

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

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The colonization dynamics of *Moraxella catarrhalis* were studied in a population comprising 1079 healthy children living in Rotterdam, The Netherlands (the Generation R Focus cohort). A total of 2751 nasal swabs were obtained during four clinic visits timed to take place at 1.5, 6, 14 and 24 months of age, yielding a total of 709 *M. catarrhalis* and 621 *Haemophilus influenzae* isolates. Between January 2004 and December 2006, approximate but regular 6-monthly cycles of colonization were observed, with peak colonization incidences occurring in the autumn/winter for *M. catarrhalis*, and winter/spring for *H. influenzae*. Co-colonization was significantly more likely than single-species colonization with either *M. catarrhalis* or *H. influenzae*, with genotypic analysis revealing no clonality for co-colonizing or single colonizers of either bacterial species. This finding is especially relevant considering the recent discovery of the importance of *H. influenzae*-*M. catarrhalis* quorum sensing in biofilm formation and host clearance. Bacterial genotype heterogeneity was maintained over the 3-year period of the study, even within this relatively localized geographical region, and there was no association of genotypes with either season or year of isolation. Furthermore, chronological and genotypic diversity in three immunologically important *M. catarrhalis* virulence genes (*uspA1*, *uspA2* and *hag/mid*) was also observed. This study indicates that genotypic variation is a key factor contributing to the success of *M. catarrhalis* colonization of healthy children in the first years of life. Furthermore, variation in immunologically relevant virulence genes within colonizing populations, and even within genotypically identical *M. catarrhalis* isolates, may be a result of immune evasion by this pathogen. Finally, the factors facilitating *M. catarrhalis* and *H. influenzae* co-colonization need to be further investigated.

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## INTRODUCTION

*Moraxella catarrhalis* is part of the normal bacterial flora in the nasopharynx of children, although over the past two decades, it has emerged as a significant bacterial pathogen

**Abbreviations:** AOM, acute otitis media; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; LOS, lipooligosaccharide; MLST, multilocus sequence typing; OMP, outer-membrane protein; OR, odds ratio; OMV, outer-membrane vesicle; URTI, upper respiratory tract infection.

and not simply a commensal colonizer (Verduin *et al.*, 2002). Studies have shown that the bacterium rapidly colonizes the nasopharynx soon after birth and that the carriage rate of *M. catarrhalis* in healthy children varies between 7 and 36% (Verhaegh *et al.*, 2010). However, in children with upper respiratory tract infections (URTI), including acute otitis media (AOM), the carriage rate increases to approximately 50% (Berner *et al.*, 1996; Konno *et al.*, 2006; Pettigrew *et al.*, 2008). Otitis media itself is a particularly important URTI during early

childhood and the primary reason for children to visit a physician (Freid *et al.*, 1998; Plasschaert *et al.*, 2006). Furthermore, in many countries, AOM is the most common reason for prescribing antibiotics (Gonzales *et al.*, 2001; Leibovitz, 2003; Pichichero, 2000; Plasschaert *et al.*, 2006) or for undergoing surgery (for the placement of grommets) (Vlastarakos *et al.*, 2007). The most common bacterial species cultured from the nasopharynx of children during otitis media episodes are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *M. catarrhalis* and *Staphylococcus aureus*, either as single pathogens or as co-cultures (Berner *et al.*, 1996; Pettigrew *et al.*, 2008), with the patterns of nasopharyngeal colonization by micro-organisms being important determinants for otitis media disease (Faden *et al.*, 1991, 1997; Murphy & Parameswaran, 2009).

With respect to *M. catarrhalis*, it has been shown that many factors affect nasopharyngeal carriage of this human-specific pathogen, including, for example, the presence of siblings, day care attendance and respiratory illness (Faden *et al.*, 1997; Hendley *et al.*, 2005; Peerbooms *et al.*, 2002; Principi *et al.*, 1999; Verhaegh *et al.*, 2010). Furthermore, there is increasing information regarding the biological mechanisms facilitating *M. catarrhalis*-mediated colonization and disease development, with most publications stressing the importance of bacterial adherence as an essential first step in this process. To date, several important *M. catarrhalis* adhesins have been described, including the ubiquitous surface proteins UspA1 and UspA2, and the haemagglutinin/*Moraxella* IgD-binding protein Hag/MID (Bullard *et al.*, 2005; Helminen *et al.*, 1994). UspA1 and UspA2 acquire their adherence characteristics through multifunctional binding sites, which include domains that have the ability to attach to epithelial cells via cell-associated fibronectin, laminin and vitronectin (Tan *et al.*, 2006b). Furthermore, UspA1 includes a critical binding site for carcinoembryonic antigen-related cell adhesion molecules, which are expressed in various human tissues including respiratory epithelia (Hill & Virji, 2003; Slevogt *et al.*, 2008). The *Moraxella* IgD-binding protein, also referred to as Hag (human erythrocyte agglutinin), is also important for host attachment. MID is an outer-membrane protein (OMP) with specific affinity for soluble and cell-bound human IgD and is the only IgD-binding protein in *Moraxella* (Forsgren *et al.*, 2001).

Several other virulence-associated genes have been identified that may be associated with colonization and infection of *M. catarrhalis*, including the OMPs CopB, OMPCD, McaP, MhaB, MhaC, Msp22, Msp75, Msp78 and lipooligosaccharide (LOS) (Akimana & Lafontaine, 2007; Balder *et al.*, 2007; Lipski *et al.*, 2007; Liu *et al.*, 2007; Riesbeck *et al.*, 2006; Ruckdeschel *et al.*, 2008, 2009; Tan *et al.*, 2006a). Furthermore, several of these virulence genes have also been associated with the induction of the humoral immune response, making them potential vaccine candidate genes. However, relatively little is known about the frequency and the extent of genotypic variation within these virulence genes, and in particular how this variation may be associated with colonization over time.

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

## METHODS

**Study population.** This study was embedded within the Generation R Focus cohort, part of a population-based prospective cohort study from fetal life until young adulthood based in Rotterdam, The Netherlands (The Generation R Study) (Jaddoe *et al.*, 2007, 2008). Within the Generation R Focus cohort, detailed assessments of fetal and postnatal growth and development were performed on 1232 pregnant Dutch women. The mothers gave birth to 1244 infants between February 2003 and August 2005, in Rotterdam, The Netherlands, of whom 138 were excluded from the study analysis because consent was withdrawn after birth. Twins were also excluded from the analysis because of genetic relatedness, leaving a total of 1079 infants. The medical ethics committee of the Erasmus Medical Center approved the study. Written informed consent was obtained from the parents of all the participants.

Infants visited the Generation R Focus cohort research centre at 1.5, 6, 14 and 24 months after birth, resulting in 630 swabs being taken at 1.5 months, 787 swabs at 6 months, 717 swabs at 14 months and 617 swabs at 24 months between November 2003 and September 2007. From 623 children, at least three swabs were available for bacterial culturing.

**Bacterial isolates.** Bacterial isolates were cultured from nasal swabs taken from infants at 1.5, 6, 14 and 24 months of age by a trained research nurse, using a sterile transport swab immersed in Amies transport medium. Swabs were cultured within 6 h of sampling, using blood agar plates containing 5% sheep blood, chocolate agar and *Haemophilus*-selective agar at an incubation temperature of 35 °C in 5% CO<sub>2</sub> for 48 h. Plates were examined daily for the growth of *M. catarrhalis* and/or *H. influenzae*.

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

PFGE for *M. catarrhalis* was performed as detailed by Verduin *et al.* (2000). Briefly, *M. catarrhalis* plug digestions were performed using *Spe*I at 20 U per reaction and an electrophoresis protocol comprising a first block with a constant voltage of 6 V cm<sup>-1</sup> and a pulse time from 3.5 to 25 s during the first 12 h, followed by a second block of 8 h where the pulse time increased linearly from 1 to 5 s. The PFGE protocol for *H. influenzae* was adapted from the work of Moor *et al.* (1999). Digestions were performed using *Sma*I at 30 U per reaction and an electrophoresis protocol comprising a first block with a constant voltage of 6 V cm<sup>-1</sup> and a pulse time from 6 to 8 s during the first 7 h, followed by a second block of 17 h where the pulse time

increased linearly from 1 to 38 s. All PFGE patterns were analysed using BioNumerics (Applied Maths), with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set at 1.5%. PFGE products between 48.5 and 339.5 kb for *M. catarrhalis* and 48.5 and 485 kb for *H. influenzae* were included in the band matching analysis.

MLST genotyping was performed to determine whether *M. catarrhalis* genotypes circulating in the Rotterdam area were similar to *M. catarrhalis* genotypes worldwide and to establish genetic relationships with other internationally recognized clones available in the *M. catarrhalis* MLST database. MLST was performed on 12 *M. catarrhalis* isolates that had been cultured serially on three separate occasions from four children. Briefly, PCR was performed to detect and sequence the *abcZ*, *adk*, *efp*, *fumC*, *glyRS*, *mutY*, *ppa* and *trpE* genes as described in the guidelines available at the MLST website (<http://mlst.ucc.ie/>). A touchdown thermocycling programme was used for *glyRS* and *adk*. The touchdown protocol used an initial annealing temperature of 70 °C, which was reduced by 1 °C per cycle over 15 PCR cycles. The following 20 cycles of amplification used an annealing temperature of 55 °C. For *ppa*, *efp*, *abcZ* and *trpE*, a standard PCR protocol comprising an annealing temperature of 52 °C and an elongation time of 2 min at 72 °C for 25 cycles was used, whilst for *fumC* and *mutY* an annealing temperature of 55 °C was used. PCR primer pairs were used for amplicon sequencing, except for the genes *glyRS*, *fumC* and *mutY*, which required separate sequencing primers (primers are available at <http://mlst.ucc.ie/>). Allelic profiles were defined and compared with the MLST types of isolates from different geographical regions available at <http://mlst.ucc.ie/>.

**PCR screening of virulence genes.** *M. catarrhalis* isolates were grown from the glycerol stock overnight at 37 °C on blood agar plates. DNA was extracted using the MagNA Pure LC System (Roche Applied Science). PCR was performed to detect the *uspA1*, *uspA2*, *uspA2H*, *hag/mid* and *ompJ* genes and 16S rRNA and LOS types as described previously (Verhaegh *et al.*, 2008). A touchdown thermocycling programme was used for all PCRs except for *hag/mid* and LOS PCRs. The touchdown protocol used an initial annealing temperature of 70 °C, which was reduced by 1 °C per cycle over 15 cycles of PCR. The following 20 cycles of amplification used an annealing temperature of 55 °C. All isolates negative for both *uspA2* and *uspA2H* genes were tested using primers *uspA2end.f* and *uspA2end* (Verhaegh *et al.*, 2008), which amplify a conserved region found at the 3'-end of both *uspA2* and *uspA2H* genes.

For the *hag/mid* gene PCR, a standard PCR protocol comprising an annealing temperature of 55 °C and an extension time of 8 min at 68 °C for 25 cycles was used. The LOS-typing PCR protocol was performed as described by Edwards *et al.* (2005).

After amplification, 16S rRNA PCR products were digested using the enzymes *FspBI* (10 U) and *HhaI* (10 U) according to Verhaegh *et al.* (2008), to identify the 16S rRNA types (Verhaegh *et al.*, 2008).

**Virulence gene variation.** Virulence gene variation was assessed using PCR-RFLP typing on three major *M. catarrhalis* virulence genes. These genes comprised the ubiquitous surface protein genes A1 and A2 (*uspA1* and *uspA2*), and the haemagglutinin/IgD-binding protein gene *hag/mid*. PCR products of these genes were digested using the restriction enzyme *HaeIII* (*BsuRI*), using 10 U per reaction mix, and incubated overnight at 37 °C. The *HaeIII* restriction enzyme was chosen because its recognition site(s) is found within immunologically important regions of the *uspA1*, *uspA2* and *hag/mid* genes.

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

**Data analysis.** To evaluate potential predictors of colonization, logistic regression analysis was performed with general estimating equations using the logit link function as implemented in the GENMOD procedure in SAS version v9.1.3 (SAS Institute, 2002, SAS 9.1.3.). Separate analyses were conducted for the binary dependent variables of presence or absence of either *M. catarrhalis* or *H. influenzae* in a repeated measures design. The repeated variable was children, each of whom had 1–4 swabs in the study period. Predictor variables were the presence/absence of the non-target species, year and season. Seasons were defined as winter (December–February), spring (March–May), summer (June–August) and autumn (September–November). An independent correlation matrix structure among predictor variables was used but there were no differences in assessment of significances using exchangeable or unstructured correlation matrices. Two- and three-way interaction terms were not included in the final model because (i) all covariances between model variables in all analyses were low ( $r < 0.04$ ), (ii) exploratory analyses that excluded the repeated design showed interaction terms that did not contribute to the efficiency of the models and (iii) all, except one, of the 64 potential interaction terms for each target species were not significant.

## RESULTS

### Bacterial isolates

A total of 709 *M. catarrhalis* isolates and 621 *H. influenzae* isolates were cultured from a total of 2751 nasal swabs obtained during the 3-year study period. The ages of the subjects when the cultures were taken are given in Table 1.

### Population dynamics

Approximate, but regular, 6-monthly cycles of peak colonization prevalence were observed over the 3-year study period for *M. catarrhalis*, with a less regular pattern observed for *H. influenzae*. Peak seasonal prevalence differed between *M. catarrhalis* and *H. influenzae*, with *M. catarrhalis* prevalence highest in autumn and winter [odds ratio (OR)=1.3–2.0 compared with spring or summer with  $P < 0.03$  for each comparison] and *H. influenzae* prevalence highest in winter and spring (OR=1.5–1.9;  $P < 0.01$ ). Each species seasonal peak represented a significantly higher risk of carriage compared with non-peak seasons and the odds of a child being colonized by one of the two pathogens were significantly increased if

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

Total number in study population=2751.

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

the other species was present (OR=1.5;  $P<0.0001$ ). The peak in joint occurrences occurred in autumn. If the product of the individual species occurrence proportions is considered an indicator of the expected proportion of joint occurrences, chi-squared analyses showed significant excesses over expectations of joint occurrences for spring ( $P<0.01$ ), summer ( $P<0.01$ ) and autumn ( $P=0.04$ ). Both pathogens were at or near their peak prevalence in 2006 and so the joint prevalence was highest that year at 10.8%. Conversely, the combined absence of both species in children was at its lowest in 2006 at 49.3%. For *M. catarrhalis*, there were significantly higher risks in 2006 and 2007 compared with 2004 and 2005 (OR=1.5–2.1;  $P<0.01$ ). In contrast, the risk for *H. influenzae* was highest in 2006 compared with other years (OR=1.3–1.7;  $P<0.03$ ) (Fig. 1).

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

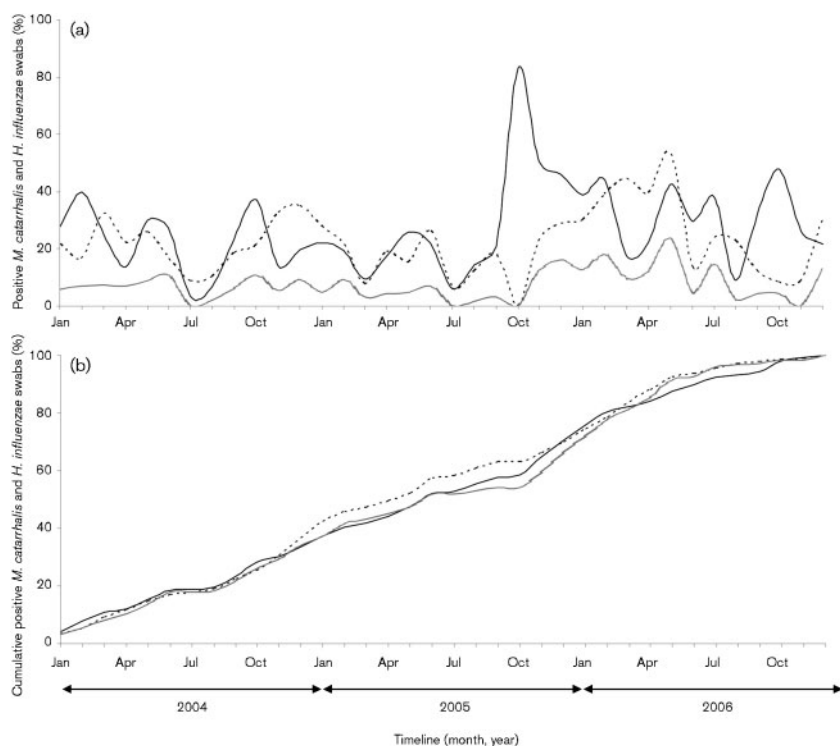
A selection of 112 isolates were arbitrarily chosen for PFGE genotyping from the 709 *M. catarrhalis* isolates cultured in this study, including isolates from children colonized only once during the study period and serial isolates cultured from the same child. A high degree of genotypic heterogeneity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

MLST genotyping of 12 *M. catarrhalis* isolates was performed in order to determine the global context of colonizing isolates obtained from Rotterdam, The Netherlands. These 12 isolates originated from four children that were colonized with *M. catarrhalis* on three separate occasions, and represented different PFGE genotypic clusters. The Rotterdam isolates tested were found to be non-clonal in nature with many (8 out of 12) singleton MLST types, further indicating the genetic heterogeneity of *M. catarrhalis* isolates, even within a relatively restricted geographical region (Fig. 3).

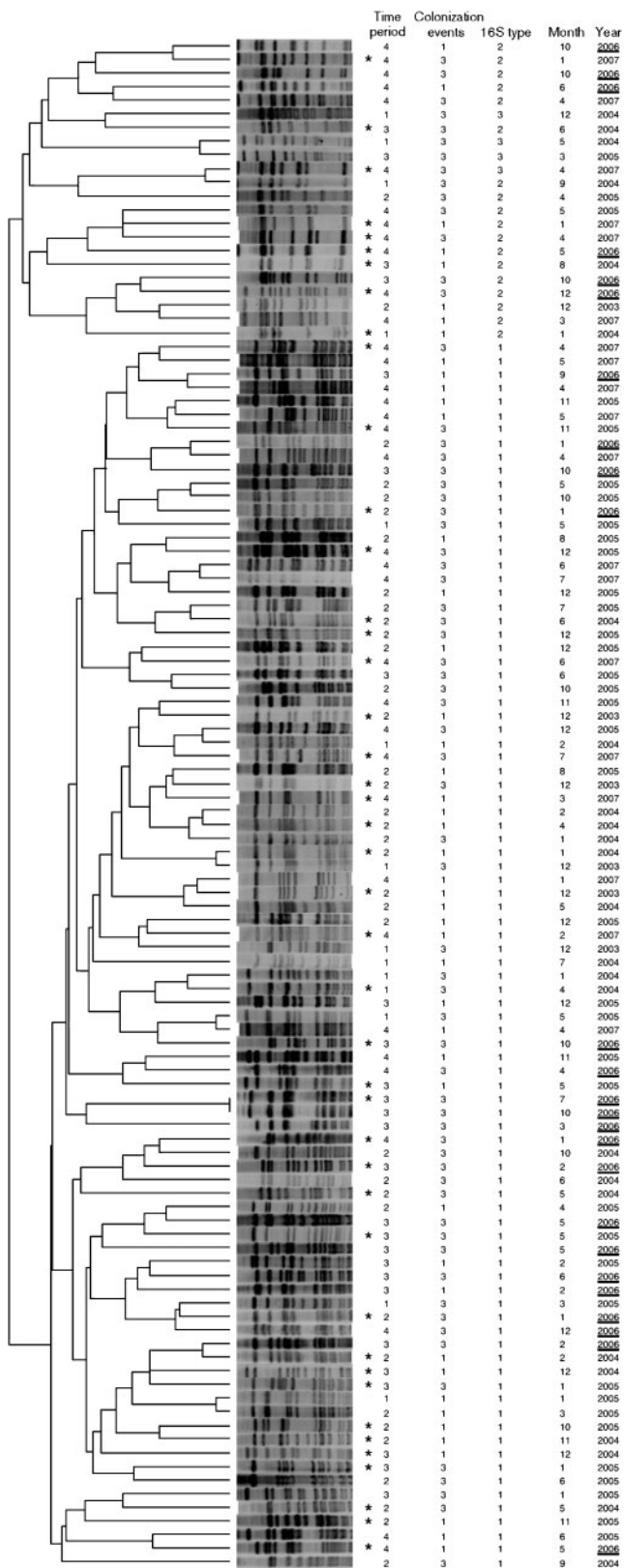
Due to the significant association between *M. catarrhalis* and *H. influenzae* colonization, 36 *H. influenzae* isolates (including both co-colonizing and single-species isolations) were chosen for PFGE genotyping to study their genetic diversity. No single *H. influenzae* genotype or clonal cluster was associated with co-colonization events (Fig. 4).

### PCR screening of virulence genes

PCR screening of the same 112 *M. catarrhalis* isolates for which PFGE was performed revealed an incidence of 97% (109/112) for *uspA1*, 80% (90/112) for *hag/mid*, 83% (85/102) for *uspA2*, 17% (17/102) for *uspA2H* and 100% (112/112) for *ompJ*. Ten isolates were found to be PCR-negative using previously published *uspA2*- and *uspA2H*-specific primers, but positive using primers designed to amplify a conserved region of both *uspA2* and *uspA2H*. These 10 isolates were not included in subsequent *uspA2* and *uspA2H* prevalence calculations, though their inclusion as



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



**Fig. 2.** Dendrogram based on PFGE genotypic variation of 112 colonizing *M. catarrhalis* isolates obtained from 74 children aged 1.5–24 months between November 2003 and September 2007 in Rotterdam. Time period after birth when nasal swabs were taken:

1, 6 weeks; 2, 6 months; 3, 14 months; 4, 24 months. Colonization events: 1, children colonized in a single sampling period only during the course of the study; 3, children colonized three times during the study period. 16S rRNA type: 1, lineage 1; 2/3, lineage 2. The month and year of isolate culture from the nose of children are also shown (year of peak prevalence underlined). Co-colonization with *H. influenzae* is indicated with asterisks (\*). No clustering of serial isolates cultured from the same child, or co-colonizing (with *H. influenzae*) isolates, was observed.

either *uspA2* or *uspA2H* would not have affected the significance of the results (Table 2).

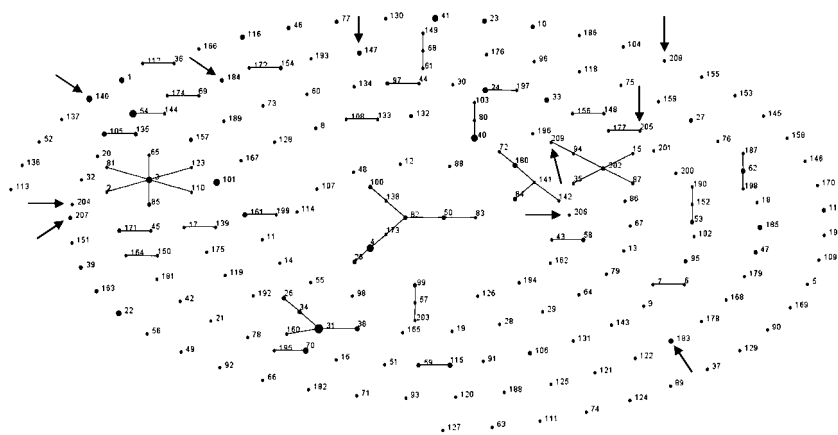
16S rRNA gene type analysis revealed an incidence of 80 % for 16S rRNA type 1 (lineage 1), and a combined 20 % for types 2 and 3 (lineage 2). All isolates that belonged to lineage 1 were associated with the presence of the *hag/mid* gene and *OmpJ* type 2.

### Virulence gene diversity

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

### DISCUSSION

The colonization dynamics of *M. catarrhalis* and *H. influenzae* were studied in a population of 1079 healthy children living in Rotterdam, The Netherlands. Over the 3-year study period, a trend towards a regular 6-monthly *M. catarrhalis* colonization cycle (peak to peak) was observed for *M. catarrhalis* (which was less evident for *H. influenzae*), without a pronounced seasonal peak for either. This observation tends to contrast with other studies reporting a seasonal influence on *M. catarrhalis* colonization, although these studies also reported that higher detection rates of *M. catarrhalis* during the colder months could be a side effect of nasal hyper-secretion induced by viral illness or cold weather (Hendley *et al.*, 2005). Marchisio *et al.* (2001) concluded that the seasonal influence (comparison between spring and autumn) on nasopharyngeal carriage of respiratory tract pathogens in healthy children was negligible (Marchisio *et al.*, 2001). However, their study was limited in describing seasonal variations due to lack of observations in other years, which would have allowed a better understanding of the dynamics



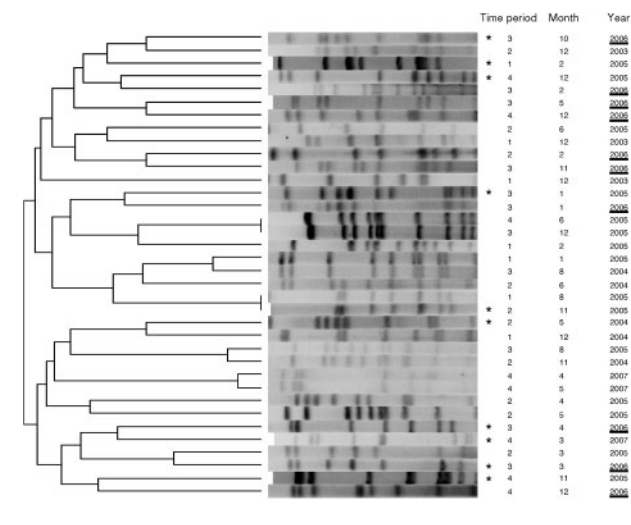
**Fig. 3.** MLST genotyping of 12 *M. catarrhalis* isolates. The sequence types assigned to the isolates are indicated with black arrows, showing the non-clonal nature of the isolates. Each dot represents a distinct MLST type, and MLST types connected with a black line possess similar allelic profiles,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

of the individual pathogens. Our results suggest that colonization with *M. catarrhalis* (and to a lesser extent *H. influenzae*) occurs via regular cycles of colonization and clearance, with the apparent increase in *M. catarrhalis* and *H. influenzae* detection during the winter months probably being a consequence of increased viral respiratory infections, e.g. influenza, resulting in increased opportunity for secondary bacterial infection by bacterial pathogens. The link between seasonal virus infections and resultant secondary bacterial infections has been indicated by several publications (Bakaletz, 1995; Pettigrew *et al.*, 2008; Ruuskanen *et al.*, 1991). However, reports by Meijer *et al.* (2007) and Arkema *et al.* (2008), who collected clinical and virological data via the European Influenza

Surveillance Scheme (EISS), concluded that the 2005–2006 and 2006–2007 influenza seasons in Europe were characterized by moderate clinical activity (Arkema *et al.*, 2008; Meijer *et al.*, 2007), and as a consequence may have resulted in less viral predisposition of the middle ear epithelium to bacterial infection.

At least two studies have suggested that the presence of a particular bacterial species during URTIs may create a more hospitable niche for co-colonization by a second distinct bacterial species (Armbruster *et al.*, 2010; Pettigrew *et al.*, 2008). Armbruster *et al.* (2010) showed that *H. influenzae* promotes *M. catarrhalis* persistence within polymicrobial biofilms via inter-species quorum signalling, and that co-infection with both species promotes the increased resistance of biofilms to antibiotics and host clearance (Armbruster *et al.*, 2010). In fact, the process of biofilm formation has been demonstrated for numerous pathogens and is clearly an important microbial survival strategy.

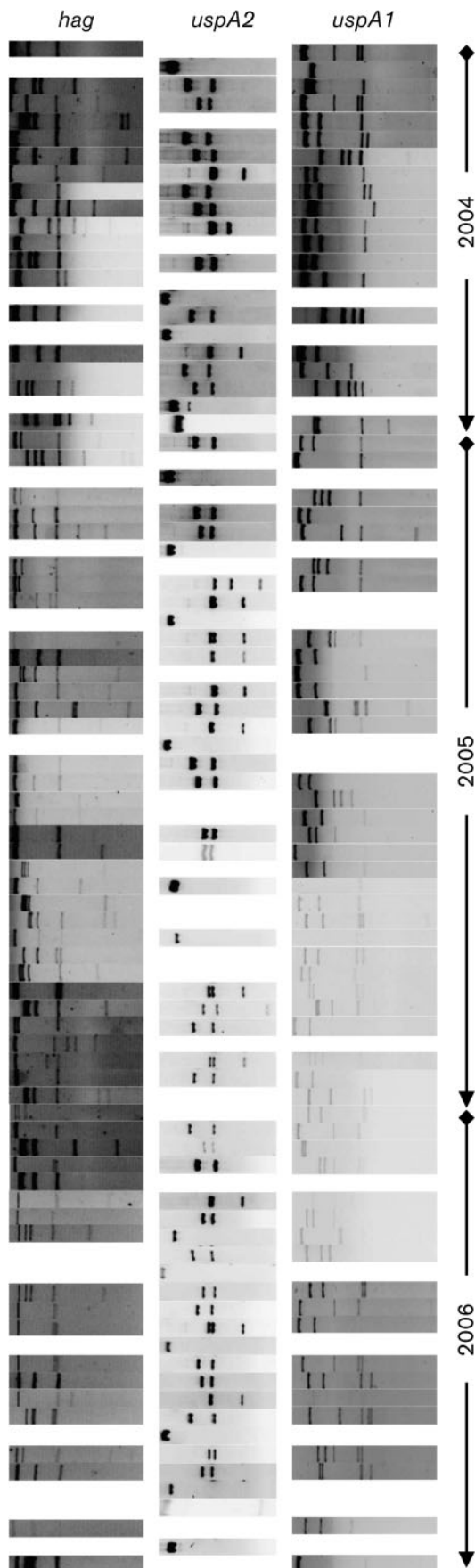
In our present study, co-colonization between *M. catarrhalis* and *H. influenzae* was significantly more likely than colonization with either bacterial species alone. This is an interesting result, as (for example) competition for cellular binding sites may be expected to occur in the presence of two distinct bacterial species, although this hypothesis needs further study. In detail, both species can bind to carcinoembryonic antigen-related cell adhesion molecules (CEACAM)-1, which is a receptor for the OMPs UspA1 and P5 of *M. catarrhalis* and *H. influenzae*, respectively



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

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

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



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

(Hill *et al.*, 2001). CEACAM-1 is a specific innate immune receptor and although the CEACAM-binding ligands of respiratory pathogens are structurally diverse, they target a common site on the receptor (Hill *et al.*, 2005; Sarantis & Gray-Owen, 2007). However, competition for binding sites could be outweighed by non-receptor-mediated factors associated with co-colonization events. For example, *M. catarrhalis* releases outer-membrane vesicles (OMVs) or ‘blebs’ from its surface during growth in several environments, e.g. liquid culture, solid culture and biofilms. The OMVs that are secreted by *M. catarrhalis* carry UspA1 and UspA2, which are known to interfere with the activation of the complement cascade. Tan *et al.* (2007) showed that blebs carrying UspA1/A2 protected *H. influenzae* from complement-mediated killing, suggesting that *M. catarrhalis* may promote the survival of *H. influenzae* during co-colonization (Tan *et al.*, 2007). Deich & Hoyer (1982) demonstrated the generation and release of DNA-binding vesicles by *H. influenzae* (Deich & Hoyer, 1982), although this study did not mention the effect of *H. influenzae* blebs on the complement cascade. At this time, no other studies have reported the presence of *H. influenzae* blebs and their possible beneficial effect on other bacterial species in co-culture.

Whatever the exact cause of co-colonization dominance, neither co-colonizing *M. catarrhalis* nor *H. influenzae* belonged to specific genotypic clones, suggesting that a ubiquitous system is facilitating the significant difference in co-colonization (versus single-species colonization) observed in this study. As recently described by Armbruster *et al.* (2010), the most likely (non-clonal-genotype-related) ubiquitous system driving co-colonization events of these two bacterial pathogens appears to be the quorum sensing system (Armbruster *et al.*, 2010). However, complement-evading systems, e.g. via OMV-mediated protection, may also play a significant role.

Genotypic analysis revealed a high degree of diversity in colonizing *M. catarrhalis* isolates, an observation which is in agreement with previous studies showing that genotypic heterogeneity is typical of worldwide *M. catarrhalis* isolates colonizing both children and adults (Hays *et al.*, 2003; Verhaegh *et al.*, 2008; Wolf *et al.*, 2000). Also, no significant association between genotype (cluster) and season, or year of isolation was observed, which is in contrast with a previous publication by Levy *et al.* (2009) who reported that winter and spring season was a significant risk factor for clustering. However, Levy *et al.* (2009) studied transmission between patients within a

relatively confined hospital setting (Levy *et al.*, 2009), whereas the focus of our study was related to community transmission.

The prevalence of the virulence genes *uspA1*, *uspA2* and *hag/mid* within our Rotterdam isolates was very similar to that previously reported for global *M. catarrhalis* isolates (Verhaegh *et al.*, 2008). These virulence genes encode OMPs that play an important role in bacterial adherence to human epithelia and complement resistance, as well as being immunogenic (Helminen *et al.*, 1994, 1995; Meier *et al.*, 2003). This finding suggests that equilibrium has been reached with respect to virulence gene distribution in global *M. catarrhalis* isolates, as local geographical factors do not appear to generate significant differences in virulence gene prevalence.

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

## Conclusion

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

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

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