Pathogenic *Mannheimia haemolytica* invades
differentiated bovine airway epithelial cells

Running title: *Mannhaeimia haemolytica* invasion of epithelial
cells

Daniel Cozens¹, Erin Sutherland¹, Miquel Lauder¹, Geraldine Taylor²,
Catherine C. Berry³ and Robert L. Davies¹#

¹ Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life
Sciences, University of Glasgow, Glasgow, UK

² The Pirbright Institute, Pirbright, Surrey, UK

³ Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life
Sciences, University of Glasgow, Glasgow, UK

#Corresponding author e-mail: robert.davies@glasgow.ac.uk
ABSTRACT

The Gram-negative bacterium *Mannheimia haemolytica* is the primary bacterial species associated with bovine respiratory disease (BRD) which is responsible for significant economic losses to the livestock industries worldwide. Healthy cattle are frequently colonised by commensal serotype A2 strains, but disease is usually caused by pathogenic strains of serotype A1. For reasons that are poorly understood, a transition occurs within the respiratory tract and a sudden explosive proliferation of serotype A1 bacteria leads to the onset of pneumonic disease. Very little is known about the interactions of *M. haemolytica* with airway epithelial cells of the respiratory mucosa which might explain the different abilities of serotype A1 and A2 strains to cause disease. In the present study, host-pathogen interactions in the bovine respiratory tract were mimicked using a novel differentiated bovine bronchial epithelial cell (BBEC) infection model. In this model, differentiated BBECs were inoculated with serotype A1 or A2 strains of *M. haemolytica* and the course of infection followed over a five-day period by microscopic assessment and measurement of key proinflammatory mediators. We have demonstrated that serotype A1, but not A2, *M. haemolytica* invades differentiated BBECs by transcytosis and subsequently undergoes rapid intracellular replication before spreading to adjacent cells and causing extensive cellular damage. Our findings suggest that the explosive proliferation of serotype A1 *M. haemolytica* that occurs within the bovine respiratory tract prior to the onset of pneumonic disease is potentially due to bacterial invasion of, and rapid proliferation within, the mucosal epithelium. The discovery of this previously unrecognised mechanism of pathogenesis is important because it will allow the serotype A1-specific virulence determinants responsible for invasion to be identified and thereby provide opportunities for the development of new strategies for combatting BRD aimed at preventing early colonisation and infection of the bovine respiratory tract.
Mannheimia haemolytica is a Gram-negative bacterium and is the primary bacterial species associated with bovine respiratory disease (BRD), a multifactorial condition of cattle involving poorly understood interactions between various bacterial and viral pathogens and the host (1-3). Bovine respiratory disease is responsible for significant economic losses (>$1-3 billion annually in the USA alone) to the livestock industries worldwide (1, 4-6).

Antibiotics play an important role in the control of BRD but the incidence of multi-drug resistant bacterial strains is increasing (7-12) and there are serious public health concerns associated with the increased use of antimicrobial drugs in food-producing animals (1, 3, 13-15). Therefore, alternative less drug-dependent strategies are required to control disease.

Vaccination is widely used for the prevention of BRD but the efficacy of currently available vaccines is inconsistent and improved vaccines are required (15, 16). However, the development of improved vaccines and other control measures is hindered by our limited understanding of the pathogenesis of BRD.

Mannheimia haemolytica occurs naturally as a commensal in the upper respiratory tract (URT) of healthy cattle but, under circumstances which are poorly understood, is frequently associated with disease (3, 5, 17). The bacterium comprises 12 capsular serotypes (18).

Healthy cattle are often colonised by commensal strains of serotype A2 but disease is almost always caused by pathogenic isolates of serotype A1 (1, 3, 5, 6, 9, 14). For reasons that are unclear, but associated with crowding, stress and/or viral infection, a sudden explosive proliferation occurs in the number of serotype A1 bacteria present in the URT of susceptible animals (5, 6, 17, 19). The colonisation of the mucosal surfaces leads to inhalation of bacteria-containing aerosol droplets into the lungs and predisposes to the onset of pneumonic disease (20, 21). Thus, pneumonia appears to be the consequence of two events – the first in the URT and the second in the lungs (19).
Events within the lungs are relatively well-defined. The secretion of leukotoxin and release of lipopolysaccharide together play a central role in the migration of neutrophils into the lungs and these immune cells are largely responsible for the excessive pulmonary inflammation and tissue damage associated with BRD (5, 6, 22-24). In contrast, the reasons for the very different behaviour of serotype A1 and A2 strains within the URT during the early stages of colonisation, and indeed for their differing abilities to cause disease, are not known. Serotype A1 and A2 strains of *M. haemolytica* differ in a wide range of virulence-associated characteristics (25-31) but there is little clear-cut evidence that any of them have specific roles which might explain unequivocally the differences in pathogenicity of these strains. Due partly to the unavailability of physiologically-relevant and reproducible *in vitro* methodologies, there has been very little focus on improving our understanding of the early interactions of *M. haemolytica* with the respiratory epithelium prior to the onset of disease.

We believe that understanding these early host-pathogen interactions is key to explaining the differential responses of serotype A1 versus A2 *M. haemolytica* strains with respect to high-level nasopharyngeal colonisation and/or disease causation.

Airway epithelial cells (AECs) play important roles in defence of the respiratory tract. The respiratory epithelium provides a physicochemical barrier against inhaled microorganisms and particulates which involves the presence of intercellular junctions and mucociliary clearance (32, 33). Furthermore, AECs are involved in the innate immune response and, during BRD, are the source of proinflammatory mediators which stimulate the activation and regulation of neutrophils and macrophages (22, 34, 35). Submerged AEC cultures, using either primary cells or immortalised cell lines, have been used to investigate interactions of *M. haemolytica* (34, 36-38) with the bovine respiratory tract but these have various limitations: they do not reflect the multicellular complexity of the parental tissue *in vivo*, they lack its three-dimensional (3-D) architecture, and the physiological conditions are
not representative of those found within the bovine URT. However, those characteristics that are lacking in submerged cultures can be recapitulated using differentiated AECs grown at an air-liquid interface (ALI) and such cell culture approaches have been used to study the interactions of various bacterial and viral pathogens with different host species (39-57). Indeed, with a view to similarly studying the interactions of *M. haemolytica* with bovine respiratory epithelium, we have established optimum culture conditions for the growth and differentiation of bovine bronchial epithelial cells (BBECs) grown at an ALI (58) and have identified a 21- to 42-day window during which these cultures are fully differentiated, healthy and suitable for infection studies (59).

In the present study, we hypothesized that serotype A1 and A2 strains of *M. haemolytica* interact with and stimulate differentiated BBECs in different ways. In particular, since various bacterial pathogens of the human respiratory tract are known to invade human AECs (55, 56, 60-63), we wished to explore the possibility that serotype A1 *M. haemolytica* invades bovine AECs. Here, we demonstrate that pathogenic serotype A1, but not commensal serotype A2, *M. haemolytica* invades, and replicates within, bovine AECs. Importantly, the discovery of this invasion process provides a possible explanation for the explosive proliferation of serotype A1 bacteria that occurs in the bovine URT before the onset of pneumonic disease and thereby opens avenues for the development of new disease intervention strategies.

**RESULTS**

*Mannheimia haemolytica* serotype A1 but not A2 colonises differentiated BBECs.

The ability of *M. haemolytica* serotype A1 (PH2) and A2 (PH202) isolates to adhere to and colonise differentiated BBECs was first assessed by bacterial enumeration at selected time-points from 0.5 to 120 h post-infection (pi) (Fig. 1). Adherence of both isolates was similar at very early time-points (0.5 and 2 h pi). A relatively small number of bacteria, representing
~1% of the inoculum, adhered to the BBECs at each time-point (Fig. 1A); conversely, a very high number of bacteria, comprising the majority of the inoculum, was present in the apical washes at each time-point (Fig. 1B). However, the fate of bacteria representing each of the two isolates associated with the BBECs was subsequently very different. From 6 h pi, bacterial numbers representing serotype A1 isolate PH2 increased exponentially and achieved a maximum (>100% of inoculum) at 24 h pi; thereafter, the numbers declined to ~100% of inoculum by 120 h (Fig. 1A). The number of serotype A1 bacteria within the washes increased marginally over the same time-points (Fig. 1B) but this increase did not match the exponential increase observed for the bacteria associated with the BBECs between 6 and 24 h (Fig. 1A). These results suggest that a high proportion of the increased bacterial numbers observed in Fig. 1A (between 6 and 24 h) remained associated with the tissue and were not removed by washing. In contrast, bacterial numbers of serotype A2 isolate PH202, both those associated with the BBECs (Fig. 1A) and those present in the washes (Fig. 1B), either decreased (animals 2 and 3) or increased marginally (animal 1) from 6 h onwards. Thus, serotype A2 bacteria were completely cleared by 16 h from animal 2 cultures, were cleared by 120 h from animal 3 cultures, and were present in low numbers (~10% of inoculum) after 120 h in animal 1 cultures. Taken together, these results clearly demonstrate marked differences in the ability of the *M. haemolytica* isolates to colonise differentiated BBEC cultures: the serotype A1 isolate was able to rapidly colonise the epithelial layer whereas the serotype A2 isolate was unable to do so.

*Mannheimia haemolytica* invades differentiated BBECs and forms foci of infection.

Colonisation of the differentiated BBEC cultures was next evaluated using immunofluorescence microscopy (IFM) and scanning electron microscopy (SEM). Immunofluorescence microscopy of infected cultures revealed very little evidence of adherence by either isolate over the first six hours of infection (Figs. 2A and S1) and this
observation was generally confirmed by SEM (Figs. 2B and S2). However, further scrutiny by SEM revealed low levels of sporadic bacterial adherence over the apical surface (Fig. 3).

Thus, small numbers of serotype A1 bacteria (typically 2-3 per cell) were observed to adhere to patches of epithelial cells (Fig. 3A, arrowheads), as well as occasionally to mucus (Fig. 3A, arrows), at early time-points. Notably, serotype A1 bacteria adhered to the apical surfaces of non-ciliated cells but not to the cilia of ciliated cells (Fig. 3B, arrow).

As expected from the viable counts data (Fig. 1), the outcome of infection with the serotype A1 and A2 strains was very different after six hours. Immunofluorescence microscopy demonstrated that bacterial numbers of serotype A1 isolate PH2 progressively increased in abundance from 6 h pi at successive time-points up to 24 h (Fig. 2A); these observations corresponded with the increasing numbers of bacteria recovered from the cultures between 6 and 24 h (Fig. 1A). However, the bacteria were not evenly distributed across the epithelial surface but, rather, were initially present in relatively small numbers of clusters, or foci of infection, that appeared to be associated with non-ciliated regions (Fig. 2A). The number, size as well as density of these foci increased between 12 and 24 h, and later time-points were associated with progressively diminished staining of cilia indicating increased damage and destruction of the epithelial layer; this was most clearly observed at 72 and 120 h (Fig. S1). The identification of well-established and extensive foci of infection from 16 h pi by IFM was confirmed by SEM (Fig. 2B). Moreover, SEM revealed that from 16 h pi these infection foci were characterised by invasion and disruption of the epithelial layer; large numbers of bacteria were clearly visible within deeper regions of infected, fractured tissue (Fig. 2B, arrowheads). At later time-points (i.e. from 48 h), SEM revealed severe destruction of the epithelial layer such that the underlying membrane was exposed (Fig. S2). In striking contrast to PH2, serotype A2 isolate PH202 continued to exhibit little or no sign of adherence from 6 h pi and there was no evidence of the colonisation and invasion...
Colonisation of the differentiated BBEC cultures was also assessed by haematoxylin and eosin (H&E) and immunohistochemical (IHC) staining of histological sections. As expected, there was little evidence of bacterial adherence and colonisation by either isolate over the first six hours of infection but the fate of bacteria representing each of the two isolates was again very different after six hours (Fig. 4). At 12 h pi, serotype A1 (PH2) bacteria were not discernible by H&E staining (Fig. 4A) but IHC staining revealed numerous small clusters of bacteria at the epithelial cell surface (Fig. 4B, arrowheads). By 16 h, bacteria were clearly observed within distinct foci of infection by H&E staining (Fig. 4A, arrow) and this was confirmed by IHC staining (Fig. 4B, arrow). Notably, this rapid increase in bacterial numbers between 12 and 16 h was supported by the IFM and SEM imaging (Fig. 2A and B). These observations highlight that the four-hour period between 12 and 16 h pi represents a key transition stage during which infection rapidly progresses from small clusters of bacteria present at the apical surface to extensive, deep-seated foci of infection which extend the full depth of the epithelial layer. Over the following eight hours (16 to 24 h pi) the foci of infection became larger (due to the lateral spread of bacteria) and more numerous (Fig. 4A and B). Furthermore, from 16 h pi, epithelial cells in the vicinity of the infection foci displayed cytopathic effects; large numbers of rounded and apoptotic cells were present (Fig. 4A, arrowheads). By 48 and 72 h, the integrity of the epithelial layer was significantly disrupted (Figs. S3 and S4) and epithelial fragments were removed during washing; these observations most likely account for the decline in bacterial numbers described above after 24 h (Fig. 1A). In contrast, and consistent with IFM and SEM, there was (with one exception) no evidence of bacterial colonisation and tissue invasion by serotype A2 isolate PH202 (Fig. 4). Indeed, the epithelial layer remained intact until day 5 (Figs. S3 and S4) and...
maintained its ciliation and barrier function (described below). The single exception was the 120 h time-point for animal 1 which showed some signs of bacterial colonisation and tissue disruption (results not shown); this observation was in agreement with the recovery of low numbers of bacteria from cultures infected with isolate PH202 (Fig. 1A). A semiquantitative assessment of bacterial infection of the BBEC cultures from each of the three animals is shown in Table 1.

**Invasion of BBECs is independent of tight junction integrity.** Tight junctions, together with adherens junctions and desmosomes, are involved in creating the hallmark barrier function of the respiratory tract and are specifically targeted and degraded by certain bacterial pathogens during paracellular infection processes (64-67). Tight junction integrity of BBECs was assessed following challenge with isolates PH2 and PH202 by ZO-1 staining and transepithelial electrical resistance (TEER) determination. Normal tight junction staining was observed within BBEC cultures infected with serotype A1 isolate PH2 at early time-points (0.5 to 16 h pi); the integrity of the junctional complexes was unaffected by early *M. haemolytica* colonisation (Figs. 5A and S5). In particular, the tight junctions of those epithelial cells which bacteria had most likely invaded at 12 and 16 h remained intact (Fig. S6A). However, as epithelial cells were damaged and disrupted at later time-points (e.g. at 20 and 24 h), there was a simultaneous loss of tight junction staining within infection foci although the surrounding cells still maintained intact tight junctions (Fig. 5A). Confocal microscopy confirmed that tight junctions remained intact in areas adjacent to infection foci (Fig. S6B). At 48, 72 and 120 h, there was a loss of tight junction staining over large areas of the epithelial layer which corresponded with the extensive tissue destruction described above (Fig. S5). These observations were reflected in the TEER measurements for isolate PH2 (Fig. 5B). TEER values were maintained until 16 h pi but there was a significant reduction in TEER between 16 and 48 h pi (*p* < 0.001, Two-way ANOVA). Furthermore, transmission
electron microscopy (TEM) of infected BBECs confirmed the presence of intact tight
junct ions between epithelial cells whose paracellular spaces contained numerous bacteria
(Fig. 5C). In contrast, infection with serotype A2 isolate PH202 had no effect on tight
junction integrity over the five-day time-course as determined by ZO-1 staining (Figs. 5A
and S5) and TEER measurement (Fig. 5B).

Based on the above observations, we hypothesized that the serotype A1 isolate PH2 was
not invading the epithelial layer by direct targeting of the tight junctions (i.e. via paracytosis)
as occurs in some bacterial pathogens (64-67). To test this hypothesis, we treated BBECs
with lipoxin A₄ (LXA₄) prior to infection with PH2. Lipoxin A₄ is a biologically active
eicosanoid which stimulates tight junction formation and repair in bronchial epithelial cells
(68, 69). The TEER of BBEC cultures pre- and post-infection increased with increasing
concentrations of LXA₄ (Fig. S7A), confirming improved barrier function, but this was not
accompanied by a reduction in the numbers of colonising bacteria 24 h pi (Fig. S7B) as might
be expected if bacteria were invading by this route.

*Mannheimia haemolytica* invades differentiated BBECs by transcytosis and rapidly
repli cates intracellularly. The association of fluorescently-labelled bacteria of serotype A1
isolate PH2 with the centres of infected BBECs, and their complete absence from cell
peripheries (Fig. S6A, arrowheads), provided preliminary evidence that transcytosis, and not
paracytosis, was the route of cellular invasion. Confocal microscopy was subsequently used
to further identify the distribution of bacteria within infected BBEC cultures. Z-stack
projections of infected cultures displayed high densities of bacteria confined within epithelial
cell boundaries (Fig. 6A, arrowheads). The intracellular location of bacteria was confirmed
using a gentamicin protection assay (Fig. S8). Following infection with isolate PH2, a small
intracellular subpopulation of gentamicin-surviving bacteria was present at 12 h pi which had
increased substantially by 24 h pi. The number of intracellular bacteria had decreased by 48

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h, a finding which is explained by the destruction and removal of the epithelium described above. Gentamicin-surviving (internalised) bacteria of serotype A2 isolate PH202 were not detected at any time-points following challenge.

To address the key question of how serotype A1 bacteria are internalised, more extensive SEM and TEM analyses were performed. Potential evidence for bacterial uptake by epithelial cells was provided by SEM although such events were observed infrequently; this may be due to the polarisation of the cells (51, 60) and/or selective binding to a limited repertoire of cells (46). Large numbers of bacteria were occasionally observed to be closely associated with invaginations of the epithelial cell surface (Fig. 3C and D, white arrows). The close proximity of extruded mucus to some of these invaginations (Fig. 3D, black arrow) suggests that these epithelial cells are, in fact, goblet cells and that this cell type represents a potential portal of entry for *M. haemolytica*. There was also evidence of single bacterial cells being taken up by epithelial cells (Fig. 6B [i]). However, evidence for the intracellular location and replication of serotype A1 bacteria was more readily obtained. Thus, large numbers of internalised bacteria were observed within epithelial cells by SEM (Fig. 6B [ii], arrow), suggesting that rapid intracellular replication occurs after uptake. Bacterial uptake and intracellular replication within BBECs were confirmed by TEM (Fig. 6C). Numerous membrane-bound vesicles containing bacteria were observed within BBECs infected with serotype A1 isolate PH2 at timepoints between 16 and 24 h pi. Many vesicles contained only a single bacterium and were often located just beneath the cell surface (Fig. 6C [i], arrow) whereas other vesicles were much larger and contained numerous (>50) bacteria (Fig. 6C [ii], arrow). In contrast, there was no evidence for the internalisation and replication of serotype A2 bacteria.

*Mannheimia haemolytica* stimulates the release of key pro-inflammatory mediators by BBECs. To provide insight into the proinflammatory innate immune response of BBECs
following challenge with *M. haemolytica* and assess differences in the response to serotype A1 and A2 isolates, the production of four key proinflammatory cytokines/chemokines was assessed by ELISA. Following challenge with isolate PH2 or PH202, the release of the cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) and the chemokine CXCL8 was quantified at selected time-points pi from both the basal (Fig. 7) and apical (Fig. S9) surfaces. All cytokines/chemokines exhibited a significant increase (*p* ≤ 0.0001, Two-way ANOVA) in production from the basal surface following infection by either PH2 or PH202 in comparison to uninfected controls (Fig. 7). Notably, the production of IL-1β and TNFα was significantly higher (*p* ≤ 0.0001, Two-way ANOVA) in cultures challenged with serotype A1 isolate PH2 compared to serotype A2 isolate PH202, peaking at 16 and 24 h, respectively; conversely, the IL-6 and CXCL8 response to both isolates was very similar. Production of IL-6 was more rapid and peaked much earlier than the other three cytokines/chemokines at 6 h pi. Notably, the response of the chemokine CXCL8 was delayed in comparison to that of the three cytokines; there was virtually no expression at 2 h but a sudden increase in activity occurred at 6 h and production continued to increase until 48 h pi. The amount of CXCL8 produced was also generally 10- to 40-fold higher than that of the three cytokines. The release of the same cytokines/chemokines from the apical surface followed the same trend as that from the basal surface (Fig S9).

**DISCUSSION**

In the present study, we have investigated the interactions of serotype A1 and A2 strains of *M. haemolytica* with differentiated BBECs with the aim of improving our understanding of host-pathogen interactions within the bovine respiratory tract during the early stages of BRD. Differentiated BBEC cultures recapitulate the 3-D, multicellular architecture of the *in vivo* airway epithelium; in particular, they possess the hallmark barrier functions of the bovine respiratory tract, including active mucociliary clearance and intact junctional complexes (58,
These mechanisms represent the first line of defence against invading pathogens and are important considerations when modelling bacterial interactions within the airways (32, 65, 70). Here, we have presented detailed BBEC infection data for single serotype A1 and A2 isolates at frequent time-points over 5 days but, importantly, our results were confirmed in further infection experiments in which we analysed an additional isolate representing each serotype at less frequent time-points (results not shown).

The initial adherence of the serotype A1 and A2 isolates to differentiated BBECs (up to 2 h pi) was very similar; enumeration demonstrated that adherence corresponded to approximately 1% of the inoculum. This level of adherence was in agreement with that previously observed for *M. haemolytica* to *ex vivo* URT tissue (3% of inoculum)(71) and BBECs (4% of inoculum) (38) maintained under submerged conditions. Microscopy revealed that adherence was generally sporadic, of relatively low frequency and, notably, occurred primarily to non-ciliated cells, rather than to ciliated cells or cilia. Interestingly, the related nontypeable *Haemophilus influenzae* (NTHI) also exhibits tropism towards non-ciliated AECs (46), whereas other more distantly related bacterial respiratory tract pathogens, including *Moraxella catarrhalis* (40), *Mycoplasma pneumoniae* (48, 51), *Pseudomonas aeruginosa* (72) and *Bordetella pertussis* (73), bind preferentially to ciliated AECs.

The fate of the serotype A1 and A2 isolates was very different at 6 h pi and later time-points. Enumeration of bacteria together with immunofluorescence, electron and light microscopy data provided clear evidence that *M. haemolytica* serotype A1 invades the epithelial cell layer by 12 h pi and rapidly replicates between 12 and 24 h pi to form characteristic foci of infection. The infection foci subsequently expand by lateral spread of bacteria which leads to severe disruption and destruction of the epithelial layer by 48 to 72 h pi. In contrast, *M. haemolytica* serotype A2 shows no, or very limited, capability for infection of BBEC cultures; this strain is unable to invade and replicate within the epithelium.
under the described conditions. Indeed, the clearance of this isolate from BBEC cultures suggests the presence of antimicrobial activity which is discussed further below. Evidence derived from both SEM and TEM, as well as from gentamicin protection assays, confirmed that serotype A1 *M. haemolytica* is internalised by BBECs and subsequently undergoes rapid intracellular replication. Invasion of AECs by *M. haemolytica* has not previously been described and represents a new facet of disease pathogenesis and a new virulence mechanism for this bacterial species. In a previous study, submerged BBECs were infected with *M. haemolytica* for 3 h but invasion was not observed (38). Our findings highlight two significant advantages of using differentiated BBECs compared to submerged cultures in infection studies. First, the cultures are, by definition, differentiated and comprise the different cell types (e.g. ciliated, goblet and basal cells) that occur in native respiratory epithelium; the cultures also possess the 3-D architecture of native epithelium and are growing under similar physiological conditions as occur in vivo. Second, it is possible to perform long-term infection experiments (up to 5 days in our case) with differentiated ALI cultures; this is not possible with submerged cultures due to bacterial growth within the culture medium. Thus, invasion was likely not observed in the previous study (38) because the epithelial cells were undifferentiated and the incubation time insufficient. Although invasion of AECs by *M. haemolytica* has not previously been described, it was not a complete surprise because this process is involved in the pathogenesis of various human respiratory tract infections caused by NTHI (46, 74-76), *M. catarrhalis* (61, 62) and *Neisseria meningitidis* (55, 60). The discovery of this invasion process led us next to question how *M. haemolytica* is traversing the epithelium. The airway epithelium acts as a physical barrier against infection and microbes have evolved various strategies for crossing this barrier; these include passing between cells (paracytosis), entering and passing through cells (transcytosis), or simply killing cells to
eliminate the barrier (32, 70, 77). Bacteria that cross epithelia by paracytosis typically possess mechanisms that target the tight junctions and other intercellular junctions (64, 65, 78, 79). In the present study, several lines of evidence suggest that the serotype A1 \( M. \) \textit{haemolytica} isolate PH2 is not traversing the epithelial layer \textit{via} paracytosis by disrupting tight junctions. First, IFM revealed that bacteria were adhering to and entering cells at a central location; there was no evidence that bacteria were associated with the cell periphery and this was confirmed by SEM. Second, there was no evidence that the integrity of the epithelium, as assessed by TEER, was adversely affected during the early stages of infection as would be expected if secreted bacterial factors were targeting tight junctions prior to invasion. The rapid decline in TEER between 16 and 48 h and associated disruption of tight junctions was clearly due to the destruction of the epithelial layer. Third, TEM analysis identified intact tight junctions between epithelial cells whose paracellular spaces contained numerous bacteria. Fourth, the addition of LXA\(_4\) to BBEC cultures prior to infection increased the integrity of the tight junctions but did not reduce tissue invasion. Lipoxin A\(_4\) stimulates the expression of ZO-1, prevents tight junction disruption and reduces the invasion of bronchial epithelial cells by \( P. \) \textit{aeruginosa} (68, 69). Thus, the addition of LXA\(_4\) to BBECs might be expected to reduce colonisation if tight junctions were being targeted and represented the route of entry but this was not the case.

Of the major human respiratory tract pathogens, there is some evidence that NTHI traverses respiratory epithelium \textit{via} paracytosis (56, 63, 80) but transcytosis plays a more prominent role in the traversal of bacterial pathogens across the respiratory epithelium. Thus, \( M. \) \textit{catarrhalis} invasion occurs \textit{via} macropinocytosis involving microfilaments and the formation of lamellipodia (61, 62) and \( N. \) \textit{meningitidis} traverses the respiratory epithelial barrier \textit{via} an intracellular microtubule-dependent route (55, 60). Micropinocytosis involving the formation of microvilli and lamellipodia (46), receptor-mediated endocytosis (81, 82) and
lipid raft-independent endocytosis (74) have been cited as mechanisms for the internalisation of NTHI by AECs. Assessing the precise mechanism of BBEC invasion by *M. haemolytica* was beyond the scope of the present study but SEM and TEM analyses of infected BBECs failed to identify any evidence of the membrane ruffling and lamellipodia formation that is characteristic of NTHI (46, 83) and *M. catarrhalis* (61) interactions with human AECs. However, SEM imaging did reveal evidence for the potential uptake of *M. haemolytica* by non-ciliated epithelial cells in the form of large numbers of bacteria associated with membrane invaginations. These invaginations may be linked with mucus extrusion and it is interesting to speculate that goblet cells are perhaps involved in bacterial uptake but further evidence is required to confirm this. A role for goblet cells in *M. haemolytica* invasion would not be entirely surprising because *Listeria monocytogenes* specifically targets this cell type to gain entry to the intestinal epithelium (84). Although the precise mechanism remains to be elucidated, we propose that transcytosis rather than paracytosis represents the most likely route of epithelial invasion by serotype A1 *M. haemolytica*.

After internalisation, TEM analysis demonstrated that serotype A1 isolate PH2 became enclosed within membrane-bound vacuoles or endosomes. The presence of large numbers of bacteria within these vacuoles, together with the very rapid increase in bacterial numbers associated with the BBEC cultures between 12 and 24 h, suggest that *M. haemolytica* serotype A1 is capable of very rapid intracellular replication after internalisation. Thus, AECs appear to provide a suitable micro-environment for which *M. haemolytica* is adapted. The ability to invade, survive and replicate within AECs provides *M. haemolytica* with a potential intracellular niche that will shield the bacterium from antibodies, complement and antibiotics, potentially allowing persistence for extended periods of time (74, 75, 80). Although persistence within AECs has not previously been considered as a survival strategy for *M. haemolytica*, our findings nonetheless raise the possibility that bacteria internalised...
within AECs could act as a reservoir of infection (74, 75, 85) and potentially lead to recurrent or reemergent colonisation of the URT (17).

Based on the observations described above, we propose a model for the invasion of differentiated BBECs by serotype A1 M. haemolytica (Fig. 8). After initial adherence to the epithelial cell surface (1) bacteria are taken up by non-ciliated epithelial cells via an endocytotic-type mechanism (2). Rapid bacterial replication (3) subsequently occurs within endosomes present in the cytoplasm of infected epithelial cells and these likely fuse with the lateral membranes releasing bacteria into the paracellular spaces (4). Bacteria are taken up laterally by adjacent epithelial cells (5) and further replication within the paracellular spaces (6) and in secondarily-infected cells (7) occurs. These events very quickly lead to disruption of tight junctions, rupturing and death of epithelial cells, and the release of large numbers of bacteria onto the epithelial surface (8). Thus, the initial uptake of a relatively small number of bacteria leads to their rapid replication and spread to adjacent cells which is manifested in the observed formation of foci of infection and destruction of the epithelial cell layer. The rapid replication and release of large numbers of bacteria onto the epithelial surface, as proposed in this model, provides a potential mechanism which might explain the explosive proliferation of serotype A1 M. haemolytica that occurs in the URT of cattle prior to the onset of disease. Although in vivo evidence for invasion of respiratory airway epithelium by M. haemolytica is lacking, it is noteworthy that descriptions of the pathology and histopathology of BRD focus almost exclusively on lesions of the lungs (2, 17, 24, 86, 87). In addition, experimental challenge studies involving M. haemolytica typically involve intratracheal inoculation of bacteria and subsequent assessment again focusing on changes in lung pathology (13, 88-92). This challenge method does not replicate natural infection and host-pathogen interactions involving airway epithelia of the trachea and upper respiratory tract are completely by-passed.
In contrast to PH2, serotype A2 isolate PH202 was unable to colonise differentiated BBECs and, by day 5 pi, was cleared from cultures derived from two of the three animals. These observations suggest that PH202 was susceptible to the anti-bacterial activity of AECs. An important function of airway epithelium is the production of antimicrobial peptides in response to infection, including defensins and cathelicidins (32, 77, 93). Indeed, it is becoming increasingly clear that the production of antimicrobial peptides, such as tracheal antimicrobial peptide, by ruminant respiratory epithelial cells plays an important role in combatting BRD (22, 94-97). Thus, our observations highlight further potential differences between serotype A1 and A2 isolates of *M. haemolytica* in terms of their response to exposure to the antimicrobial activity of bovine AECs.

In addition to their barrier function, AECs also play an important role in orchestrating the host innate immune response to infection through the release of proinflammatory cytokines and chemokines (33, 77, 98, 99). The proinflammatory cytokines IL-1β, IL-6 and TNFα as well as the proinflammatory chemokine CXCL8 are produced by bovine AECs during BRD (23, 34, 35, 100). In the present study, challenge of differentiated BBECs stimulated the release of IL-1β, IL-6, TNFα and CXCL8 by strains of both *M. haemolytica* serotypes. Notably, the serotype A1 isolate PH2 resulted in significantly higher responses of IL-1β and TNFα than did the serotype A2 strain PH202. Insights into the kinetics of proinflammatory mediator release were also gained. Thus, IL-6 was produced very rapidly and peaked at 6 h whereas induction of the other cytokines/chemokines, especially CXCL8, was slower and production peaked later. There were also quantitative differences in cytokine/chemokine production: in particular, production of CXCL8 was 10- to 40-fold above levels for the three cytokines. Importantly, the earlier production of IL-1β and TNFα compared to CXCL8, and the higher levels of CXCL8 production, agree with kinetic profile and quantitative data on cytokine/chemokine production in the airways of calves infected with *M. haemolytica* (23).
Thus, the proinflammatory innate immune response of differentiated BBECs challenged with
*M. haemolytica* closely mimics the *in vivo* response of bovine AECs in infected calves and
provides partial validation for use of the model in such studies.

In summary, we have demonstrated that serotype A1, but not A2, *M. haemolytica* invades
differentiated AECs and subsequently undergoes rapid intracellular replication before
spreading to adjacent cells and causing extensive cellular damage. The differing abilities of
serotype A1 and A2 *M. haemolytica* isolates to invade and damage the airway epithelium
correlates with the behaviour of these strains *in vivo* and supports the relevance of using
differentiated BBECs for studying the pathogenesis of *M. haemolytica* disease. In particular,
our findings may provide insight into the previously unexplained and sudden explosive
proliferation of serotype A1 bacteria that occurs within the bovine respiratory tract prior to
the onset of pneumonic disease. The identification of an invasion mechanism in serotype A1,
but not A2, *M. haemolytica* represents a significant step forward in understanding why the
former, but not the latter, is responsible for the majority of disease outbreaks. Our findings
suggest that serotype A1 strains possess previously unrecognised virulence determinants
associated with invasion that may represent potential new vaccine and/or drug targets.
Understanding the molecular basis of AEC invasion may provide opportunities for the
development of new and improved prevention and treatment strategies that target early
colonisation of the bovine URT. Finally, we have demonstrated that differentiated BBECs
are an excellent mimic of the bovine respiratory epithelium and represent a realistic and
potentially powerful *in vitro* tool for studying the interactions of *M. haemolytica* and other
BRD-associated pathogens with their bovine host. Thus, the BBEC infection model
described herein has broad applications and significant potential for replacing and reducing
the use of cattle in BRD research.
MATERIALS AND METHODS

Bacterial strains and growth conditions. Two key *M. haemolytica* reference strains, PH2 and PH202, were used in this study. Isolate PH2 is of serotype A1 and was isolated from the lungs of a confirmed case of bovine pneumonic pasteurellosis, whereas isolate PH202 is of serotype A2 and was recovered from the nasopharynx of a clinically healthy calf on a disease-free farm. Both isolates have been characterised in previous comparative studies of *M. haemolytica* (27, 28, 31, 101-103). The bacterial isolates were stored at -80°C in 50% (v/v) glycerol in brain heart infusion broth (BHIB; Oxoid) and were subcultured on brain heart infusion agar (Oxoid) containing 5% (v/v) defibrinated sheep’s blood (blood agar) overnight at 37°C. Broth cultures were prepared by inoculating 25-ml volumes of BHIB from overnight growth on blood agar and incubating at 37°C and 120 rpm.

Isolation and culture of differentiated BBECs. Differentiated BBECs were prepared from primary bronchial epithelial cells recovered from the lungs of freshly-slaughtered, 24-30 month-old cattle as described previously (58). Briefly, the lungs were transported to the laboratory on ice and the left and right bronchi were dissected and sections incubated overnight at 4°C in digestion medium (DM). Epithelial cells were recovered from the bronchial sections and resuspended in submerged growth medium (SGM) at a cell density of 5.0 x 10^5 cells/ml. Ten-ml of cell suspension were seeded into T75 tissue culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 14% O₂. The BBECs were grown until 80-90% confluent (~4 days), trypsinised, and resuspended in SGM to a density of 5.0 x 10^5 cells/ml. Subsequently, 0.5 ml of the cell suspension were seeded onto the apical surface of 12-mm diameter, PET Thincerts of 0.4 µm pore diameter and containing 1.0 x 10^8 pores per cm² (Greiner, #665640). The epithelial cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and 14% O₂ and fed every 2 to 3 days. The TEER of the cultures was measured daily using an EVOM2 Epithelial Voltohmmeter (World
Precision Instruments, UK) according to the manufacturer’s instructions. When the TEER reached 200 Ω.cm$^2$ or above (~2 days), the growth medium was replaced with a 50:50 mixture of SGM and air-liquid interface (ALI) medium (containing 10 ng/ml epidermal growth factor and 100 nM retinoic acid). When the TEER reached 500 Ω.cm$^2$ (indicating successful barrier formation), an ALI was generated (this represented day 0 post-ALI) and the cells were fed exclusively from the basal compartment with ALI medium every 2 to 3 days until a well-differentiated epithelial layer was obtained (Fig. S10).

**Infection of bovine bronchial epithelial cells.** Differentiated BBEC cultures were infected on day 21 post-ALI (59). Twenty-four hours prior to infection, the basal medium was removed, the apical and basal compartments were washed twice with PBS, and the basal compartment replenished with 1.0 ml of antibiotic-free ALI medium. On the day of infection, bacterial broth cultures were grown to exponential phase (4-5 h), and the bacteria were harvested by centrifugation, washed and resuspended in PBS to a cell density of 1.0 x 10$^9$ cfu/ml. The apical surface of each BBEC culture was washed with 0.5 ml PBS and inoculated with 25 µl of bacterial suspension (2.5 x 10$^7$ cfu/insert) and the infected cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO$_2$ and 14% O$_2$. The BBEC cultures were assessed 0.5, 2, 6, 12, 16, 20, 24, 48, 72 and 120 h pi.

**Quantification of bacterial adherence and colonisation.** Bacterial enumeration was performed at each time-point following infection by performing viable counts. The ALI medium was removed from the basal compartment and the apical surface of the insert was washed three times with 1 ml PBS to remove unattached bacteria. The washes were pooled and the number of viable bacteria present determined as described below. The BBEC layer was disrupted by the addition of 0.5 ml of 1% Triton X-100 in PBS to the apical surface for 10 min followed by mechanical scraping and pipetting. Numbers of viable bacteria in both the apical washes and BBEC lysates were quantified, in triplicate, using the Miles and Misra...
method of bacterial counting (104). Bacterial numbers were expressed as a percentage of the inoculum. In some experiments, intracellular bacteria were enumerated using the gentamicin protection assay. In this case, the apical surface of the epithelial layer was incubated with 0.5 ml of gentamicin (200 µg/ml) for one hour at 37°C prior to disruption and counting as described above. Bacterial enumeration was performed in three independent BBEC cultures at each time-point and using cells from three different animals.

**Histology and immunohistochemistry.** Infected BBEC cultures representing each time-point pi were fixed, processed and sectioned as previously described (58); sections were stained either with H&E or by IHC. In the latter case, bacteria were identified by incubation for 30 min with a 1:800 dilution of rabbit anti-OmpA antibody (103), application of an anti-rabbit HRP-labelled polymer and visualisation with a REAL EnVision Peroxidase/DAB+ Detection System (Dako; #K3468); samples were subsequently counterstained with Gill’s haematoxylin. Tissue sections were viewed with a Leica DM2000 light microscope.

**Immunofluorescence microscopy.** Infected BBEC cultures representing each time-point pi were fixed and processed for IFM as previously described (58). Tight-junction formation and cilia were detected with anti-ZO-1 and anti-β-tubulin antibodies, respectively. Bacteria were detected with a 1:50 dilution of bovine anti-*M. haemolytica* whole-cell antibodies and visualised with goat anti-bovine-FITC antibodies used at a dilution of 1:400 (Thermo Fisher #A18752). Standard IFM images were acquired with a Leica DMi8 microscope. Z-stack orthological representation was performed on a Zeiss AxioObserver Z1spinning disk confocal microscope. Analysis of captured images was performed using ImageJ software.

**Scanning electron microscopy.** Infected BBEC cultures representing each time-point pi were fixed and processed for SEM as previously described (58). The cultures were analysed with a Jeol 6400 scanning electron microscope at 10 kV.
Transmission electron microscopy. Infected BBEC cultures representing selected time-points pi were fixed and processed for TEM as previously described (59). The cultures were analysed on a FEI Tecnai transmission electron microscope at 200 kV and images captured with a Gatan Multiscan 794 camera.

Proinflammatory cytokine/chemokine analysis. Production of IL-1β, IL-6, TNFα and CXCL8 (IL-8) from both the apical and basal surfaces of infected and uninfected (PBS alone added to the apical surface) BBEC cultures was assessed at each time-point pi. To measure cytokine/chemokine production from the basolateral surface, 1 ml of medium was removed from the basolateral compartment, centrifuged at 5000xg for 5 min, and the supernatant was immediately frozen at -80°C. To measure cytokine/chemokine production from the apical surface, 0.5 ml of antibiotic-free ALI medium was added to the apical surface of each culture and these were returned to the incubator for 30 min. The medium was subsequently removed, centrifuged at 5000xg for 5 min, and the supernatant was immediately frozen at -80°C. Cytokine/chemokine production was quantified using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s instructions as follows: IL-1β - bovine IL-1β ELISA reagent kit (Thermo Fisher; #ESS0027); IL-6 - bovine IL-6 ELISA reagent kit (Thermo Fisher; #ESS0029); CXCL8 - bovine IL-8 ELISA development kit (Mabtech; 3114-1A-6); and TNFα - bovine TNFα DuoSet ELISA development system (R&D systems; DY2279). Triplicate samples were measured for each insert and two individual cultures were analysed for each donor animal (n = 6).

Data Analysis. Unless otherwise stated, all experiments were independently performed three times using epithelial cells derived from three individual donor animals and, for quantitative analysis, three separate cultures from each donor animal were analysed (n = 9). Results are presented as the mean ± standard deviation. Data were statistically analysed using One- or Two-way ANOVAs for comparison of one or two independent variables,
respectively. Significance was determined by a $p$-value less than 0.05. Analyses were performed using GraphPad Prism (GraphPad Software Inc.).

**ACKNOWLEDGEMENTS**

The authors would like to thank Margaret Mullin and Lynne Stevenson (both University of Glasgow) for assistance with electron microscopy and histology, respectively.
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FIGURE LEGENDS

FIG 1 Bacterial enumeration of differentiated BBEC cultures infected with *M. haemolytica* isolates PH2 and PH202 over 5-day time-course. Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. Adherence and colonisation were assessed by enumeration at the indicated time points of (A) bacteria associated with the epithelial cells after apical washing and (B) bacteria present in the apical washes. The bacteria were enumerated by viable counts and the numbers are expressed as percentage of the original inoculum. Three inserts were analysed per time point and the data represent the mean +/- standard deviation for cultures derived from three different animals (■, animal 1; ■, animal 2; ■, animal 3).

FIG 2 Microscopic analysis of differentiated BBEC cultures infected with *M. haemolytica* isolates PH2 and PH202 over 5-day time-course. Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. At the indicated time points pi, the cultures were washed to remove unbound bacteria and fixed. Bacterial colonisation was subsequently assessed using (A) IFM and (B) SEM. In (A), increasing numbers of PH2 but not PH202 bacteria were associated with BBECs over time (bacteria - green; cilia [β-tubulin] - red; nuclei - blue); in (B), increasing numbers of PH2 (arrowheads) but not PH202 bacteria were associated with damaged tissue from 16 h post-infection. Further time points are shown in Figs S1 and S2.

FIG 3 Scanning electron microscopy of differentiated BBEC cultures infected with *M. haemolytica* isolate PH2. Differentiated BBEC cultures were infected with *M. haemolytica* isolate PH2 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. At selected time points pi, the cultures were washed to remove unbound bacteria, fixed and examined by SEM. (A) shows bacteria adhering to non-ciliated epithelial cells (arrowheads) and to mucus (arrows); (B) shows bacteria (arrow) adhering to the centre of a non-ciliated...
epithelial cell but not to cilia; (C) shows large numbers of bacteria (arrows) associated with an invagination of the cell membrane; and (D) shows bacteria (white arrow) associated with an invagination of the cell membrane which may be the result of mucus extrusion (black arrow).

**FIG 4** Histological analysis of differentiated BBEC cultures infected with *M. haemolytica* isolates PH2 and PH202 over 5-day time-course. Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. At the indicated time points pi, the cultures were washed to remove unbound bacteria, fixed, paraffin-embedded and sectioned using standard histological techniques. Bacterial colonisation and invasion were assessed using (A) H&E staining and (B) IHC (OmpA-labelled bacteria are stained brown). In (A), PH2 but not PH202 bacteria were observed within the epithelial layer from 16 h pi (arrow) and apoptotic and rounded cells were apparent at later time-points (arrowheads). In (B), PH2 but not PH202 bacteria were identified within the epithelial layer from 12 h pi (arrowheads) and discrete foci of infection (arrow), penetrating the full depth of the epithelial layer, were visible by 16 h. Further time points are shown in Figs S3 and S4.

**FIG 5** Tight junction integrity of differentiated BBEC cultures infected with *M. haemolytica* isolates PH2 and PH202 over 5-day time-course. Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. At the indicated time points pi, the cultures were washed to remove unbound bacteria and fixed. Bacterial colonisation and tight junction integrity were subsequently assessed using (A) IFM (bacteria - green; ZO-1 - red; nuclei - blue), (B) TEER determination and (C) TEM. In (A), increasing numbers of PH2 but not PH202 bacteria were associated with BBECs over time and tight-junctions remained intact until severe damage of epithelial cells occurred at later time points. In (B), infection with PH2 but not PH202
bacteria caused a rapid decline in tight junction integrity (TEER) of BBEC cultures between 16 and 48 h pi. In these experiments, three inserts were analysed per condition and the data represent the mean +/- standard deviation for cultures derived from three different animals (■, animal 1; ■, animal 2; ■, animal 3). In (C), TEM image of 24 h, PH2-infected BBECs shows bacteria (arrows) within the paracellular spaces between epithelial cells which possess intact tight junctions (arrowheads).

**FIG 6** Internalisation of *M. haemolytica* isolate PH2 by differentiated BBECs.

Differentiated BBEC cultures were infected with *M. haemolytica* isolate PH2 (2.5 x 10^7 cfu/insert) at day 21 post-ALI and maintained for five days. At selected time points pi, the cultures were washed to remove unbound bacteria and fixed. Internalisation of bacteria was subsequently assessed using (A) IFM (bacteria - green; cilia [β-tubulin] - red; nuclei - blue), (B) SEM and (C) TEM. In (A), a Z-stack orthogonal representation (630x magnification) of a 24 h-infected BBEC culture shows bacteria located intracellularly within rounded epithelial cells (arrowheads). In (B), representative images are shown of (i) potential bacterial uptake by a non-ciliated epithelial cell (arrow) and (ii) an epithelial cell (with apical membrane partially removed) containing large numbers of internalised bacteria (arrow). In (C), representative images are shown of (i) a single bacterium (arrow) within a small vesicle (endosome) in the cytoplasm of an infected cell (note intact tight junction [arrowhead]) and (ii) numerous bacteria within a larger vesicle (endosome) in the cytoplasm of an infected cell. Both images at 20 h pi.

**FIG 7** Proinflammatory innate immune response of differentiated BBECs from the basal surface following infection with *M. haemolytica* isolates PH2 and PH202. Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10^7 cfu/insert) at day 21 post-ALI. At the indicated time points pi, the expression of IL-1β, TNFα, IL-6 and CXCL8 within the basolateral medium was measured by ELISA.
Cytokine/chemokine expression was quantified in two inserts at each time point and the data represent the mean +/- standard deviation of cultures derived from three different animals (■, uninfected control; ▲, infection with isolate PH2; ▼, infection with isolate PH202).

**FIG 8** Proposed model for the internalisation and infection of differentiated BBECs by serotype A1 *M. haemolytica*. Bacteria adhere to the apical surface of non-ciliated epithelial cells (1) and are taken up via an endocytic-type mechanism (2). Rapid replication of internalised bacteria occurs within endosomes (3) and these fuse with the lateral membranes releasing bacteria into the paracellular spaces (4). Bacteria may gain entry into adjacent cells (5) and further replication within the paracellular spaces (6) and cytoplasm (7) lead to the development of infection foci, disruption of tight junctions, rupture and death of epithelial cells, and to the release of large numbers of bacteria onto the epithelial cell surface (8).
TABLE 1  Semi-quantitative assessment of bacterial invasion of, and epithelial damage to, differentiated BBEC cultures infected with *M. haemolytica* isolates PH2 and PH202 over 5-day time-course.

| Time post-infection (h) | Isolate PH2 | Isolate PH202 |
|------------------------|-------------|---------------|
|                        | Animal 1    | Animal 2      | Animal 3    | Animal 1 | Animal 2 | Animal 3 |
| 0.5                    | -           | -             | -           | -         | -         | -         |
| 2                      | -           | -             | -           | -         | -         | -         |
| 6                      | -           | -             | -           | -         | -         | -         |
| 12                     | +           | +             | -           | -         | -         | -         |
| 16                     | ++          | ++            | ++          | -         | -         | -         |
| 20                     | ++          | ++            | ++          | -         | -         | -         |
| 24                     | ++          | ++            | ++          | -         | -         | -         |
| 48                     | +++         | +++           | +++         | -         | -         | -         |
| 72                     | +++         | +++           | +++         | -         | -         | -         |
| 120                    | +++         | +++           | +++         | -         | -         | -         |

*Differentiated BBEC cultures were infected with *M. haemolytica* isolates PH2 and PH202 (2.5 x 10⁵ cfu/insert) at day 21 post-ALI and maintained for five days. At the indicated time points pi, sections of infected BBEC cultures were analysed using H&E staining and IHC (see Figs 4, S3 and S4). Assessment of bacterial colonisation/invasion and epithelial integrity of the histological sections were made semi-quantitatively as follows: - , no evidence of infection, healthy epithelial layer; +, evidence of minor degree of infection, a small number of foci of infection present, no tissue damage; ++, evidence of moderate degree of infection, large numbers of foci of infection present, some epithelial damage; ++++, evidence of high degree of infection affecting entire culture, extensive epithelial damage.*
| (A) | (B) |
|-----|-----|
| PH2 | PH202 |
| PH2 | PH202 |
| 6h 20µm | 6h 20µm |
| 12h 20µm | 12h 20µm |
| 16h 20µm | 16h 20µm |
| 20h 20µm | 20h 20µm |
| 24h 20µm | 24h 20µm |

*Note: Images depict samples stained with H&E and representative images are shown for each time point.*
