ORIGINAL ARTICLE 10.1111/j.1469-0691.2006.01582.x

High-throughput amplification fragment length polymorphism (htAFLP) analysis identifies genetic lineage markers but not complement phenotype-specific markers in *Moraxella catarrhalis*

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ABSTRACT

Comparative high-throughput amplified fragment length polymorphism (htAFLP) analysis was performed on a set of 25 complement-resistant and 23 complement-sensitive isolates of *Moraxella catarrhalis* in order to determine whether there were complement phenotype-specific markers within this species. The htAFLP analysis used 21 primer-pair combinations, generating 41 364 individual fragments and 2273 fragment length polymorphisms, with an average of 862 polymorphisms per isolate. Analysis of polymorphism data clearly indicated the presence of two phylogenetic lineages and 40 (2%) lineage-specific polymorphisms. However, despite the presence of 361 (16%) statistically significant complement phenotype-associated polymorphisms, no single marker was 100% complement phenotype-specific. Furthermore, no complement phenotype-specific marker was found within different phylogenetic lineages. These findings agree with previous results indicating that the complement resistance phenotype within *M. catarrhalis* is probably defined by multiple genes, although not all of these genes may be present within all *M. catarrhalis* isolates.

Keywords AFLP, complement resistance, genetic lineage, *Moraxella catarrhalis*, phylogenetic analysis, polymorphisms

Original Submission: 15 February 2006; **Revised Submission:** 9 June 2006; **Accepted:** 24 July 2006

Clin Microbiol Infect 2007; 13: 55–62

INTRODUCTION

Moraxella catarrhalis is a commensal organism in the human respiratory tract, but is also recognised as a pathogen for both children and adults. Carriage rates differ widely between children and adult populations, being *c.*77.5% and 2%, respectively [1,2]. In children, the organism has been associated with acute otitis media [3], sinusitis [4] and, infrequently, pneumonia [5] and bacteraemia [6]. The maximum rate of *M. catarrhalis* colonisation appears to occur in children aged 1–48 months [7], with strains appearing to spread among children in nursery schools and day-care centres [8]. Nosocomial spread has also been reported [9], although respiratory tract colonisation by a new *M. catarrhalis* genotype does not necessarily result in the production of overt disease [10]. In adults, the organism has been associated with exacerbations of chronic obstructive airways disease/bronchitis [11,12] and pneumonia [13]. At the present time, there appears to be an almost universal (> 90%) incidence of chromosomally-encoded β-lactamase-mediated resistance to penicillin-like antibiotics among clinical isolates [14,15]. Previous studies using a variety of different DNA typing techniques, including pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) [16], single adapter probe-generated RFLP (SAR-FLP), probe-generated restriction fragment length polymorphisms (pRFLP) and 16S rRNA sequencing [17], have suggested that genetic lineages or

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sub-populations of *M. catarrhalis* exist, and that these lineages exhibit different virulence potential, including the expression of complement-resistant and complement-sensitive phenotypes. However, these genotyping techniques have not been used previously to identify complement phenotype-specific polymorphisms *per se*.

Complement resistance has been established previously as a virulence trait in *M. catarrhalis* [18,19], with evidence suggesting that the mechanism of resistance may be facilitated via inhibition of membrane attack complex (MAC) formation, possibly following binding of the complement inhibitor vitronectin to the bacterial ubiquitous surface protein A2 (UspA2) [20–25]. However, this is not the only mechanism that has been described, as the iron-acquisition protein CopB [26,27], a 50-kDa outer membrane protein OmpE [28], a lipooligosaccharide P(k) epitope [29] and a ferric uptake regulator (*fur*) [30] have also been implicated in facilitating complement resistance within this species. In addition, several reports have indicated that clinical isolates of *M. catarrhalis* are more likely to be complement-resistant than are isolates obtained from healthy individuals [31,32].

In the present study, comparative genomic polymorphism analysis was performed on a set of complement-resistant and complement-sensitive isolates of *M. catarrhalis* in order to determine whether there are complement resistance-specific markers within this species. At the same time, a *uspA2* PCR-RFLP method was used to provide a standard internal genetic marker for the complement resistant/sensitive phenotype. The identification of complement phenotype-specific markers would: (i) help to confirm any relationship between complement resistance and genetic lineage; (ii) help to identify novel complement resistance-associated genes; and (iii) possibly assist in the development of rapid tests (including other virulence markers) to distinguish between virulent and non-virulent *M. catarrhalis* isolates.

MATERIALS AND METHODS

Bacterial isolates

Twenty-five complement-resistant and 23 complement-sensitive isolates of *M. catarrhalis* were chosen from the collection of isolates stored at −80°C by the Department of Medical Microbiology and Infectious Diseases at the Erasmus MC, Rotterdam, The Netherlands (Table 1). These isolates had been characterised previously by Verduin *et al*. [16] using several different genotyping techniques.

Table 1. *Moraxella catarrhalis* isolates used in high-throughput amplified fragment length polymorphism (htAFLP) analysis

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

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

Complement resistance testing

The complement-resistant or -sensitive phenotype of the 48 *M. catarrhalis* isolates studied was determined previously using the culture-and-spot test by Verduin *et al*. [33]. This is a rapid and simple test for determining the complement resistance phenotype of *M. catarrhalis*, and exhibits a statistically significant concordance with the serum bactericidal assay. For the purposes of the present study, any isolate

Table 2. 3′-end discriminating bases added to high-throughput amplified fragment length polymorphism (htAFLP) linker primers

	*Mbo*I primer			
***Mse*I Primer**	**+A**	**+C**	**+G**	**+T**
+ AA		+		
+ AC	+			+
+ AG				+
+ AT		+	+	
+ CA	+			
+ CT	+			+
+ GA	+			+
+ GT	+			+
+ TA		+	+	
+ TC	+			+
+ TG	+			+
+ TT		+	+	

found to express an intermediate complement-resistant phenotype (isolates F5.82, 1.24 and F6.92) was regarded as complement-resistant.

High-throughput AFLP analysis (htAFLP)

htAFLP analysis was performed at Keygene N.V. (Wageningen, The Netherlands), using primers labelled with radioactive phosphorous-33 as described previously [34]. Genomic DNA was digested using the restriction enzymes *Mbo*I and *Mse*I and heat-inactivated, followed by ligation of the restriction products to *Mse*I and *Mbo*I adapters. htAFLP amplification was performed using: (i) an *Mbo*I primer (5′-GTAGACTGCGTACCGATC) incorporating an extra selective nucleotide at its 3′-end (labelled with radioactive phosphorous-33); and (ii) an *Mse*I primer (5′-GACGATGAGTCCTGAGTAA) incorporating two extra selective nucleotides at its 3′-end (both unlabelled). In total, 21 primer combinations were used in the htAFLP analysis of the 48 *M. catarrhalis* isolates tested (Table 2). Analysis of htAFLP gel fingerprints used 1 μL of each htAFLP reaction, which was loaded on a denaturing polyacrylamide sequencing gel (5% w/v) along with a radioactively-labelled internal size marker. Fragments were separated by electrophoresis for 2 h at constant power (110 W). After electrophoresis, gels were fixed for 30 min in acetic acid 10% v/v, dried on glass plates, and exposed to phosphor image plates (Fuji, Rotterdam, The Netherlands). Fingerprint patterns were visualised using a BAS-2000 phosphor image plate scanner (Fuji) and the scanned images were analysed using AFLP-QuantarPro software (Keygene). Any fragment found to be present in 100% of *M. catarrhalis* isolates (after exclusion of equivocal results) was discarded from the total set of fragments generated and was not used for comparative analysis.

Phylogenetic analysis

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

htAFLP polymorphic fragment sequencing

A PCR sequencing methodology was used to identify DNA sequences associated with particular htAFLP polymorphic fragments. Essentially, a small piece of the relevant polymorphic fragment was recovered from the htAFLP gel and added directly to a PCR sequencing reaction mix containing the relevant htAFLP linker primer. PCR sequencing was performed using dye terminators on an ABI 3700 capillary sequencer (Applied Biosystems, Warrington, UK). The sequence data from each polymorphic fragment were then compared with the *M. catarrhalis* genome sequences available at GenBank (accession numbers AX067426 – AX067466, comprising 41 contigs ranging in size from 429 to 261 300 bp in length) in order to identify homologous open reading frame (ORF) sequences. The corresponding homologous ORF was then translated into protein, and the resulting protein sequence was used to search publicly available protein sequence databases to identify the nature of the original gene.

uspA2 PCR-RFLP

The *uspA2* gene of all 48 *M. catarrhalis* isolates was analysed by PCR-RFLP as a means of providing an internal genetic control for the complement-resistant or -sensitive phenotype, based on the findings of Attia *et al*. [24], who revealed that a 102-amino-acid region of the *uspA2* gene was important in conferring complement resistance in *uspA2*-carrying *M. catarrhalis* isolates. Initial *uspA2* gene amplification was achieved using PCR primer pair uspA2start (5′-CGCTGTAACCAGTGCCATGA) and uspA2end (5′-ACGATAGCCAGCACCGATAG), followed by amplimer digestion using restriction enzyme *Hae*III. Following gel electrophoresis, a dendrogram of the restriction fragment patterns was constructed using BioNumerics v.3.0 software (Applied Maths, Sint-Martens-Latem, Belgium). All *uspA2* PCRs (including negative reactions) were shown to contain PCR-amplifiable *M. catarrhalis* DNA via a confirmatory *ompJ* PCR using primer pair 19 kDaresf (5′-CTAACGCTGCCATCAGCTAT) and 19 kDaresr (5′-GTTGCATTACGGCTGGTAAC) [35].

RESULTS

htAFLP analysis

In total, 41 364 fragments were obtained using 21 htAFLP primer combinations with a set of 48 *M. catarrhalis* isolates. Further analysis revealed the presence of 2273 different fragment length polymorphisms, ranging in size from 67 to 493 bp, with an average fragment length of *c*. 200 bp. Using these values, the theoretical genome coverage of the polymorphic markers was estimated at $(2273 \times 200/1\ 912\ 671) \times 100 = 23.7\%$, where 1 912 671 is the number of bp present in the unannotated *M. catarrhalis* genomic sequence available at GenBank (accession numbers AX067426 – AX067466). The largest number of polymorphic markers (176 markers) was generated

using the selective nucleotide combination A/TC, and the fewest (61 markers) using the selective nucleotide combination T/CT.

Phylogenetic analysis

Comparative phylogenetic analysis of the htAFLP data obtained from the 48 isolates revealed a bifurcation in the distribution of htAFLP polymorphisms into two distinct phylogenetic lineages, exhibiting *c*. 5% difference in their branch depth. These two lineages were differentiated by *c*. 250 and 242 lineage-defining polymorphisms, respectively, with 40 (1.9%) htAFLP polymorphisms found to be 100% specific for one or the other of the two genetic lineages identified. Twenty-three of these lineage-specific polymorphisms mapped to the lineage with a shorter branch depth, while the remaining 17 polymorphisms mapped to the lineage with the longer branch depth. Interestingly, 22 of the 23 lineage 1-specific polymorphisms were also associated significantly with the complement-resistant phenotype ($p < 0.05$), while 16 of 17 cluster 2-specific polymorphisms were associated significantly with the complement-sensitive phenotype. Furthermore, there was a significant correlation between the complement phenotype and the two phylogenetic lineages (Fisher's exact test, p 0.016; Pearson's correlation $r = 0.38$ (0.104–0.597), p 0.008). However, an artificial rearrangement of the isolates into complement-resistant and complement-sensitive groups failed to reveal a polymorphism that was 100% specific for either complement phenotype (i.e., found in all isolates of either the complement-resistant or complement-sensitive phenotype only), with both complement-resistant and complement-sensitive groups clearly composed of multiple genotypes. Furthermore, no 100%-specific complement phenotype markers could be identified, even when the genetic lineages were considered separately. However, 361 (16%) polymorphisms were found to be associated significantly with the complement phenotype at $p < 0.05$ (Fisher's exact test), including 172 polymorphisms associated with complement resistance and 189 associated with complement sensitivity.

htAFLP polymorphic fragment sequencing

Using the results obtained from phylogenetic analysis, 13 polymorphic fragments found to be 100%-associated with the genetic lineage, and 17 polymorphic fragments found to be associated significantly with the complement phenotype, were chosen arbitrarily and sequenced. Of the genetic lineage-specific markers, 11 were mapped to putative housekeeping genes, while two were mapped to putative virulence-associated genes (Table 3). The majority of the complement phenotype-associated markers that were sequenced were found to map to putative housekeeping genes, although polymorphic marker F3121.511 <N was found to reside within the *copB* gene, which has been associated with iron-acquisition [36], is a target for antibodies involved in pulmonary clearance [37], and has been implicated in altering serum resistance *in vivo* [26].

The sequence data for the htAFLP fragments sequenced in this study are available from GenBank under accession numbers AY771621–AY771641 and AY944724–AY944731.

uspA2 PCR-RFLP

In total, 35 (73%) of the 48 isolates generated PCR products for PCR-RFLP analysis using the uspA2start/uspA2end primer-pair combination, although all 48 isolates were positive using a confirmatory *ompJ* PCR protocol (data not shown). Once assembled into a dendrogram, two main clusters of *uspA2* genes were observed (Fig. 1). For the 35 isolates that yielded *uspA2* PCR amplification products, the correlation between *uspA2* PCR-RFLP cluster and complement phenotype was highly significant (Pearson $r = 0.944$ (0.888–0.972), $p < 0.0001$), as was the correlation between *uspA2* PCR-RFLP cluster and htAFLP genetic lineage (Pearson $r = 0.542$ (0.255–0.741), $p < 0.0008$).

DISCUSSION

Complement resistance is a virulence trait of *M. catarrhalis* that appears to increase the pathogenic potential of this organism. Furthermore, the expression of the complement-resistant phenotype has been associated previously with distinct sub-populations or lineages of isolates, and the identification of complement phenotype-specific markers would help to further define the relationship between complement resistance and genetic lineage within this species. A range of such virulence markers would also be useful in

Table 3. High-throughput amplified fragment length (htAFLP) polymorphic fragments associated with *Moraxella catarrhalis* lineage and complement phenotype

htAFLP fragment	Size (bp)	Lineage	Putative gene description	Predicted function of identified ORF	Accession number
100% specific for genetic lineage					
A / AC F-134.585 < N[a]	109	1	Probable carboxyl-terminal protease	Protein metabolism	AY771621
A / AC F-379.208 < N[a]	353	1	N-acetylglutamate synthase	Protein metabolism	AY771622
A / CA F-166.034 < N[a]	145	1	Phosphatase domain-containing protein	Unknown	AY771623
A / CA F-174.909 < N[a]	151	1	Lipid A disaccharide synthase (*lpxB*)	Biosynthesis of lipopolysaccharide	AY771624
A / CA F-177.244 < N[a]	163	1	Elongation factor G	Protein metabolism	AY771625
A / CA F-266.454 < N[a]	245	1	DnaJ domain-containing protein	Molecular chaperone	AY771626
A / GA F-189.403 < N[a]	160	1	Signal transduction histidine kinase	Signal transduction	AY771629
A / GA F-256.710 < N[a]	228	1	Colicin tolerance gene (*tolB*)[#]	Colicin A sensitivity/resistance	AY771631
A / GA F-375.414 < N[a]	352	1	Ferredoxin-NADP+ reductase	Protect against oxidative stress	AY771632
A / TC F-227.958 < N[a]	201	1	Lipoprotein precursor *(nlpD)*	Cell wall formation	AY771634
A / AC F-239.018 < N[b]	217	2	Flavodoxin reductase	Protects against oxygen radicals	AY771638
A / CA F-173.831 < N[b]	144	2	Putative competence factor[c]	Bacterial competence	AY771639
A / CT F-301.112 < N[b]	280	2	Hypothetical protein	Unknown	AY771640
Associated with the complement-resistant phenotype ($p < 0.05$)					
A / CA F-315.261 < N	287		Putative monooxygenase	Energy production + conversion	AY771627
A / GA F-112.386 < N	86		Mg-dependent DNase	DNase	AY771628
A / GA F-217.923 < N	135		Response regulator (*gacA*)	Transcription/response regulator	AY771630
A / CT F-321.511 < N	29		Major outer membrane protein CopB	Iron acquisition / Serum resistance	AY944723
A / TC F-360.194 < N	260		Adenylosuccinate synthetase	Purine ribonucleotide synthesis	AY771635
A / TC F-217.777 < N	193		Macrophage infectivity potentiator protein - Macrophage infection - Acyl-coA dehydrogenase (intergenic)	Energy production	AY771633
T / TG F-536.245 < N	501		Homoserine dehydrogenase	Protein metabolism	AY944724
C / TA F-194.337 < N	163		16S rRNA processing protein (RimM)	Maturation of 30S rRNA	AY944725
C / TA F-224.476 < N	174		Membrane fusion protein - Cation/multidrug efflux pump (intergenic)	Membrane fusion / efflux pump	AY771636
G / TA F-391.095 < N	363		ParA ATPase	Partitioning of bacterial plasmids	AY771637
G / AT F-191.261 < N	121		Leucyl, phenylalanyl-tRNA-protein transferase	Protein metabolism	AY944726
Associated with the complement-sensitive phenotype ($p < 0.05$)					
A / AC F-316.046 < N	289		Succinate-semi-aldehydedehydmgenase	Energy production+conversion	AY944727
A / TC F-318.602 < N	303		Dihydrolipoamide acetyltransferase	Energy production + conversion	AY771641
T / AG F-168.547 < N	142		Pyridoxamine 5-phosphate oxidase	Co-enzyme metabolism	AY944728
C / AT F-214.578 < N	189		Guanosine-3′, 5- bis (diphosphate)	Purine metabolism	AY944729
G / AT F-142.047 < N	115		Replication helicase	DNA unwinding	AY944730
G / AT F-310.917 < N	285		Promoter for acetaldehyde dehydrogenase II	Energy production + conversion	AY944731

[a] also associated with the complement resistant phenotype ($p < 0.05$).
[b] also associated with the complement sensitive phenotype ($p < 0.05$).
[c] putative virulence gene; putative genes assigned via in-silico translation of htAFLP fragment followed by BLASTp searching (http://www.ncbi.nih.gov/BLAST/), as well as by reference to the *M. catarrhalis* genome sequence available at GenBank (accession numbers AX067426 - AX067466).

the development of rapid tests to distinguish between *M. catarrhalis* isolates exhibiting increased virulence potential, and could possibly influence the choice of treatment (antibiotic therapy vs. 'watchful waiting'). Such markers would also help to identify novel complement resistance-associated genes, and the mechanisms influencing their expression, e.g., mutations associated with gene promoter regions or nonsense shifts in mRNA reading-frames.

In order to achieve this aim, an intensive genetic analysis of *M. catarrhalis* was performed, utilising 2273 polymorphic markers from 48 isolates, in an attempt to identify complement phenotype-specific markers (i.e., markers found in 100% of either complement-resistant or complement-sensitive phenotypes). However, although phylogenetic analysis revealed several hundred lineage-associated genetic markers, i.e., markers associated significantly with genetic lineage (including 40 lineage-specific markers), no marker was found that was 100% specific for the complement phenotype. Furthermore, no complement phenotype-specific marker could be found when the two major genetic lineages were compared separately. However, the data did reveal a similar number of lineage-associated and complement phenotype-associated genetic markers, with a statistically significant correlation between genetic lineage and complement phenotype, and 38 of the 40 lineage-specific markers were also found to be associated significantly with the complement phenotype.

The spread of complement resistance-conferring genes through *M. catarrhalis* sub-populations could occur via either the horizontal or vertical (including clonal expansion of an individual isolate) transfer of genes. It is also conceivable that complement resistance could be conferred by a single gene, several individual genes, or by

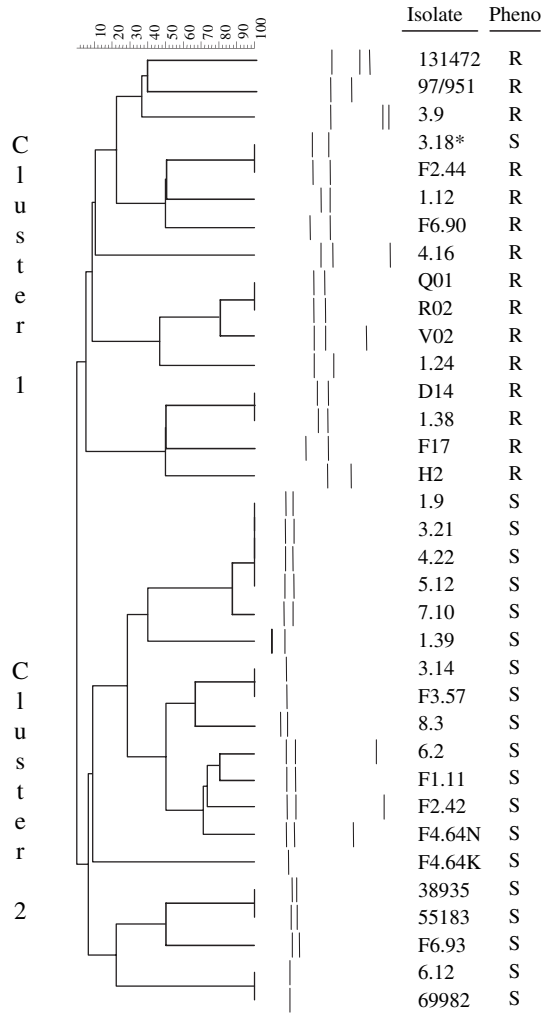

Fig. 1. Dendrogram showing the relationship between *Moraxella catarrhalis uspA2* genes after *Hae*III PCR-RFLP. The dendrogram was created using the DICE coefficient with the band tolerance set at 1%. Of the 48 isolates tested, 13 (27%) failed to generate *uspA*2 PCR amplification products, and are therefore not included in the analysis. Isolate, *M. catarrhalis* isolate; Pheno, complement phenotype, where R = resistant and S = sensitive. * = outlier.

multiple genes working in cooperation. However, if multiple genes, working in cooperation, were required to achieve the complement-resistant phenotype, then the likelihood of horizontal transfer of these genes would be reduced unless the genes were in linkage disequilibrium. From the present results, the absence of complement phenotype-specific markers indicates that polymorphisms associated with complement resistance-conferring genes are spread throughout the complement-resistant and complement-sensitive phenotypes of *M. catarrhalis*, suggesting that complement resistance is actually conferred by multiple genes (not universally present in all isolates), as the presence of a single homologous complement resistance-conferring gene would lead to gene-specific, and hence complement resistance-specific, polymorphisms being detected. Furthermore, even if heterologous forms of the same complement resistance-conferring gene were found to be present within complement-resistant and complement-sensitive isolates (as is the case with the *uspA2* gene, which may comprise a hybrid *uspA1/uspA2* gene in up to 20% of isolates [38]), then polymorphisms associated with complement resistance-conferring sequences (including hybrid sequences) would be observed. This conclusion agrees with previous reports suggesting that several different genes are associated with the complement-resistant phenotype in *M. catarrhalis*, although only the UspA2 (and closely related) UspA1 proteins, to date, have been shown to interact functionally with complement system components.

With regard to the htAFLP methodology *per se*, sequence analysis of a small proportion of polymorphic fragments identified polymorphisms in three putative virulence genes, indicating that the htAFLP methodology allowed detection of polymorphism in virulence-associated (e.g., complement resistance-associated) genes. In addition, an internal control PCR-RFLP strategy using a gene associated previously with complement resistance in *M. catarrhalis* (i.e., *uspA2*) verified the significant association between htAFLP genetic lineage and the complement-resistant phenotype (as reported previously following the use of other genotyping techniques by Verduin *et al*. [16] and Bootsma *et al*. [17]). Finally, in-silico restriction digestion and htAFLP analysis of the *uspA2* gene sequence of a patent *M. catarrhalis* isolate (GenBank accession numbers AX067426 – AX067466) indicated that the htAFLP protocol used in this study would be expected to generate at least 20 bands for polymorphic marker analysis in this gene alone. Taken together, these results show that the htAFLP protocol used to analyse this *M. catarrhalis* population yielded genotyping results consistent with previous reports, and was, theoretically, sufficiently sensitive to detect polymorphic markers associated with complement resistance-conferring genes.

In the context of the development of rapid tests to distinguish virulent *M. catarrhalis* isolates, 40 lineage-specific polymorphic markers and 13

genes that carry these markers, but no complement phenotype-specific markers, were identified. Moreover, no multiplex combination of markers (maximum of three different markers) could be found among the 361 significant complement phenotype markers that would distinguish completely between complement-resistant and complement-sensitive isolates. htAFLP analysis does not, therefore, appear to be a suitable methodology for identifying genetic polymorphisms that could be useful in the development of rapid diagnostic tests for determining the complement resistance phenotype in *M. catarrhalis*.

Finally, it was found that the htAFLP strategy tended to identify polymorphism within putative housekeeping genes as being associated with complement phenotype. This association is most likely to occur as a consequence of a shared genetic lineage among *M. catarrhalis* isolates already possessing complement resistance-associated genes, and does not indicate that the putative housekeeping genes identified are involved in complement resistance *per se*.

In conclusion, the present study provides the most detailed polymorphism analysis of the *M. catarrhalis* species published to date, utilising comparative genomic polymorphism analysis to search for complement phenotype-specific markers within sub-populations of *M. catarrhalis*. The fact that 40 lineage-specific markers, but no complement phenotype-specific markers, were found, suggests that *M. catarrhalis* utilises multiple (combinations of) genes, which may not be present in all isolates, as a means of achieving the complement-resistant phenotype. The study revealed that *M. catarrhalis* is predominantly clonal for the complement-resistant isolates tested, with two major phylogenetic lineages. However, isolates expressing the complement resistance phenotype may be observed within both of these major lineages.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Ministry of Economic Affairs (BTS 00145) and the SOPHIA Foundation for Medical Research, The Netherlands (Project: 397).

REFERENCES

1. DiGiovanni C, Riley TV, Hoyne GF, Yeo R, Cooksey P. Respiratory tract infections due to *Branhamella catarrhalis*: epidemiological data from western Australia. *Epidemiol Infect* 1987; **99**: 445–453.
2. Faden H, Harabuchi Y, Hong JJ. Epidemiology of *Moraxella catarrhalis* in children during the first 2 years of life: relationship to otitis media. *J Infect Dis* 1994; **169**: 1312–1317.
3. Kilpi T, Herva E, Kaijalainen T, Syrjanen R, Takala AK. Bacteriology of acute otitis media in a cohort of Finnish children followed for the first two years of life. *Pediatr Infect Dis J* 2001; **20**: 654–662.
4. Wald ER. The microbiology of chronic sinusitis in children. A review. *Acta Otorhinolaryngol Belg* 1997; **51**: 51–54.
5. Claesson BA, Leinonen M. *Moraxella catarrhalis* – an uncommon cause of community-acquired pneumonia in Swedish children. *Scand J Infect Dis* 1994; **26**: 399–402.
6. Abuhammour WM, Abdel-Haq NM, Asmar BI, Dajani AS. *Moraxella catarrhalis* bacteremia: a 10-year experience. *South Med J* 1999; **92**: 1071–1074.
7. Ejlertsen T, Thisted E, Ebbesen F, Olesen B, Renneberg J. *Branhamella catarrhalis* in children and adults. A study of prevalence, time of colonisation, and association with upper and lower respiratory tract infections. *J Infect* 1994; **29**: 23–31.
8. Prellner K, Christensen P, Hovelius B, Rosen C. Nasopharyngeal carriage of bacteria in otitis-prone and non-otitis-prone children in day-care centres. *Acta Otolaryngol* 1984; **98**: 343–350.
9. Morgan MG, McKenzie H, Enright MC, Bain M, Emmanuel FX. Use of molecular methods to characterize *Moraxella catarrhalis* strains in a suspected outbreak of nosocomial infection. *Eur J Clin Microbiol Infect Dis* 1992; **11**: 305–312.
10. Hays JP, Eadie K, Verduin CM, Hazelzet J, Verbrugh H, van Belkum A. Changes in genetic types and population dynamics of *Moraxella catarrhalis* in hospitalized children are not associated with an exacerbation of existing disease. *J Med Microbiol* 2003; **52**: 815–820.
11. Lieberman D, Ben-Yaakov M, Lazarovich Z *et al.* Infectious etiologies in acute exacerbation of COPD. *Diagn Microbiol Infect Dis* 2001; **40**: 95–102.
12. Sethi S, Evans N, Grant BJ, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *N Engl J Med* 2002; **347**: 465–471.
13. Lim WS, Macfarlane JT, Boswell TC *et al.* Study of community acquired pneumonia aetiology (scapa) in adults admitted to hospital: implications for management guidelines. *Thorax* 2001; **56**: 296–301.
14. Bootsma HJ, van Dijk H, Vauterin P, Verhoef J, Mooi FR. Genesis of BRO beta-lactamase-producing *Moraxella catarrhalis*: evidence for transformation-mediated horizontal transfer. *Mol Microbiol* 2000; **36**: 93–104.
15. Chaibi EB, Mugnier P, Kitzis MD, Goldstein FW, Acar JF. Beta-lactamases of *Branhamella catarrhalis* and their phenotypic implications. *Res Microbiol* 1995; **146**: 761–771.
16. Verduin CM, Kools-Sijmons M, van der Plas J *et al.* Complement-resistant *Moraxella catarrhalis* forms a genetically distinct lineage within the species. *FEMS Microbiol Lett* 2000; **184**: 1–8.
17. Bootsma HJ, van der Heide HG, van de Pas S, Schouls LM, Mooi FR. Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. *J Infect Dis* 2000; **181**: 1376–1387.

18. Hol C, Verduin CM, Van Dijke EE, Verhoef J, Fleer A, van Dijk H. Complement resistance is a virulence factor of *Branhamella (Moraxella) catarrhalis FEMS Immunol Med Microbiol* 1995; **11**: 207–211.
19. Hol C, Verduin CM, van Dijke E, Verhoef J, van Dijk H. Complement resistance in *Branhamella (Moraxella) catarrhalis*. *Lancet* 1993; **341**: 1281.
20. Nordstrom T, Blom AM, Forsgren A, Riesbeck K. The emerging pathogen *Moraxella catarrhalis* interacts with complement inhibitor c4b binding protein through ubiquitous surface proteins A1 and A2. *J Immunol* 2004; **173**: 4598–4606.
21. Verduin CM, Jansze M, Verhoef J, Fleer A, van Dijk H. Complement resistance in *Moraxella (Branhamella) catarrhalis* is mediated by a vitronectin-binding surface protein. *Clin Exp Immunol* 1994; **97**: 50.
22. Aebi C, Lafontaine ER, Cope LD *et al*. Phenotypic effect of isogenic *uspA1* and *uspA2* mutations on *Moraxella catarrhalis* 035e. *Infect Immun* 1998; **66**: 3113–3119.
23. McMichael JC, Fiske MJ, Fredenburg RA *et al*. Isolation and characterization of two proteins from *Moraxella catarrhalis* that bear a common epitope. *Infect Immun* 1998; **66**: 4374–4381.
24. Attia AS, Lafontaine ER, Latimer JL, Aebi C, Syrogiannopoulos GA, Hansen EJ. The UspA2 protein of *Moraxella catarrhalis* is directly involved in the expression of serum resistance. *Infect Immun* 2005; **73**: 2400–2410.
25. Nordstrom T, Blom AM, Tan TT, Forsgren A, Riesbeck K. Ionic binding of c3 to the human pathogen *Moraxella catarrhalis* is a unique mechanism for combating innate immunity. *J Immunol* 2005; **175**: 3628–3636.
26. Helminen ME, Maciver I, Paris M *et al*. A mutation affecting expression of a major outer membrane protein of *Moraxella catarrhalis* alters serum resistance and survival in vivo. *J Infect Dis* 1993; **168**: 1194–1201.
27. Aebi C, Stone B, Beucher M *et al*. Expression of the CopB outer membrane protein by *Moraxella catarrhalis* is regulated by iron and affects iron acquisition from transferrin and lactoferrin. *Infect Immun* 1996; **64**: 2024–2030.
28. Murphy TF, Brauer AL, Yuskiw N, Hiltke TJ. Antigenic structure of outer membrane protein ε of *Moraxella catarrhalis* and construction and characterization of mutants. *Infect Immun* 2000; **68**: 6250–6256.
29. Zaleski A, Scheffler NK, Densen P *et al*. Lipooligosaccharide p(k) (galalpha1–4galbeta1–4glc) epitope of *Moraxella catarrhalis* is a factor in resistance to bactericidal activity mediated by normal human serum. *Infect Immun* 2000; **68**: 5261–5268.
30. Furano K, Campagnari AA. Inactivation of the *Moraxella catarrhalis* 7169 ferric uptake regulator increases susceptibility to the bactericidal activity of normal human sera. *Infect Immun* 2003; **71**: 1843–1848.
31. Enright MC, McKenzie H. *Moraxella (Branhamella) catarrhalis* – clinical and molecular aspects of a rediscovered pathogen. *J Med Microbiol* 1997; **46**: 360–371.
32. Wolf B, Kools-Sijmons M, Verduin C *et al*. Genetic diversity among strains of *Moraxella catarrhalis* cultured from the nasopharynx of young and healthy Brazilian, Angolan and Dutch children. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 759–764.
33. Verduin CM, Hol C, Van Dijke E *et al*. Assessment of complement-mediated killing of *Moraxella (Branhamella) catarrhalis* isolates by a simple method. *Clin Diagn Lab Immunol* 1995; **2**: 365–368.
34. Vos P, Hogers R, Bleeker M *et al*. AFLP. A new technique for DNA fingerprinting. *Nucl Acids Res* 1995; **23**: 4407–4414.
35. Hays JP, van Selm S, Hoogenboezem T *et al*. Identification and characterization of a novel outer membrane protein (OMP J) of *Moraxella catarrhalis* that exists in two major forms. *J Bacteriol* 2005; **187**: 7977–7984.
36. Bonnah RA, Yu RH, Wong H, Schryvers AB. Biochemical and immunological properties of lactoferrin binding proteins from *Moraxella (Branhamella) catarrhalis*. *Microb Pathog* 1998; **24**: 89–100.
37. Helminen ME, Maciver I, Latimer JL, Cope LD, McCracken GH, Hansen EJ. A major outer membrane protein of *Moraxella catarrhalis* is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model. *Infect Immun* 1993; **61**: 2003–2010.
38. Lafontaine ER, Cope LD, Aebi C, Latimer JL, McCracken GH, Hansen EJ. The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella catarrhalis* to human epithelial cells in vitro. *J Bacteriol* 2000; **182**: 1364–1373.