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

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

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INTRODUCTION

Moraxella catarrhalis has been associated with a number of respiratory-associated infections affecting both children and adults, including laryngitis (Hol *et al.*, 1996), bronchitis (Ahmad, 1998) and pneumonia (Kobayashi, 2000; Manfredi *et al.*, 2000). However, the bacterium is mostly associated with upper respiratory tract infections in children, e.g. otitis media (Faden *et al.*, 1994), and lower respiratory tract infection in adults, e.g. exacerbations of chronic obstructive pulmonary disease (COPD) (Murphy *et al.*, 2005a).

M. catarrhalis populations may be subdivided into two distinct genetic lineages, phenotypically characterized by

(1) their ability to resist the destructive effect of human serum (i.e. complement resistant versus complement sensitive), and (2) differences in their ability to adhere to human epithelial cells (Bootsma *et al.*, 2000; Wirth *et al.*, 2007). Recent research indicates that a population expansion (including the acquisition of virulence genes) probably occurred within the seroresistant lineage of *M. catarrhalis* around the time of hominid expansion some 5 million years ago (Wirth *et al.*, 2007). Further, the existence of these two subpopulations has implications for vaccine design, economics and vaccine development.

Several virulence-associated genes (and potential vaccine candidates) have been identified in *M. catarrhalis*, including the outer-membrane proteins UspA1 (N'Guessan *et al.*, 2007), UspA2 (Attia *et al.*, 2006), Hag (Pearson *et al.*, 2006), OMPCD (Akimana & Lafontaine, 2007), CopB (Sethi *et al.*, 1997) and lipooligosaccharide (LOS) (Akgul *et al.*, 2005). All of these vaccine candidates have been

Abbreviation: LOS, lipooligosaccharide.

A supplementary table with details of the test results for the 195 isolates is available with the online version of this paper.

associated with the production of antibody (Bakri *et al.*, 2002; Faden, 1995; Meier *et al.*, 2003; Murphy *et al.*, 2003, 2005b, c), although little is known about the frequency and expression of the corresponding genes in global isolates recovered from children and adults.

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

METHODS

Bacterial isolates. The bacterial isolates comprised 195 *Moraxella catarrhalis* isolates cultured between 2001 and 2002 from children and adults presenting with respiratory disease (GR Micro, London, UK). Approximately 40 random isolates from five world regions (11 countries) were included in the study (Tables 1 and 2). All isolates were cultured on Columbia blood agar at 37 °C.

Autoagglutination assay. *M. catarrhalis* autoagglutination was measured according to Pearson *et al.* (2002). Briefly, isolates were grown overnight on brain heart infusion (BHI) agar, washed and resuspended in 4 ml PBS to an OD₄₀₅ of 1.0 ± 0.1 (Pharmacia Biotech Novaspec II). The suspension was then allowed to stand undisturbed at room temperature. After 1 h, 1 ml aliquots were taken from the surface, the OD₄₀₅ measured, and the difference in the OD₄₀₅ between the two time points calculated.

Biofilm assay. Biofilm formation was assessed according to Pearson *et al.* (2006). *M. catarrhalis* isolates were grown in BHI broth overnight at 37 °C, resuspended in BHI broth to approximately $2-4 \times 10^8$ cells ml⁻¹ (OD₆₀₀ approximately 0.2 using a Pharmacia Biotech Novaspec II spectrophotometer) and 2 ml volumes were pipetted into 24-well cell culture plates (Corning, COSTAR) in duplicate. Plates were incubated for 19 h at 37 °C, then the BHI broth was replaced with 2 ml 199 medium/Earle's balanced salt solution/crystal violet followed by incubation at room temperature for 15 min. After washing three times with deionized water, 2 ml 95% ethanol was added and the plate gently rocked for 15 min. The absorbance of

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

Source	No. of isolates	
	Children	Adults
Ear fluid	14	1
Nasopharyngeal aspirate	27	13
Sinus	11	6
Blood	3	0
Throat	6	3
Unspecified	0	1
Sputum	33	61
Bronchoalveolar lavage	4	12

the ethanol solution was measured at 570 nm. Isolates whose duplicate absorbances differed by >25% were retested and an average from four A₅₇₀ readings was taken.

Serum resistance assay. Serum resistance was determined using the culture-and-spot test (Verduin *et al.*, 1995). A suspension of *M. catarrhalis* was made in PBS to an OD₆₀₀ of 1.0 ± 0.1 and 100 µl evenly spread over a Columbia blood agar plate. After drying, 50 µl pooled human serum (obtained from 10 healthy adult volunteers) and 50 µl heat-inactivated serum (30 min at 56 °C) were spotted onto the plate and the plate was incubated overnight at 37 °C. Isolates were considered serum resistant (no effect on growth), intermediate (effect on growth visible but >50 colonies growing in active serum zone), or sensitive (<50 colonies growing in active serum zone). No effect on growth should be observed for the heat-inactivated (control) serum.

PFGE typing. PFGE was performed as detailed by Verduin *et al.* (2000). Digestions were performed using *SpeI* at 20 U per reaction and the following electrophoresis protocol: the first block consisted of a constant voltage of 6 V cm⁻¹, with the pulse time increased from 3.5 to 25 s during the first 12 h; it was followed by a second block of 8 h during which the pulse time increased linearly from 1 to 5 s. PFGE patterns were analysed using GelCompar software (Applied Maths) with gel lanes normalized against a lambda DNA ladder (Bio-Rad) and band tolerance set at 1.5%. PFGE products between 48.5 and 339.5 kb were included in the analysis.

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

For the *hag* 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 with *FspBI* (2 U) and *HhaI* (2 U) according to the manufacturer's instructions. Gel electrophoresis revealed the following 16S rRNA types: type 1, *FspBI* 439/124 bp and *HhaI* 563 bp; type 2, *FspBI* 332/124/107 bp and *HhaI* 563 bp; type 3, *FspBI* 332/124/106 bp and *HhaI*

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

World region	Country	No. of isolates
North America	USA	20
	Canada	20
Western Europe	Belgium	4
	Germany	16
	Spain	20
Latin America	Brazil	14
	Ecuador	10
	Peru	5
	Venezuela	10
Far East	Japan	36
South Africa	South Africa	40

Table 3. Primer sequences used in this study

Primer	Sequence (5'–3')	Gene
uspA2start	cgctgtaaccagtgcattga	<i>uspA2</i>
uspA2end	acgatagccagcaccgatag	<i>uspA2/uspA2H</i>
uspA2end.f	gacagcagacgcattacca	<i>uspA2/uspA2H</i>
MeierRTB2-10	ttgagccatagccaccaagtgc	<i>uspA2H</i>
MeierRTF1-9	ctgaatttgccaaaggctcat	<i>uspA2H</i>
MeierRTF1-8	cgttatgcactaaaagacaggtc	<i>uspA1</i>
MeierRTB1-8	gcattctgaccagcttagaccaatc	<i>uspA1</i>
UspA1tl	tgtgagcaaatgactggc	<i>uspA1</i>
Pr406LOS	caaaagaagacaaacagcagc	LOS
Pr408LOS	catcaaaaacccccctacc	LOS
Pr649LOS	atcctgtctcaactgactttc	LOS
McCOPB2F	ggcgtgcgtgtgacgcttttg	<i>copB</i>
McCOPB2R	gtttggcaggcgataggcgacat	<i>copB</i>
Mcat.ompCD.f	acgcactggcaagaagctaga	<i>ompCD</i>
Mcat.ompCD.r	gacctgcaccaaccaagacat	<i>ompCD</i>
McatHag-2	gtcagcatgtatcatttttaagg	<i>hag</i>
McatHagR4	tgagcggtaaatggttaagtgc	<i>hag</i>
EUB-R	agagtttgatcctggttcag	16S rRNA
EUB-L	ctttacgcccttaataatccg	16S rRNA
mcaP-f	cgcaataagatcacatgcttg	<i>mcaP</i>
mcaP-r	cgggatcccgctgacacattgcataaa	<i>mcaP</i>

370/192 bp. The digestion method was based on the 16S rRNA type sequence results published by Bootsma *et al.* (2000).

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

The restriction digestion site for *ompCD* PCR product (*BseI*) was situated within the first A549 cell-binding domain (amino acids 16–236) of OMPCD (Akimana & Lafontaine, 2007).

UspA1 and Hag ELISA. The presence or absence of UspA1 and Hag expression was assessed in *uspA1* and *hag* PCR-positive isolates using a standard ELISA methodology and specific anti-UspA1 mAb 24B5 and anti-Hag 5D2 antibodies (kindly supplied by Professor E. Hansen, UT South-Western Medical Center at Dallas, Texas, USA). Briefly, *M. catarrhalis* isolates were grown overnight on Columbia blood agar at 37 °C and resuspended in PBS to an OD₆₀₀ of 0.5 (equivalent to approximately 5×10^8 cells ml⁻¹). One hundred microlitres of each suspension was then loaded into 96-well MaxiSorp ELISA plates (NUNC), and allowed to dry by overnight incubation at 37 °C. The following morning, plates were washed four times with PBS, blocked in 150 µl PBS with 2% (w/v) BSA and 5% (w/v) sucrose for 2 h at 37 °C, and then rewashed four times in PBS with 0.05% (v/v) Tween 20. One hundred microlitres of primary antibody was then added (at a 1:10 dilution of stock in PBS/BSA/sucrose for both 24B5 and 5D2) and incubated for 1 h at 37 °C, before being washed four times in PBS/Tween 20. One hundred microlitres of secondary antibody (1:5000 dilution of stock of polyclonal goat anti-mouse for 24B5 or 1:10 000 dilution in PBS/BSA/sucrose for 5D2) was added and incubated for 1 h at 37 °C before washing four times in PBS/Tween 20. Finally, 50 µl tetramethylbenzidine substrate was

added to each well and colour allowed to develop at room temperature; two PCR-negative isolates were included in triplicate in each ELISA plate. The geometric mean absorbance plus 4 times the standard deviation for the six negative control wells was calculated per plate and used as a negative cut-off value. Results were recorded as either positive or negative, dependent on this cut-off value.

RESULTS

Autoagglutination and biofilm assays

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

Results from the biofilm assay revealed no significant difference between upper versus lower respiratory tract infection, or world region (unpaired *t*-test $P=0.94$ and Kruskal–Wallis test $P=0.18$). Further, biofilm-forming ability was not significantly affected by the presence/absence of the *hag* or *uspA1* genes, *copB* type, 16S rRNA type, *ompCD* PCR RFLP type or LOS type. However, a significant difference in biofilm formation was observed between *M. catarrhalis* isolates cultured from children and adults ($P=0.002$) and between isolates carrying the mutually exclusive *uspA2* and *uspA2H* genes ($P<0.0001$) (Fig. 1).

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

Serum resistance assay

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

PFGE and 16S RNA types

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

PCR screening of genes

PCR screening of the 195 *M. catarrhalis* isolates revealed an incidence of 99 % (193/195) for *uspA1*, 90 % (176/195) for *hag*, 78 % (141/180) for *uspA2*, 22 % (39/180) for *uspA2H* and 99 % (194/195) for *mcaP*. The *copB* and *ompCD* genes were present in all the isolates screened. When divided into age groups, *uspA1* was found to be present in 99 % (97/98) of child isolates and 99 % (96/97) of adult isolates, and *hag* in 90 % (88/98) and 91 % (88/97), respectively. Interestingly, *uspA2* was present in 95 % (88/93) of child-associated but only 61 % (53/87) of adult-associated isolates, whilst the mutually exclusive *uspA2H* was present in only 5 % (5/93) of child-associated and 39 % (34/87) of adult-associated isolates (Fisher's exact test $P<0.0001$). Fifteen isolates (8 %) were found to be PCR-negative for *uspA2*- and *uspA2H*-specific PCRs, but positive using primers *uspA2end.f* and *uspA2end*. Although these isolates were not included in the above *uspA2* and *uspA2H* calculations, their inclusion as either *uspA2* or *uspA2H* would not have affected the significance of the results. All *uspA2H* PCR-positive isolates belonged to the 16S rRNA type 1, irrespective of age group and specimen source.

The majority of isolates belonged to LOS type A, representing 81 % (79/98) and 63 % (61/97) of child and adult isolates, respectively. The incidence of the LOS types is shown in Table 4. Within the age groups, 4 % (4/98) and

Table 4. Incidence of LOS types

<5, isolates from children; >20, isolates from adults.

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

*Incidence of 16S rRNA types 1/2/3 with respect to individual LOS types. A significant difference is observed between LOS types A and B and age group (Fisher's exact test $P=0.01$). All LOS B isolates and the great majority of LOS A isolates belonged to 16S rRNA type 1.

7 % (7/97) of isolates were untypable (generated no PCR products). All negative PCRs were tested twice by PCR and were shown to contain DNA using a 16S rDNA PCR.

copB and *ompCD* PCR RFLP

The distribution of CopB types was not significant between age groups, although there appeared to be a trend towards an increase in CopB types I/III (54/99) and a decrease in CopB type II (31/75) in adult-associated *M. catarrhalis* isolates. Further, CopB types I/III and II were almost exclusively associated with 16S rRNA type 1 (173/174 isolates), and CopB types 0 and IV were mainly associated with 16S rRNA types 2 and 3 (18/21 isolates).

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

UspA1 and Hag ELISA

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

All of the results described above are given in Supplementary Table S1, available with the online version of this paper.

DISCUSSION

The ability to autoagglutinate has been linked to virulence in several Gram-negative bacterial species, including *Moraxella bovis* (Gil-Turnes, 1983; Kapperud & Lassen, 1983; Misawa & Blaser, 2000). Further, the ability to autoagglutinate has been shown to be independent of the strain isolation site (i.e. lower versus upper respiratory

Table 5. Incidence of UspA1 and Hag expression

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

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

tract), which was also indicated by our own results (Janicka *et al.*, 2002). The loss of autoagglutination ability has previously been linked to the insertional inactivation of the haemagglutinin (*hag*) gene, but not the *uspA1* or *uspA2* genes, in *M. catarrhalis* isolate O35E (Aebi *et al.*, 1998; Pearson *et al.*, 2002). From our own population-based study, it appears that the *hag* gene may not be equally important in influencing autoagglutination ability in all isolates of *M. catarrhalis*.

Bacterial biofilms on the middle-ear mucosa are associated with chronic otitis media (Fergie *et al.*, 2004; Vlastarakos *et al.*, 2007). Further, *M. catarrhalis* is an important aetiological agent of otitis media in children that has been shown to be capable of forming such biofilms (Pearson *et al.*, 2006). Published evidence suggests a role for both UspA1 and UspA2H in biofilm formation (Pearson & Hansen, 2007; Pearson *et al.*, 2006). From our results, only two isolates were found to be *uspA1*-negative by PCR, which makes comparisons between *uspA1*-positive and -negative populations in our study difficult. Further, we found that isolates positive for *uspA2* (a gene always found to be mutually exclusive of *uspA2H*) were better biofilm formers than isolates carrying the mutually exclusive *uspA2H* gene, suggesting that *uspA2* could also have a role in biofilm formation. In fact, several recently described genes have also been associated with biofilm formation, including *mcmA*, *mcaP* (Lipski *et al.*, 2007a, b) and *pil* genes for type IV pili (TFP) (Luke *et al.*, 2004). In this study, the gene *mcaP* was equally distributed within child and adult age groups; *mcmA*, however, was not tested, as only one sequence is available (GenBank accession no. EF017300), meaning that conserved primers could not be designed. Luke *et al.* (2007) recently showed that TFP play an important role in nasopharyngeal colonization of *M. catarrhalis* and that biofilm formation is enhanced by TFP expression; and that TFP genes are ubiquitous within *M. catarrhalis* (Luke *et al.*, 2004). So it seems likely that a combination of several genes is important in biofilm formation by *M. catarrhalis*. Whatever the true situation, our results indicate that *M. catarrhalis* isolates from children are better biofilm formers than isolates from adults, possibly related to differences in *hag* gene

expression levels. The fact that no significant difference in biofilm formation was observed between isolates cultured from the upper and lower respiratory tract indicates that biofilm formation is probably not reliant on a particular cell type, unless that particular cell type or a particular receptor is present in both upper and lower respiratory tract tissues.

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

In this study, 99% of isolates possessed the *uspA1*, 78% *uspA2* and 22% *uspA2H* genes, an incidence similar to that previously reported by Meier *et al.* (2002) (99% *uspA1*, 79% *uspA2* and 21% *uspA2H*). However, significant differences in the incidence of *uspA2* (increased) and *uspA2H* (decreased) were observed between the child and adult groups. One possible scenario arising from our results is that children are initially immuno-naïve towards the various *M. catarrhalis* outer-membrane proteins (and especially *uspA2*), but gain a repertoire of neutralizing antibodies over time. Non-naïve adults on the other hand would generally possess neutralizing antibody (and in this scenario, especially anti-UspA2 antibodies), resulting in selection pressure for non-*uspA2*-expressing isolates. The reduced incidence of *uspA2* in adults could therefore be a consequence of immune evasion in *M. catarrhalis*. However, it should be noted that the presence of the *uspA2* or *uspA2H* gene does not necessarily indicate UspA2 or UspA2H expression (Wang *et al.*, 2007). At present there is unfortunately no antibody available that can specifically distinguish between the UspA2 and UspA2H proteins.

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

One of the major findings of this study was a statistically significant decrease in the incidence of *M. catarrhalis* *hag* gene expression (in isolates proven to possess the *hag* gene)

in adult-associated versus child-associated isolates. In contrast, a significant difference in the incidence of the *hag* gene between child- and adult-associated *M. catarrhalis* isolates was not found.

Hag expression appears therefore to be downregulated in *M. catarrhalis* isolates infecting adults, a phenomenon that could again possibly occur as part of an immune-evasion response. Hag (and indeed UspA1) have been previously shown to be immunogenic in children and adults (Meier *et al.*, 2003; Murphy *et al.*, 2005a, b, c). It is, however, possible that UspA1 and Hag expression *in vivo* may differ from that observed *in vitro*, meaning that further research is necessary to verify the clinical significance of our observations.

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

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