A 200 kDa protein is associated with haemagglutinating isolates of
Moraxella (Branhamella) catarrhalis

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Abstract

Moraxella catarrhalis adheres to human erythrocytes by means of a proteinaceous, trypsin sensitive, heat modifiable
haemagglutinin. A 200 kDa protein was found to be associated with haemagglutinating isolates of M. catarrhalis. This protein
was present on all haemagglutinating isolates (n = 17), but was absent on the non-haemagglutinating isolates (n = 23) examined.
This protein demonstrated heat-modifiable properties in sodium dodecyl sulfate and was degraded by trypsin. Immunoblot
assays with polyclonal antiserum indicated that the 200 kDa protein was associated exclusively with haemagglutinating isolates
and antibodies to this protein did not recognise epitopes on non-haemagglutinating isolates. This protein, which appears to be
a surface expressed protein may be a haemagglutinin of M. catarrhalis.

Keywords: Moraxella (Branhamella) catarrhalis; Haemagglutination; Surface protein; Heat modifiability; Immunoblotting

1. Introduction

In recent years Moraxella (Branhamella) catarrhalis has been increasingly recognised as an important human pathogen [1,2]. Previously dismissed as a commensal of the oro-nasopharyngeal tract, it is
now reported as the third most isolated after Haemophilus influenzae and Streptococcus pneumoniae as the causative agent in lower respiratory tract infections such as bronchitis and pneumonia in the

elderly, especially in those with underlying pulmonary disease [3,4]. M. catarrhalis has been sporadically implicated in invasive infections including septicaemia in both the immunocompetent [5] and the
immunocompromised patient [6].

The recent recognition of M. catarrhalis as an important upper and lower respiratory tract pathogen has prompted researchers to investigate the possible virulence mechanisms employed by the organism in
order to understand the pathogenesis of M. catarrhalis infection. Bacterial adherence is believed to be the
initial event in most infectious processes [7]. There have been a number of studies on the adherence of
M. catarrhalis to human oropharyngeal cells and human erythrocytes [8–11]. The adhesive processes

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Abbreviations: PBS A, Dulbecco A phosphate buffered saline;
NOG, N-octyl-β-D-glucopyranoside; TBS, Tris buffered saline
involved remain unresolved. It has been established that the haemagglutinin is a trypsin sensitive protein [11,12] and is associated with tracheal cell adherence [11]. Two phenotypes of the haemagglutinin have been identified, phenotype I agglutinates human erythrocytes only, while phenotype II agglutinates human and rabbit erythrocytes [12]. A recent study has reported that a significantly higher percentage of *M. catarrhalis* isolates recovered from infected elderly patients, with respiratory tract infection, haemagglutinated human erythrocytes when compared with the haemagglutinating activity of isolates recovered from colonised healthy elderly people, suggesting that the haemagglutinin may play a role in the infectious process in respiratory tract infection in the elderly population [13].

In this present study 40 isolates of *M. catarrhalis* were examined by SDS-PAGE and a 200 kDa protein, which was found to be associated with haemagglutinating isolates was investigated.

2. Materials and methods

2.1. Bacterial isolates and growth conditions

Forty isolates of *M. catarrhalis* were examined. These isolates were from a collection acquired during a study of the epidemiological aspects of *M. catarrhalis* infection and colonisation [13]. Isolates were identified according to the criteria previously outlined [14] and were stored at −70°C using the Protect Bead System (Technical Service Consultants). Prior to all tests isolates were grown on Columbia blood agar at 37°C for 18 h, unless otherwise stated.

2.2. Haemagglutination

The ability of the 40 isolates to agglutinate human group O red blood cells and rabbit erythrocytes was determined by a micotitre method [12].

2.3. SDS-PAGE of crude whole-cell protein preparations

Whole cell preparations of 17 haemagglutinating and the 23 non-haemagglutinating isolates of *M. catarrhalis* were analysed by SDS-PAGE. Bacterial suspensions were centrifuged in a microfuge at 10,000×g for 5 min. The pellet was boiled in 200 μl sample buffer containing 0.065 M Tris, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol and 0.001% (w/v) bromphenol blue. The resulting protein extracts were resolved by SDS-PAGE on 10% polyacrylamide gels by the technique of Laemmli [15]. The resolved proteins were visualised by staining with Coomassie brilliant blue.

Isolate K48 showed variability on repeated haemagglutination testing. Separate colonies were subcultured and three phenotypes were identified. These were titled W, X and Y and gave titres of 4, 2 and 0 against human erythrocytes and 32, 16 and 1 against rabbit erythrocytes. These phenotypes were tested by SDS-PAGE, using standardised suspensions.

Bacterial suspensions of phenotype I and phenotype II haemagglutinating isolates (B4, K29, S109, S407 and S580) were treated with an equal volume of trypsin solution (2 mg ml⁻¹) in PBS A at 37°C for 2 h. Following washing in PBS A the bacterial pellets were prepared for SDS-PAGE analysis as described above. Control preparations were treated identically, using PBS A instead of trypsin solution.

2.4. Heat modifiability of the 200 kDa protein

The heat modifiability of the 200 kDa protein was examined by incubating crude whole-cell protein extracts of isolate S407 in SDS-PAGE sample buffer at various temperatures ranging from 20°C to 100°C for 5 min, before resolving the preparations by SDS-PAGE on 5% polyacrylamide gels.

2.5. Isolation of outer membranes

The outer membranes of two haemagglutinating isolates (S407 and S580) and two non-haemagglutinating isolates (K16 and S89) were obtained using the broth culture supernatant technique previously described [16], with some slight modifications. Brain Heart Infusion broth (100 ml) was inoculated with several colonies of bacteria from a Columbia blood agar plate and incubated at 37°C for 24 h, with shaking. The cells were centrifuged at 10,000×g for 15 min at 4°C. The supernatant fraction was collected and centrifuged again at 10,000×g for 15 min at 4°C. The resulting supernatant fraction was
then centrifuged at 100,000×g for 90 min at 4°C. The pellet was suspended in sample buffer and the proteins present in these outer membrane vesicles were resolved by SDS-PAGE using 10% polyacrylamide gels.

These blebs consist of outer membrane vesicles that can be observed by electron microscopy; they contain OMPs and lipooligosaccharide and are free of cytoplasmic membrane contamination [17].

2.6. Preparation of anti-serum

Polyclonal antiserum against whole cells was obtained by immunisation of a rabbit with a formalin treated suspension of isolate S407. In addition, polyclonal antiserum was prepared against a protein extract of isolate S407, obtained by ion-exchange chromatography. Following elution of the proteins from the ion-exchange column, fractions containing protein were analysed by SDS-PAGE. The fractions containing the 200 kDa protein were pooled, dialysed with several changes of sterile water at 4°C for 48 h and used in the production of antiserum. In the preparation of each anti-serum a New Zealand white rabbit was given three injections, subcutaneously, of the whole cell preparation or the protein extract (800 µg protein per dose) in Freund's complete adjuvant on day 0, and in Freund's incomplete adjuvant on days 14 and 35.

Following immunization, antisera were obtained on day 49 and the immunoglobulin fraction of the antiserum was separated by the method of Harboe and Ingild [18]. Antisera were adsorbed by using (i) a cocktail of non-haemagglutinating isolates (21, 25, K2, K16, S22, S68, S75, S76, S89) and (ii) a cocktail of haemagglutinating isolates (B4, K38, K44, K51, S109, S274, S407, S540, S580).

2.7. Immunoblot analysis

Both adsorbed and unadsorbed antisera were studied using immunoblot analysis. Western immunoblots were performed, with polyclonal anti-serum to whole cell preparation of isolate S407, by electrophoretic transfer of crude protein extracts of haemagglutinating isolates (B4 and S407) and a non-haemagglutinating isolate (isolate 21) of M. catarrhalis resolved by SDS-PAGE to nitrocellulose, employing the technique described by Bollag and Edelstein [19]. The membranes were blocked in 2% (w/v) skim milk (Difco) prepared in Tris buffered saline (TBS) for 1 h. Following washing, the membrane was probed with the polyclonal antiserum as the primary antibody. The membranes were washed in TBS, incubated in 1:1000 dilution of swine anti-rabbit, horseradish peroxidase-conjugated antibody for 2 h, washed again and developed in a solution of 4-chloro-1-naphthol for 10 min.

Antisera to the protein extract of isolate S407 which had been adsorbed with the cocktail of non-haemagglutinating isolates (described above) was examined in more detail using immunoblot analysis by probing against a selection of haemagglutinating isolates (B4, K29, K38, K48, S64, S109, S407, S540 and S580) and non-haemagglutinating isolates (21, K1, K3, K5, K10, S68 and S89) of M. catarrhalis.

3. Results

3.1. Haemagglutination and SDS-PAGE analysis

Seventeen of the 40 isolates examined agglutinated human erythrocytes, while the remaining 23 isolates were non-haemagglutinating. Of the 17 haemagglutinating isolates, 10 isolates haemagglutinated human erythrocytes (phenotype I), while 7 isolates haemagglutinated both human and rabbit erythrocytes (phenotype II). The protein profiles obtained following the resolution of crude protein extracts of the 17 haemagglutinating and the 23 non-haemagglutinating isolates of M. catarrhalis by SDS-PAGE demonstrated the presence of a 200 kDa protein in all preparations of haemagglutinating isolates which was absent in all preparations of non-haemagglutinating isolates examined (Fig. 1). The three phenotypes of K48 showed differences in the amount of the 200 kDa protein that was present. The strength of the 200 kDa band was related to the haemagglutinating activity of the phenotypes (Fig. 2). The trypsin-treated haemagglutinating strains showed no 200 kDa protein. The apparent molecular mass (Mr) of the 200 kDa protein varied slightly between some isolates from 185–205 kDa. However, the Mr of the '200 kDa' of a particular isolate was repeatedly the same when different preparations of the same
isolate were compared. In addition, no apparent difference in protein profile was noted when phenotype I isolates were compared with phenotype II isolates, thus inter-strain variability in $M_r$ was unrelated to haemagglutination phenotype.

The 200 kDa protein demonstrated heat-modifiability, it migrated as a protein of higher $M_r$ when solubilised at temperatures less than 80°C. Determination of the $M_r$ of this protein proved difficult, but it appeared to be greater than 400 kDa.

OMP preparations were obtained using the broth culture supernatant technique. The purified outer membranes of two haemagglutinating (S407 and S580) and of two non-haemagglutinating (K16 and

![Fig. 1. SDS-PAGE analysis of crude whole-cell protein extracts of M. catarrhalis. Lanes 2–6, haemagglutinating isolates and lanes 7–11, non-haemagglutinating isolates. The presence of a 200 kDa protein in the extracts of haemagglutinating isolates is indicated by '>'. The 10% polyacrylamide gel was stained with Coomassie brilliant blue. $M_r$ markers are shown in lane 1.](image)

![Fig. 2. SDS-PAGE analysis of phenotypes W, X and Y of isolate K48. Lane 3, phenotype W (titre against rabbit erythrocytes 32 and human erythrocytes 4); lane 2, phenotype X (titre against rabbit erythrocytes 16 and human erythrocytes 2) and lane 4, phenotype Y (titre against rabbit erythrocytes 1 and human erythrocytes 0). The 200 kDa protein is marked '>'. Proteins were stained with Coomassie brilliant blue on a 10% polyacrylamide gel. $M_r$ markers are shown in lane 1.](image)
Fig. 3. SDS-PAGE analysis of outer membrane preparation of isolate S407 (bottom panel) and isolate S 580 (top panel). In (bottom) upper lane, outer membrane bleb preparation; middle lane, whole cell preparation. In (top) middle lane, outer membrane bleb preparation; bottom lane, whole cell preparation. The 200 kDa protein is marked '>'. Proteins are stained with 1% Coomassie blue on a 10% polyacrylamide gel. M, markers are shown in the bottom lane in the bottom panel and in the top lane in the top panel.

S89) *M. catarrhalis* isolates were resolved by SDS-PAGE. The protein band patterns were similar to those obtained by Bartos and Murphy [16] and Murphy and Loeb [17] with the eight major outer membrane proteins (A–H) being present in all preparations. However, in the haemagglutinating isolates

Fig. 4. Western blot analysis of crude whole-cell protein preparations of *M. catarrhalis*. Haemagglutinating isolate S407 (lane 1) and isolate B4 (lane 2) and non-haemagglutinating isolate 21 (lane 3) probed with unadsorbed polyclonal antiserum (left), polyclonal antiserum adsorbed with a cocktail of non-haemagglutinating isolates (middle) and polyclonal antiserum adsorbed with a cocktail of haemagglutinating isolates (right). The 200 kDa protein is indicated by '>'. 
examined (S407 and S580), the 200 kDa protein observed in the crude protein whole cell extracts was also evident, albeit weakly in the outer membrane preparations. (Fig. 3). This band was not found in the outer membrane preparations of the non-haemagglutinating isolates (K16 and S89).

3.2. Western immunoblotting

The polyclonal antiserum to a whole cell preparation of *M. catarrhalis* (isolate S407) was analysed in immunoblot assays (Fig. 4a). When this antiserum was adsorbed with a cocktail of non-haemagglutinating isolates and examined by immunoblotting analysis, the adsorbed antiserum recognised epitopes on the 200 kDa protein on the two haemagglutinating isolates studied (S407 and B4), while antibodies recognising epitopes on other proteins had been significantly adsorbed out (Fig. 4b). Adsorption of the antiserum with a cocktail of haemagglutinating isolates resulted in the significant removal of antibodies recognising the 200 kDa protein (Fig. 4c).

To determine if antiserum adsorbed with a cocktail of non-haemagglutinating isolates, which contained antibodies predominantly to the 200 kDa protein, demonstrated reactivity with a larger selection of *M. catarrhalis* isolates, a total of 16 isolates were examined by immunoblot analysis, using antiserum to the protein extract of *M. catarrhalis* (isolate 407). This adsorbed antiserum recognised epitopes on the 200 kDa protein in all nine haemagglutinating isolates examined (B4, K29, K38, K48, S64, S109, S407, S540 and S580), but failed to detect a 200 kDa protein in all seven non-haemagglutinating isolates (21, K1, K3, K5, K10, S68 and S89) of *M. catarrhalis* studied (Fig. 5).

4. Discussion

*M. catarrhalis* is now a well recognised cause of upper and lower respiratory tract infections in children and in the elderly, respectively. Due to the increased awareness of the pathogenic role played by *M. catarrhalis* in infection, much work has evolved around establishing the virulence factors employed by the organism. Adherence of bacteria to host cells is generally considered the initial step in the patho-
genesis of many infections [7]. It has been proposed that the adherence of *M. catarrhalis* to oropharyngeal cells and human erythrocytes may be mediated by fimbriae [8]. While Ahmed et al. [9] report an association between nasopharyngeal adherence of strains and haemagglutination, this association is tenuous as a number of strains, which were highly fimbriated, were non-adherent to nasopharyngeal cells and were non-haemagglutinating. Similarly, Rikitomi et al. [10] have observed no significant difference in the adhesive capacity to oropharyngeal cells or in haemagglutination between fimbriated and non-fimbriated strains of *M. catarrhalis*. In the latter study, it was also noted that no correlation between haemagglutination and oropharyngeal cell adherence was evident. In an ongoing study no correlation between haemagglutination and adherence to HEp-2 cells by *M. catarrhalis* has also been observed (M. Fitzgerald, unpublished observations). Thus it would appear that haemagglutination and epithelial cell adherence are mediated by separate adhesins and that non-fimbrial adhesins, may be involved in haemagglutination.

Recently, a correlation was found between the haemagglutination titre of isolates of *M. catarrhalis* and their ability to adhere to tracheal epithelial cells [11]. This finding may be of importance as the organism is commonly associated with tracheobronchitis in the elderly [20]. Furthermore, the fact that 75% of strains isolated from the sputum of the elderly with respiratory tract infections were haemagglutinating in comparison to 15% of the strains isolated from healthy elderly individuals would suggest a role for the haemagglutinin in the aetiology of *M. catarrhalis* infection in the elderly [13].

A recent study has established that *M. catarrhalis* has at least two haemagglutination phenotypes [12]. Phenotype I isolates haemagglutinate human erythrocytes, while phenotype II isolates haemagglutinate human and rabbit erythrocytes. Haemagglutination by both phenotypes was mediated by trypsin-sensitive proteins that were heat-labile at 70°C. The observation that haemagglutination is mediated by proteinaceous structures has also been reported by others [11]. To date no study characterising these haemagglutinins has been documented.

In this present study it was established that a 200 kDa protein is exclusively associated with haemagglutinating isolates of *M. catarrhalis*. There was some variability of the *M* of this protein from one isolate to another, but the *M* was consistent for each isolate. This protein was found on both haemagglutinating phenotypes and there was no correlation between *M* and phenotypes. In addition, it was established that in the SDS-PAGE of the three phenotypes of K48, the density of the 200 kDa band correlated closely with the haemagglutinating titre of the phenotype. SDS-PAGE analysis of the bleb preparations of haemagglutinating isolates demonstrated the presence of the 200 kDa protein, indicating that it may be an outer membrane protein. However, while it was identified in the outer membrane vesicles, the relative amount was not greater than the lysate, thus the possibility exists that this protein may not be an outer membrane protein, but may be a surface protein bound to the membrane. Evidence that the 200 kDa protein was surface expressed was provided by the fact that: (a) antibodies recognising epitopes on this protein were removed following adsorption of the antiserum with haemagglutinating isolates, as demonstrated by immunoblot analysis and (b) ongoing studies have shown that antibodies to the 200 kDa protein are present in the serum of children and adults when analysed by immunoblotting, indicating that the protein is expressed in vivo (R. Mulcahy, unpublished observations).

Electron microscopy studies have shown that haemagglutinating strains have an outer fibrillar coat which is removed by trypsin treatment and adherence to red blood cells appears to be mediated by this outer fibrillar coat [12]. Haemagglutination appears to be mediated by a trypsin sensitive, heat sensitive, protein haemagglutinin. The 200 kDa protein is heat sensitive and trypsin sensitive and because of the association between the 200 kDa protein and haemagglutinating strains it is possible that this protein may be a haemagglutinin. Further work is currently underway to investigate this.

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References