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Development of an aerosolized *Mannheimia haemolytica* experimental pneumonia model in clean-catch colostrum-deprived calves

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ABSTRACT

Mannheimia haemolytica is an important cause of bovine respiratory disease (BRD). BRD is usually a multifactorial disease with host factors and viral infections influencing pathogenesis. Previous studies that have attempted to experimentally induce pneumonia using aerosolized *M. haemolytica* alone have produced inconsistent results, yet an aerosol model would be useful to study the details of early infection and to investigate the role of innate defences in pathogenesis. The objective of these studies was to develop and characterize an aerosolized *M. haemolytica* disease model. In an initial study, conventionally raised calves with higher levels of antibody against *M. haemolytica* leukotoxin developed acute respiratory distress and diffuse alveolar damage, but did not develop bronchopneumonia, following challenge with *M. haemolytica* serotype 1. Clean-catch colostrum-deprived calves challenged with 1×10^{10} colony forming units of *M. haemolytica* serotype 1 consistently developed bronchopneumonia, with elevations in rectal temperature, serum haptoglobin, plasma fibrinogen, and blood neutrophils. *Mannheimia haemolytica* serotype 1 was consistently isolated from the nasal cavities and lungs of challenged calves. Despite distribution of aerosol and isolation of *M. haemolytica* in all lung lobes, gross lesions were mainly observed in the cranioventral area of lung. Gross and histologic lesions included neutrophilic bronchopneumonia and fibrinous pleuritis, with oat cells (necrotic neutrophils with streaming nuclei), and areas of coagulative necrosis, which are similar to lesions in naturally occurring BRD. Thus, challenge with *M. haemolytica* serotype 1 and use of clean-catch colostrum-deprived calves with low or absent antibody titres allowed development of an effective aerosol challenge model that induced typical clinical disease and lesions.

1. Introduction

Bovine respiratory disease (BRD) causes significant economic losses to the North American beef industry due to morbidity, mortality, and decreased production and meat quality (Griffin, 1997). A number of bacterial and viral pathogens contribute to its development, but of these, *Mannheimia haemolytica* is the most important bacterial agent in acute pneumonia of feedlot calves (Griffin et al., 2010). This bacterium can be frequently isolated from the nasopharynx of healthy cattle, although it is more prevalent in those with respiratory disease (Allen 1991; Timsit 2017). It is therefore considered an opportunistic pathogen, associated with disease under circumstances when host defenses have been altered such as following stressors or viral infection (Caswell 2014).

Experimental models of *M. haemolytica* pneumonia include: i) prior viral infection such as bovine herpesvirus 1 (BHV-1) followed by aerosolized *M. haemolytica* challenge (Jericho and Langford, 1978, Stockdale et al. 1979) or bovine viral diarrhea virus infection followed by *M. haemolytica* challenge (Burciaga-Robles et al., 2010; Potgieter et al., 1984), ii) inoculation of *M. haemolytica* directly into the trachea or bronchi (Amrine et al., 2014; Gershwin et al., 2015; Hewson et al., 2011b; Hodgins and Shewen, 2000), and iii) transthoracic challenge with *M. haemolytica* (Panciera and Corstvet, 1984). However, these experimental models are not well-suited to investigating the role of pulmonary innate defenses in the establishment and progression of *M. haemolytica* infection. Intra-tracheal, intra-bronchial or trans-thoracic fluid inoculation is expected to bypass and dilute many of the innate immune defenses that are important in preventing the initial establishment of infection, but these are not the natural mode by which bacteria enter the lungs. Infection with viruses such as BHV-1 alters innate immune responses, either by damaging or altering the function of epithelial cells or leukocytes, or by induction of inflammatory

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responses (Levings, 2013). Although pre-infection with virus might model the events in some cases, viral infection is not necessary for the development of BRD. For example, animals treated for BRD did not differ from untreated animals with respect to detection of viruses in nasal swabs or seroconversion to viral pathogens (Martin and Bohac, 1986; Martin et al., 1999; Mitra et al., 2016). Furthermore, in Ontario feedlots during the first 28 days after arrival, seroconversion to BHV-1 was infrequent (less than 5%) and was not associated with BRD. Thus, although BHV-1 is used in an *M. haemolytica* model, the findings suggest that BHV-1 might not be a common predisposing risk factor for BRD in this high-risk period (Martin and Bohac, 1986; Martin et al., 1989, 1999).

Existing models of *M. haemolytica* pneumonia either bypass upper respiratory tract defenses or use pre-infection with respiratory viruses. Therefore, they do not allow investigation of early innate immune responses of the respiratory tract to a pure *M. haemolytica* infection and they are problematic when the research objective is to understand the early stages of bacterial colonization of the lungs, or the effect of induced innate immune responses to prevent disease caused by *M. haemolytica*. Thus, our goal was to develop an experimental disease model in calves using aerosolized *M. haemolytica* without prior viral infection. Such a model would be relevant to natural disease because *M. haemolytica* has been demonstrated in exhaled air from infected calves (Jericho et al., 1986), and thus aerosolization of droplets containing bacteria is a biologically plausible route of infection.

Published experiments using aerosolized *M. haemolytica* alone have not consistently produced respiratory disease (Bielefeldt Ohmann and Babiuk, 1985; Filion et al., 1984; Jericho, 1989; Jericho and Langford, 1978; Yates et al., 1983). Only one of these studies created post-mortem lesions of pneumonia, and only in 2 of 3 challenged calves (Jericho, 1989). Here we present and characterize a novel model of *M. haemolytica* pneumonia in clean-catch colostrum-deprived calves using aerosol bacterial challenge.

2. Materials and methods

2.1. Animals

The use of animals in this study was approved by the Animal Care Committee of the University of Guelph (#3286) in accordance with the Canadian Council on Animal Care.

Sequential studies were conducted to develop a disease model using aerosolized *M. haemolytica* in calves. The first experiment (i.e. *Mannheimia haemolytica* serotype 1 challenge in conventional calves) used a single pair of 3-month old conventionally raised male Holstein calves, obtained from the Elora Dairy Research Centre, University of Guelph. These calves were transported to the Isolation Unit of the Central Animal Facility, University of Guelph and were housed together in a pen, allowing 10 days of acclimatization prior to pathogen challenge.

Subsequent studies used 8 clean-catch colostrum-deprived calves that were obtained and housed similar to that previously described (Hodgins and Shewen, 2000). Briefly, male Holstein calves were obtained at birth from the Elora Dairy Research Centre, University of Guelph, and were delivered by members of the research team onto single-use plastic sheets and immediately removed from the calving pen. The muzzle and umbilicus were disinfected with 7% tincture of iodine. Calves received 2 mg/kg of ceftiofur (Excenel RTU, Zoetis, Kirkland, Quebec) subcutaneously and were transported to the University of Guelph Central Animal Facility Isolation Unit.

Calves were housed indoors, in separate rooms in groups of 2–3 similarly aged animals until the time of challenge. Lab-grade shavings were used as bedding and all personnel entering the room followed a biosecurity protocol. On arrival, calves received 1 mL/45 kg vitamin E and selenium (Dystosel, Zoetis, Kirkland, Quebec). In the first 48 h of life, calves were fed 2 L of commercially available evaporated milk (Carnation Evaporated Milk, Markham, Ontario) mixed 50:50 with water, three times daily. Colostrum and milk replacer were not offered until 48 h after birth to ensure that calves did not absorb antibody to *M. haemolytica*. From 48 h, calves were bottle-fed milk replacer (Grober High Conversion 20/15, Grober Nutrition, Cambridge, Ontario) three times daily until they were able to transition to a nipple bucket feeder. At one week of age, unmedicated calf starter pellets (Sharpe Farm Supplies, Guelph, Ontario) and hay were offered *ad libitum*. The calves received 1 mg/kg ceftiofur hydrochloride (Excenel RTU, Zoetis, Kirkland, Quebec) subcutaneously twice daily for 7 days. From birth to the start of the experiments calves were assessed three times daily at feeding, including measurements of rectal temperature, assessment of stool consistency and evaluation of demeanor and appetite. During the first 2 weeks after birth, joints and umbilical structures were palpated once daily.

2.2. Preparation and characterization of Mannheimia haemolytica

A *Mannheimia haemolytica* isolate (B158) from the lung of an adult female Holstein cow with fatal bronchopneumonia was kindly provided by the Animal Health Laboratory (AHL), Guelph, Ontario and stored at −80 °C. The serotype was determined by multiplex polymerase chain reaction (PCR) (Klima et al., 2017) using a no-template negative control, with comparison to known isolates of serotypes 1, 2 and 6, and sequencing of the amplified products.

Frozen bacterial stock was inoculated onto Columbia agar with 5% sheep blood (Oxoid Canada, Nepean, Ontario, Canada) and incubated overnight at 37 °C. A single colony was inoculated in 10 mL brain-heart infusion broth (BHIB; Bacto Brain Heart Infusion, Sparks, MD) and incubated overnight on a rotary shaker at 37 °C. On the morning of challenge, 10 mL of the overnight culture was placed in 150 mL of BHIB and incubated until an OD_{600} of 0.5 (approximately 1×10^{10} CFU/mL) was reached. A calculated volume of log-phase *M. haemolytica* at OD_{600} 0.5, based on inoculation dose, was centrifuged for 20 min at 1500 x *g* and the pellet was re-suspended in 10 mL of cold phosphate-buffered saline (PBS) and kept on ice until the time of challenge (within 1 h). Purity of the inoculum was verified by examining a Gram-stained smear. The targeted challenge doses, based on the outcome of prior studies, were as follows: conventionally raised calves received 2×10^{10} and 1×10^{11} cfu, and clean-catch colostrum-deprived calves received 1×10^{10} cfu. The data presented are from 6 separate challenge studies; calves C2 and C6 were challenged in the same study, as were C5 and C6.

The actual challenge dose was subsequently determined by plating serial dilutions of each inoculum on blood agar and incubating overnight at 37 °C. For each challenge, representative colonies were analyzed by matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) to confirm the identity and genotype of the *M. haemolytica* (Loy and Clawson, 2017).

The *M. haemolytica* aerosol challenge was done using a compressor (Precision Medical, Northampton, PA) that delivered approximately 25 psi of air pressure through a Whisper Jet nebulizer (Marquest, Englewood, Colo) to a large canine anesthesia mask with an added one-way exhalation valve that was placed over the calves' muzzles. In a preliminary study in a single calf, the experimental apparatus delivered aerosolized dye throughout the respiratory tract: deposits were abundant in the nasal passages and larynx, minimal in the trachea, and increased at bronchial bifurcations; dye was histologically visible in bronchioles and rarely alveoli, and was similar across the histologic sections of cranioventral, caudodorsal and caudoventral lung (data not shown).

2.3. Clinical data and sampling

Immediately prior to challenge, baseline clinical parameters including temperature, heart rate, respiration rate, demeanor and appetite were recorded. Calves were visually assessed multiple times daily, as indicated by the severity of disease following pathogen challenge. Full evaluation and scoring were performed in a blinded manner by the same investigator twice daily. In addition to the parameters described above, the presence or absence of coughing and nasal discharge were also recorded. Calves were scored based a system used to identify respiratory disease on-farm in pre-weaned calves (McGuirk and Peek, 2014).

Once-daily blood samples were collected from the jugular vein for measurement of complete blood counts, and serum haptoglobin and plasma fibrinogen concentrations (AHL, University of Guelph). Antibodies to *M. haemolytica* leukotoxin were measured (Prairie Diagnostic Laboratory, Saskatoon, Saskatchewan) in pre-challenge serum as previously described (Harland et al., 1992). ELISA values were expressed as the reciprocal of the final dilution, giving a positive value compared to background. Right and left nasal swabs at a depth of 7 cm were collected at baseline and once daily thereafter, stored in transport medium, and aerobic bacterial cultures were routinely prepared (AHL, University of Guelph).

Baseline and subsequent daily systematic thoracic ultrasound examination of clean-catch colostrum-deprived calves was performed using a portable linear rectal ultrasound unit (Ibex Pro, E. I. Medical, Loveland, CO), with a frequency of 6.2 MHz, depth set to 6 cm, and an 18 dB gain (near 31 dB, far 35 dB) as previously described (Ollivett et al., 2015). Isopropyl alcohol (70%) was applied to the unshaved skin, and the lungs were visualized by moving the transducer dorsal to ventral, beginning at the 10th intercostal space and proceeding cranially, to examine each intercostal space. Lung consolidation was identified as hypoechoic regions within the parenchyma, with a loss of the normally observed parallel horizontal lines (known as A-lines, or reverberation artifacts). The location of lesions was identified based on the intercostal space and vertical position (dorsal, mid, and ventral lung field). Any identified lung lesions or pleural fluid were recorded along with location and approximate size and depth of the lesion.

2.4. Euthanasia and post-mortem evaluation and sampling

Calves were euthanized prior to the planned study endpoint if they were in respiratory distress or were unwilling to rise or eat. All surviving calves were euthanized 3 days after *M. haemolytica* inoculation.

Gross postmortem examination was performed within one hour of euthanasia, and lesions were evaluated in a blinded manner. Several measures of the severity of pneumonia were determined. First, the ratio of lung-to-heart weight was determined by separating the heart from the lungs, removing the blood and major vessels from the heart, removing the lungs from the aorta, esophagus, vena cava and trachea cranial to the bifurcation, and weighing the heart and lungs separately. Second, the lungs were examined visually and by palpation, and areas were considered to be pneumonic if they were discoloured and firmer than normal and if the red-purple discoloration was sharply demarcated from normal lung and typically in an angular or lobular pattern. The portion of each lung lobe that was diseased was recorded on a schematic map of the lung and the proportion of diseased lobe was multiplied by the relative size of the lobe to calculate the total proportion of lung that was diseased (Jericho and Kozub, 2004). Additional findings such as pleuritis or pleural effusion were also recorded. Third, digital photographs of the lateral surface of the left and right lungs were obtained after they were removed from the thoracic cavity. Identifiers were removed, and the images were assigned random numbers for blinded analysis. Image analysis was used (with knowledge of the notes taken at postmortem examination) to calculate the extent of lesions in the left and right lungs of each calf (ImageJ software, Schneider et al., 2012). Each focus of consolidated lung was traced and their areas were added together to estimate the total affected surface area, which was then divided by the total surface area of the lung in the image.

Fresh and formalin-fixed samples of the left and right cranioventral, caudodorsal and caudoventral lung lobes and fresh samples of accessory lung lobes were collected in a standardized manner, focusing on lesional lung if it was present. The right kidney was also sampled. All fresh samples were kept on ice and then frozen until aerobic bacterial culture (AHL, University of Guelph). The formalin-fixed tissues were routinely processed and histologic sections were stained with hematoxylin and eosin. Histologic sections were scored for the presence or absence of bronchopneumonia (neutrophils in alveoli and bronchioles), oat cells (necrotic neutrophils with streaming chromatin), coagulation necrosis, pleuritis (fibrin and neutrophils on the pleural surface), and fibrin in alveoli.

2.5. Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) was conducted using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA) and Dunnett's test was used to compare pre-challenge to post-challenge parameters measured every 12 h (temperature, heart rate, respiratory rate, clinical scores) or every 24 h (fibrinogen, haptoglobin). The correlation between various measures of postmortem lung lesion severity including visual analysis of the proportion of affected lung, image analysis of proportion of affected lung, and lung-to-heart weight ratio was determined by linear regression and an R-squared value was calculated.

3. Results

3.1. Characterization of Mannheimia haemolytica challenge strains

The *M. haemolytica* strain used in these studies (strain B158) was identified by multiplex PCR as serotype 1. The amplification products were sequenced and results were consistent with that expected for *M. haemolytica*. The strain was determined by MALDI-TOF MS to be genotype 2.

3.2. Aerosolized Mannheimia haemolytica serotype 1 challenge in conventional calves

Two conventionally raised calves with similar positive anti-leukotoxin antibody titres of 19,800 (Calf A1) and 13,700 (Calf A2) were challenged by aerosol with 2×10^{10} cfu (Calf A1, lower dose) and 1×10^{11} cfu (Calf A2, higher dose) of *M. haemolytica* serotype 1. Within 1 h of administration of the challenge dose, both calves displayed similar clinical signs, with increased rectal temperature, depression, increased respiratory rate and effort. One hour following challenge, both calves had increased respiratory effort. By 2 h, both were moderately depressed, continued to have increased respiratory rate and effort, and were reluctant to rise when researchers entered the pen. At 6 h, the calf that received the higher dose of *M. haemolytica* became severely dyspneic, refused to rise when researchers entered the pen, and was euthanized. The second calf's demeanor and respiratory effort improved between 6 and 12 h after challenge, and remained normal from this point until euthanasia 3 days post challenge.

On gross postmortem examination of the calf that received the higher dose of *M. haemolytica* (euthanized 6 h after challenge), the lungs were diffusely wet and heavy, with marked interlobular and subpleural edema and emphysema. Histologically, the lungs had diffuse alveolar damage with hyaline membranes lining alveoli, a large amount of proteinaceous fluid and variable amounts of fibrin strands, neutrophils and hemorrhage filling alveoli, and occasional thrombi (Fig. 1). In contrast, the calf that received the lower dose (euthanized at 3 days after challenge) had grossly normal lungs. Histologically, the lungs of this calf had occasional areas of atelectasis with small numbers of neutrophils and rare bronchioles filled with neutrophils. *Mannheimia haemolytica* was isolated from the cranioventral and dorsocaudal areas of the lungs of both calves. Neither *Mannheimia* nor other pathogens were isolated from the kidney of either calf.

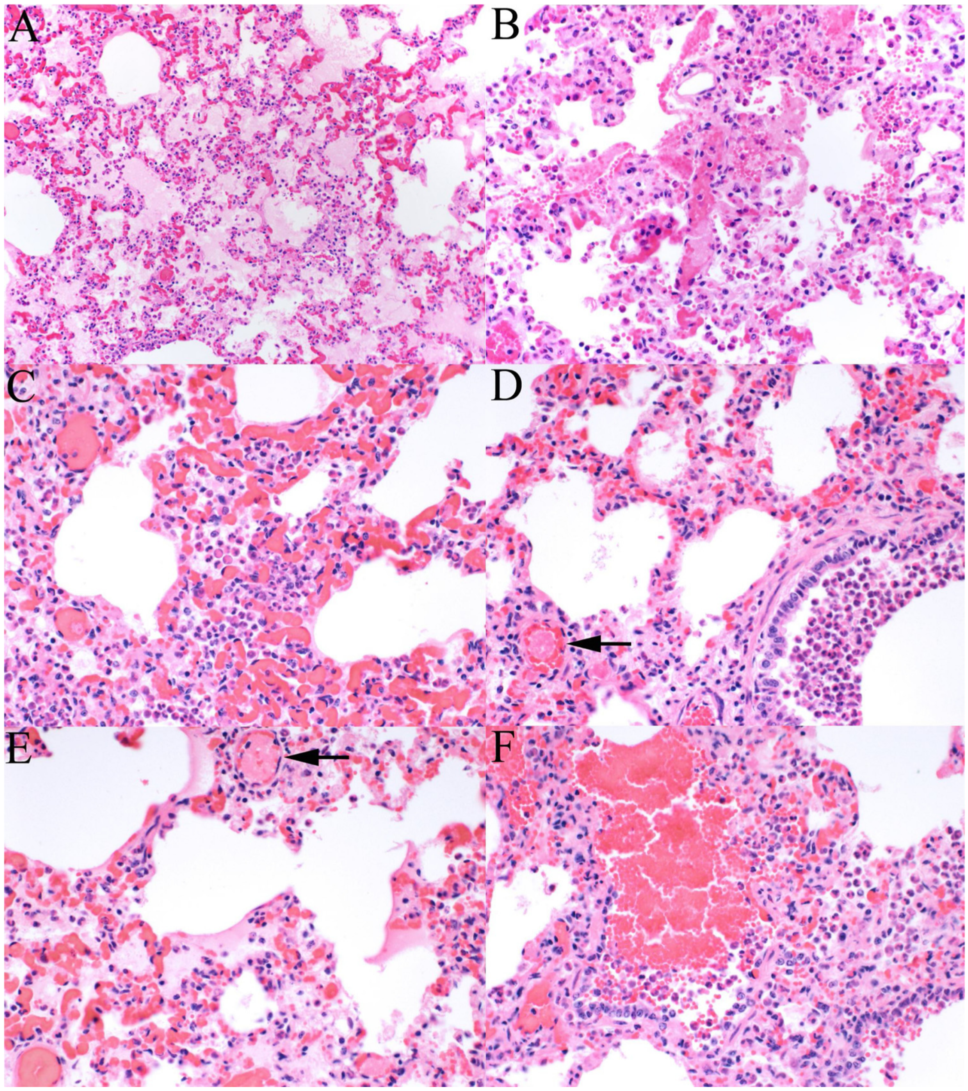

Fig. 1. Lung tissue, conventionally raised calf (Calf A2), euthanized 6 h after challenge with 5×10^{10} cfu aerosolized *Mannheimia haemolytica* serotype 1. All examined sections had alveolar edema, fibrin and neutrophils. Lungs had eosinophilic (proteinaceous) alveolar fluid (A), alveolar fibrin and hyaline membranes (B), intact and necrotic neutrophils in alveoli (C), fibrin thrombi in blood vessels (arrows) (D, E), and alveolar and bronchiolar hemorrhage (F).

3.3. Aerosolized Mannheimia haemolytica serotype 1 challenge in clean-catch colostrum-deprived calves

Eight clean-catch colostrum-deprived calves were used in this study. Anti-leukotoxin antibodies in serum were measured by ELISA, and values ranged from 100-2225. By comparison, 2 samples of commercial fetal calf serum (Invitrogen, San Diego, CA) had ELISA values of 50 and 4700. Calves were challenged by aerosol with a target dose, based on OD_{600}, of 1×10^{10} cfu *M. haemolytica* serotype 1 and the challenge dose was subsequently confirmed using colony counts to range from 2×10^9 to 9×10^{10} cfu. All calves developed elevated rectal temperature by 12 h after inoculation, and this persisted until euthanasia 3–4 days later ($P < 0.001$ compared to baseline) (Fig. 2A). The mean respiratory rate was not affected by *M. haemolytica* challenge (p = 0.35) (Fig. 2B). Heart rate increased after challenge, but this was only significant at 36 h following inoculation ($P < 0.05$) (Fig. 2C). Clinical signs are summarized in Table 1. The most frequently identified clinical signs were (in order of decreasing frequency) cough, increased respiratory effort, depression and reduced appetite. Infrequently identified parameters (observed in 1 or 2 calves) included nasal discharge, ear flicking or droop, and ocular discharge. Three of the 8 calves developed severe clinical signs and were euthanized two days after challenge, prior to the study endpoint.

Blood indicators of inflammation increased on the first day following challenge with *M. haemolytica* serotype 1. Serum haptoglobin and plasma fibrinogen concentrations increased at 24 h following challenge and remained elevated until euthanasia ($P < 0.01$) (Fig. 3). Total blood leukocyte counts increased at 24 h following challenge ($P < 0.001$) (Fig. 4) primarily due to an increase in blood neutrophils ($P < 0.001$). The number of blood monocytes did not increase significantly during the course of the study ($P = 0.176$). Blood lymphocytes decreased following challenge and remained lower than baseline until euthanasia ($P < 0.001$). The ratio of neutrophils: lymphocytes in blood increased following challenge and this persisted to the end of the study (data not shown).

Bilateral nasal swabs were collected daily from each calf and aerobic bacterial culture was performed. *Mannheimia haemolytica* was not isolated prior to challenge. However, all post-challenge swabs contained moderate to large numbers of *M. haemolytica* (reported as 3+ to 4+).

Thoracic ultrasound evaluation of calves prior to challenge demonstrated occasional comet tails but there was no evidence of lung consolidation (Fig. 5). Evidence of consolidation (hypoechoic regions lacking the white pleural band and reverberation lines) appeared one

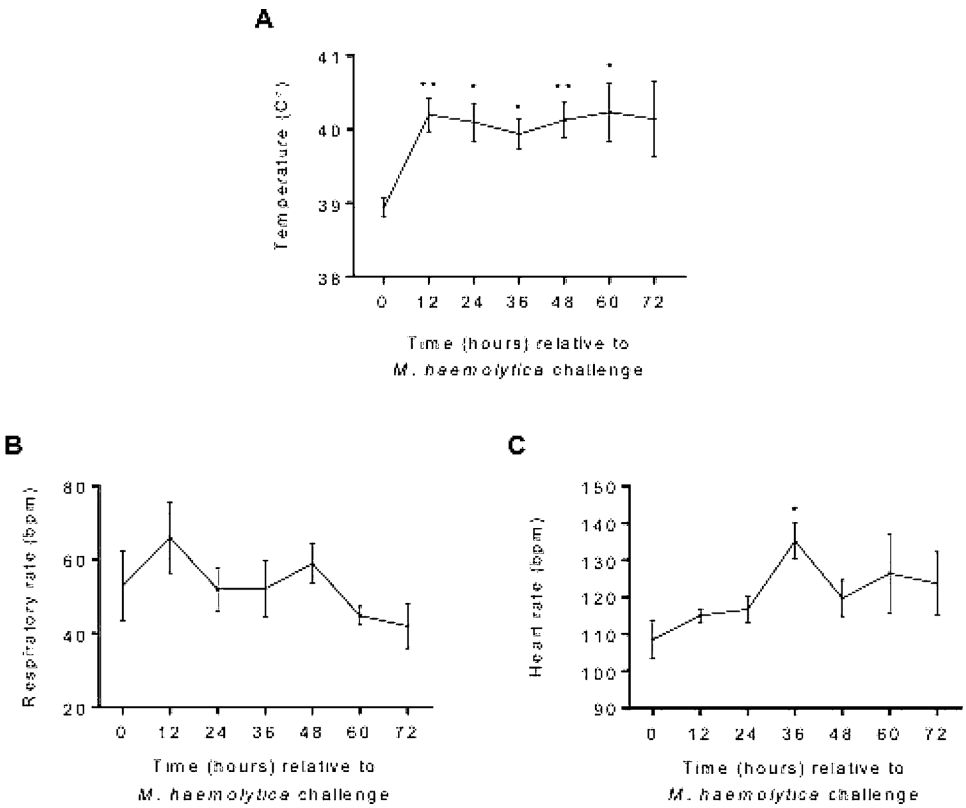

Fig. 2. Clinical parameters over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1. The data are mean ± standard error of the mean from 8 clean-catch colostrum-deprived Holstein bull calves. The data at 60 and 72 h are from the 6 and 5 surviving calves, respectively. A) Rectal temperature. B) Respiratory rate. C) Heart rate. Data were compared to pre-challenge (time = 0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$.

Table 1
The prevalence of clinical signs prior to and following aerosol challenge with 1×10^{10} colony forming units *Mannheimia haemolytica* serotype 1 in eight clean-catch colostrum-deprived Holstein bull calves.

Clinical sign	Pre-challenge	0-24 h after challenge	25-48 h after challenge
Cough	0	6	5
Increased respiratory effort	0	5	4
Depression	0	3	5
Reduction in strength	0	2	4
Loss of appetite	0	1	3
Nasal discharge	0	1	2
Ear flicking or droop	0	2	1
Ocular discharge	0	0	1

day following challenge in 7/8 calves and at 2 days following challenge in the remaining calf. Once identified, lesions persisted and the amount of affected lung increased over time until the calves were euthanized. One calf had pleural fluid detected by ultrasound examination on day 2 after challenge and was euthanized due to the severity of the clinical signs.

At postmortem, all calves had some degree of consolidation of the cranioventral lung (Fig. 6). The proportion of affected lung ranged from 1% to 55% (mean 20%; 95% confidence interval 3–36%). The consolidation primarily affected the cranial and right middle lung lobes (Table 2). Most frequently, the lesions appeared as angular (lobular) areas of red-purple discoloration that were neither raised nor collapsed. When these coalesced, they formed larger lesions that were palpably firmer than normal. Less frequently, lesions affected most or all of a lobe. On cut section, the affected lung was homogeneously red-purple or contained irregular foci delineated by a white line (coagulation necrosis). Three calves had prominent pleuritis, with sheets of fibrin covering a large portion of the lung surface, while 3 of 8 calves had focal areas of pleuritis measuring less than 2 cm, which appeared as

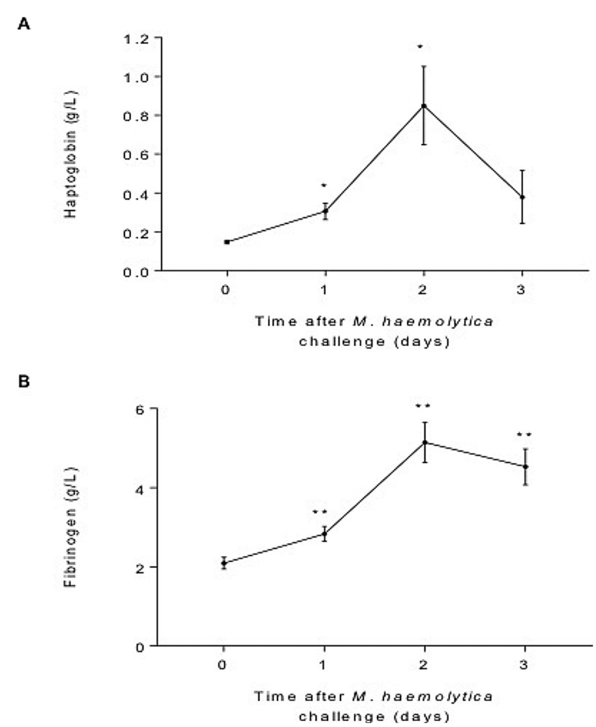

Fig. 3. A) Serum haptoglobin and B) plasma fibrinogen concentrations following challenge with aerosolized *Mannheimia haemolytica* serotype 1. The data are mean ± standard error of the mean from 8 clean-catch colostrum-deprived Holstein bull calves. Data at day 3 are from the 5 surviving calves at this time point. Data were compared to pre-challenge (time = 0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$. ** $P < 0.01$.

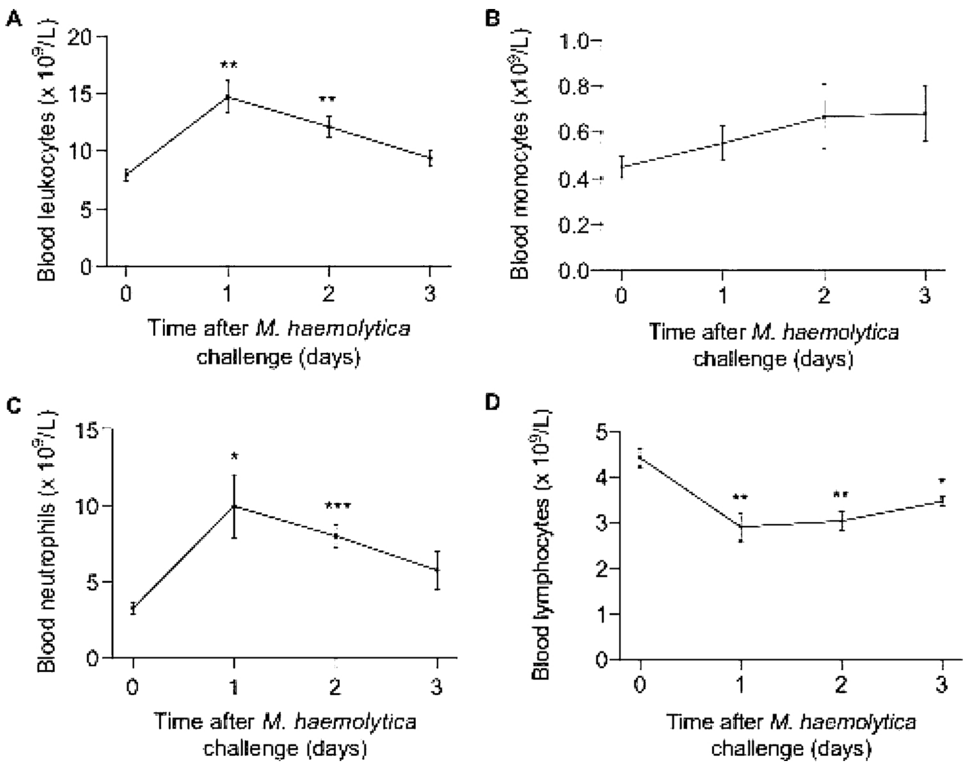

Fig. 4. Blood leukocyte counts over time after challenge with aerosolized *Mannheimia haemolytica* serotype 1. A) Total leukocytes. B) Monocytes. C) Neutrophils. D) Lymphocytes. The data are mean ± SEM from 8 colostrum-deprived Holstein bull calves. The data at day 3 are from the 5 surviving calves at this time point. Data were compared to pre-challenge (time = 0) using repeated measures one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$. ** $P < 0.01$.

pleural opacity with a ground-glass appearance and a rough texture. All measures of the severity of postmortem lung disease were significantly correlated with one another ($P < 0.05$) including visual estimates of the proportion of diseased lung (including and excluding the accessory lobe), software-based image analysis, and lung-to-heart weight ratios (Fig. 7).

Histologic examination of lung tissue from all animals with consolidation confirmed the presence of acute bronchopneumonia (Table 3), These areas often contained irregularly shaped foci of coagulation necrosis, and large numbers of degenerate leukocytes with

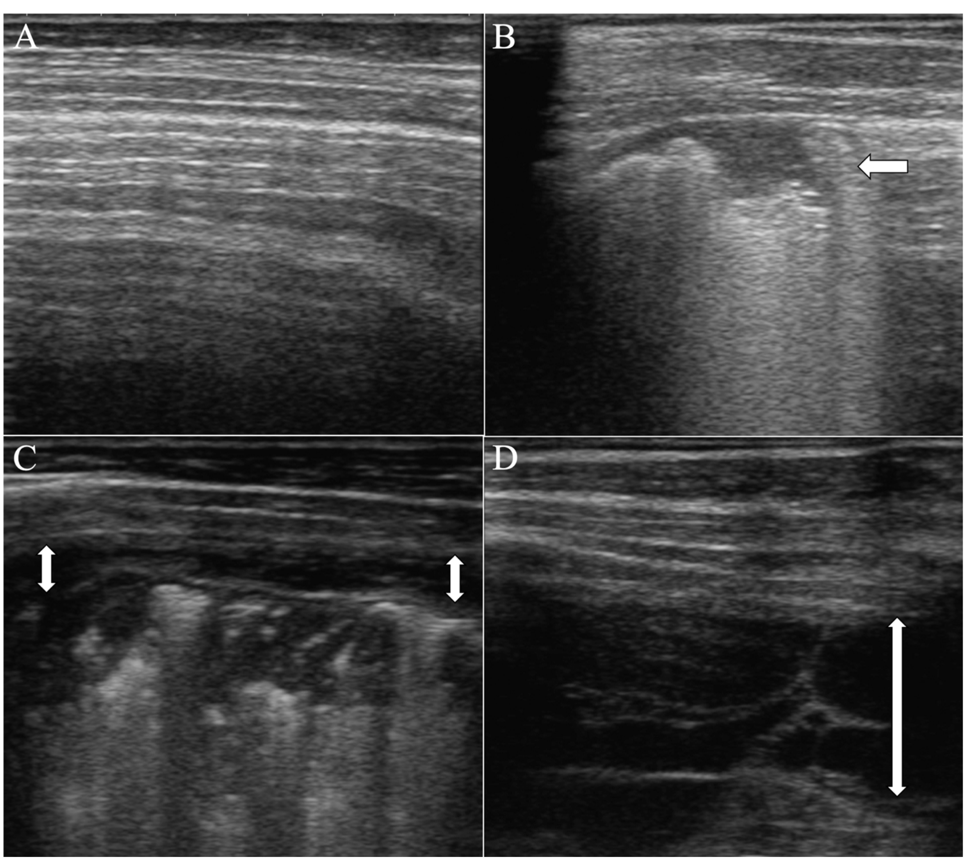

Fig. 5. Ultrasonographic findings in the lungs of calves. A. Normal lung. The parallel horizontal white lines are A-lines (reverberation artifact) and are characteristic of healthy lungs. B. Lung (Calf B8), 24 h after *M. haemolytica* infection. The lung contains an area of consolidation (arrow) that is hypoechoic and lacking reverberation artifact. C. Lung (Calf B6), 48 h after *M. haemolytica* infection. The entire lung in this field is consolidated (hypoechoic and lacking reverberation artefact) and there is a small amount of pleural effusion identified as a black area (double arrows) between the body wall and the pleural surface of the lungs. D. Lung (Calf B6), 48 h after *M. haemolytica* infection. Some areas had a large amount of pleural fluid (double arrow) and fibrin strands between the pleural surfaces.

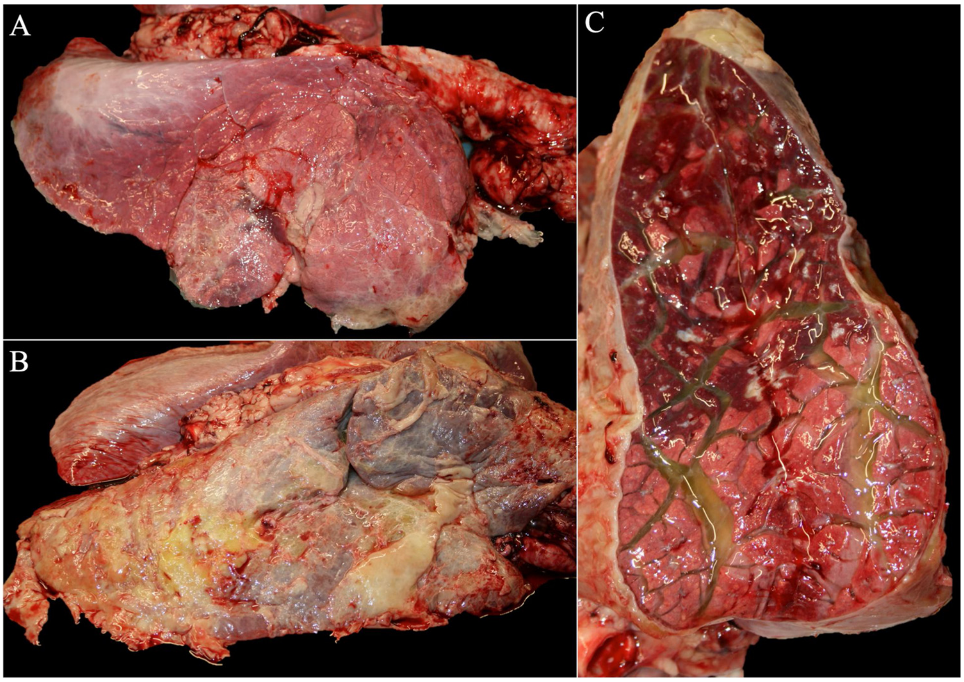

Fig. 6. Lung lesions following aerosol challenge of clean-catch colostrum-deprived calves with 1×10^{10} cfu of *Mannheimia haemolytica* serotype 1. A) Calf B4, 2 days post-infection. There is lobular consolidation and purple-red-tan discoloration of the left cranioventral lung. B) Calf B6, euthanized 3 days post infection. Fibrin covers the entire pleural surface of the right lung, and there is consolidation of the cranial and middle lobes. C) Calf B6, euthanized 3 days post infection. On cut section, there is lobular consolidation, interlobular septa are markedly expanded by edema, and fibrinous pleuritis is present (at the top of the image). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

streaming nuclei (oat cells) were frequently present within alveoli and bronchioles particularly at the periphery of the foci of necrosis (Fig. 8). In other areas, the alveoli and bronchioles contained non-necrotic neutrophils and mononuclear cells, and some also had fibrin and hemorrhage with occasional intravascular thrombi or pleuritis. Cranioventral lung sections tended to have more severe histologic lesions than dorsocaudal lung sections.

Large numbers of *M. haemolytica* were isolated from one or more samples from lungs of all calves (8/8), and pleural fluid when sampled (2/2) (Table 2). *Mannheimia haemolytica* was isolated from 15/16 cranial lung lobes and 12/16 caudal lung lobes. Discrepencies between culture data and gross findings were as follows: *M. haemolytica* was isolated from 2 of the 7 lung lobes that had no gross lesions; conversely, *M. haemolytica* was not isolated from 3 of the 25 lung samples that had gross lesions. In 1 out of 8 calves, *M. haemolytica* was isolated from kidney, indicating bacteremia.

4. Discussion

This study describes the development of a novel experimental model in which clean-catch colostrum-deprived calves were challenged by aerosol with a virulent strain of *M. haemolytica* without prior viral challenge. Inhalation of *M. haemolytica* within aerosolized droplets from the nasopharynx is considered the natural route of infection and prior research has demonstrated the presence of *M. haemolytica* in exhaled air and in tracheal air from infected cattle (Jericho et al., 1986). In the current study, the aerosol challenge consistently resulted in *M. haemolytica* nasal and lung infection, with clinical and pathologic changes that were comparable to the natural disease (Rehmtulla and Thomson, 1981). We expect this model will be useful to evaluate how modulating innate immune responses affects the development of *M. haemolytica* pneumonia.

In the ELISA for antibody to *M. haemolytica* leukotoxin, the ELISA values in clean-catch colostrum-deprived calves were uniformly low (100–2225) and were comparable to those measured in fetal bovine serum (50 and 4700) suggesting that these ELISA values reflect an absence of anti-leukotoxin antibody. Furthermore, the ELISA values measured in the colostrum-deprived calves were significantly lower than those measured in the conventionally raised calves from the same farm (13,700 and 19,800), as well as from those reported in unvaccinated 5-week-old beef calves (geometric mean of 20,770) and in on-arrival feedlot calves (geometric mean of 40,468) (Harland et al., 1992; Van Donkersgoed et al., 1993, 1994). Disinfection of the nares and the prevention of contact with adult cattle prior to removal from the farm of origin was intended to prevent colonization of the upper respiratory tract by *M. haemolytica*, and this was supported by its

Table 2
Gross lesions and isolation of *Mannheimia haemolytica* from the lung, after aerosol challenge of 8 clean-catch colostrum-deprived calves. The data show the percentage of lung with gross lesions, and whether *M. haemolytica* was isolated (+) or not (-).

	Left lung lobes				Right lung lobes						
Calf ID	Cranial		Caudal		Cranial		Middle[a]	Accessory		Caudal	
B1	68%	+	10%	+	77%	+	80%	50%[a]		80%	+
B2	8%	+	5%	+	10%	+	2%	2%	–	0%	+
B3	80%	+	10%	+	30%	+	25%	95%	+	1%	+
B4	50%	+	15%	+	53%	+	50%	2%	+	5%	+
B5	0%	–	0%	–	3%	+	1%	0%	–	0%	–
B6	3%	+	3%	+	70%	+	100%	5%	+	3%	+
B7	31%	+	0%	+	3%	+	10%	0%	–	1%	–
B8	30%	+	1%	+	3%	+	15%	30%	–	1%	+
Total with lesions	7/8		6/8		8/8		8/8	6/8		7/8	

[a] Culture data were not available from the right middle lung lobe, nor from the accessory lobe of calf B1.

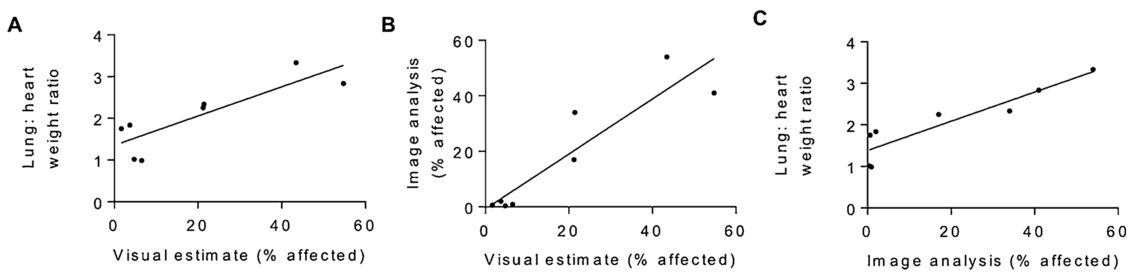

Fig. 7. Relationship among 3 quantitative measures of gross lung lesions including subjective visual estimation of the percentage of lung affected, software-based image analysis, and lung: heart weight ratios. A) Lung: heart weight ratio versus visual estimate (R^2 = 0.72, P = 0.008). B) Image analysis versus visual estimate (R^2 = 0.838, P = 0.001. C) Lung: heart weight ratio versus image analysis (R^2 = 0.846, P = 0.001).

Table 3
Histologic lesions in clean-catch colostrum-deprived calves 2–3 days following challenge with 1 × 10^{10} colony forming units of aerosolized *Mannheimia haemolytica*. The data indicate the presence (+) or absence (-) of bronchopneumonia, oat cells, coagulation necrosis, pleuritis and fibrin in alveoli, in histologic sections of cranioventral (CV) and caudodorsal (CD) lung.

Calf ID	Day of euthanasia relative to challenge	Broncho-pneumonia		Oat cells		Coagulative necrosis		Pleuritis		Fibrin in alveoli		*M. haemolytica* cultured from lungs	
		CV	CD	CV	CD	CV	CD	CV	CD	CV	CD	CV	CD
B1	2	+	+	+	+	+	–	+	–	+	+	+	+
B2	3	+	+	–	–	–	–	–	–	–	+	+	+
B3	2	+	+	–	–	–	–	+	–	–	–	+	+
B4	2	+	+	+	–	+	–	+	–	+	+	+	+
B5	3	+	–	+	–	–	–	+	+	+	–	+	+
B6	3	+	+	+	–	+	–	+	–	+	–	+	+
B7	3	+	–	+	–	+	–	+	–	+	–	+	–
B8	3	+	–	+	–	+	–	+	–	+	–	+	+
Number of calves with lesion (n = 8)		8	5	6	1	5	0	7	1	6	3	8	7

absence in bacterial culture of paired nasal swabs at the time of challenge.

The dose of *M. haemolytica* in this study was higher than reported in studies that inoculated a bacterial suspension directly into the trachea or bronchus (Gershwin et al., 2015; Hewson et al., 2011a). However, it is likely that only a small percentage of the nebulized *M. haemolytica* reached the lungs. One study estimated that only a maximum of 0.42% of *M. haemolytica*-containing aerosols reached the trachea (Jericho et al., 1986). In several previous studies, lower challenge doses of aerosolized *M. haemolytica* failed to produce disease (Bielefeldt Ohmann and Babiuk, 1985; Jericho and Langford, 1978; Yates et al., 1983), while one study used an identical challenge dose but failed to produce disease (Filion et al., 1984). In another study, calves received 1 × 10^8 cfu of *M. haemolytica* by intrabronchial aerosolization; 3 days later, *M. haemolytica* was isolated from bronchoalveolar lavage fluid from only 1 of 8 calves (McBride et al., 1999). There are several potential differences from the current study, including the experimental apparatus used to produce and deliver the aerosols (which might affect the number of bacteria reaching the lungs), the use of calves with low or absent antibody titres to *M. haemolytica*, and the use of fresh log-phase *M. haemolytica* serotype 1 bacteria (Filion et al., 1984). It is noteworthy that in the one study where aerosolization of *M. haemolytica* produced pneumonia, the challenged calves did not have detectable *M. haemolytica* antibody in serum and that fresh log-phase bacteria were used for challenge, providing further evidence for the importance of these methods (Jericho, 1989).

One limitation of this model is the variability in the severity of the lung lesions, with visual estimates of the proportion of affected lung ranging from 2 to 55%. This variability has also been reported in other models. In the study reporting pneumonia induced by aerosolized *M. haemolytica*, one of the three challenged calves did not develop any evidence of pneumonia, and in the other two calves the lesions affected only 5% of the left anterior cranial lobe in one calf and 40% of the right anterior cranial and 25% of the posterior cranial in the other calf (Jericho, 1989). Similar variability was observed among calves in our study, which might have resulted from differences among individuals in innate defences, the nature of the inflammatory response, or breathing patterns at the time of bacterial challenge. Amrine (2014), using an intratracheal challenge with a suspension of *M. haemolytica*, also reported a variable proportion of lung with pneumonia following challenge (range of 13–40% in non-antibiotic-treated calves). A retrospective analysis of the combined BHV-1 and *M. haemolytica* challenge

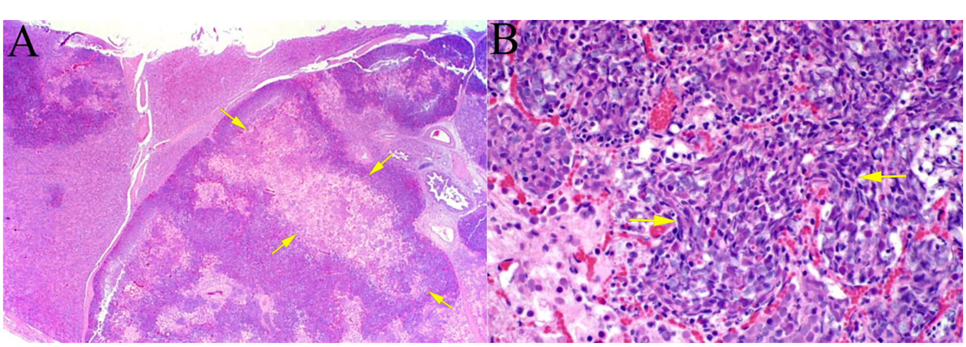

Fig. 8. Histologic lesions in lung from a colostrum-deprived calf (Calf B4) challenged with 1 × 10^{11} cfu of *Mannheimia haemolytica*. A) The lung contains irregularly shaped areas of coagulation necrosis (outlined with arrows) surrounded by leukocytes. B) Alveoli contain necrotic neutrophils with streaming nuclei (arrows). Hematoxylin and eosin.

studies conducted at one institute over 15 years also identified a high variance of pneumonia within and between experiments (Jericho and Kozub, 2004). Interestingly, these authors reported that there was not a linear relationship between the challenge dose of *M. haemolytica* and the severity of clinical signs (Jericho and Kozub, 2004). Similarly, in our experiments, although attempts were made to standardize the challenge dose of *M. haemolytica* using OD_{600} measurements, subsequent counts of colony forming units indicated that the actual challenge dose differed between experiments, but dose was not correlated with the severity of disease. One of the most severely affected calves (Calf B6) received the lowest challenge dose. An experiment where lambs were challenged with *M. haemolytica* (*Pasteurella haemolytica* serotype A2, the most common serotype in pneumonic sheep) reported no difference in the proportion of lambs developing gross lesions of pneumonia at 5 days after challenge with a 1000-fold difference in challenge dose (Gilmour et al., 1984). Thus, although variance in disease severity is problematic because it increases the sample size needed to demonstrate a statistically significant difference between groups, it may not be an inherent problem with the model because it reflects the true nature of the biologic responses in outbred animals.

The culture data showed widespread distribution of *M. haemolytica* throughout the lungs, supported by a widespread distribution of aerosolized dye in one calf. In contrast, the pneumonic lesions were primarily in the cranioventral area of the lung. In the combined BHV-1/*M. haemolytica* model, a similar pattern of pneumonia developed, with ventral distribution of bacterial pneumonia, despite generalized petechial and ecchymotic viral lesions seen throughout the lung (Jericho and Kozub, 2004). These findings suggest that the cranioventral distribution of lesions in bacterial bronchopneumonia is not the result of gravity-dependent distribution of inhaled pathogens, but other differences between these lung regions at the time of bacterial challenge.

In the first experiment, using conventionally raised leukotoxin antibody-positive calves that received 1×10^{10} or 5×10^{10} cfu of *M. haemolytica* serotype 1, both calves developed severe dyspnea in the first few hours following challenge that required euthanasia of one of the calves. This severe acute reaction did not occur in the clean-catch colostrum-deprived calves that received a similar dose. The pathogenesis of this reaction is not certain, but similar patterns have been reported. For example, in an investigation of *M. haemolytica* vaccination, cattle with greater titres had more severe lung lesions following challenge (Friend et al., 1977). In another study, 36% of calves vaccinated with capsular polysaccharide developed an anaphylactoid reaction at the time of challenge (Rice Conlon and Shewen, 1993). In another study, although antibody levels were not reported, increased BALF neutrophils were evident only after repeated exposures to intrabronchial aerosols of *M. haemolytica* (McBride et al., 1999). It is possible that in these calves with positive antibody titres and an intense and generalized exposure to *M. haemolytica*, there was excessive activation of inflammatory pathways. The affected calves developed severe dyspnea resembling acute respiratory distress syndrome requiring euthanasia of one of the calves. This pronounced inflammatory response to *M. haemolytica* was associated with histologic lesions of diffuse alveolar damage, which differ from the lesions in naturally occurring *M. haemolytica* bronchopneumonia. In the calf that received the lower dose, the dyspnea resolved within 12 h after challenge, and by the time of postmortem evaluation there were no lesions of bronchopneumonia although *M. haemolytica* was isolated from the lung. In summary, the calf that received the higher dose of *M. haemolytica* became moribund and had to be euthanized, and the calf that received the lower dose developed severe but transient dyspnea and then failed to develop bronchopneumonia by day 3 following inoculation. Thus, aerosol challenge of conventional calves was not considered a useful model of bronchopneumonia, and the subsequent studies used clean-catch colostrum-deprived calves.

Multiple clinical and pathologic outcomes were evaluated in the aerosol-challenged colostrum-deprived calves. The most consistent clinical signs were cough, increased respiratory effort, depression, and reduced appetite. Nasal discharge, ear flicking or droop, and ocular discharge were only infrequently identified, and thus were not considered useful measures of disease in this experimental model. All challenged calves in our studies developed an elevation in rectal temperature early after challenge that persisted until euthanasia. In contrast, Jericho (1989) reported only early and transient temperature elevations following aerosolized *M. haemolytica* challenge, which may be due to a lower challenge dose, differences in the age of the calves, immune status or other factors. Complete blood counts were informative as blood neutrophil counts increased whereas blood lymphocyte counts decreased following challenge. Similarly, serum haptoglobin and plasma fibrinogen concentrations increased following challenge. Thoracic ultrasound was useful to rule out the presence of lesions prior to challenge and to follow development of pneumonia over time. Quantification of ultrasound lesions was not attempted.

Several quantitative measures of postmortem disease severity were compared. There were significant correlations between visual estimates of the proportion of lung affected, image analysis (using software) to assess the proportion of lung affected, and the ratio of lung to heart weights. Visually estimating the proportion of lung affected is quick and results are immediately available; however, variation in the estimated proportion of diseased lung is expected among operators. Analysis of lung photographs using image analysis software does not consider the depth or volume of the lesions or lesions not visible on the dorsolateral surfaces, and requires additional time. Furthermore, colour changes in the lung frequently develop after death, so notes of postmortem findings were useful to determine whether discolored areas in the digital images were considered to be antemortem lesions. However, image analysis is more objective than visual estimation. A study examining the correlations between image analysis, visual analysis and lung:body weight ratios in pigs challenged with *Actinobacillus pleuropneumoniae* found similar results (Sibila et al., 2014). The percent of organ to body weight is frequently used to assess the true proportional size of organs but we could not measure carcass weight in the isolation facility and carcass weight is highly influenced by the degree of rumen fill and hydration. Thus, heart weight may be proxy for lean body weight and a better standard for comparison of lung weights among variably sized calves.

5. Conclusion

Aerosolization of *M. haemolytica* serotype 1 to clean-catch colostrum-deprived calves caused clinical signs and gross and histological lesions similar to those seen in natural disease. This model may be useful for studying early innate immune responses, virulence factors necessary for colonization and disease, and interventions to prevent *M. haemolytica* pneumonia. In contrast, conventional calves that had higher titres of antibodies against *M. haemolytica* developed acute respiratory distress with diffuse alveolar damage, but no evidence of progressive bronchopneumonia, suggesting the importance of using clean-catch colostrum-deprived calves with low antibody titers to *M. haemolytica* leukotoxin.

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Conflict of interest statement

The authors report no conflict of interest.

Declaration of interest

None.

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