Characterisation of hospital isolates of *Moraxella* (*Branhamella*) *catarrhalis* by SDS-PAGE of whole-cell proteins, immunoblotting and restriction-endonuclease analysis

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Summary. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell proteins (WCP), immunoblot analysis and DNA restriction-endonuclease analysis (REA) were applied as potential typing methods to 31 clinically significant strains of *Moraxella* (*Branhamella*) *catarrhalis*, five of which came from a suspected outbreak of nosocomial infection in a respiratory-diseases ward. Twelve of 31 isolates were placed in four groups, each of which contained strains indistinguishable by the three typing techniques used. Each of a further two groups contained two strains, and they were similar by at least one technique; the remaining 15 strains were unique by all three methods. Four of five strains from the suspected outbreak were indistinguishable by SDS-PAGE of WCP, immunoblotting and REA. Results show that SDS-PAGE of WCP, immunoblotting and REA are suitable techniques for characterising *M. catarrhalis* and that there is a considerable degree of strain heterogeneity. Nosocomial infection with *M. catarrhalis* may be relatively common and further epidemiological studies with a combination of typing techniques are indicated.

Introduction

Moraxella (*Branhamella*) *catarrhalis* is now generally accepted as a respiratory pathogen, ranking third in frequency of isolation after *Streptococcus pneumoniae* and *Haemophilus influenzae*. It is most commonly involved in acute exacerbations of chronic bronchitis, but is occasionally responsible for pneumonia,¹ otitis media,² sinusitis³ and eye infections.⁴ Nosocomial infection with this organism has previously been suspected,^{5,6} but has been difficult to prove because of the absence of a typing system allowing epidemiological investigation of putative outbreaks.

SDS-PAGE of outer-membrane proteins from 50 different strains of *M. catarrhalis* revealed a high degree of homogeneity⁷ and plasmid analysis was of little value in differentiating strains^{6,8} because plasmids are found infrequently in *M. catarrhalis*. Iso-electric focusing of β -lactamase enzymes⁹ and bacteriocin typing⁵ have also been investigated and found to be of little value. Sixteen different lipopolysaccharide antigens were described in a study of 302 *M. catarrhalis* strains, but 93% of isolates fell into only three common antigen types.¹⁰ However, strains have been

successfully differentiated on the basis of esterase electrophoresis¹¹ and a hospital outbreak of *M. catarrhalis* infection has been confirmed by restriction-endonuclease analysis.¹²

We have explored the use of SDS-PAGE analysis of whole-cell proteins (WCP), immunoblotting with pooled normal human serum and DNA restriction-endonuclease analysis (REA) as means of differentiating strains of *M. catarrhalis*. These techniques were applied to 31 clinically significant isolates including five strains from a suspected outbreak of nosocomial infection.

Materials and methods

Organisms

Thirty-one clinically significant isolates of *M. catarrhalis* were studied, including five strains from a suspected outbreak of nosocomial infection in a respiratory ward (in Edinburgh) and a further 26 strains selected from routine hospital isolates (in Aberdeen). The only criteria of selection were that isolates were from symptomatic patients and *M. catarrhalis* was the only, or predominant, isolate. In addition, the type strain of *M. (B.) catarrhalis* (NCTC 11020) and two non-pathogenic strains of *Neisseria* (*N. pharyngis* NCTC 4590 and *N. lactamica* NCTC

Fig. 1. SDS-PAGE of WCP of control strains and 31 clinical isolates of *M. catarrhalis*: *M. catarrhalis* strain NCTC 11020 (lane 1); *N. pharyngis* strain NCTC 4590 (2); *N. lactamica* strain NCTC 1061 (3); five strains of *M. catarrhalis* from a suspected nosocomial outbreak of infection in a respiratory-diseases ward (4–8); other clinical isolates of *M. catarrhalis* (9–34). Mol.-wt markers (kDa) are shown to the right of each figure.

1061) were also examined. All *M. catarrhalis* strains gave negative reactions in standard sugar-fermentation tests and positive ones for DNAase production, nitrate reduction and tributyrin hydrolysis.

SDS-PAGE and immunoblotting

These were performed broadly as described before,¹³ except as detailed below. All strains were cultured on

Fig. 2. Immunoblot profiles of organisms described, and in same lane order as, in fig. 1. Mol.-wt markers (kDa) are shown.

blood agar for 24 h at 37°C in CO₂ 5% in air and harvested, washed and resuspended in sterile saline before sonication. Samples were cooled in an ice bath during sonication for 2 min (Lucas Dawe Ultrasonics Soniprobe). Sonicated extracts were stored at -70°C until use. For SDS-PAGE and immunoblotting, sonicated extracts were mixed with three volumes of 0.01 M-Tris-hydrochloride, pH 6.8, containing SDS

4.4% w/v, glycerol 20% v/v, 2-mercaptoethanol 20% v/v and bromophenol blue 0.001% w/v, and boiled for 5 min in a water bath. Samples were centrifuged for 5 min at 13000 rpm in a Micro-centaur centrifuge (MSE) and 30 µl of supernate containing *c.* 1 mg of protein/ml, as determined by the method of Bradford,¹⁴ was subjected to electrophoresis on an acrylamide 10% separating gel with a 4% stacking

Fig. 3. DNA restriction-endonuclease analysis of 31 clinical isolates of *M. catarrhalis* described, and in same lane order (4-34) as, in figs. 1 and 2.

gel. Electrophoresis was continued until the leading edge of the dye reached the end of the gel and polypeptide bands were stained with Coomassie Brilliant Blue R.

Immunoblotting was performed by overnight electrophoretic transfer of material from acrylamide gels to nitrocellulose membranes (Hybond-C, Amersham, Bucks). Membranes were incubated for 1 h at 37°C in a blocking buffer of 0.1 M phosphate-

buffered saline, pH 7.4, containing Skimmed-Milk Powder (Oxoid) 3% w/v. A single batch of pooled normal human serum (Blood Transfusion Service, Aberdeen Royal Infirmary), diluted 1 in 100 in blocking buffer, was used as the primary antibody and peroxidase-labelled anti-human IgG (SAPU, Law Hospital, Carlisle), diluted 1 in 200 in blocking buffer, was used to detect antibody binding.

DNA extraction and restriction analysis

Total cellular DNA was extracted by a modification of a method described elsewhere.¹⁵ Briefly, bacterial colonies from an overnight culture on a blood-agar plate were harvested in 300 µl of 50 mM Tris (pH 8) with sucrose 25% in a microfuge tube. Tubes were placed on ice and the following solutions were added: lysozyme (50 mg/ml) 100 µl, 0.2 M EDTA 60 µl, lysing solution (containing 10 mM EDTA, Triton X-100 2% v/v and 50 mM Tris, pH 8) 300 µl and proteinase K (5 mg/ml) 10 µl. Resulting suspensions were mixed by inversion, stored on ice for at least 1 h and extracted once with phenol-chloroform and four times with water-saturated phenol. DNA was precipitated with an equal volume of cold (−20°C) isopropanol followed by storage at −20°C for 30 min, and collected by centrifugation for 2.5 min. DNA was resuspended in 400 µl of 10 mM Tris (pH 8) with 1 mM EDTA and 30 µl of 5 M NaCl; 5 mM spermidine 10 µl was added and DNA was reprecipitated by storage at −20 °C and centrifugation, as before. Final DNA pellets were resuspended in sufficient 10 mM Tris with 1 mM EDTA to saturate the DNA (c. 400 µl) and left at 4°C overnight to dissolve.

DNA (5 µg) was digested with c. 10 units of *Taq* I restriction endonuclease in a total volume of 10 µl under conditions recommended by the manufacturer (Boehringer Mannheim) and in the presence of RNAase 0.1 mg/ml. The resulting fragments were separated on horizontal agarose 0.8% gels in 89 mM Tris, 89 mM borate, 2 mM EDTA, pH 8.3. Electrophoresis was terminated when the bromophenol-blue marker reached the end of the gel. Gels were stained with ethidium bromide and photographed under ultraviolet transillumination.

Results

The WCP profiles of all strains determined by SDS-PAGE are shown (fig. 1). The patterns obtained were complex, but clear differences between strains of *Neisseria* and *M. (B.) catarrhalis* were evident, particularly in relation to the characteristic, heavily-staining bands of 48–60 kDa found in all *M. catarrhalis* strains. Considerable variation among *M. catarrhalis* strains was evident; 23 different WCP patterns were observed among 31 clinical isolates, 19 of which had unique patterns. The remaining 12 strains were classified in four groups, each of indistinguishable WCP pattern, although there were minor differences only among strains of groups 2 and 3 (fig. 1a).

Immunoblotting of the same strains with pooled normal human serum detected a range of *M. catarrhalis* antigens (fig. 2), usually with 1–3 prominent antigenic bands per strain. Major antigens were not detected in strains of *N. lactamica* and *N. pharyngis* (fig. 2a). Comparison of immunoblot patterns confirmed the four SDS-PAGE groupings, with clear differences among strains of groups 2 and 3. Immunoblotting also revealed a fifth grouping of two strains which had shown some minor differences in WCP patterns (group 5; lanes 18 and 20, figs. 1b and 2b). The remaining 17 strains had unique immunoblot patterns.

DNA-restriction analysis with *Taq* I enzyme was also performed on the 31 clinical isolates of *M. catarrhalis* and the results are shown (fig. 3). Comparison of REA patterns confirmed the five groupings previously obtained from SDS-PAGE and immunoblotting for 14 strains, but also showed similar patterns of restriction fragments for two strains (group 6; lanes 25 and 30; fig. 3b) which were not identical by SDS-PAGE or immunoblotting. Repeat tests on strains in group 6 confirmed that they were indistinguishable by REA but not by other methods. The

Table 1. Comparison of results for 31 strains of *M. catarrhalis* examined by SDS-PAGE of WCP, immunoblotting and REA

Group	Number of isolates	Lane nos.*	Source	Identity confirmed by			Unique by all methods
				SDS-PAGE of WCP	immuno-blotting	REA	
1	4	4, 6–8	Respiratory ward, Edinburgh	+	+	+	—
2	3	9–11	Medical unit, Aberdeen	+	+	+	—
3	2	12, 13	Unrelated wards	+	+	+	—
4	3	14–16	Unrelated wards	+	+	+	—
5	2	18, 20	Adjoining paediatric wards, Aberdeen	—	+	+	—
6	2	25, 30	Unrelated wards	—	—	+	—
7	15	5, 17, 19, 21–24, 26–29, 31–34	Various	—	—	—	+

* Lane nos., fig. 1.

Table II. Sources of *M. catarrhalis* strains from a suspected outbreak of nosocomial infection in a respiratory ward in Edinburgh

Isolate/ lane no.*	Patient	Disease	Date of	
			admission	isolation
4	M 69	COAD	23 Feb.	25 Feb.
5	M 47	Bronchiectasis	20 Feb.	26 Feb.
6 } 7 }	M 63	COAD	23 Feb. }	27 Feb.
8	M 51	Asthma	21 Feb.	2 March

COAD, chronic obstructive airways disease.

* Lane nos., see fig. 1.

extent of agreement among the three methods used to characterise the 31 strains is summarised (table I).

Four of five suspected outbreak strains (from Edinburgh) were indistinguishable by SDS-PAGE, immunoblotting and REA (group 1). These strains were isolated from four male patients who were in a respiratory-diseases ward at the same time and for whom clinical details of the origin and date of sampling are shown (table II). *M. catarrhalis* strains were not isolated from other patients on that ward during that period. A further group of three strains was isolated from three different patients in a medical unit in Aberdeen in the absence of previous suspicion of nosocomial infection (group 2). The 15 strains that were found to be unique by all three techniques came from a variety of wards.

Discussion

Our results confirm previous reports of strain heterogeneity within *M. catarrhalis*.¹⁰⁻¹² Two of the three techniques used—SDS-PAGE of WCP and immunoblotting—have not previously been applied successfully to the characterisation of *M. catarrhalis* strains. The complexity of WCP profiles obtained makes detailed comparison of strains difficult and immunoblotting produces much simpler patterns which, nevertheless, still have sufficient variability to form the basis of a suitable typing method. The main disadvantage of immunoblotting is its requirement for a source of antibody that is difficult to standardise without batch-to-batch variation. The variety of antigens detected by pooled normal human serum is surprising in view of the apparent homogeneity of outer-membrane proteins reported by other workers.⁷ Our blotting procedure was designed to detect the most prominent antigens and further work is required

to establish their relationship to previously described outer-membrane proteins. However, the present results suggest that serological studies of the immune response to *M. catarrhalis* may give variable results with different strains because of antigenic variation. The absence of demonstrable IgG antibody to *N. lactamica* and *N. pharyngis* strains in normal human serum is consistent with a non-pathogenic role for these organisms.

Previous studies have shown that REA may be a useful technique for characterising strains of *M. catarrhalis*.^{12,16} The enzyme used in the present study produced simple patterns of 5–7 bands in the upper region of the gel allowing easy comparison of different strain patterns. If REA is the only method of typing to be used, it is probably necessary to use more than one enzyme to confirm the groupings obtained.¹⁶ In the present study, REA in combination with two protein-based typing techniques, provided confirmation of strain groupings. Thus, for the group-6 strains, despite the identical REA patterns obtained with *Taq* 1, WCP and immunoblot results suggested that these strains are quite different. However, SDS-PAGE and immunoblotting are susceptible to variable results due to phenotypic change which may account for minor differences in WCP profile between the two group-5 strains that appeared identical by other methods. All three techniques discriminated among strains of *M. catarrhalis*, but no single method was wholly effective and our results confirmed the value of using more than one method for typing. For ease of interpretation, REA and immunoblotting were more acceptable than SDS-PAGE of WCP and the former would form a useful combination for further investigation of the epidemiology of *M. catarrhalis* infection.

Circumstantial evidence that *M. catarrhalis* is an important nosocomial pathogen has been confirmed by REA on only one occasion to date.¹² We have demonstrated that three of four patients implicated in a suspected outbreak of infection yielded *M. catarrhalis* strains indistinguishable by the typing methods used. Furthermore, in the absence of any suspicion of nosocomial infection, we have found indistinguishable strains from three different patients in the same medical unit, results suggesting that nosocomial infection with *M. catarrhalis* may be relatively common. More detailed clinical studies based on the typing methods now available are required to assess the epidemiology of this increasingly recognised pathogen.

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