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*.

**INTRODUCTION**

*M. 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...
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 × 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 the ethanol solution was measured at 570 nm. Isolates whose duplicate absorbances differed by >25 % were retested and an average from four A_{570} 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 (BioRad) and band tolerance set at 1.5 %.

**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 FspI (2 U) and Hhal (2 U) according to the manufacturer’s instructions. Gel electrophoresis revealed the following 16S rRNA types: type 1, FspBI 439/124 bp and Hhal 563 bp; type 2, FspBI 332/124/107 bp and Hhal 563 bp; type 3, FspBI 332/124/106 bp and Hhal...
Table 3. Primer sequences used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Gene</th>
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<tbody>
<tr>
<td>uspA2start</td>
<td>cgctgtacaccgtcgcataga</td>
<td>uspA2</td>
</tr>
<tr>
<td>uspA2end</td>
<td>acgatgccacgcagattag</td>
<td>uspA2/uspA2H</td>
</tr>
<tr>
<td>uspA2end.f</td>
<td>gacgacgaagccatcaca</td>
<td>uspA2/uspA2H</td>
</tr>
<tr>
<td>MeierRTB2-10</td>
<td>ttgagctacagccaacagtc</td>
<td>uspA2H</td>
</tr>
<tr>
<td>MeierRTF1-9</td>
<td>tctgattgcaaaagctcat</td>
<td>uspA2H</td>
</tr>
<tr>
<td>MeierRTF1-8</td>
<td>gcttgactacaatagcaggg</td>
<td>uspA1</td>
</tr>
<tr>
<td>MeierRTB1-8</td>
<td>gactgcagcttgaccaaactc</td>
<td>uspA1</td>
</tr>
<tr>
<td>UspA1H</td>
<td>tggagcaaatgagctgc</td>
<td>uspA1</td>
</tr>
<tr>
<td>PrR406L</td>
<td>cacaagaagcaacaaagcatc</td>
<td>LOS</td>
</tr>
<tr>
<td>PrR408L</td>
<td>catcaaaaaacccctcacc</td>
<td>LOS</td>
</tr>
<tr>
<td>PrR649L</td>
<td>atctgcctcaacatgcttc</td>
<td>LOS</td>
</tr>
<tr>
<td>McCOPB2F</td>
<td>ggccgtgcgttggtgactgg</td>
<td>copB</td>
</tr>
<tr>
<td>McCOPB2R</td>
<td>gttggacgcctgcagcgcacat</td>
<td>copB</td>
</tr>
<tr>
<td>Mcat ompCF.d</td>
<td>acgacggaagaagacaga</td>
<td>ompCD</td>
</tr>
<tr>
<td>Mcat ompCD.r</td>
<td>gacgtcgcacaaacaaagcat</td>
<td>ompCD</td>
</tr>
<tr>
<td>Mcat Hag2</td>
<td>gtcagctagatcatttttaag</td>
<td>hag</td>
</tr>
<tr>
<td>Mcat HagR4</td>
<td>tgagcgcgtatgatcatttt</td>
<td>hag</td>
</tr>
<tr>
<td>EUB-R</td>
<td>aagagttcgtgctgctggctg</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>EUB-L</td>
<td>ctcttgacctttattacccg</td>
<td>16S rRNA</td>
</tr>
<tr>
<td>mcaP.F</td>
<td>cgcataaaagataccatgtgcttg</td>
<td>mcaP</td>
</tr>
<tr>
<td>mcaP-R</td>
<td>cgggtatccggctgacattctgctagaa</td>
<td>mcaP</td>
</tr>
</tbody>
</table>

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 I; 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 (BsaBI) 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 x 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 rewarshed 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 of stock of PBS/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).

Table 1. Differences in biofilm-forming ability (A570) 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).

<table>
<thead>
<tr>
<th>Age group</th>
<th>uspA2 vs uspA2H</th>
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<tr>
<td>&lt;5</td>
<td>0.75 (P=0.002)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>0.50 (P=0.0001)</td>
</tr>
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</table>

Fig. 1. Differences in biofilm-forming ability (A570) 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 7% (7/97) of isolates were untyptable (generated no PCR products). All negative PCRs were tested twice by PCR and were shown to contain DNA using a 16S rDNA PCR.

Table 4. Incidence of LOS types

<table>
<thead>
<tr>
<th>LOS type</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
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<tbody>
<tr>
<td>&lt;5, isolates from children</td>
<td>79</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>&gt;20, isolates from adults</td>
<td>61</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>16S type*</td>
<td>127/6/7</td>
<td>40/0/0</td>
<td>2/2/0</td>
</tr>
</tbody>
</table>

*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 $P=0.001$). All LOS B isolates and the great majority of LOS A isolates belonged to 16S rRNA type 1.

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).

<table>
<thead>
<tr>
<th></th>
<th>UspA1</th>
<th>Hag</th>
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<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&lt;5</td>
<td>95 (98 %) 2 (2 %)</td>
<td>81 (92 %) 7 (8 %)</td>
</tr>
<tr>
<td>&gt;20</td>
<td>93 (97 %) 3 (3 %)</td>
<td>64 (73 %) 24 (27 %)</td>
</tr>
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</table>

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 pilus (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 untypeable. Interestingly, LOS type B was twice as prevalent in isolates from adults compared to children and was exclusively associated with 16S rRNA type 1 isolates. This result could also possibly be a consequence of immune evasion by adult-infecting M. catarrhalis isolates, and has, in any case, negative consequences with respect to the development of single-type LOS vaccines.

One of the major findings of this study was a statistically significant decrease in the incidence of M. catarrhalis hag gene expression (in isolates proven to possess the hag gene)
in adult-associated versus child-associated isolates. In contrast, a significant difference in the incidence of the hag gene between child- and adult-associated *M. catarrhalis* isolates was not found.

Hag expression appears therefore to be downregulated in *M. catarrhalis* isolates infecting adults, a phenomenon that could again possibly occur as part of an immune-evasion response. Hag (and indeed UspA1) have been previously shown to be immunogenic in children and adults (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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