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# Mannheimia haemolytica biofilm formation on bovine respiratory epithelial cells



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## ABSTRACT

*Mannheimia haemolytica* is the most important bacterial agent associated with the bovine respiratory disease complex (BRDC), which causes worldwide economic losses to the cattle industry. *M. haemolytica* cells initially colonize the tonsillar crypts in the upper respiratory tract of cattle, from where they can subsequently descend into the lungs to cause disease. Many bacteria exist as biofilms inside their hosts. We hypothesize that *M. haemolytica* colonization of cattle during its commensal state may include biofilm formation. To begin to assess this possibility, we developed an *in vitro* system to study biofilm formation directly on bovine respiratory epithelial cells. Using fixed primary bovine bronchial epithelial cells, we observed *M. haemolytica* biofilm formation after a 48 h incubation period at 37 °C. Addition of mucin, the main component of mucus present in the upper respiratory tract, decreased *M. haemolytica* biofilm formation on bovine epithelial cells. We investigated the effects of prior viral infection of the epithelial cells on subsequent biofilm formation by *M. haemolytica* and found negligible effects. Utilization of this model system will provide new insights into the potential role of biofilm formation by *M. haemolytica* in the pathogenesis of BRDC.

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## 1. Introduction

Bovine Respiratory Disease Complex (BRDC) causes hundreds of millions of dollars per year in losses to the cattle industry (Bowland and Shewen, 2000). The disease is associated with multiple bacterial and viral pathogens including *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis*, bovine viral diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV), and bovine herpes virus (BHV) (Caswell, 2014; Moore et al., 2015). The most important bacterial species associated with BRDC is *Mannheimia haemolytica*, a member of the family *Pasteurellaceae*. *M. haemolytica* exists as a commensal in the upper respiratory tract of healthy cattle, from where it can descend into the lungs to cause pneumonia (Frank and Smith, 1983; Frank et al., 1993). Previous studies have shown the ability of *M. haemolytica* to colonize bovine tonsils for extended periods (Frank et al., 1993). Severe BRDC is often preceded by infection of the respiratory epithelium with viruses like BVDV-1, BRSV, BHV-1, or bovine coronavirus (Frank et al., 1993). The mechanisms by which these viral infections predispose the host to *M. haemolytica* are unclear. One possibility is

that viral infections alter the respiratory epithelial lining to favor bacterial colonization (Selinger et al., 1981).

Commensal bacteria often exist in their hosts as biofilms, which are microcolonies of bacteria encased in an extracellular polymeric substance (EPS) (Olson et al., 2002). These biofilms provide protection for the bacterial cells against the host immune response and antibiotic therapy. It has been shown that bacteria within a biofilm can be more resistant than planktonic cells to antibiotics (Olson et al., 2002). As a result, biofilms pose an added concern when treating infections with antibiotics.

A previous report provided scanning electron micrographs suggesting that microcolonies of *M. haemolytica* cells can be observed in the lungs of cattle with severe respiratory disease (Morck et al., 1989). Other members of the family *Pasteurellaceae* have been shown to produce biofilms *in vitro*. For example, the closely related porcine respiratory pathogen *Actinobacillus pleuropneumoniae* forms biofilms within 4 h, under static growth conditions *in vitro* (Tremblay et al., 2013). The bovine respiratory pathogen *H. somni* can form biofilms that are thought to allow this commensal to persist on the bovine mucosa (Sandal et al., 2011; Elswaifi et al., 2012). The EPS in *H. somni* biofilms was found to consist largely of polysaccharides, which contribute to persistence of the bacteria in its biofilm state during systemic infection (Elswaifi et al., 2012). In a previous study, we showed that *M.*

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*haemolytica* will form biofilm on plastic *in vitro*, and that protein is the major constituent in the EPS (Boukahil and Czuprynski, 2015).

To effectively study biofilm formation in the context of the host, it is necessary to examine their interactions with underlying epithelial cells (Tran et al., 2014). Some bacterial species require 48 h to form a biofilm (Boukahil and Czuprynski, 2015), which presents a technical challenge because the underlying host cells will detach as a result of bacterial overgrowth in a closed system. By fixing epithelial cells in tissue culture wells, we have devised a system to study biofilm formation directly on bovine respiratory epithelial cells.

We used variations of this model system to investigate how various conditions affect biofilm formation. We found that at concentrations similar to those found in the oral cavity (Kejriwal et al., 2014), mucin decreased *M. haemolytica* biofilm formation on bovine epithelial cells. In an effort to explain the link between viral infection and *M. haemolytica* pathogenesis, we infected the epithelial cells with several respiratory viruses to determine if biofilm formation was altered. Results obtained with our model system will help to elucidate how *M. haemolytica* persists as a commensal in the upper respiratory tract.

## 2. Materials and methods

### 2.1. Bacterial strains

*M. haemolytica* A1 (derived from a pneumonic bovine lung) (Gioia et al., 2006), was grown without shaking for 18 h in 10 ml of brain heart infusion broth (BHI broth, EMD Millipore, MA) at 37 °C to achieve a final concentration of 10<sup>9</sup> CFU/ml.

### 2.2. Primary cells

Primary bovine bronchial epithelial cells (BBEC) were kindly provided by Dr. D.S. Allen-Gipson (Omaha, NE) and maintained in DMEM-F12 media (Corning, VA) containing 10% fetal bovine serum, 2 mM L-glutamine, and 10 µg/ml of epidermal growth factor (Sigma, MO). These cells were harvested from lungs of a healthy cow obtained at an abattoir (Allen-Gipson et al., 2006) and have been used by our lab in previous studies (N'jai et al., 2013).

### 2.3. Biofilm assay

BBEC were seeded into wells of a flat bottom polystyrene 24 well plate (Corning, NY) and incubated at 37 °C with 5% CO<sub>2</sub> until they reached confluency. The cells were then washed once with 1 ml sterile water, and incubated with 0.75 ml 0.1% glutaraldehyde (diluted in water) (Sigma) at room temperature for 10 min (Bollinger et al., 2003). The fixative was removed and the wells washed three times with 1 ml sterile water. *M. haemolytica* cells were suspended in RPMI to achieve a final concentration of 10<sup>5</sup> CFU/ml, and 0.8 ml of the bacterial suspension was added to each well. A control group of fixed BBEC incubated with RPMI that did not contain bacterial cells (Lonza, MD) was included as a negative control. The plate was incubated at 37 °C with 5% CO<sub>2</sub> for 48 h. Every 12 h, the medium was removed from the wells, the wells rinsed with 1 ml warm PBS, and 0.8 ml of RPMI media dispensed into the wells. Following the 48 h incubation, the wells were rinsed twice with deionized water, and allowed to air dry for 1 h. The wells then were incubated with filtered crystal violet (Harleco, EMD Millipore) at room temperature for 15 min. After two washes with 1 ml of water, the plate was air dried for 1 h, the retained dye solubilized with 30% glacial acetic acid (Sigma) and absorbance at 595 nm determined using a plate reader (DTX 880 Multimode Detector, Beckman Coulter, CA). For scanning electron microscopy (SEM), biofilms were formed on fixed bovine

respiratory epithelial cells in Lab-Tek slides (Thermo Scientific, MA), fixed overnight in 2.5% glutaraldehyde, and processed as previously described (Shippy et al., 2012). Images were taken at the Biological and Biophysical Preparation, Imaging and Characterization Laboratory at the University of Wisconsin-Madison on a Hitachi S900 microscope (Hitachi, Japan).

### 2.4. Adhesion assay

Confluent BBEC cells were seeded into wells of a 24 well plate, washed once with 1 ml sterile water, and either fixed with glutaraldehyde (as described in the biofilm assay above) or left unfixed. *M. haemolytica* cells (10<sup>5</sup> CFU/ml) in RPMI 1640 were added to each well and the cells incubated for 2 h at 37 °C with 5% CO<sub>2</sub>. Afterwards, the wells were rinsed twice with 1 ml PBS and adherent bacterial cells were removed by adding 1 ml PBS and disrupting the BBEC monolayer through scraping and vigorous pipetting. The bacteria were enumerated via serial dilution and plating onto blood agar plates. Each group was run in quintuplicate wells.

### 2.5. Mucin assay

Bovine submaxillary mucin (purified from the submaxillary mucin clot using the procedure described in Nisizawa and Pigman (1959) (Worthington Biochemical, Lakewood, NJ)) was prepared in PBS (10 mg/ml stock solution) and UV sterilized for six hours. The solution was diluted to the desired concentration in RPMI, combined with 10<sup>5</sup> *M. haemolytica* CFU/ml, and added to fixed BBEC as described above for the biofilm assay. The plates were incubated at 37 °C with 5% CO<sub>2</sub> for 48 h. Every 12 h, the wells were rinsed and the media replaced with the appropriate mucin concentrations. As a control to mimic the viscosity of mucin, methyl cellulose of a similar concentration was added to one group of wells (Kavanaugh et al., 2014). After incubation, the wells were rinsed two times with deionized water and biofilm quantified with crystal violet as described in the biofilm assay.

To assess the effect of mucin on *M. haemolytica* adhesion, an adherence assay was performed similar to the conditions described above. Various concentrations of mucin were mixed with *M. haemolytica* cells (10<sup>5</sup> CFU/ml) and added to fixed BBEC as described above for the biofilm assay. The plates were incubated at 37 °C with 5% CO<sub>2</sub> for 2 h, at which time samples were removed by adding 1 ml PBS and disrupting the BBEC monolayer through scraping and vigorous pipetting. Next, serial dilution and plating was used to estimate CFU of *M. haemolytica* adherent to the monolayers. To determine if mucin inhibits growth of *M. haemolytica*, 10<sup>5</sup> CFU/ml of *M. haemolytica* were combined with various concentrations of mucin (or methyl cellulose as a control) in RPMI. The solutions were dispensed into triplicate wells containing fixed BBEC cells and incubated at 37 °C with 5% CO<sub>2</sub> for 24 h.

### 2.6. Effect of viral infection on biofilm formation

BRSV and BVDV-1 were kindly provided by Dr. Kathy Kurth (University of Wisconsin-Madison). BHV-1 was provided by Dr. Ronald Schultz (University of Wisconsin-Madison). To propagate the virus, confluent BBEC in a T75 flask were infected with virus and subsequently the viruses were concentrated using a procedure described previously (Eisfeld et al., 2014). To initiate viral infection, uninfected BBEC were seeded at a density of 10<sup>5</sup> per well in a 24 well plate. After incubation for 24 h at 37 °C with 5% CO<sub>2</sub>, the cells were infected with 10<sup>5</sup> PFU of virus (MOI of 1) in DMEM-F12 media containing 2% fetal bovine serum and 2 mM L-glutamine. Because viral infection of cells is based on a Poisson distribution we assume

that at an MOI of 1 63% of the BBEC cells were virally infected (Knipe and Howley, 2013). A control group of uninfected BBEC was included as a control. BBEC were incubated at 37 °C with 5% CO<sub>2</sub> until the first signs of CPE were observed (i.e. 12 h for BHV-1, 60 h incubation for BVDV-1, 72 h for BRSV). The cells were then fixed and used for the biofilm assay described above.

### 2.7. Antibiotic susceptibility assays

We assessed the effects of antibiotics on *M. haemolytica* using both minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) assays. The MIC assay was performed by suspending 10<sup>5</sup> CFU/mL planktonic cells of *M. haemolytica* in RPMI. Next, two-fold dilutions (.0625 µg/ml to 64 µg/ml) of gentamicin (Sparhawk Laboratories Inc., KS), florfenicol (Hospira Inc., IL), chlortetracycline (MP Biomedicals, CA), erythromycin (Sigma), or tulathromycin (Pfizer, NJ) were added to the bacterial suspensions and the mixtures dispensed into duplicate wells (800 µl per well) of a 24 well plate. The plate was incubated at 37 °C with 5% CO<sub>2</sub> for 24 h and the MIC determined to be the lowest dilution that showed no turbidity in the wells.

Minimum biofilm eradication concentration (MBEC) assays were performed as described previously (Boukahil and Czuprynski, 2015), with some modifications. BBEC were washed once with 1 ml of sterile water, fixed in wells of a 24 well plate, rinsed three times with 1 ml water, and *M. haemolytica* biofilms allowed to form as described above in the biofilm assay. Biofilms were rinsed once with 1 ml PBS, and 0.8 ml of RPMI containing serial two-fold dilutions (2–1024 µg/ml) of various antibiotics (florfenicol, gentamicin, chlortetracycline, erythromycin, or tulathromycin) were added to wells. The plates were incubated for 24 h at 37 °C with 5% CO<sub>2</sub>, rinsed once with 1 ml PBS, and replaced with 0.8 ml RPMI. The biofilms were dispersed from the well surface by scraping and vigorous pipetting and the plate incubated for an additional 24 h at 37 °C with 5% CO<sub>2</sub>. MBEC was defined as the lowest antibiotic concentration that did not result in visible turbidity or produce any colonies after plating (100 µl) onto blood agar.

### 2.8. Statistical analysis

Data were analyzed by either one way analysis of variance followed by the Tukey test for significance, or by a paired *t*-test using Prism 5 Software (GraphPad, CA). Statistical significance was set at *P* < 0.05.

## 3. Results

### 3.1. *M. haemolytica* forms biofilm on bovine bronchial epithelial cells

We previously reported that *M. haemolytica* produces the greatest amount of biofilm on polystyrene after a 48 h incubation *in vitro* (Boukahil and Czuprynski, 2015). The extended incubation period posed a challenge to our goal to form biofilm on epithelial cells because unfixed epithelial cells detached from the well when incubated with *M. haemolytica* for this amount of time (unpublished). To overcome this obstacle, we fixed the epithelial cells with 0.1% glutaraldehyde for 10 min prior to adding 10<sup>5</sup> CFU *M. haemolytica* cells. The plate then was incubated for 48 h at 37 °C with 5% CO<sub>2</sub>. During this time, the wells were washed once every 12 h to remove planktonic cells. After 48 h, the wells were stained with crystal violet and absorbance measured at 595 nm.

Using this method, we found that *M. haemolytica* formed biofilm on the fixed BBEC (Fig. 1A). Representative examples of fixed epithelial cells and cells containing biofilms are illustrated in Fig. 1B. Fig. 1C shows scanning electron micrograph images of

extensive EPS being produced by the bacteria compared to the epithelial cells alone. To ensure that the wash steps did not enhance biofilm formation we included a control group, without epithelial cells, that received *M. haemolytica* cells and was washed every 12 h. We found this treatment group produced less biofilm on plastic than a static culture of *M. haemolytica*. Another control group, glutaraldehyde-treated plastic, was tested to show that the glutaraldehyde did not somehow enhance biofilm formation on the plastic wells.

Bacterial adhesion is the critical first step in biofilm formation. To see if adhesion of bacteria was adversely affected by glutaraldehyde fixation of the BBEC, we examined the adhesion of *M. haemolytica* to both living and fixed epithelial cells. After incubating at 37 °C, unattached bacteria were washed away and the remaining adherent bacteria were quantified by plating onto agar. We recovered similar CFU of *M. haemolytica* from live and fixed cells (Fig. 2), suggesting that glutaraldehyde fixation does not alter bacterial adhesion to BBEC.

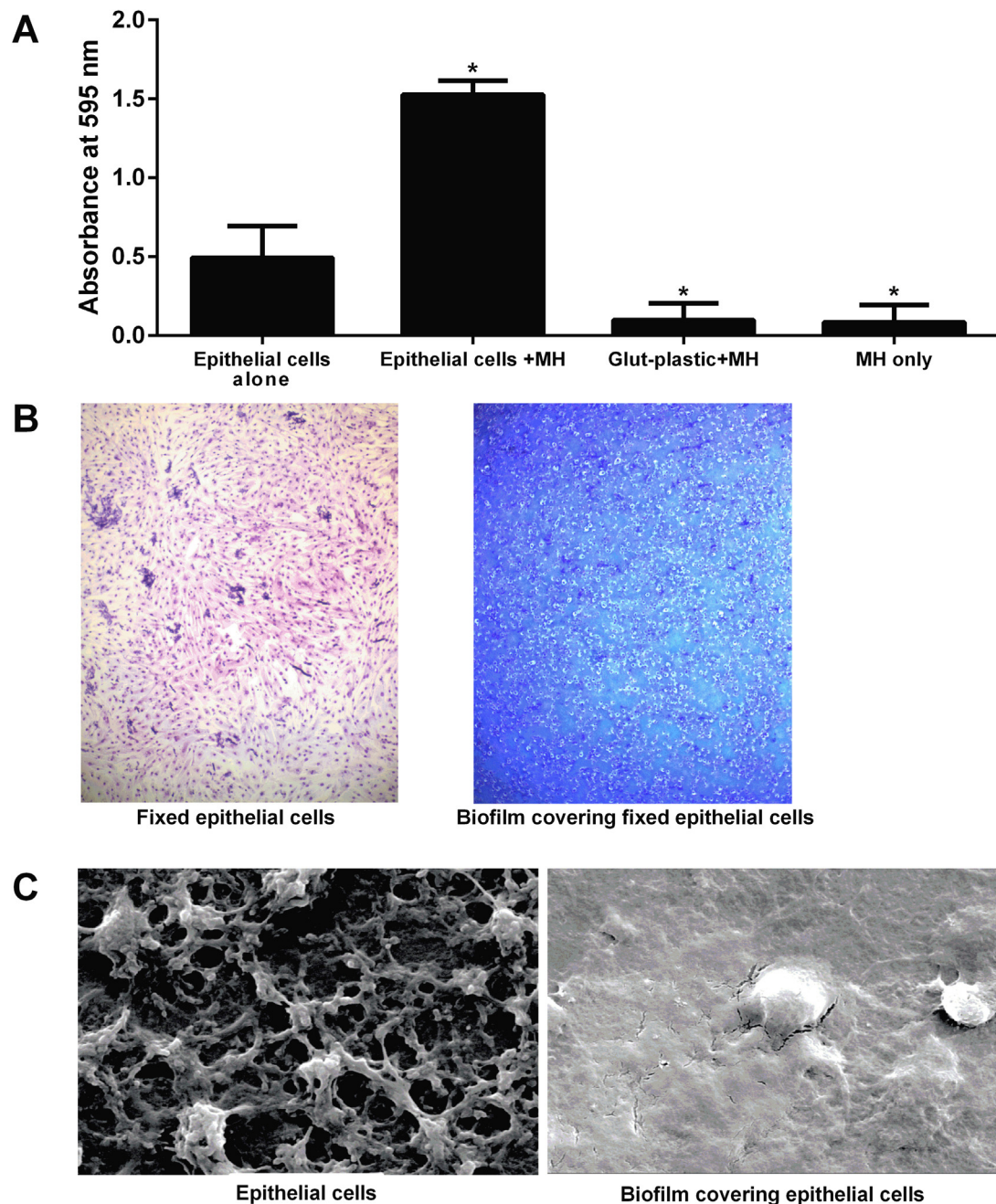
### 3.2. Mucin decreases *M. haemolytica* biofilm formation

Mucin is present in the healthy respiratory tract, but it also plays a detrimental role in diseases like cystic fibrosis and periodontal disease (Landry et al., 2006). We tested whether mucin affects *M. haemolytica* biofilm formation on bovine epithelial cells by adding various concentrations of bovine mucin to the suspension of bacterial cells, prior to adding them to BBEC in the biofilm assay described above. We found that at 1 mg/ml, mucin led to less biofilm formation on BBEC than the control group without mucin (Fig. 3). As an additional control we also examined methyl cellulose, which has a similar viscosity to mucin, and found that it did not decrease biofilm formation. This finding suggests that the effect of mucin on biofilm formation is not simply because of increased viscosity of the medium.

To determine if mucin affected adherence, we performed an adhesion assay as done earlier, in the presence of various concentrations of mucin. We found that mucin did not inhibit adhesion compared to no mucin or methyl cellulose controls (see Supplementary Fig. S1 in the online version at DOI: <http://dx.doi.org/10.1016/j.vetmic.2016.11.012>). We also examined mucin in a MIC assay and found it was not bacteriostatic or bactericidal to *M. haemolytica* at the concentrations used (data not shown).

### 3.3. Viral infection does not enhance *M. haemolytica* biofilm formation

Viral infection can predispose cattle to *M. haemolytica* infection and cause severe BRDC (van der Sluijs et al., 2010; Moore et al., 2015). To test whether viral infection increases biofilm formation on epithelial cells, we used bovine viral diarrhea virus-1 (BVDV-1), bovine respiratory syncytial virus (BRSV), and bovine herpesvirus-1 (BHV-1), all of which have been shown to worsen *M. haemolytica* infection *in vivo* (Potgieter et al., 1984; van der Sluijs et al., 2010; Hodgson et al., 2012). We first infected BBEC with the indicated viruses at an MOI of 1 and incubated the cells until the first signs of cytopathic effects were evident (Fig. 4A). We then fixed the cells with 0.1% glutaraldehyde, added 10<sup>5</sup> CFU *M. haemolytica*, and quantified biofilm formation as described earlier. We found that biofilm formation on virally infected cells did not differ from uninfected cells (Fig. 4B–D). When we quantify *M. haemolytica* biofilms forming on BBEC after 48 h absorbance values in the crystal violet assay can range up to 2.7. We considered the possibility that these relatively high values might confound our ability to detect inhibition by viral infection of the BBEC. To evaluate this possibility we quantified biofilm formation on BVDV infected fixed BBEC at 36 and 48 h after addition of *M. haemolytica*. As in Fig. 4, here too we did not observe any inhibitory effect of viral



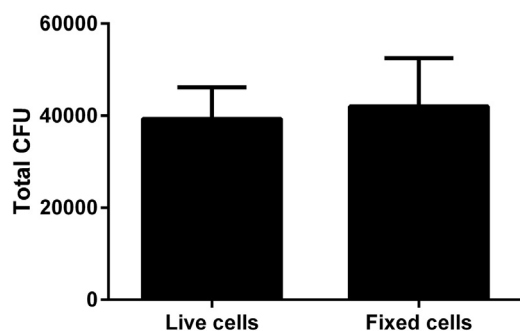
**Fig. 1.** *M. haemolytica* can form biofilms on glutaraldehyde-fixed bovine respiratory epithelial cells. (A) Bovine respiratory epithelial cells grown in the wells of a 24 well plate were fixed with 0.1% glutaraldehyde, washed, and  $10^5$  CFU *M. haemolytica* (MH) in RPMI medium added to each well. Wells that contained fixed bovine respiratory epithelial cells but did not receive bacteria were used to determine background staining. The wells were then incubated at 37 °C with 5% CO<sub>2</sub> for 48 h. Biofilm was stained with crystal violet and absorbance at 595 nm was determined. Data are the mean  $\pm$  SEM of triplicate wells from three independent experiments. \*,  $P < 0.05$  compared to epithelial cells alone. (B) Fixed bovine respiratory epithelial cells without (left panel) and with (right panel) *M. haemolytica* biofilm were stained with Diff-Quik<sup>®</sup> and viewed by light microscopy (40 $\times$ ). Images are representative of three independent experiments. (C) Fixed epithelial cells alone (left panel) and cells supporting *M. haemolytica* biofilm (right panel) were imaged by scanning electron microscopy (2000 $\times$ ). Images are representative of those from three independent experiments that were performed.

infection on biofilm formation at 36 h at these lower absorbance values (See Supplementary Fig. S2 in the online version at DOI: <http://dx.doi.org/10.1016/j.vetmic.2016.11.012>).

#### 3.4. Antibiotic resistance profile of *M. haemolytica* biofilms on epithelial cells

Biofilms pose a challenge to clinicians seeking to treat infections with antibiotics (da Costa Krewer et al., 2015). Biofilm cells generally have greater resistance to antibiotics than planktonic bacterial cells. We were interested in whether *M.*

*haemolytica* biofilms on epithelial cells would have an even greater tolerance to antibiotics than biofilms formed on polystyrene. To answer this question, we performed a minimum biofilm eradication concentration (MBEC) assay to test the effects of chlortetracycline, erythromycin, gentamicin, tulathromycin, and florfenicol on *M. haemolytica* biofilms formed on plastic and BBEC. Chlortetracycline and erythromycin exhibited the biggest increases in MBEC on cells versus plastic (8 and 16 fold, respectively). We observed a four-fold increase in MBEC for gentamicin, and a two-fold increase for tulathromycin, for biofilms formed on epithelial cells compared to plastic (Table 1). Florfenicol showed no



**Fig. 2.** Similar numbers of *M. haemolytica* bind to live and glutaraldehyde fixed epithelial cells. Bovine respiratory epithelial cells were seeded into wells of a 24 well plate and grown to confluence. One group was fixed with 0.1% glutaraldehyde for 10 min at room temperature and the other group remained unfixed. *M. haemolytica* ( $10^5$  CFU/ml) in RPMI were then added to both groups and incubated at 37 °C with 5% CO<sub>2</sub> for 2 h. Wells were washed with PBS, the adherent bacteria disrupted by vigorous scraping and pipetting, and the bacterial cells resuspended in 1 ml PBS. Bacteria were enumerated by serial dilution and plating onto blood agar plates. Data are the mean ± SEM from three independent experiments.

difference in MBEC for biofilms on polystyrene and BBEC, although the concentration needed was much greater than the other antibiotics tested. For comparative purposes we include MIC values for planktonic cells in Table 1. The MIC value for all antibiotics tested (planktonic state) were several-fold less than their respective MBEC values. This was particularly true for florfenicol where the difference was quite large.

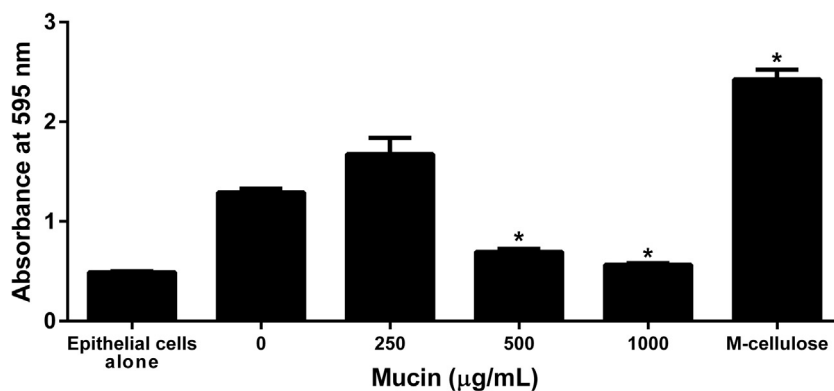
#### 4. Discussion

In this study, we demonstrate that *M. haemolytica* can form biofilms on bovine respiratory epithelial cells. These findings are consistent with a previous report that *M. haemolytica* cells adhere and form a biofilm-like structure *in vivo* (Morck et al., 1989), and on plastic *in vitro* (Boukahil and Czuprynski, 2015). Other members of the *Pasteurellaceae* family, including *A. pleuropneumoniae* and *H. somni*, have been shown to form biofilms *in vitro* and *in vivo* (Sandall et al., 2011; Tremblay et al., 2013).

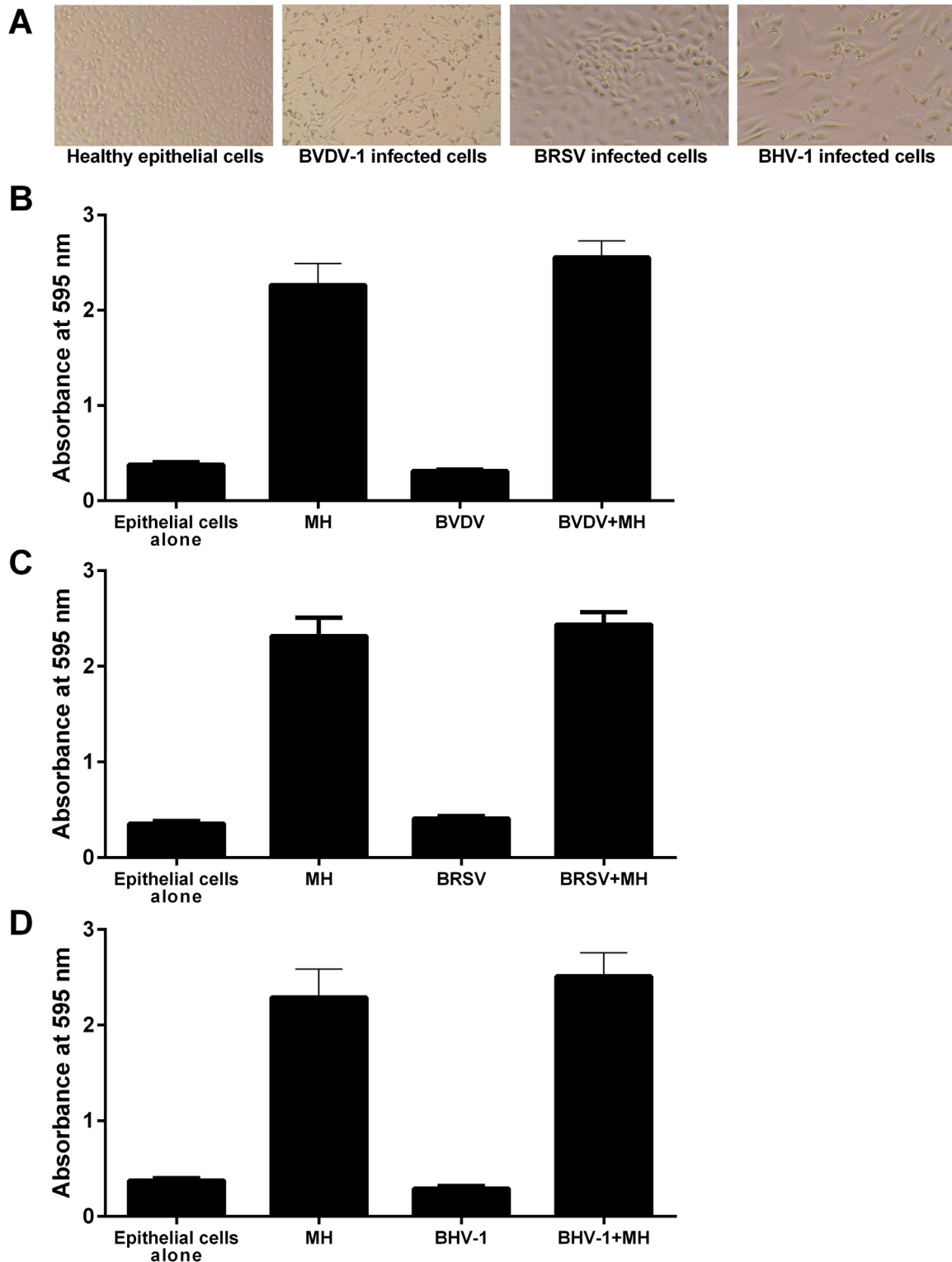
Our model system was driven by a desire to investigate the ability of *M. haemolytica* to form biofilms on respiratory epithelial cells. As with other bacterial species, *M. haemolytica* requires 48 h to form a biofilm *in vitro*. This incubation period poses technical challenges associated with controlling numbers of planktonic

bacterial cells and maintaining the integrity of the underlying epithelial cells. One group of investigators used a transwell membrane barrier between the biofilm being formed and the underlying epithelial cells (Tran et al., 2014). Although this approach allowed the epithelial cells to survive, the lack of direct contact between the biofilm cells and epithelial cells offers limited insights into the likely process of biofilm formation *in vivo*. Other investigators have reported model systems that allow for direct contact between the bacterial and epithelial cells, however these studies used bacterial species that rapidly formed biofilms (i.e. 9 h) (Anderson et al., 2008). Incubation beyond 12 h resulted in detachment of the underlying eukaryotic cells due to the toxicity of bacterial products in the culture system. Our model is the first to report biofilm formation directly on host cells during incubation periods of up to 72 h (See Supplementary Fig. S3 in the online version at DOI: <http://dx.doi.org/10.1016/j.vetmic.2016.11.012>). To allow the underlying epithelial cells to support the growing biofilm, we gently fixed the epithelial cells with glutaraldehyde, which is reported to have limited effects on the tertiary structures of proteins (Chui and Wan, 1997). Indeed, others have shown that the protein trypsin retains catalytic activity after being similarly treated with glutaraldehyde (Chui and Wan, 1997). We confirmed fixation had limited effects on the epithelial cells by showing equivalent adhesion of *M. haemolytica* cells to fixed and living epithelial cells (Fig. 2).

Our finding that mucin decreases biofilm formation is consistent with studies, which showed that mucin significantly decreased biofilm formation by *Streptococcus mutans* and *P. aeruginosa* (Haley et al., 2014; Frenkel and Ribbeck, 2015). It has been proposed that mucin, the main glycoprotein of mucus, acts as an anti-biofouling agent that impairs pathogen colonization of epithelial cells lining the respiratory tract (Hollingsworth and Swanson, 2004; Linden et al., 2008). We found that at mucin concentrations similar to those reported *in vivo*, biofilm formation was nearly eliminated (Fig. 3). There are several possible mechanisms by which mucin protects the epithelium from bacterial colonization. Bergstrom et al. showed that mucin-deficient mice had a greater commensal bacterial load in the intestinal epithelium when infected with the pathogen *Citrobacter rodentium* than did wildtype mice (Bergstrom et al., 2010). Mucin prevented over-colonization of commensal and pathogenic organisms alike, by enabling cilia to sweep away the bacterial cells (Bergstrom et al., 2010). A similar mechanism of bacterial clearance is believed to occur in the upper respiratory tract



**Fig. 3.** Mucin decreases *M. haemolytica* biofilm formation on fixed bovine respiratory epithelial cells.  $10^5$  CFU *M. haemolytica* were incubated with either mucin (0–1000 μg/ml) or methyl cellulose (1000 μg/ml) and then added to fixed bovine respiratory epithelial cells. The plates were incubated at 37 °C with 5% CO<sub>2</sub> for 48 h with washing every 12 h to remove planktonic cells. The resultant biofilms were stained with crystal violet and absorbance read at 595 nm. Data are the mean ± SEM from three independent experiments. \*,  $P < 0.05$  compared to control (0 μg/ml).



**Fig. 4.** Viral infection of BBEC does not affect *M. haemolytica* biofilm formation. (A) Bovine respiratory epithelial cells were first infected with either BVDV-1, BRSV, or BHV-1 and incubated until the first signs of cytopathic effects were observed. A control was included that contained uninfected epithelial cells. Images were taken at 40 $\times$  magnification. BBEC infected with BVDV (B), BRSV (C), or BHV-1 (D), were fixed and *M. haemolytica* suspended in RPMI added to the monolayers. The cells were incubated for 48 h at 37  $^{\circ}$ C with 5% CO $_2$  and the resultant biofilms quantified by crystal violet staining. Data are the mean  $\pm$  SEM of triplicate wells from three independent experiments.

(Thornton et al., 2008). In humans, chronic oral disease leads to increased mucin levels, which in turn are thought to contribute to inflammation during infection (Hollingsworth and Swanson, 2004). As infection proceeds, the resulting inflammation

stimulates overproduction of mucin (Thornton et al., 2008). Consistent with this finding, one of the characteristic signs of severe *M. haemolytica* respiratory infection is excess mucus emanating from the nostrils of cattle (Poulsen and McGuirk,

**Table 1**Increased antibiotic resistance of *M. haemolytica* biofilms (MBEC) on bovine respiratory epithelial cells compared to polystyrene.

Antibiotic	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>	Polystyrene ( $\mu\text{g/ml}$ )	Epithelial cells ( $\mu\text{g/ml}$ )	Fold Increase
Chlortetracycline	8	32	256	8
Erythromycin	$\leq 0.0625$	32	512	16
Gentamicin	1	16	64	4
Tulathromycin	0.0625	2	4	2
Florfenicol	1	1024	1024	0

<sup>a</sup> MIC data for the same antibiotics used in the MBEC assays.

2009). If mucin has antimicrobial properties, then the question remains as to why commensal bacteria can colonize the respiratory tract in humans and animals. This apparent paradox may be explained by evidence that mucin helps to maintain consistent levels of commensal bacteria while suppressing colonization by pathogenic bacteria (Frenkel and Ribbeck, 2015). Because *M. haemolytica* is regarded as part of the microflora in the upper respiratory tract of healthy cattle, perhaps mucin allows *M. haemolytica* to colonize at low levels, as a commensal biofilm.

The link between viral infection and increased susceptibility to *M. haemolytica* infection is well-documented (van der Sluijs et al., 2010; Caswell, 2014). We found that viral infection of the bovine respiratory epithelial cells did not have an effect on biofilm formation. While our findings do not provide evidence for a direct link between viral infection and biofilm formation, perhaps viruses may affect *M. haemolytica* colonization of host epithelia through indirect means such as by weakening the host's innate immune system's ability to remove the bacterial cells.

Growth as a biofilm can alter resistance to antibiotics. Standard antibiotic susceptibility assays may not reflect the susceptibility of bacterial cells enmeshed in a biofilm (Olson et al., 2002). Furthermore, bacterial cells in biofilms may have an increased mutation rate, and the close proximity of bacterial cells can facilitate transfer of antibiotic resistance, allowing resistant cells to emerge (Driffield et al., 2008). Some research laboratories have investigated antibiotic resistance by biofilms formed on abiotic surfaces to predict antibiotic doses that are effective *in vivo* (Hengzhuang et al., 2011). Here we showed that biofilm formation by *M. haemolytica* on epithelial cells enhanced antibiotic resistance relative to biofilm formation on polystyrene. One simple explanation for the enhanced resistance is that *M. haemolytica* cells in the extracellular matrix exuded by both biofilm cells and BBEC are physically shielded from the antibiotic. Hence, a greater amount of antibiotic is needed to kill these embedded bacterial cells.

Future work will examine the ability of *M. haemolytica* to form co-biofilms with other bacterial species on BBEC. *M. haemolytica* has been shown to share its niche in the upper respiratory tract with other bacterial species, such as *P. multocida* and *H. somni*, that are also associated with BRDC (Moore et al., 2015), and have been shown to form biofilms (Olson et al., 2002; Sandal et al., 2011; Elswaifi et al., 2012). These studies may elucidate interactions among these bacterial species when they are in close proximity on the respiratory epithelium.

## 5. Conclusion

We developed a novel *in vitro* model system that allows *M. haemolytica* to form biofilms on fixed bovine respiratory epithelial cells *in vitro*. We confirm that adhesion of the bacteria cells to living and fixed epithelial cells was similar. Addition of mucin (0.5 to 1.0 mg/ml), the main glycoprotein of mucus, significantly reduced biofilm formation. Prior viral infection of the epithelial cells did not alter subsequent biofilm levels, suggesting that BRDC viruses enhance bacterial infection by other mechanisms. Finally, *M.*

*haemolytica* biofilms formed on epithelial cells had greater resistance to several antibiotics than biofilms formed on polystyrene. Taken together, this model system provides a new tool for understanding the relationship of *M. haemolytica* with its host.

## Conflict of interest

None.

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