten die betrokken zijn bij kolonisatie en het ontstaan van ziekten bij de mens. Niettemin moeten mensen voorzichtig zijn met hun conclusies wanneer er een statistische significante associatie tussen een bepaald gen en een bepaald fenotype blijkt te zijn, want deze associatie hoeft niet te betekenen dat het betrokken gen een reële rol speelt in het ontstaan van een bepaalde fenotype. Het complement resistente fenotype is een goed voorbeeld daarvan, omdat dit fenotype bij verschillende specifieke groepen van *M. catarrhalis* genotypen is aangetroffen. Ook zijn vele andere genen of verschil in gen samenstelling met dezelfde groepen van genotypen geassocieerd. De overgrote meerderheid van deze genen hebben niets met complement resistentie te maken.

Op dit moment is veel onderzoek gericht op het determineren en evalueren van mogelijke vaccineiwitten tegen *M. catarrhalis*. Onlangs is er een wisselende expressie van sommige oppervlakte eiwitten geconstateerd met mogelijke gevolgen voor de respons van het immuun systeem bijvoorbeeld op isolaten die potentiële vaccin eiwit kandidaten niet tot expressie brengen. In elk geval zal antibioticumtherapie en niet vaccinatie de voorkeur hebben in de behandeling van *M. catarrhalis* infecties in de nabije toekomst.

Bovendien is er op dit moment een opmerkelijke vooruitgang geboekt in het *M. catarrhalis* onderzoek, zoals de studie naar *biofilms* en de relatie hiervan met betrekking tot het ontstaan van middenoorontsteking. Een *biofilm* is een gemeenschap van bacteriële soorten omhuld door een complexe “suiker” laag, die zich vasthechten aan weefsel oppervlakten. Zulke *biofilms* zijn slecht met antibiotica behandelbaar. Verder onderzoek naar de relatie tussen *M. catarrhalis* en andere bacteriële ziekteverwekkers in *biofilms* is noodzakelijk

Tot slot blijven twee grote vragen over *M. catarrhalis* infecties in de toekomst open staan. Ten eerste, zou het toenemen van het aantal rokers in de “Derde Wereld” en Aziatische landen, een toename in *M. catarrhalis* veroorzaakte luchtweginfecties

betekenen? Ten tweede, zou het invoeren van vaccins tegen *Streptococcus pneumoniae* en *Haemophilus influenzae* leiden tot een vermindering van deze bacteriën als veroorzakers van luchtweginfecties en tegelijkertijd leiden tot een toenemende rol van *M. catarrhalis* bij luchtweginfecties?

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Five years ago when I first started this project, I was an unmarried, mortgage-free “Engelsman”, of respectable age, possessing a less than respectable knowledge of the Dutch language and customs. Well not much has changed since then, except that I now find myself married, mortgaged and middle-aged, sharing my life with three companions, namely my wife Cindy, our son Thomas, and a cat called (mad) Max. During the same period, my knowledge of the Dutch language has (slightly) improved as I can now confidently distinguish between a “man in de rij” and a “mandarijn” (a useful skill when shopping in the supermarket!). More importantly perhaps, I have had the great pleasure of meeting and working with a wide range of individuals who have contributed to the completion of this thesis (and therefore my current state of euphoria!). So here goes for all of those individuals

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CURRICULUM VITAE

John Hays was born in Sutton-in-Ashfield, Nottinghamshire, England on the 27th December 1963 and completed his secondary education at Quarrydale Comprehensive school in the same town. After 3 years (1983-1986) studying for a BSc degree (achieving an Upper Second Class) in Biology/Food Science and Nutrition at the Oxford Polytechnic, Oxford, he spent 2 years working for DuPont (plastics division!). The following move was to the Public Health Laboratory Service (PHLS) laboratory at the Queen's Medical Centre in Nottingham, performing routine microbiological testing (2 years in the bacteriology section and 3 years in the virology/serology sections), during which time he studied part-time (2 years) for an MSc in Biomedical Science (with distinction) and was awarded the John Adams memorial prize for his project on HHV-6 and CMV infection in renal transplant recipients (supervisors Mr. Alan Pawley and Dr. Will L. Irving). The following 3 years were spent studying for a PhD degree on "The Molecular Epidemiology of Human Coronaviruses 229E" at the School of Medicine, University of Leicester, Leicester, under the leadership of Prof. Steven H. Myint. After completing his PhD studies (just!), he moved to the Central Science Laboratory, York (supervisor Dr. Nigel Cook), working on a short-term project using molecular techniques (NASBA[®]) to detect viruses and bacteria in food. At the end of this period, he was employed for 2 years (1997- 1999) on a GlaxoWellcome funded phase 3 trial using multiplex RT-PCR to investigate the efficacy of the anti-influenza drug Relenza[®] (Zanamivir). This work was performed at the Central Public Health Laboratory, Colindale, London under the guidance of Dr. Maria Zambon. During this period of time, he met his future wife and emigrated to The Netherlands, eventually finding a position in the department of Medical Microbiology and Infectious Diseases at the Erasmus MC, Rotterdam, researching into the bacterium *Moraxella catarrhalis* under the supervision of Prof. Dr. Dr. Alex van Belkum.

LIST OF PUBLICATIONS

- 1) **John P. Hays**, Saskia van Selm, Theo Hoogenboezem, Silvia Estevão, Helena Kayhty, Alex van Belkum, Peter W. M. Hermans. Investigations into the immune response and pulmonary clearance after passive immunization using the novel outer membrane proteins OMP J1 and OMP J2 of *Moraxella catarrhalis*. *In preparation*.
- 2) **John P. Hays**, Roy Gorkink, Guus Simons, Justine K. Peeters, Kim Eadie, Cees M. Verduin, Henri Verbrugh, Alex van Belkum. Whole-cell genomic analysis and one-dimensional protein expression profiling in the search for lineage-specific and virulence-associated markers of *Moraxella catarrhalis*. *Submitted*.
- 3) **John P. Hays**, Saskia van Selm, Theo Hoogenboezem, Silvia Estevão, Kimberly Eadie, Peter van Veelen, Jan Tommassen, Alex van Belkum, Peter W. M. Hermans. 2005. The identification and characterization of a novel outer membrane protein (OMP J) of *Moraxella catarrhalis* that exists in two major forms. *J. Bacteriol.* Dec; 187(23):7977-7984.
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- 8) **Hays JP**, Eadie K, Verduin CM, Hazelzet J, Verbrugh H, van Belkum A. 2003. Changes in genetic types and population dynamics of *Moraxella catarrhalis* in hospitalized children are not associated with an exacerbation of existing disease. *J. Med. Microbiol.* Sep;52:815-20.
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BOOKS

- 1) **Hays JP** “Hays J.P. The Genus *Moraxella*. In: Dworkin, M.; Falkow, S.; Rosenberg, E.; Schleifer, K.-H.; Stackebrandt, E. (Eds.), *THE PROKARYOTES - An Evolving Electronic Resource for the Microbiological Community*”. Springer-Verlag (New York). ISBN: 0-387-14254-1. ©Springer-Verlag. <http://link.springer-ny.com/link/service/books/10125/>. In Preparation.
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