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Research article

Adherence of *Mannheimia haemolytica* to ovine bronchial epithelial cells

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The adherence of six isolates of *Mannheimia haemolytica* to ovine bronchial epithelial cells (OBECs) was examined via immunofluorescence, scanning electron microscopy (SEM) and quantification assays; isolates used represented different capsular, outer membrane protein A (OmpA), and LPS types and species of isolation. The study revealed that both the capsular and LPS types of the isolates affected the level of adherence displayed; however there was no difference displayed in the level of adherence between ovine and bovine isolated strains, implying the presence of non-specific adhesins. Isolates were further tested for their ability to adhere to the extracellular matrix component, collagen, using a collagen-coated transwell insert seeded with OBECs, mimicking the *in vivo* environment of the respiratory tract. SEM results showed that all six isolates tested adhered to collagen. Additionally, the role of OmpA in attachment was investigated through inhibition assays using cross-absorbed OmpA-specific antibodies, with results showing that OmpA failed to have any effect on the level of attachment to OBECs achieved by all but the two virulent isolates tested, insinuating its potential as a virulence factor. Additionally, SEM revealed an unknown substance on the surface of the OBECs and decorating all six isolates. Subsequent analysis, entailing further SEM imaging and the growth of the isolates on Congo red agar indicated that this may be extracellular polysaccharide, implying that *M. haemolytica* possesses the potential to form biofilms, a previously undocumented ability.

Key words: *Mannheimia haemolytica*, outer membrane protein A, transwell inserts, biofilm, ovine bronchial epithelial cells, adherence.

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Introduction

Mannheimia haemolytica; the aetiological agent of pneumonic pasteurellosis

The non-motile Gram-negative gamma-proteobacterium *Mannheimia haemolytica* is the aetiological agent of pneumonic pasteurellosis; one of the most economically significant respiratory diseases in worldwide cattle and sheep industries, with an annual economic loss to the USA cattle industry alone being estimated to be $640 million.[1]

Mannheimia haemolytica is a commensal resident of the nasopharyngeal microflora of ruminant animals and is capable of causing infection when the body's defence mechanisms are impaired. The organism is mainly confined to ruminants with the most adequately characterized strains originating from cattle, sheep and goats[2] of all ages. Most outbreaks of pneumonic pasteurellosis in Europe occur in May to July, mainly due to stress induced by excessive temperatures or stress associated with weaning, dehorning and transportation of livestock.[3]

The pathogenesis of pneumonic pasteurellosis remains a subject of considerable speculation and controversy due to the complex nature of the disease and the lack of consistency in experimental results. It is believed by many that *M. haemolytica* cannot act alone as the causative agent of disease in the absence of a well-defined predisposing factor such as viral infections (parainfluenza virus 3), bacterial infections (*Pasteurella multocida*) and environmental stresses.[3] It is believed that these factors seem to alter the upper respiratory tract (URT) epithelium allowing the sequential down-regulation of local pulmonary defence mechanisms such as ciliation and mucous production, resulting in

M. haemolytica colonizing, escaping clearance in the nasopharynx, and moving to the lungs by gravitational drainage. Due to the rapid growth, multiplication and endotoxin production of the bacteria in infected lobules, extensive intravascular thrombosis of pulmonary veins, capillaries and lymphatics occurs. Histologically, the alveoli will mainly consist of elongated cells with basophilic spindle-shaped nuclei (oat cell), masses of which form whorls that stream between adjacent alveoli.[4]

Strain characterization

Mannheimia haemolytica has been the subject of extensive reclassification in the past: first called *Bacterium bipolare multocidum* by Theodore Kitt in 1885, it was renamed *Pasteurella haemolytica* in 1932[5] and classified into two biotypes (A and T) based on its ability to ferment the sugars arabinose and trehalose, respectively. These biotypes could be further subdivided into 13 A serotypes (A1, A2, A5, A6, A7, A8, A9, A11, A12, A13, A14, A16 and A17) and 4T capsular serotypes, based on results from an indirect haemagglutination test.[2] Of the 13 A serotypes described, A1 and A2 are established worldwide, although there have been reports of morbidity and mortality accounted to serotypes A6, A7, A9 and A12.[6–8] Both A1 and A2 possess the ability to colonize the URT of cattle and sheep, they are however often species specific. Healthy cattle frequently carry serotype A2 in their URT but following stress or coinfection, A1 quickly takes the place of A2 as the main serotype probably due to horizontal transfer from ill animals.[3,9]

Virulence and control

Numerous attempts have been made to develop efficacious vaccines against *M. haemolytica*[10–12] all of which have been partially successful. Further, injectable, edible and mucosal vaccines against pneumonic pasteurellosis are currently commercially available and a recent vaccine preparation containing *M. haemolytica* lacking leukotoxin has been shown to improve cattle immunity to the pathogen.[13] However, it should be stressed that vaccine development is problematic and there is no single vaccine that provides protection from all the various strains. Furthermore, the imprudent use of antibiotics has driven, and continues to drive, the spread of antibiotic-resistance genes on plasmids and transposon, resulting in the reduced efficacy of these agents.[14]

Regardless of the economic importance of this pathogen, relatively little is understood about the precise mechanisms of pathogenesis, specifically how *M. haemolytica* adheres to the respiratory tract epithelium. Previous studies have demonstrated the ability of *M. haemolytica* to adhere to epithelial cells *in vitro*.[15]

The first stage of infection is the adherence of a pathogen to the surface of target cells, usually facilitated by specific interactions between a cell surface protein on the bacterium and the extracellular matrix (ECM) in the target cell.[16] The ECM is composed of a mixture of many glycoproteins including, fibronectin and collagen, with the former recently shown to form a specific interaction with *M. haemolytica*'s OmpA.[16] Similar investigations have not been carried out with any of the other ECM components. OmpA has been shown in other pathogens[17] to be extremely important in host-specific ligand binding and there is indirect evidence that *M. haemolytica* achieves its host specificity in the same manner. This hypothesis is strongly supported by the fact that bovine and ovine strains possess different OmpA types (OmpA1 and OmpA2, respectively). The differences between the two classes are located at the ends of the four hypervariable loops (regions that may come into contact with host cells). Hence, differences in these regions could contribute to the host specificity exhibited by bovine and ovine strains.[18] Furthermore, OmpA has subsequently been shown to significantly increase *M. haemolytica*'s adherence to bovine bronchial epithelial cells (BBEC),[15] although no such work has been carried out on OBECs. However, adhesion is normally a multifactorial process that involves several components of the bacterium and the host. In the case of *M. haemolytica*, OmpA is probably one of the several factors, which plays a role in adherence. Recent genomic sequencing of *M. haemolytica* has discovered several adhesin-like proteins, many of which are pili, which have been implicated in adhesion.[19] Additionally, several non-pilus adhesion proteins that could modify host mucosal surfaces are also present in *M. haemolytica*. These include an ortholog (40%) to *Bordetella pertussis*'s filamentous haemagglutinin (FhaB)[19] and an adhesin complex protein found only in *N. meningitidis* and *Eikenella corrodens*. It is not known whether these proteins are expressed by *M. haemolytica* cells or play any role in its adherence to BBEC and OBEC.

The present study aimed to determine if there were differences in the adherence of bovine and ovine isolates of *M. haemolytica* to OBECs and to elucidate whether OmpA is involved in such adherence. In addition, the ability of these isolates to adhere to collagen was investigated.

Materials and methods

Bacterial strain selection and growth conditions

Six *M. haemolytica* isolates (Table 1), kindly provided by R.L. Davies (University of Glasgow), were selected to allow comparison between ovine and bovine strains possessing specific capsular types. The strains were stored at −85°C in 50% (vol/vol) glycerol in brain heart infusion (BHI) broth and were grown on 5% (vol/vol) sheep's blood BHI agar by overnight incubation at 37°C. Liquid cultures were prepared by inoculating three to five colonies into 10 ml BHI broth and incubating overnight at 37°C with agitation (120 rpm). Four hundred microlitres of overnight culture was added to conical flasks containing 20 ml BHI broth

Table 1. Properties of six *M. haemolytica* isolates used

Isolate	Capsular serotype	Host species	*ompA* allele	LPS type	Anti-LPS antibodies
PH2	A1	Bovine	OmpA1.1	1A	PH2 (Anti-1A)
PH8	A1	Ovine	OmpA2.1	1B	PH8 (Anti-1B)
PH202	A2	Bovine	OmpA1.3	3B	PH8 (Anti-1B)
PH278	A2	Ovine	OmpA2.3	3B	PH8 (Anti-1B)
PH284	A6	Ovine	OmpA2.1	1A	PH2 (Anti-1A)
PH376	A6	Bovine	OmpA1.1	1A	PH2 (Anti-1A)

(1:50 dilution) and incubated at 37°C, 120 rpm until late logarithmic phase (OD_{600} of 0.4–0.5). An OD_{600} of 0.4 is equivalent to $\sim 2 \times 10^8$ cfu ml^{-1} The cells were collected by centrifugation (2500×*g*, for 10 min), washed three times with M199 and resuspended in M199 containing 10% foetal calf serum (FCS). Serial dilutions of 10^{-5}, 10^{-6} were also prepared for each bacterial suspension used. Plate counts were performed on 1.2% BHI agar plates, to retrospectively determine the true concentration of inoculum given to OBECs. Plates were incubated at 37°C and colonies counted at 24 h.

Tissue culture

Collection and preparation of OBEC

Ovine bronchial tissue was collected from Sandyford Abattoir, Paisley Ltd. and bronchi dissected aseptically in a laminar flow cabinet. Bronchi were subsequently cut into 2 cm sections and immersed into filter sterilized M199 [Gibco (Invitrogen)] containing 0.1% protease XIV (Sigma Chemicals), 2 μg/ml fungizone and 50–100 μg/ml penicillin and streptomycin [Gibco (Invitrogen)] and incubated overnight at 4°C. The bronchi sections were held with a hemostat and vigorously rinsed 15 times with 10 ml M199/10% heat-inactivated FCS above a sterile beaker. The collected bronchial rinse solution was passed through a 70 μm Nitex mesh (BD Biosciences), which was aseptically stretched across a sterile beaker. OBECs were retrieved and centrifuged at 1500 g for 10 min. The resulting supernatant was aspirated and the pellet thoroughly resuspended in 50 ml M199/10% FCS. Viable cell counts were performed using Trypan blue (200:200 μl) and adjusted to give a cell density of 2×10^5 cells/ml. Adjusted OBECs were pipetted into 50-ml tissue culture flasks and incubated at 37°C/5% CO_2 until 100% confluence was reached, the uniformity and sterility of the OBEC monolayers were checked daily and media replaced every 72 h. Fully confluent OBEC were either frozen, passaged or seeded.

Seeding and passaging OBEC

Culture media were discarded and the monolayers washed with 10 ml phosphate buffer saline (PBS) to remove trace FCS. Five millilitres of prewarmed 0.5% trypsin–ethylenediaminetetraacetic acid (EDTA) was added and incubated at 37°C for 5 min. Ten millilitres of M199 containing 10% FCS, 2 μg/ml fungizone and 50–100 μg/ml penicillin and streptomycin was added and the sides of the culture flasks were gently tapped to facilitate cell removal. The media were subsequently eluted into Falcon tubes and centrifuged at 1000 g for 10 min. The supernatants were removed and the pellets resuspended in 10 ml M199 containing 10% FCS, 2 μg/ml fungizone and 50–100 μg/ml penicillin and streptomycin and centrifugation was repeated as above. The OBECs were either passaged to new flasks or seeded onto 13 mm rounded glass coverslips or onto 12 mm 0.4 μm collagen type I- and III-coated transwell inserts.

Coverslips: Seeding was carried out by placing sterile 10 mm glass coverslips into 24-well transwell plates and adding 1 ml of the OBEC solution. Coverslips were incubated at 37°C 5% CO_2 for 24 h.

Transwell inserts: Seeding was carried out by adding 1.5 ml of M199 containing 10% FCS, 2 μg/ml fungizone and 50–100 μg/ml penicillin and streptomycin to each well in a 12-well transwell plate and incubating for 1 h at 37°C. Sterile inserts were then placed into each well and 0.5 ml of OBEC was added and incubated at 37°C for 168 h, uniformity and sterility of the OBEC monolayers were checked daily and media replaced every 72 h.

Adherence assay on coverslips

All isolates (Table 1) were prepared as mentioned previously for analysis via immunofluorescence and SEM. One millilitre of the bacterial suspension was pipetted onto seeded and washed (M199 to remove antibiotics) glass coverslips. Two coverslips were left unexposed to bacteria to allow basic cell cytoskeleton structure to be examined and a negative control (PBS) was used. Coverslips were incubated for 1 h at 37°C/5% CO_2 after which the bacterial suspension was removed and the coverslips were washed twice with M199, after which the coverslips were fixed for either immunofluorescence staining or SEM (see the sections on Immunofluorescene and SEM).

Adherence assay on collagen gel inserts

All strains (Table 1) with the exception of PH202 and PH376 were prepared as described in the section 'Adherence assay on coverslips' for analysis via SEM, with the exception that 0.5 ml bacterial suspension were added to the insert.

Immunofluorescence staining

Cell cytoskeleton

Coverslips or collagen transwell inserts were washed several times with 1 ml PBS, then 1 ml of 4% formaldehyde/PBS fix was added under a laminar flow hood and incubated at

37°C for 15 min. The fixative was removed and coverslips or inserts were washed several times with PBS. One millilitre of Perm buffer was added and incubated for 5 min at 4°C. This was replaced with 1% BSA/PBS and incubated at 37°C for 5 min. The BSA/PBS was removed and 200 μl of 1:50 phalloidin/anti-cytokeratin was added and incubated for 1 h at 37°C (whilst wrapped in foil). The stain was removed and the samples washed several times with 1 ml PBS/0.5% Tween, followed by a wash with PBS. Two hundred microlitres of 1:50 of the secondary antibody (anti-mouse) were added and incubated for 1 h at 37°C. The cells were washed several times with 1 ml PBS/0.5% Tween and the tertiary streptavidin component added for 30 min at 4°C. The cells were mounted in a fluorescent mountant containing 4′,6-diamidino-2-phenylindole and a 20 mm × 20 mm coverslip placed on top. After a few minutes the cells were viewed under a Leica DMRA2 fluorescent microscope at ×10 000 (Leica Microsystems).

Immunofluorescence staining of *M. haemolytica*

Performed as for cytoskeleton staining with the exception that the primary antibody used was either phalloidin/anti-LPS (PH2/PH8) (Table 1) or phalloidin/anti-OmpA at a concentration of 1:100, with a secondary anti-rabbit antibody.

SEM

Performed as,[20] briefly, samples were fixed with 1.5% glutaraldehyde buffered in 0.1 M sodium cacodylate. Samples were then fixed in 1% osmium tetroxide for 1 h with 1% tannic acid used as a mordant. Samples were then dehydrated using a series of alcohol concentrations, stained in 0.5% uranyl acetate, followed by further dehydration with 90, 100% alcohol and finally hexamethyl-disilanzane. Once dry, samples were coated with gold before examination with a Jeol 6400 scanning microscope at an accelerating voltage of 6 keV.

Adhesion quantification assay

Adapted from,[15] briefly, plate-grown bacteria (Table 1) were suspended in M199 with 10% FCS and incubated with OBEC (MOI of 100:1) for 1 h at 37°C in 5% CO_2. Epithelial cells were washed five times with PBS to release non-adherent bacteria and lysed with 1% Saponin (Sigma) in M199 for 20 min at room temperature to release adherent bacteria. The number of CFU in each well was quantified by plating out serial dilutions of cell lysates on blood agar plates. Each bacterial strain was tested six times; PBS was used as a negative control.

Adhesion inhibition assay

Performed as in quantification assay, with the exception that prior to bacterial incubation with OBECs, bacteria were pre-incubated with a 1:50 dilution of either cross-absorbed rabbit anti-OmpA1 or anti-OmpA2 antibodies (Table 1) for 45 min at room temperature. Preimmune rabbit sera were incubated with PH2 as a control.

Biofilm formation

Adhesion on coverslips

This was performed as in the adherence assay on coverslips, with the exception that only PH2 and PH278 were tested. The infected OBECs were incubated for 2 and 24 h after which coverslips were processed for SEM.

Congo red agar method

Performed as.[21] The constituents of the media were brain heart infusion broth (37 g/l), sucrose (0.8 g/l), agar (10 g/l) and Congo red stain (0.8 g/l). The Congo red stain was prepared as a concentrated aqueous solution, autoclaved separately and added to the media when the agar had cooled to 55°C. Congo red plates were inoculated with all the isolates in Table 1 and incubated aerobically for 48 h at 37°C. Biofilm positive strains produced black coloured colonies while biofilm negative strains were pink coloured. Plates were checked and the colour noted after 24 and 48 h. *Staphylococcus epidermidis* (GDH 2346) was used a positive control.

Statistical analysis

Data are presented as the mean percentage ± standard error of bacteria attached to epithelial cells relative to the inoculum used to infect monolayers. Analysis of variance followed by Mann–Whitney tests for significance was performed using Minitab. p values of <0.05 were reported as statistically significant.

Results

Immunofluorescence staining and microscopy

OBEC cytoskeleton and morphology

OBEC morphology is illustrated by immunofluorescence and SEM imaging (Fig. 1A and B, respectively). Figure 1A clearly depicts normal OBECs showing the actin cytoskeleton radiating around the surface of cell with the intermediate filament cytokeratin being localized more centrally within the cell. Furthermore, a confluent epithelial cell monolayer demonstrating classical epithelial cell morphology was observed (Fig. 1B): cells were isodiametric and well compacted with precise limits and tight junctions at the interface between cells were clearly visible. Additionally, individual cells were decorated with microvilli and filopodia were observed around the circumference further confirming the cell type. This typical morphology was maintained for all assays.

Mannheimia haemolytica immunofluorescence

OBECs grown on coverslips were infected with an actual bacterial dose of $3.3 \times 10^7 \pm 9.6 \times 10^6$cfu, confirmed by

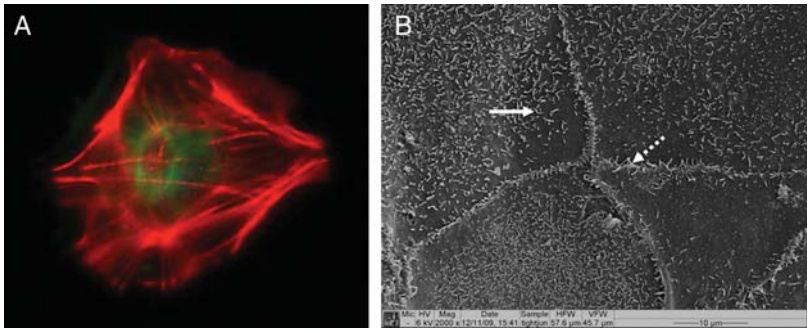

Figure 1. OBEC immunofluorescence and morphology. (**A**) Immunofluorescence OBEC at ×10 000, red = actin, green = cytokeratin and blue = nucleoid. (**B**) SEM of OBEC at ×2000 depicting classical epithelial cell morphology, solid arrow indicates microvilli and dashed arrow indicates filopodia.

plate counts (MOI ~100:1). All six strains tested adhered to OBECs (Fig. 2) as judged by immunofluorescence using both anti-ompA and anti-LPS sera (data for latter not shown). With regards to immunofluorescence all strains were found to be present, but only in small-specific locations on the OBEC surface and not randomly dispersed over the entire cell. Subjectively, there were no obvious differences in the levels of adherence between the ovine and bovine isolates to OBECs. However, there were differences in the level of adherence between the strains, with PH2 and PH278 exhibiting the greatest level of adherence and PH284 and PH376 showing the least adherence.

Mannheimia haemolytica SEM analysis

OBECs grown on coverlsips were infected with an actual bacterial dose of $3.28 \times 10^7 \pm 9.6 \times$ and OBECs grown on transwells were infected with $1.0 \times 10^7 \pm 1.1 \times 10^6$ cfu confirmed by plate counts (MOI ~100:1). Figures 3–5 show that all six isolates tested adhered to both monolayers of OBECs seeded to glass coverslips and to collagen-coated transwell membranes.

From Figs 3–5 it is not possible to draw any conclusions with regard to the levels of adherence. However, it is obvious that a significant proportion of the bacterial inocula bind to the OBEC, in particular to OBECs which have microvilli decorating their surface, although binding to smoother cells was also demonstrated, albeit to a lesser degree. Furthermore, there appears to be no difference in the level of adherence between bovine and ovine isolates.

An interesting finding that has not been previously observed to the best of the author's knowledge in *M. haemolytica* was the formation of long filamentous bacteria. This pleomorphism was exhibited by all the strains to varying degrees and can be seen on both OBECs and collagen transwell membranes. When viewing under SEM, it was further noted that there was also an unknown substance (absent in the negative control) on the surface of the OBECs, located around the bacteria. Whether this is protein debris from the cells or the remnants of biofilm extracellular polymeric substance is not known.

Adhesion quantification and inhibition assay

OBECs were grown and incubated with strains (Table 1) with and without prior incubation with anti-ompA at MOI of 100:1 for 1 h, after which non-adherent bacteria were removed by washing. The remaining adherent bacteria were lysed and quantified by plate counts, Fig. 6 depicts the mean adherence levels achieved by each strain based on six replicas. Referring to Fig. 6 it can be seen that there is a statistically significant difference (p-value < 0.001) in the percentage adherence displayed by the strains, with PH2 and PH278 having the greatest adherence and PH284 and PH376 having the lowest adherence. Furthermore, there is a statistical difference between the level of adherence displayed by the various capsular types (p-value < 0.001), with capsular type A1 isolates displaying the greatest adherence and capsular type A6 isolates displaying the lowest adherence. Interestingly, there was no significant difference between the percentage adherence of bovine and ovine isolates (p-value 0.153).

After pre-incubation with anti-OmpA specific antibodies, the levels of adherence were not statistically different (p-value 0.433) from the adherence levels achieved without pre-incubation, with the exception of the ovine strain PH278 (p-value 0.004) and the bovine strain PH2 (p-value 0.010). Again, there were no statistically significant differences in the percentage adherence of bovine and ovine isolates before and after pre-incubation with anti-OmpA specific antibodies (p-values 0.726 and 0.265, respectively).

Biofilm formation

Strains in Table 1 and *S. epidermidis* were grown on Congo red agar plate in order to determine their biofilm-forming potential. Referring to Table 2, it can be seen that all six isolates produced black colonies after 48 h, with isolate PH284

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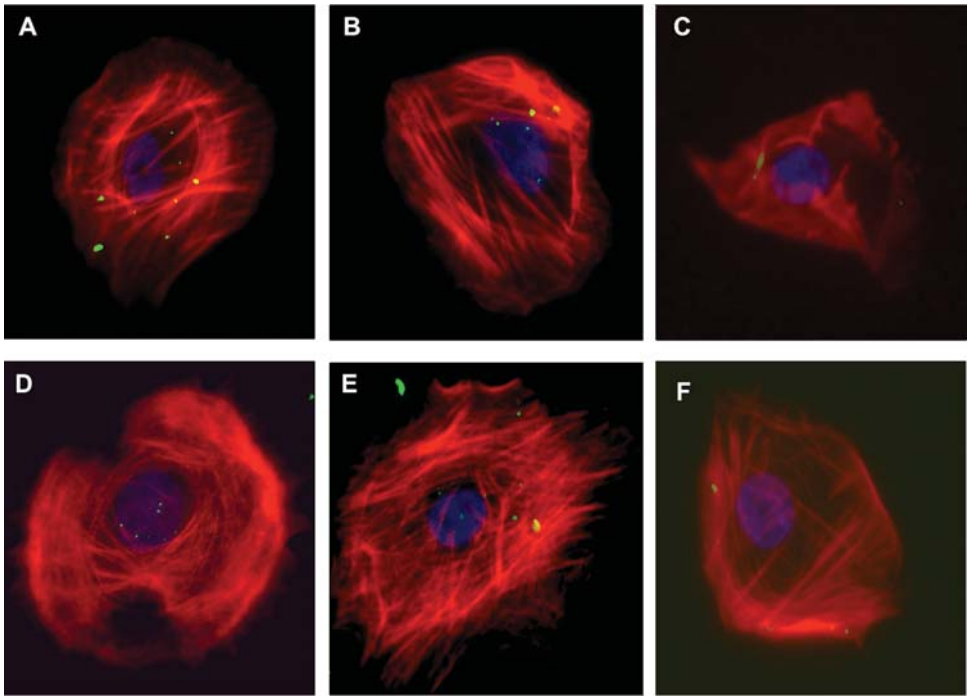

Figure 2. Immunofluorescence staining of various strains of *M. haemolytica* incubated with OBEC for 1 h at an MOI of 100:1. Images visualized at ×10 000 using an immunofluorescent microscope. Images depict a single OBEC. Red = actin, green = anti-OmpA of *M. haemolytica* and blue = nucleoid. Top row represents bovine isolates strains and bottom row ovine isolates. (**A**) PH2 (**B**) PH202 (**C**) PH376 (**D**) PH8 (**E**) PH278 (**F**) PH284.

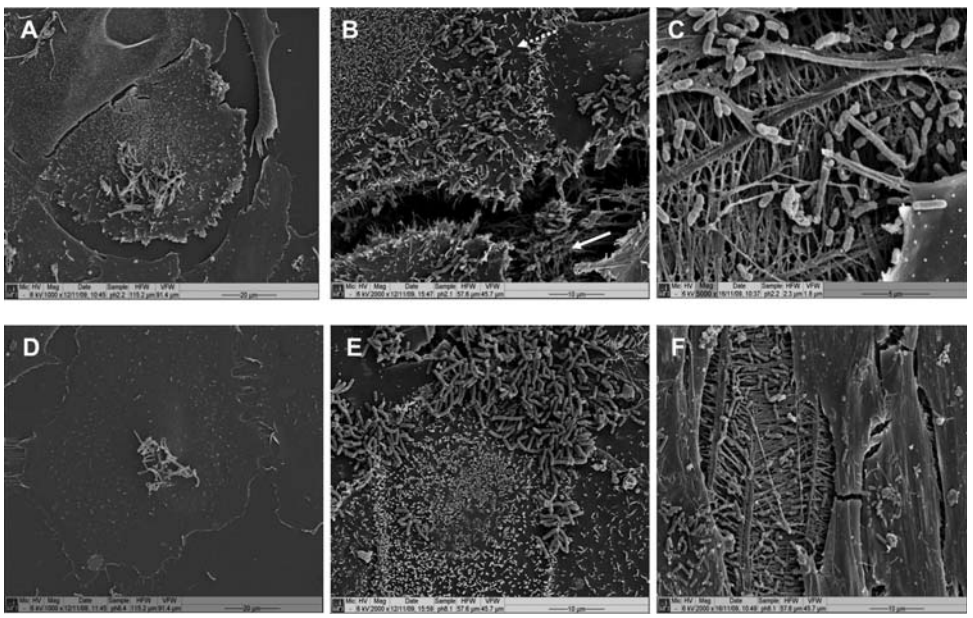

Figure 3. SEM images of *M. haemolytica* adhering to OBEC after 1 h incubation with the cells at an MOI of 100:1. Top panel represents isolate PH2 and bottom panel isolate PH8. (**A**) and (**D**) are coverslip images, (**B**), (**C**), (**E**) and (**F**) are collagen transwell images. White dashed arrow indicates bacteria on OBEC. Solid white arrow indicates bacteria on collagen matrix.

forming the densest lawn of very black colonies. Furthermore, Fig 7 shows that both isolates PH2 and PH278 appear to be encapsulated by a 'glue-like' substance after 2 h incubation. Images produced after 24 h (data not shown) revealed a patchy substance decorating the OBECs and surrounding the bacteria.

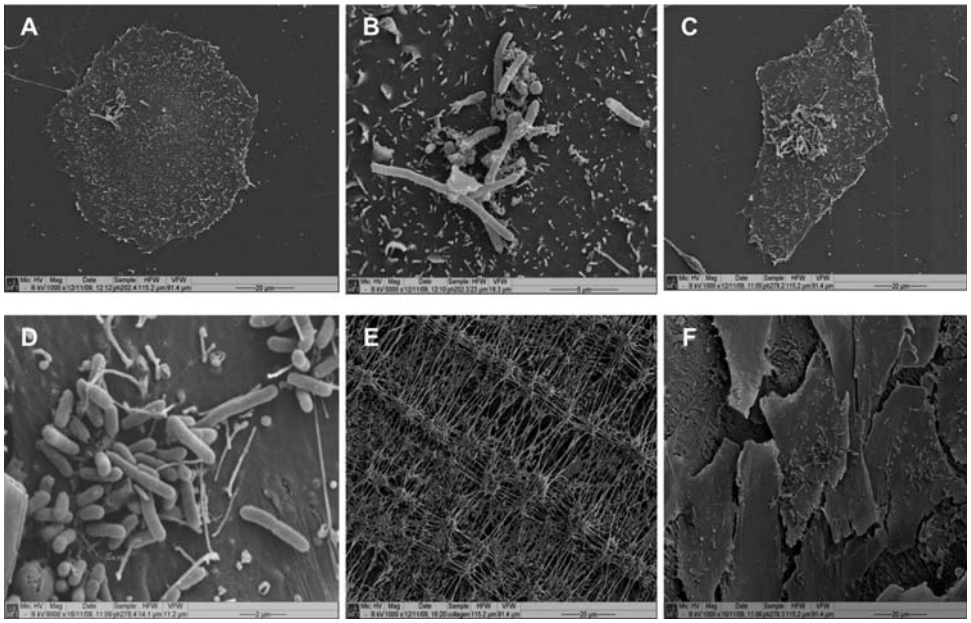

Figure 4. SEM images of *M. haemolytica* adhering to OBEC after 1 h incubation with the cells at an MOI of 100:1. (**A**) and (**B**) represent isolate PH202 on coverslips and the remaining images represent isolate PH278. (**C**) and (**D**) are coverslip images, (**E**) and (**F**) are collagen transwell images.

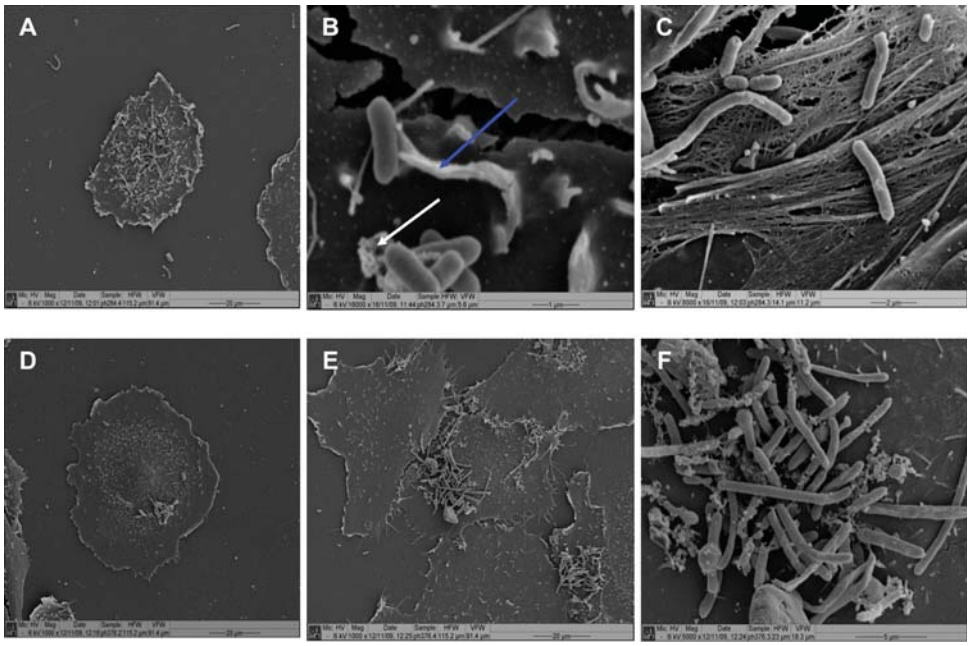

Figure 5. SEM images of *M. haemolytica* adhering to OBEC after 1 h incubation with the cells at an MOI of 100:1. Top panel represent isolate PH284 and bottom isolate PH376. (**A**), (**D**), (**E**) and (**F**) are coverslip images, (**B**) illustrates PH284 binding to a microvilli and (**C**) is a collagen transwell image. White arrow indicates unknown material. Blue arrow indicates microvilli with bound bacterium.

Discussion

Adherence of *M. haemolytica* to OBECs

Previous studies have shown that *M. haemolytica* adheres to different types of cultured epithelial cells.[15] However, adhesins that facilitate such binding to target cells are poorly described. In the present study, it was demonstrated that bovine and ovine *M. haemolytica* isolates adhered with equal affinity to OBECs, suggesting that the bacteria possess non-specific adhesins involved in such attachment.

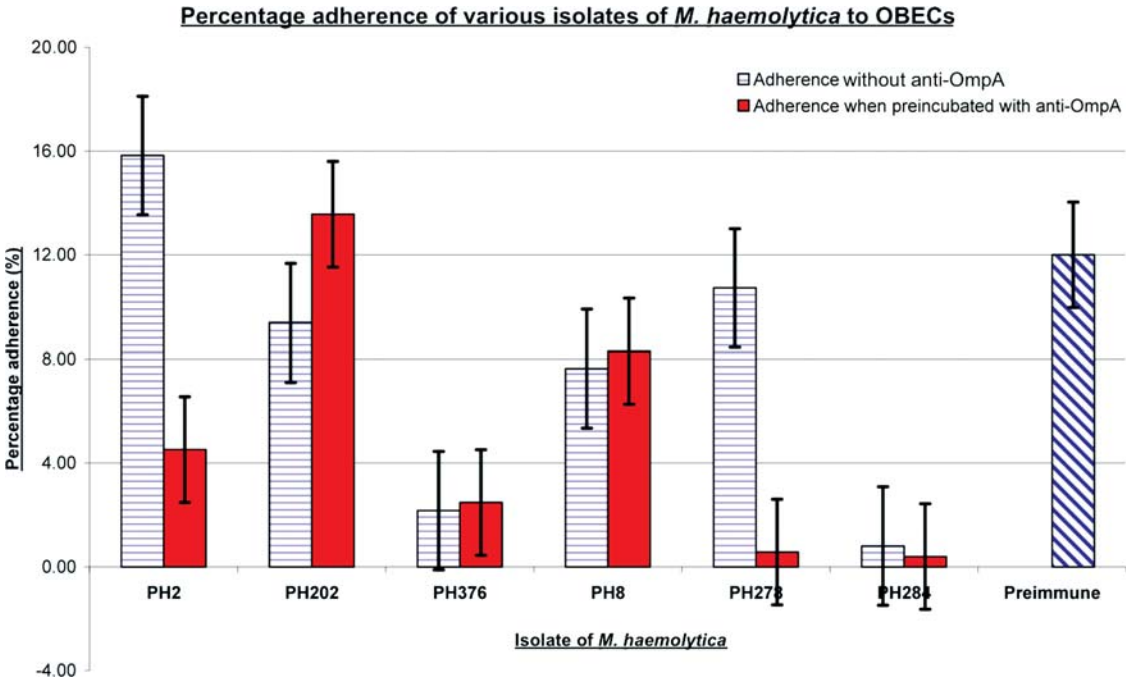

Figure 6. Mean percentage adherence ± standard error of various isolates of *M. haemolytica* to OBECs with and without prior incubation with anti-OmpA antibodies. Data are the mean percentage adherence of six repetitions for each bacterial isolate, expressed as a percentage of the original inoculums. *$p < 0.05$ compared with adherence without prior pre-incubation with anti-OmpA.

Table 2. Biofilm formation of *M. haemolytica*, determined by the presence of black colonies on Congo red agar after 24 and 48 h incubation at 37°C

Species of isolation	Isolate	Presence of black colonies after	
		24 h	48 h
Bovine	PH2	+	++
Ovine	PH8	++	++
Bovine	PH202	+	++
Ovine	PH278	−	++
Ovine	PH284	++	+++
Bovine	PH376	−	++
Human	*S. epidermidis* (control)	+++	+++

S. epidermidis (GDH 2346) was used as a positive control. Data are presented as the presence (+) or absence (−) of black colonies. Species of isolation are shown. '+' means slightly darkened colonies, '++' denotes lots of defined black colonies, '+++' dense growth of black colonies and '−' no black colonies.

Interestingly, the level of adherence with all isolates tested was found to be greater on OBECs, which were more heavily decorated with microvilli. This is consistent with findings of numerous other respiratory pathogens such as *Klebsiella pneumoniae*, *Moraxella catarrhalis* and *Pseudomonas aeruginosa*[22,23] In essence cells with a greater surface area, decorated with microvilli or cilia, allow greater regions for attachment.[24]

However, there were differences in the levels of adherence displayed by different isolates with the two virulent bovine and ovine isolates (PH2 and PH278) exhibiting the greatest level of attachment. This could be explained by the fact that in order for these isolates to cause significant disease in the lower respiratory tract they must adhere to the URT in order to overcome the mucocillary escalator. Furthermore, capsular type appeared to affect the level of adherence achieved, with capsular type A1 exhibiting a 10-fold greater level of attachment compared with A6 isolates. Possible reasons for such differences may lie in the composition and thickness of the capsules; with thicker capsules potentially shrouding any adhesins and thus reducing the level of attachment achieved, something well documented in *Streptococcus suis*.[25,26]

Interestingly, two previously undocumented observations were made in this study, first, the filamentation of all six isolates tested on adherence to OBEC. It is unknown whether this pleomorphism is due to media growth conditions. However, it has been documented[27,28] that exposure of UV light, peroxides or heavy metals can result in the inhibition of septum formation in *E. coli*, resulting in filamentation.

Second, the study also demonstrated, for the first time, that *M. haemolytica* possesses the potential to form biofilms. Distinct microcolonies encased in an extracellular polymeric substance (ECP) were visible after 2 h incubation with OBEC. However, after 24 h incubation the images retrieved showed only remnants of the ECP. Potential reasons for the

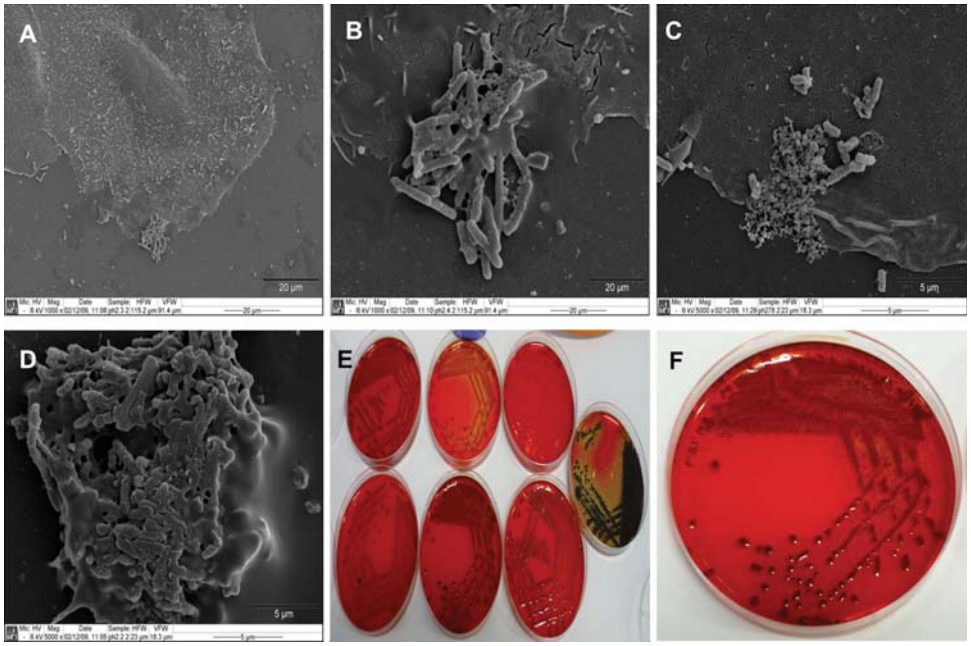

Figure 7. Images confirming biofilm formation of *M. haemolytica*. Top panel are SEM images of *M. haemolytica* isolates PH2 and PH278 adhering to OBEC (MOI of 100:1) after 2 h incubation with the OBECs. (**A**) and (**B**) PH2 and (**C**) PH278. Bottom panel is a photograph of the Congo red plates after 48 h incubation, isolates arranged in numerical order from left to right. Far right middle image is the positive control.

lack of congruence in the images may include nutrient deprivation as, after 24 h, nutrients in the media are likely to be exhausted, thus halting the production and renewal of ECP. Such deprivation could be overcome by adapting the approach of,[29] by using a flow-through chamber that permits the insertion of a coversli coated with OBECs and adherent *M. haemolytica*, hence providing constant replenishment of media and preventing nutrient starvation. Additionally, there are several improvements that could be made to the SEM protocol. For example, Alcian Blue could be added to the SEM fixative.[30] This has been shown to help preserve ECP composition, allowing the integrity of the biofilm structure to be preserved and viewed.

OmpA involvement in the adherence of *M. haemolytica* to OBECs

Preincubation of *M. haemolytica* isolates with anti-OmpA specific antibodies failed to have any affect on the level of adherence achieved by four of the six isolates tested. However, the level of attachment achieved by the two virulent bovine (PH2) and ovine (PH278) isolates to OBECs was significantly inhibited, implying that OmpA is a potential virulence factor. The role of OmpA as a virulence factor can be seen in *Klebsiella pneumoniae* binding to human bronchial epithelial cells,[15] *Acinetobacter baumanni* binding to, and inducing apoptosis in, epithelial cells[31]and *Actinobacillus suis* binding to porcine epithelial cells.[18] Furthermore, recent work[16] has shown that OmpA acts as a specific ligand for fibronectin, further compounding the complexity of OmpA's involvement in attachment to bronchial epithelial cells. Further work, entailing the creation of mutant OmpA is required in order to determine whether it is an important attachment facilitator, a virulence factor or a combination of both.

Adherence of *M. haemolytica* to collagen

The ECM is a complex network consisting of a diverse group of secreted extracellular macromolecules that form the scaffolding responsible for the development, growth and maintenance of mammalian tissues.[32] In epithelial tissues, certain ECM proteins function together with cells to form barriers intended to prevent penetration of these tissues by microorganisms. However, many bacterial pathogens have evolved mechanisms such as adhesins, which attach to ECM components to circumvent such defences.[33] Recently, Daigneault and Lo[34] have identified the *ahs* locus in *M. haemolytica*, which encodes two collagen-binding trimeric autotransporters (ahsA and ahsB). This *ahs* locus shares great homology with *Aggregatibacter actinomycetemcomitans* EmaA adhesin, *H. influenzae*'s Hsf and Hia proteins and *Yersinia enterocolitica's* YadA adhesin.[33]

Conclusions

Despite the growing economic importance of *M. haemolytica* as an opportunistic pathogen of ruminants, very little is understood about its pathogenesis, specifically the interaction between the bacterium and the host respiratory

tract. This study has furthered our understanding of *M. haemolytica*'s interaction with the ovine respiratory epithelium, showing that regardless of the host species the bacterial isolate originated from the levels of adherence to OBEC were the same. Furthermore, the finding that *M. haemolytica* possesses the ability to form biofilms provides further insight into the pathogenesis mechanisms deployed by the bacteria prior to entering the LRT.

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Author biography

S.-J.H. recently graduated from the University of Glasgow with an MSci. in Microbiology. Her main interests throughout these 5 years have been in antibiotic resistance acquisition, biofilm formation and the development of cell culture models as a way of investigating *in vivo* disease without the use of animal models. More recently she is about to begin a PhD in the characterization of the functional ecology of slow sand filtration through metagenomics.

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