Understanding the Diagnosis and Risk Factors for Respiratory Disease in Dairy Calves

by

Theresa Ollivett

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ABSTRACT

UNDERSTANDING THE DIAGNOSIS AND RISK FACTORS FOR RESPIRATORY DISEASE IN DAIRY CALVES

Theresa L. Ollivett
University of Guelph, 2014

Co-advisors:
Dr. David Kelton
Dr. Todd Duffield

The purpose of the work presented in this thesis was to evaluate the use of portable ultrasonography (US) for the diagnosis of respiratory disease in dairy calves. In addition to testing diagnostic accuracy of US, the efficacy of an intranasal vaccine against viral pathogens and the short-term effects of respiratory disease on calf behavior were evaluated.

In calves affected with subclinical respiratory disease, the sensitivity and specificity of US in diagnosing lung lesions was 94% (95% CI: 69 - 100%) and 100% (95% CI: 64 - 100%), respectively; and the presence of US lung lesions predicted a neutrophil proportion ≥ 4% in bronchoalveolar lavage fluid (OR = 23; 95% CI: 2.6 – 198; P < 0.01). Ultrasonographic lesions were highly correlated to post-mortem lesions (r = 0.92; P < 0.01). After experimental bacterial infection, lesions associated with bronchopneumonia developed rapidly, and progressed to maximum size over 48 hours, after which the lesions remained stable for several days. The odds of observing lung lesions were lower in calves that were vaccinated within the 1st week of life and 6 weeks of age with an intranasal viral respiratory vaccine as compared to calves subjected to a positive or negative control vaccination protocol. Intranasal vaccine was associated with average daily gain within the 1st 8 weeks of life, although this relationship was farm dependent.
The presence of lesions as detected by ultrasonography had no effect on lying behavior of young dairy calves.

The portable US machine carried by bovine practitioners provided a practical means to accurately assess lung lesions in dairy calves. This tool will help practitioners assist producers in making well-informed treatment and management decisions, hopefully serving to improve both calf health and welfare. Additionally, from a research perspective, this equipment will reduce the number of calves used to study respiratory disease and allow for new outcomes to be measured including the onset, duration, and resolution of lung lesions as we study the impact they have on future performance.
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STATEMENT OF WORK DONE

The study design, Animal Use Protocol and funding proposals were developed with the combined effort of Theresa Ollivett, the advisory committee, and Sam Deelen. Project data collection for Chapters 2, 4, and 5 was conducted by Theresa Ollivett and the following research assistants: Jolene Cyples, Jessica Cyples, Sam Deelen, Brittany Stinson, Melissa Wagner, Danielle Kelton, Viv Bielman, and Patrick Chung. Theresa Ollivett, Joanne Hewson, Jeff Caswell, and Roland Schubotz were responsible for data collection for Chapter 3.

Laboratory testing of serum samples for total protein by hand-held refractometer was completed by Theresa Ollivett and the above mentioned research assistants. All bronchoalveolar lavage fluid (BALF) cytology and cell counts, BVD PCR testing of skin samples, and histological slide preparation were completed at the Animal Health Laboratory of the University of Guelph. All BALF collections, euthanasia of animals, post-mortem examinations, post mortem sample collection, and histological slide analysis were performed by Theresa Ollivett with the assistance of Jeff Caswell.

Raw data were transcribed from audio files or paper forms into a Microsoft Access database with the help of all aforementioned research assistants as well as Sarah Stanger-Guy. Data for Chapters 2 – 5 were cleaned by Theresa Ollivett. All statistical analyses of the data were completed by Theresa Ollivett, with the help of William Sears when needed. All of the chapters included within this thesis were written entirely by Theresa Ollivett and revised by all members of her advisory committee.
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CHAPTER 1. LITERATURE REVIEW

1.1 INTRODUCTION

In contrast to the simplicity that the name suggests, bovine respiratory disease (BRD) is not a specific disease process. Instead, it is a collection of signs that when taken together with patient signalment and history, can be categorized into 3 separate clinical presentations: enzootic pneumonia of dairy calves, shipping fever of feedlot cattle, and atypical interstitial pneumonia (Lillie, 1974). Early detection of BRD in dairy calves is often poor due to the lack of consistent clinical signs such as depression, inappetance, and cough (McGuirk, 2008). There is a small body of research describing an association between the presence of lung lesions seen at post mortem and average daily gain in feedlot and veal calves that have not been treated for respiratory disease, presumably due to the lack of clinical signs (Wittum et al., 1996; Thompson et al., 2006; Leruste et al., 2012). Such findings suggest that there is a need to modify our diagnostic tests and broaden our definitions in order to identify and account for the potential effects of subclinical BRD in young dairy calves. This literature review and the subsequent research chapters will focus on our current knowledge of BRD in dairy calves and methods by which we can identify and prevent the occurrence of this disease.

1.2 ETIOLOGY OF BRD IN DAIRY CALVES

In dairy calves, BRD is a multifactorial disease in which a combination of host, agent, and environmental factors contribute to infection of the lower airways by both viral and bacterial pathogens. The common viral pathogens include bovine respiratory syncytial virus (BRSV), bovine herpes virus (BoHV), and bovine parainfluenza 3 (PI3). Controversy exists regarding the extent to which bovine corona virus (BCV; as reviewed by Love et al., 2014) and bovine viral
diarrhea virus (BVDV; Caswell et al., 2007; Ridpath et al., 2010) contribute to respiratory disease in calves.

Viral infection of the respiratory tract often causes destruction of the respiratory epithelium resulting in impaired mucociliary function and secondary bacterial infection from pathogens normally residing in the nasopharynx (Caswell, 2007; Moeller et al., 2013). Bacterial respiratory pathogens include *Pasteurella multocida*, *Mannheimia haemolytica*, *Trueperella pyogenes*, *Mycoplasma bovis* and less commonly, *Histophilus somni* and *Bibersteinia trehalosi*. Neutrophilic infiltrates in the bronchial, bronchiolar, and alveolar compartments of the lung are the main pathological changes associated with bacterial bronchitis, bronchiolitis, and bronchopneumonia (Caswell et al., 2007). These lung lesions in dairy calves typically have a cranioventral distribution, starting in the cranial aspect of the cranial lobe or right middle lung lobe and proceeding caudally (Allan et al., 1985; Reinhold et al., 2002; Dagleish et al., 2010). Such changes can occur as early as 2 hours and peak at 6 hours after experimental bacterial challenge (Ackermann et al., 1999; Radi et al., 2002).

### 1.3 EPIDEMIOLOGY AND RISK FACTORS FOR BRD IN DAIRY CALVES

Several large multi-herd studies investigating BRD have been performed in various locations over the past 30 years and are listed in Table 1.1. The calf level prevalence of BRD ranges from very low to approximately 1/3 of calves studied. In two single herd studies, approximately 14% of calves were affected (Stanton et al., 2010; Bach et al., 2011a); however, others have shown that the within-herd prevalence of BRD ranges dramatically from 20 – 90% (Heins et al., 2014), 0 – 37% (Lago et al., 2006), and 0 – 44% (Windeyer et al., 2012). These patterns suggest that BRD is much more of a problem for certain herds. Additionally, at least 20 – 30% of calves affected by BRD require multiple antimicrobial treatments (van Donkersgoed et
al., 1993; Windeyer et al., 2012; Heins et al., 2014). Interestingly, compared to veterinarians, producers are twice as likely to retreat calves, which suggests a possible discrepancy in re-treatment indicators (van Donkersgoed et al., 1993).

Over-crowding and poor air quality have long been understood to contribute to BRD (Lillie, 1974). Specifically, drafts, high ammonia levels, shared housing, housing with older animals, large herd size, diarrhea, prolonged time to dam separation, and BRSV vaccination have all been reported as risk factors (van Donkersgoed et al., 1993; Virtala et al., 1999; Lundborg et al., 2005; Gulliksen et al., 2009). Additionally, in enclosed barns, low air bacterial counts within calf pens, solid barriers between calves, and the ability of the calf to nest in deep straw protected pre-weaned calves from BRD during the winter months (Lago et al., 2006). Separating previously sick calves from healthy calves in group housing has also been associated with a lower risk of BRD in the healthy calves (Bach et al., 2011b).

Compared to the effects of housing and ventilation, the association between failure of passive transfer (FPT) and the development of BRD is not as straightforward. Several studies have documented an increased risk of BRD in calves with insufficient absorption of maternal immunoglobulins (van Donkersgoed et al., 1993; Donovan et al., 1998; Virtala et al., 1999; Windeyer et al., 2014). In contrast, FPT did not increase the risk of BRD in calves from a study conducted in Minnesota (Sivula et al., 1996). Calves approximately 2 weeks and younger were tested with radial immune-diffusion (RID; van Donkersgoed et al., 1993; Virtala et al., 1999), whereas calves under approximately 8 days of age were tested by serum total protein using a cut-off of 6.5 mg/dL (Donovan et al., 1998) or 5.7 mg/dL (Windeyer et al., 2012). In the one study that did not find an association between FPT and BRD, passive transfer was estimated in a small convenience sample of calves using a sodium sulfite turbidity test (SSTT; Sivula et al.,
The SSTT is a semi-quantitative test in which 3 concentrations of sodium are used to precipitate large proteins, such as immunoglobulin, resulting in the presence of turbidity (Weaver et al., 2000). The SSTT is known for having excessive false positive results when the 14% or 16% test solutions are used as compared to the 18% test solution (Tyler et al., 1996). It is unclear which cut-off was used by Sivula et al. (1996). It is possible that the potential association between FPT and BRD was masked by the small sample size or excessive false positive test results in the unaffected calves. Comparisons between studies are difficult as calf age and testing strategy differ between reports and the decay of maternal antibody is rarely taken into account (Virtala et al., 1999).

1.4 TESTING FOR BRD IN DAIRY CALVES

When defining BRD, it is important that we understand exactly what it is that we are trying to describe, i.e. exposure vs. infection vs. disease. As summarized by Fulton and Confer (2012), the presence of pathogens without lesions confirms infection; the presence of pathogen with lesions confirms disease (either subclinical or clinical); the presence of pathogen, lesions, and clinical signs confirms clinical disease; and the presence of antibody confirms exposure, but not necessarily disease. Historically, definitions for BRD in research studies have focused on clinical abnormalities as indicators of lung lesions, and many of these definitions center around the presence of fever and depression (Table 1.2) or are not described at all (Waltner-Toews et al., 1986; Sivula et al., 1996). Failure to define a disease reduces the ability to make inferences from that data set. However, defining disease with subjective measures also risks poor accuracy and agreement between the individuals declaring the presence or absence of that disease. Evidence of this lies in the discrepancies noted between clinical diagnoses and post-mortem results (Sivula et al., 1996), between producer and veterinarian based diagnoses (van Donkersgoed et al., 1993),
and even between veterinarians making the same diagnosis (Amrine et al., 2013). For the purposes of this report, methods of diagnosing clinical disease, lung lesions, and lung inflammation will be reviewed.

**Clinical Scoring Systems**

Systematic respiratory scoring (RS) was developed to ameliorate the problems associated with generic definitions and to hopefully reduce the impact of BRD on calf welfare and the cost of raising replacement animals by improving early detection rates and the initiation of treatment protocols (McGuirk, 2008). The most widely used system, the Wisconsin Calf Scoring Chart (WCSC, Appendix 1), divides the response to respiratory disease into 5 categories: body temperature, nasal discharge, cough, ocular discharge, and ear position. Each category is assigned 0 – 3 points corresponding to the subjective level of abnormality (0 = normal, 1 = mild, 2 = moderate, and 3 = severely abnormal) and the total number of points is summed to arrive at an overall RS. However, when ear position and ocular discharge are both abnormal, only the higher value is included in the score. Calves scoring greater than 4 are considered sick based on an unpublished comparison of RS to bronchoalveolar lavage fluid (BALF) culture, and BALF cytology (McGuirk, 2008). The recommended goal for producers is to identify and treat calves having a RS > 4. Recently, the WCSC, thoracic auscultation and treatment records were compared to thoracic US in a cross-sectional study of 106 calves from 13 Canadian dairy farms (Buczinski et al., 2014). Using an US cut-off of 1 cm lung consolidation as a case definition, the sensitivity and specificity of the WCSC was 55 and 58%, respectively; and the sensitivity of thoracic auscultation as compared to US ranged from 3 to 17% (Buczinski et al., 2014). This Canadian study provides evidence that clinical scoring alone underestimates the prevalence of
lung lesions in dairy calves; however it does not investigate the clinical or physiological significance of the US lesions.

One pitfall of scoring systems, such as the WCSC, is the subjective nature of ranking the severity of clinical signs. Recently, 3 novel respiratory scoring systems were developed using statistical methods, instead of subjective judgements, to assign weights to describe the severity of the abnormality within each category, i.e. nasal discharge (Love et al., 2014). More specifically, a conditional logistic regression model was built using forward selection. Body temperature was forced into the model as a dichotomous predictor using a cut-point of 39.2°C. The remaining components of the WCSC were categorized 3 different ways for each of the different scoring systems. For example, in the first scoring system, all components of the WCSC were entered into the model with levels of severity according to the WCSC (except for body temperature, as previously mentioned). Severity levels within each category were collapsed when odds ratios (OR) were either non-significant or when inadequate sample size prevented convergence. An additional dichotomous variable was included to account for the presence of abnormal respiratory effort. The coefficients from the final model were used as the score weights for each category and receiver operator characteristic (ROC) curves were developed to identify which cut-point correctly identified the largest proportion of calves affected with BRD according to the study definition. For the purposes of that study, 3 definitions of BRD were allowed: 1) PCR positive for respiratory virus or 2) culture positive for aerobic respiratory bacteria plus WCSC RS > 4 or 3) culture positive for *Mycoplasma bovis* plus WCSC RS > 4. Each of the 3 systems correctly classified approximately 90% of the animals and required less handling than the WCSC. The animals originally identified as potential cases for this study were selected from a dataset that identified cases only when BRD was suspected clinically and the WCSC RS > 4.
Therefore these calves are not representative of the general population of dairy calves and the authors are correct in pointing out that because of this fact, these scoring systems are not intended to act as gold standards for the diagnosis of BRD, but instead may serve as a means of identifying a large proportion of clinically affected calves under some conditions.

**Diagnostic Imaging**

Although scoring systems as described above are more organized and systematic than the definitions provided in Table 1.2, they still fail to identify the calves with lung lesions associated with BRD in the absence of clinical signs. Diagnostic images obtained by radiography, computed tomography (CT), and ultrasonography (US) are non-invasive methods of diagnosing lung lesions prior to performing a post-mortem examination. One retrospective study of 42 clinically ill adult dairy cows demonstrated that the sensitivity (Se = 94%) of radiography is excellent, but specificity (Sp = 50%) is poor for identifying thoracic lesions when compared to post-mortem findings (Masseau et al., 2008). Unfortunately, this study only evaluated clinically ill animals and did not evaluate young dairy calves. In contrast, CT has been evaluated in young dairy calves after experimental infection with *M. haemolytica* (Lubbers et al., 2007). In this particular study, there was a high correlation (r = 0.94) between CT and post-mortem levels of consolidation. Unfortunately, radiography and CT are of no practical value for diagnosing lung lesions in large numbers of calves in a farm setting due to physical equipment constraints, expense, and the potential for exposure to radiation. In contrast, US can be performed using portable, readily available machines without the fear of radiation exposure.
Ultrasonography

The pathophysiology of BRD is such that cellular infiltrates and cellular debris effectively displace air from the lung tissue, resulting in non-aerated and/or consolidated lung lesions that are detectable by US. These lesions alter the lung character, changing the US image from that of a strong reflector with reverberation artifact to a homogenous, hypoechoic structure similar to that of liver (Reef et al., 1991). These changes might make it possible to arrive at an US diagnosis of lung lesions regardless of the clinical state of the animal.

Although diagnostic US has been available since the mid-1960’s, relatively few studies were carried out in dairy cattle during the first 20 years and none involved US of the lungs (Lamb et al., 1988). Since the early 1990’s, more studies have focused on using US to diagnose BRD. One small study showed that US was a reliable method of confirming clinical bronchopneumonia in 18 Holstein calves up to 5 months of age (Rabeling et al., 1998). In a separate study in which 3 observers with varying levels of experience imaged 10 dairy calves (healthy, n = 4; treated for BRD, n = 6), the inter-observer agreement was moderate to almost perfect (kappa = 0.6 – 1.0) depending on the experience level of the observer (Buczinski et al., 2013). One other study assessed lung lesions identified by US after an experimental bacterial infection (Reinhold et al., 2002). In that study, there was excellent agreement between the post-mortem examination and US distribution of lesions; however, only one post-challenge US was performed 48 hours after inoculation.

The US studies performed in the past used few animals and did not examine the cranial thorax. More specifically, examinations focused on the 3 – 11th or 12th intercostal space (Reinhold et al., 2002; Jung and Bostedt, 2004), the 5 – 12th intercostal space (Babkine and
Blond, 2009), or the right third, fifth, and seventh intercostal space (Abutarbush et al., 2012). Others only evaluated the 7 – 11th intercostal spaces (Braun, 1997; Flock, 2004). In Flock (2004), bronchopneumonia was described as occurring predominantly in the cranio-ventral portions of the lung; however, the described technique only evaluated the lung lobes caudal to the 7th intercostal space, thereby excluding the entire left cranial lobe, the right middle lung lobe, and the entire right cranial lobe.

It is possible that the sensitivity of 85% and specificity of 98% reported by Rabeling et al. (1998) were relatively high because clinical cases were evaluated. Theoretically, clinical cases should have a greater area of consolidation than subclinical cases. The sensitivity of US might have been much lower if subclinical cases were evaluated, since lung lesions are often localized to the cranial aspect of the cranial lobe (Dagleish et al., 2010). In Rabeling et al., (1998), the imperfect sensitivity was attributed to changes located in the caudal lung lobes and consisted of a 10 cm pulmonary abscess, a pneumothorax, and one case of interstitial pneumonia.

In previous reports, probe frequency and probe design ranged from 3.5 MHz sector (Abutarbush et al., 2012), 3.5 – 13 MHz linear (Braun et al., 1996; Flöck, 2004), 7.5 MHz sector (Rabeling et al., 1998), and 5 MHz convex (Jung and Bostedt, 2004). Although these reports often used linear probes, none reported using a portable linear rectal transducer. Probe design is an important factor to consider when imaging the cranial most aspect of the thorax as probes designed for transcutaneous use are bulky, unlike transrectal probes, which permit better access to the axillary region and cranial thorax. Transrectal probes are also widely used by bovine practitioners making them suitable for practical field-based use of US in dairy calves.
**Lung fluid analysis**

A more invasive method of detecting lung inflammation and infection is through the assessment of lung fluid. Lung fluid can be collected by performing a trans-tracheal wash or through the collection of bronchoalveolar lavage fluid (BALF). Trans-tracheal wash samples represent fluid from both lungs. This is considered the ideal test to diagnose the etiologic agent associated with respiratory disease (Angen et al., 2009). In contrast, BALF is collected from deep within the bronchial tree and represents fluid from a focal area within one lung lobe. Fluid collected via bronchoalveolar lavage has less variation in cell populations than fluid collected via trans-tracheal wash and is often the preferred method for detecting pulmonary inflammation (McGuirk, 2008). In health, macrophages are overwhelmingly the predominant cell type in BALF, with neutrophils making up a very low percentage of cells. During bacterial and occasionally viral lung disease, neutrophil migration into the airway lumen occurs, resulting in greater neutrophil and lower macrophage proportions as compared to the normal state. Suggested cut-points for neutrophil proportions in BALF are variable and range from 10 – 40% (Pringle et al., 1988; McGuirk, 2008). Occasionally BALF has been used to control for the potentially confounding effect of subclinical BRD on BALF parameters in apparently normal calves (Pringle et al., 1988). In that report, 18 of 30 clinically normal calves were excluded from the study when neutrophil proportions were greater than 10% as they were considered positive for subclinical BRD.

**Sickness behavior associated with BRD**

Sickness behavior, a coordinated set of responses to an infectious or inflammatory condition (Johnson, 2002), can be assessed by analyzing lying behavior of affected animals
(Weary et al., 2009; Borderas et al., 2008). Previous work addressing lying behavior often used visual observation of video recordings (Hänninen et al., 2005; Borderas et al., 2008), whereas accelerometers are now used to objectively measure specific aspects of lying patterns without the labor of analyzing large quantities of video (Müller and Schrader, 2003; Bonk et al., 2013). Automated measures of lying behavior that have been validated in the dairy calf consist of total lying time (LT), total standing time, number and duration of individual lying and standing bouts, and the laterality of the lying position (Bonk et al., 2013).

The effect of specific diseases on lying behavior in dairy calves has not been extensively studied, although a low dose injection of bacterial endotoxin does appear to alter lying patterns (Borderas et al., 2008). This report measured behavior changes in response to endotoxin, a cell wall component of gram negative bacteria, including the common respiratory pathogens *M. haemolytica* and *P. multocida*. It is possible that automated tools, such as accelerometers, may be able to detect behavioral changes during the early stage of BRD, therefore enhancing our ability to identify and manage disease.

1.5 MUCOSAL VACCINATION

An understanding of the neonatal immune system is imperative to understanding how to incorporate proper vaccine schedules into the management of replacement heifers. Calves are born agammaglobulinemic and must ingest maternal colostrum for immune support, until their own immune system is sufficiently developed. Although maternal transfer of antibody to the newborn calf provides many great benefits (Faber et al., 2005), high levels of maternal antibodies are associated with a delayed antibody production by the neonate, and as well as selective inhibition of lymphocyte responses (Tizard, 2013). The potential for maternal blockade has caused concern regarding the practice of vaccinating calves during the first few months of
life to prevent BRD, as maternal antibodies can be present for up to 6 months of age (Menanteau-Horta et al., 1985). Mucosal vaccination via the intranasal route might provide an option to bypass this problem.

From the nasal passages to the lower airways within the lungs, the lining of the respiratory tract serves as a barrier between the internal and external environments of the dairy calf. The anatomy of this barrier is such that specialized ciliated epithelial cells function to steadily move mucus, debris, and pathogens from the lower to the upper airway for removal by coughing or swallowing. The respiratory mucosa acts as a physical obstruction to infectious pathogens associated with respiratory disease. This mucosa can also interact with these pathogens in such a way that either immediate death of the pathogen occurs, such as with the innate immune system; or by stimulating the immune system into developing an adaptive response in which memory is involved allowing for future protection. Specifically, one locally produced and secreted factor that is derived from plasma cells, Immunoglobulin A (IgA), is critical in the prevention of bacterial and viral attachment to the respiratory mucosa (Griebel, 2009). The systemic immune system differs remarkably from the mucosal immune system in a number of ways. Primarily, a particular pathogen needs to be recognized by an antigen presenting cell which will travel to the nearest lymph node, where a process will occur that results in the proliferation of a population of B cells that can recognize the antigen and produce antibody against it. Antibodies produced in this method are typically IgG and IgM and will circulate throughout the blood stream and interstitial tissues. Essentially, a spill-over of IgG onto the mucosal surface serves to protect the calf from invading viral and bacterial pathogens. The concentration of these antibodies is known to be relatively low, compared to the potential local production of IgA, if stimulated mucosally. In regards to dairy calves, the more important
difference between mucosal and systemic immunity, is the ability of the mucosal immune system to respond to antigens in the presence of high levels of circulating maternal antibody. Maternal antibody levels peak within the calf approximately 24 – 48 hours after ingestion of colostrum and are at significantly lower levels by approximately 3 weeks of age, at which point the calf’s own immune system is beginning antibody production of its own. However, during this time of circulating maternal antibodies when the calf is seropositive, several studies have shown that administration of a parenteral or systemic vaccine will not achieve the same immunologic response as that from a seronegative or colostrum deprived calf.

It has been established that 3 - 8 day old Holstein calves are capable of mounting a mucosal immune response in the face of maternal antibodies (Hill et al., 2012). However, over the last 10 years, reports regarding the potential for intranasal vaccination to protect young dairy calves with and without maternal antibodies from infection with BRSV and PI3 have been inconsistent. In one report, 2 controlled experiments were performed in which seropositive and seronegative Holstein calves were vaccinated IN at 3 – 8 days of age with a product intended for subcutaneous injection followed by challenge 21 d later or at 4.5 months of age (Ellis et al., 2010). After delayed challenge at 4.5 months, seropositive calves had wider temperature fluctuations compared to seronegative calves. However, there was no difference in maximum mean clinical score, the number of days with an elevated clinical score, the partial pressure of arterial oxygen, lung lesion score, or mortality. In that same report whereby calves were challenged 21 d after IN vaccination with a similar product containing a lower dose of BRSV antigen, seronegative vaccinated calves had less extensive lung lesions at post-mortem examination. However, no differences in clinical score or mortality were observed. Ellis (2013) demonstrated that IN vaccination between 3 – 8 days of age resulted in improved partial pressure
of oxygen (\(\text{Pa}_2\)), fewer lung lesions and lower mortality rate than unvaccinated calves following a BRSV challenge 9 weeks after vaccination, but not after 14 weeks after vaccination, suggesting that the duration of immunity to BRSV is short lived. Additionally, two doses of monovalent injectable BRSV product used intranasal completely protected calves from clinical disease. Calves receiving just one dose had minimal signs of respiratory disease, whereas all control calves required euthanasia due to severe respiratory disease (Ellis et al., 2007). These studies used either laboratory designed attenuated virus (Woolums et al., 2004) or commercially available products designed for injection (Ellis et al., 2007, 2010; Vangeel et al., 2007). Only one report studied a commercially available product designed for intranasal use (Ellis et al., 2013). None of these evaluated vaccine efficacy in a randomized clinical trial.

1.6 IMPLICATIONS OF BRD

In the United States, 22% of all pre-weaned calf deaths are the result of BRD (USDA, 2010) with case fatality rates ranging from \(\sim 2 \text{ – } 9\%\) (van Donkersgoed et al., 1993; Sivula et al., 1996); such mortality has not improved over the last twenty years despite advances in preventative and therapeutic strategies (Gorden and Plummer, 2010). Although the short terms effects of BRD, such as death and increased drug use, are felt acutely by the dairy producer and possibly the herd veterinarian, unrealized delayed effects likely end up costing more in the long run.

In an older study, BRD was associated with a 66 g reduction in ADG during the first month of life. Each additional week of BRD in the 3rd month reduced ADG by 14g and reduced total BW gain by 3.8kg (Virtala et al., 1996). This same study showed that respiratory disease that is subclinical to the caretaker, but clinical to the veterinarian, did not affect BW gain, suggesting the subclinical BRD in this case may not be of importance. The authors admit that
sample size limitations may have reduced the power of the study. Considering that the definition for veterinarian diagnosed BRD in this study was based the presence of fever, cough, depression, abnormal lung sounds on auscultation, and lack of any other body system abnormalities, it is possible that no effect was seen because of the low sensitivity of clinical diagnosis (White et al., 2009). In a recent report, calves diagnosed with BRD based on respiratory scoring (McGuirk, 2008) did not gain weight differently from normal scoring calves (Heins et al., 2014). The authors commented that the high prevalence of clinical disease and therefore a potentially high level of subclinical disease may have contributed to the inability to establish a difference between BRD positive and negative calves. In contrast, many studies have documented reductions in BW associated with BRD. In a study on 144 calves, there was a tendency for lower body weight (BW) and average daily gain (ADG) in calves that experienced 3 or more cases of BRD (Bach et al., 2011a). Additionally, Stanton et al., 2010 demonstrated an approximately 8 kg difference in BW in those calves developing BRD between weaning and 14 w of age. Additionally, in a subset of calves from the previous study, calves affected with BRD during the 60 days post-weaning had significantly lower ADG between 2 and 9 months of age compared to calves that remained healthy during that same time period (Stanton et al., 2012). Although little work has been done to define and measure subclinical BRD in dairy calves, the association between greater body weight gains after implementation of metaphylaxis suggests the presence of subclinical disease in this population of animals (Stanton et al., 2012).

In a data set of nearly 8,000 dairy animals that reached first lactation, several negative, long-term effects of BRD were documented (Bach, 2011a). In this study, calves experiencing 4 or more bouts of BRD before first calving had greater odds of not completing their first lactation as compared to calves that did not experience BRD (Bach, 2011a). This finding was supported
by survival analysis which showed a significant negative effect on survivorship after experiencing 4 cases of BRD and a tendency for decreased survivorship in those animals suffering from 1 – 3 cases of BRD. Lastly, a significant delay in age at first calving was identified in calves experiencing 4 cases of BRD, although the clinical impact of this delay is unknown as it was only an 8 day difference. It is not clear from the study what constituted one case of BRD but one can infer that relapses of BRD might have a negative impact on the future productive life of the dairy calf. In a survey of 25 New York State herds, producer diagnosed BRD within the first 90 days of life was not associated with reduced first lactation milk production, but those affected were less likely to enter the milking string (Warnick et al., 1995).

The short term costs associated with managing BRD are approximately $10 to $16 per calf (as reviewed by Gorden and Plummer, 2010). Long term effects shall increase such estimates as a result of reductions in post-weaning growth rates, longevity and future production. Early diagnosis and treatment is needed to reduce treatment failures and the subsequent negative outcomes and economic impacts associated with chronic disease (McGuirk, 2008).

1.7 CONCLUSIONS AND THESIS OBJECTIVES

In conclusion, there is evidence that the incidence of BRD in dairy calves has not improved over the last few decades and long term consequences of this disease are becoming clearer. Our ability to promptly and accurately diagnose this disease is often undermined by subtle and inconsistent clinical signs. As such, methods of diagnosing lung lesions and a better understanding of the effect of subclinical BRD should be a priority for dairy researchers and might serve to improve the welfare of dairy calves and the businesses that foster their upbringing. Therefore, the purpose of this thesis was to evaluate the ability of a clinician using readily available, portable thoracic US to diagnose the lung lesions associated with BRD in dairy
calves. In addition to testing diagnostic accuracy of this tool, a new commercially available intranasal vaccination was studied, as well as the effect of BRD on calf behavior.

Specifically, the research objectives were:

1) Evaluate BALF characteristics and develop a neutrophil proportion cut-point for determining sensitivity (Se) and specificity (Sp) of BALF and determine the Se and Sp of portable US for detecting subclinical BRD

2) Describe the progression of US lung consolidation after experimental infection with *Mannheimia haemolytica* and correlate the presence and size of the US lesions with post-mortem examination

3) Evaluate the effect of a new commercially available intranasal vaccine on health, US lung consolidation, and growth

4) Determine the effect of naturally occurring BRD on lying behavior

### 1.8 REFERENCES


### 1.9 TABLES

Table 2.1 Summary of world-wide BRD surveys in dairy calves since 1980.

<table>
<thead>
<tr>
<th>Primary author</th>
<th>Year</th>
<th>Location</th>
<th>#herds</th>
<th>#calves</th>
<th>Examiner</th>
<th>%BRD</th>
<th>Study period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waltner-Toews</td>
<td>1986</td>
<td>Ontario</td>
<td>104</td>
<td>2000</td>
<td>Producer NS</td>
<td>15</td>
<td>pre-weaning</td>
</tr>
<tr>
<td>Curtis</td>
<td>1988</td>
<td>New York</td>
<td>26</td>
<td>1200</td>
<td>Producer</td>
<td>7.4</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>van Donkersgoed</td>
<td>1993</td>
<td>Saskatchewan</td>
<td>17</td>
<td>325</td>
<td>Vet/Producer</td>
<td>29 / 39</td>
<td>&lt; 6 months</td>
</tr>
<tr>
<td>Virtala</td>
<td>1996</td>
<td>New York</td>
<td>18</td>
<td>410</td>
<td>Veterinarian</td>
<td>25</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>Sivula</td>
<td>1996</td>
<td>Minnesota</td>
<td>30</td>
<td>845</td>
<td>Producer NS</td>
<td>8</td>
<td>&lt; 4 months</td>
</tr>
<tr>
<td>Lundborg</td>
<td>2005</td>
<td>Sweden</td>
<td>122</td>
<td>3000</td>
<td>Producer</td>
<td>7</td>
<td>&lt; 3 months</td>
</tr>
<tr>
<td>Lago</td>
<td>2006</td>
<td>Wisconsin</td>
<td>13</td>
<td>225</td>
<td>Veterinarian</td>
<td>14</td>
<td>pre-weaning</td>
</tr>
<tr>
<td>Gulliksen</td>
<td>2009</td>
<td>Norway</td>
<td>135</td>
<td>5100</td>
<td>Producer</td>
<td>2.5</td>
<td>pre-weaning</td>
</tr>
<tr>
<td>USDA</td>
<td>2010</td>
<td>United States</td>
<td>2000</td>
<td>--</td>
<td>Producer</td>
<td>12</td>
<td>pre-weaning</td>
</tr>
<tr>
<td>Windeyer</td>
<td>2012</td>
<td>Ontario &amp; MN</td>
<td>19</td>
<td>2800</td>
<td>Producer</td>
<td>22</td>
<td>&lt; 3 months</td>
</tr>
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</table>
Table 2.2 Definitions of bovine respiratory disease from various scientific studies. Studies not replicated from Table 1.1 indicate failure to report a disease definition.

<table>
<thead>
<tr>
<th>Primary author</th>
<th>Year</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curtis</td>
<td>1988</td>
<td>cough, runny eyes or nose, trouble breathing</td>
</tr>
<tr>
<td>van Donkersgoed</td>
<td>1993</td>
<td>depression plus any two of the following: cough, T &gt; 39.5°C, RR &gt; 40 bpm, increased anterioventral lung sounds or wheezes</td>
</tr>
<tr>
<td>Virtala</td>
<td>1996</td>
<td>abnormal signs associated with the respiratory tract including: inducible cough, abnormal auscultation, T &gt; 39.5°C, depression, lack of other body system involvement</td>
</tr>
<tr>
<td>Lundborg</td>
<td>2005</td>
<td>coughing or sneezing for more than 2 days, or severely increased respiratory sounds on auscultation, or moderately increased respiratory sounds with coughing or nasal discharge</td>
</tr>
<tr>
<td>Lago</td>
<td>2006</td>
<td>respiratory score* ≥ 6</td>
</tr>
<tr>
<td>Gulliksen</td>
<td>2009</td>
<td>coughing or sneezing with heavy breathing or nasal discharge for at least 2 days</td>
</tr>
<tr>
<td>Stanton</td>
<td>2010</td>
<td>dull, listless with elevated RR and/or nasal discharge, T &gt; 39.5°C</td>
</tr>
<tr>
<td>Windeyer</td>
<td>2012</td>
<td>increased RR, sound, or effort and T &gt; 39.5°C with either coughing, nasal discharge, depression, decreased appetite, or rough haircoat</td>
</tr>
<tr>
<td>Heins</td>
<td>2014</td>
<td>respiratory score* &gt; 4</td>
</tr>
</tbody>
</table>

*Appendix 1 - University of Wisconsin Calf Scoring Chart
CHAPTER 2. THORACIC ULTRASONOGRAPHY AND BRONCHOALVEOLAR LAVAGE FLUID ANALYSIS IN HOLSTEIN CALVES AFFECTED WITH SUBCLINICAL LUNG LESIONS

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From the Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada (Ollivett, Kelton, Duffield, Leslie); the Department of Population Medicine and Diagnostic Sciences, Cornell University College of Veterinary Medicine, Ithaca, NY (Nydam); the Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada (Hewson); and the Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada (Caswell).

Running head: Subclinical lung lesions in dairy calves

Key words: Dairy calf; Subclinical pneumonia; Ultrasound; Bronchoalveolar lavage

Abbreviations:

BAL bronchoalveolar lavage

BALF bronchoalveolar lavage fluid

RS respiratory score

TNCC total nucleated cell count

US thoracic ultrasonography
Corresponding author: T. Ollivett, Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1; e-mail: tollivet@uoguelph.ca

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The authors have no conflicts of interest to declare.
2.1 ABSTRACT

**Background:** Ultrasonography (US) and bronchoalveolar lavage fluid (BALF) analysis are ante-mortem methods of identifying the lung lesions associated with bovine respiratory disease (BRD). The accuracy of US and the cell distributions in BALF have not been characterized in calves affected by subclinical BRD (sBRD).

**Objectives:** To evaluate the accuracy of US and BALF, and describe BALF characteristics in calves affected with sBRD.

**Animals:** 25 Holstein bull calves, 1 – 12 weeks old.

**Methods:** Calves with low respiratory scores underwent US and BALF collection followed by post-mortem exam (normal US, n = 5; comet-tails, n = 5; consolidation, n = 15). Bronchoalveolar lavage fluid was collected and analyzed for total and differential cell counts. Lung lesions were assessed with gross and histopathologic examination. Data were described using non-parametric methods and univariable logistic regression, and the accuracy of US, BALF were calculated.

**Results:** The sensitivity and specificity of US in detecting subclinical lung lesions was 94% (95% CI: 69 - 100%) and 100% (95% CI: 64 - 100%), respectively, compared to post-mortem examination. A cut-point of ≥ 4% BALF neutrophils was associated with the highest BALF sensitivity and specificity, 81% (95% CI: 56 – 94%) and 75% (95% CI: 36 – 95%). The presence of US consolidation increased the odds of having a BALF neutrophil proportion ≥ 4% (OR = 23; 95% CI: 2.6 – 198; P < 0.01).
Conclusions and clinical importance: Ultrasonography accurately detects the lung lesions associated with sBRD in young calves. Clinicians should consider using a cut-point of ≥ 4% BALF neutrophils to diagnose sBRD when US is not available.

2.2 INTRODUCTION

Clinical bovine respiratory disease (BRD) is a common cause of morbidity and mortality in young dairy heifers. Within-herd prevalence is highly variable, ranging from 0 – 90% of calves affected. This within-herd variation could result from differences in disease frequency, recording method, or scoring system. Scoring systems are used in clinical and research settings to classify calves as either healthy or sick for the purposes of treatment or analysis. The constituents of scoring systems vary, but in general they are based on the combined severity of several clinical signs associated with respiratory disease. Pitfalls of scoring systems include the subjective nature of ranking severity of clinical signs as well as the inherent failure to identify calves with subclinical bovine respiratory disease (sBRD).

The association between post-mortem lung lesions and decreased average daily gain in the absence of clinical signs is evidence of sBRD in beef and veal calves. Others have documented increased neutrophil proportions or pathogens in BALF from clinically normal calves. Although little work has been done to define and measure sBRD in dairy calves, the association between greater body weight gains after implementation of metaphylaxis suggests the presence of sBRD in this population of animals as well. Accurate ante-mortem methods of detecting sBRD through identification of lung lesions or detection of low-grade lung inflammation should allow better classification of disease status in both individual animals and herds.
Direct methods of identifying lung inflammation have relied on sampling the airways via trans-tracheal aspiration or bronchoalveolar lavage (BAL). Both methods are inherently invasive, and are seldom used in the farm setting. Also, cut points for differential cell counts have not been defined for sBRD. Indirect methods of detecting lung inflammation involve identifying the downstream effects of the inflammatory cascade, such as fever and acute phase proteins. Elevated serum haptoglobin and serum amyloid A have been documented in clinical cases of BRD when compared to healthy controls, however the presence of fever (T > 39.5°C) was included in the definition and therefore does not reflect the level of haptoglobin in afebrile or subclinical cases of BRD. Additionally, these indirect tests might suffer from a lack of specificity as other disease processes can cause fever and elevated serum haptoglobin or amyloid A resulting in an excessive false positive rate.

Recently, interest in using thoracic ultrasonography (US) to diagnose the lung lesions associated with sBRD in dairy calves has grown. Bacterial and occasionally viral respiratory diseases result in the creation of non-aerated superficial lung lobules. This change in lung character alters the US image from that of a strong reflector with reverberation artifact to a homogenous hypoechoic structure similar to that of liver making it possible to reach an ultrasonographic diagnosis of lung lesions regardless of the clinical state of the animal. The accuracy of US has been documented in clinical cases of bronchopneumonia; however, few of these reports were prospective in nature or case-controlled and none examined sBRD.

In order to improve ante mortem diagnosis of sBRD in dairy calves, the primary objective of this study was to determine the accuracy of portable US to detect sBRD in apparently healthy calves. The secondary objective was to evaluate BALF characteristics and
develop a neutrophil proportion cut-point for determining sensitivity (Se) and specificity (Sp) for detecting sBRD.

2.3 MATERIALS AND METHODS

General

This prospective study was completed between January 1, 2012 and December 15, 2012 at the Elora and Ponsonby Dairy Research Centres of the University of Guelph in south-western Ontario, Canada. Sixty-two 3–6 d old Holstein bull calves were enrolled into a separate study evaluating the effect of an intranasal respiratory vaccine over a 12 week follow-up period. Calves were raised in either individual stalls within an enclosed room with an active ventilation system, or outside tethered to individual plastic hutches. Calves were fed 6 L of whole unpasteurized milk per day until approximately 6 weeks of age, at which point they were gradually weaned and moved to group housing by 8 weeks of age. Free choice water and calf starter were available beginning at 3 days of age. Calves were monitored and treated as needed during each feeding by farm personnel for signs of acute illness including depression and inappetance. The Animal Care Committee of the University of Guelph approved this study (AUP #11R110).

Respiratory Scoring and Thoracic Ultrasonography

As part of the other study, one author (TO) and a research technician visited the research centres twice weekly to determine respiratory scores according to a standardized respiratory scoring (RS) system$^4$ (Appendix 1). Points were assigned to rectal temperature, nasal discharge, cough, and ocular discharge or ear position. Respiratory scores ranged from 0 to 12. Calves with
RS > 4 were considered sick and were excluded from inclusion in the current intensive study, which focused on evaluation of apparently healthy calves (McGuirk, 2008).

Once per week, immediately following the RS, US of each hemithorax was performed by TO. A portable variable frequency linear rectal ultrasound was used, set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 16 dB (Near 13 dB; Far 36 dB). Approximately 300 mL of 70% isopropyl alcohol was applied to the hair as the transducing agent. The hair of the thorax was not shaved or clipped. Systematic scanning started at the level of the epaxial muscles in the right/left 10th intercostal space (ICS). Within each ICS, the probe was positioned parallel to the ribs and moved ventrally towards the sternum until specified ultrasonographic landmarks were visualized (Tables 2.1 and 2.2). The probe was moved cranially to examine each ICS up to the right 1st or left 2nd ICS (Figure 2.1). The scapula marked the dorsal margin of the exam cranial to the 7th ICS bilaterally. The lung adjacent to the right 4th through 1st ICS and left 4th through 2nd ICS was examined with the US transducer between the forelimb and the cranial ventral thoracic body wall. Peripheral lung tissue was considered normal when a hyperechoic line with reverberation artifact was present signifying the interface between the high impedance tissue of the thorax and the low impedance tissue of the lung. Comet-tailing artifact was noted when a vertical hyperechoic line emanated from the pleural surface. Consolidated lung appeared hypoechoic and lacked both the bright white band at the pleural interface and reverberation artifact. Ultrasonographic lung abnormalities were documented according to location and ventral-dorsal distance. For the purpose of this study, the goal was to obtain a convenience sample of 5 calves for each category of a 5-point US lesion score (LS) based on the presence and size of the US lung lesions for a total of 25 calves. Criteria for each category are as follows: LS0 - no evidence of comet-tail artifact or consolidation (n = 4); LS1: only comet-tail artifact and no
consolidation (n = 5); LS2: < 1 cm consolidation (n = 5); LS3: 1 – 3 cm of consolidation (n = 5); LS4: > 3 cm consolidation (n = 5). All observations were spoken and digitally recorded.

Animal Selection for Intensive Study

Selection criteria for this intensive study included: RS < 5, previously normal US, and no antimicrobial treatment within the prior two weeks or since birth if they were less than 2 weeks of age. Approximately once a week, 1 – 2 calves that met the above criteria were selected for BALF collection, euthanasia, and post-mortem examination until all 25 calves were obtained.

Collection and Analysis of Bronchoalveolar Lavage Fluid.

Calves selected for BALF collection were sedated with xylazine (0.05 mg/kg IV) and butorphanol (0.1 mg/kg IV) and restrained in sternal recumbency. The external naris was cleaned with gauze and a 9 mm (outer diameter), 1.5 m flexible fibreoptic bronchoscope (E-22VGS99x17 Veterinary Fiberscope, LSVP International, Los Altos, CA) previously sterilized with a 2% glutaraldehyde solution, was introduced into the ventral nasal meatus of the clean naris. The bronchoscope was then passed through the nasopharynx into the trachea, extending into a distal airway within the lung containing the ultrasonographic evidence of consolidation or, if normal, the right lung. Once wedged into the bronchus, two aliquots of 120 mL sterile saline were sequentially dispensed into and retrieved from the airway via the biopsy channel. Once 50% of the aliquot was retrieved, the scope was removed. Diagnostic samples were taken from the last aliquot retrieved, placed in Ca-EDTA commercial blood collection tubes, and held in an ice water bath until submission to the Animal Health Laboratory at the University of Guelph. Samples were evaluated within 4 h of collection. Wright-stained sediment and cytocentrifuge preparations of BALF were evaluated to determine the 200 cell differential cell count.
Automated methods were used to determine total nucleated cell count (TNCC, Coulter ZBI, Hialeah, Florida).

**Euthanasia and Post-mortem Examination.**

Immediately following BALF collection, while still sedated and in sternal recumbency, each calf was euthanized via captive bolt and exsanguination. A respiratory system-based post-mortem examination was performed immediately after euthanasia by one author (TO). During the post-mortem examination, the carcass was in lateral recumbency with the affected lung up. The skin, subcutaneous tissues, and rib caudal to the lesion were reflected dorsally to confirm location of the lesion with respect to external landmarks as identified by prior ultrasonographic examination (Figure 2.2). Once confirmed, all of the ribs were cut at the costochondral junction and reflected dorsally to expose the whole affected lung. The heart and lung were removed *en bloc*, placed on ice, and transferred back to the University pathology suite for further evaluation. Once in the pathology suite, all lung lesions were recorded on a standard diagram after thorough palpation of the tissues to confirm consolidation and identify any pulmonary changes within the parenchyma. Tissue samples for histopathology were taken from the border zone between normal and consolidated lung. Additional histopathology samples were taken from taken from the right and left cranial lobes, the right middle lung lobe, and the right and left caudal lobes in unaffected portions of affected lungs. In unaffected lungs, histopathology samples were taken from the right and left cranial lobes, the right middle lung lobe, and the right and left caudal lobes. Routine hematoxylin and eosin stains were made of prepared histopathology sections after at least 24 hours of fixation within 10 % buffered formalin.
Statistical Analyses

Sensitivity (Se) and specificity (Sp) were estimated for US diagnosis of consolidation coded as a dichotomous result (yes = presence of dark red, firm lung tissue; no = absence of dark red, firm lung tissue), using post-mortem examination as the Gold Standard. Descriptive statistics were compiled for BALF parameters including total nucleated cell count (TNCC), and differential cell counts including neutrophil proportion, macrophage proportion and lymphocyte proportion. In addition to describing each US category individually, all calves with US consolidation (LS 2 – 4) were combined into one group (“Consolidated,” n = 15) and compared to completely normal (LS 0) calves. These BALF were described using medians (interquartile ranges) and were compared using Wilcoxon rank sum test (Kaleidograph 4.1.1, Synergy Software, Reading, PA). A receiver operator characteristic (ROC) curve was developed to determine which BALF neutrophil proportion provided the highest Se and Sp for predicting lung consolidation using post-mortem examination as the Gold Standard (Stata 12.1, Stata Corp LP. College Station, TX). Fisher’s exact test was used to compare the Se and Sp of US and BALF. A standard statistical package was used to fit the univariable logistic regression model (outcome = BALF NP ≥ 4%; predictor = US lung consolidation, LS 2 – 4) (Stata 12.1, Stata Corp LP. College Station, TX). Alpha was P < 0.05 for all comparisons. Histopathological lungs lesions were categorized as either normal, bacterial (ie- the presence of neutrophils within the bronchial, bronchiolar, or alveolar compartments), or viral (ie- the presence of mononuclear peribronchial or peribronchiolar infiltrates, with or without epithelial necrosis and lobular atelectasis).

2.4 RESULTS

Age, RS, US findings, and BALF results for each US category are summarized in Table 2.3. One BALF sample was damaged in transport, resulting in 24 calves for analysis (LS0, n = 4;
LS1, n = 5; LS2, n = 5; LS3, n = 5, LS4, n = 5). The Se and Sp of US in detecting sBRD was 94% (95% CI: 69 – 100%) and 100% (95% CI: 64 – 100%), respectively. Ultrasonographic consolidation was associated with firm, red lesions on gross examination in all cases (n = 15). Ultrasound examination failed to detect a 1 cm area of atypical consolidation located in the dorsomedial aspect of the right lung of one calf, however, severe diffuse comet-tailing artifacts were observed within the right 5th ICS of this particular calf. Histopathology results are summarized in Figure 2.4. Bronchoalveolar lavage fluid total nucleated cell counts (TNCC) were not different (P = 0.65) between Consolidated (LS2 – 4: 0.52 x 10^9 cells/L; n = 15) and completely normal lungs (LS0: 0.59 x 10^9 cells/L; n = 5). Neutrophil proportion was greater in Consolidated (LS2 – 4: 14%) versus completely normal lungs (LS0: 1.25%; P = 0.005). Neutrophil proportion in calves with comet-tailing was less than that of Consolidated lungs (2% vs 14%, respectively; P = 0.05) but not different from completely normal lungs (2% vs. 1.5%, respectively; P = 0.22). BALF macrophage proportions were less in Consolidated versus completely normal lungs (79% vs. 97%, respectively; P = 0.006). Macrophage proportion in calves with comet-tailing was not different from that of completely normal lungs (91% vs. 97%, respectively; P = 0.06) or from Consolidated lungs (91% vs. 79%, respectively; P = 0.24).

The highest BALF Se (81%, 95% CI: 56 – 94%) and Sp (75%, 95% CI: 36 – 95%) was associated with a cut-off of ≥ 4% neutrophils based on receiver operator characteristics (AUC = 0.85, 95% CI: 0.67 – 1.0; Figure 2.5) and were not significantly different from the Se (P = 0.60) and Sp (P = 0.47) of US. The presence of ultrasonographic consolidation increased the odds of having a neutrophil proportion ≥ 4% (OR = 23; 95% CI: 2.6 – 198; P < 0.01). No significant difference in sensitivity (P = 0.47) and specificity (P = 0.60) were detected between US and BALF using a cut-off of ≥ 4% neutrophils for diagnosing sBRD.
2.5 DISCUSSION

To the authors’ knowledge, this is the first study to evaluate US and BALF characteristics in dairy calves affected with sBRD; finding that identification of lung consolidation by US accurately predicted the presence of gross and histopathologic lung lesions and was associated with increased BALF neutrophils in affected calves.

The Se and Sp of ultrasound were highly acceptable in this study, generally agreeing with a previous report.\textsuperscript{16} Previous research has supported the general accuracy of US;\textsuperscript{16,18,19} however, direct comparisons between studies should be made with caution considering the differences in technique, equipment, and study design. Previous reports did not extend their examinations cranially beyond the 3\textsuperscript{rd} ICS.\textsuperscript{16-19,21-23} Considering that the most common form of BRD, bronchopneumonia, typically starts in the cranial aspect of the cranial lobe or right middle lung lobe,\textsuperscript{17,24,25} 4 out of 5 of the most severely affected calves in the current study would have been misclassified as normal had these techniques been implemented, thereby lowering the Se. Additionally, although previous reports often used linear probes, none reported using a linear rectal transducer. The streamlined design of the rectal transducer is conducive to a more thorough examination of the cranial thorax as compared to a traditional probe whereby the handle and cord extend out of the midsection of the probe instead of the end. The shape of the linear transducer allows it to slide between the forelimb musculature and the cranial thoracic body wall for evaluation of the cranial aspect of the cranial lobes, which was previously thought unreachable during ultrasonographic examination.\textsuperscript{17}

Using US, one case of atypical consolidation located in the dorsomedial aspect of the right lung was not detected. Severe diffuse comet-tailing artifacts were observed within the right 5\textsuperscript{th} ICS of this particular calf; however, since the consolidation was surrounded by aerated lung,
it could not be visualized. Another study\textsuperscript{16} observed a similar situation in which a 10 cm pulmonary abscess in the caudal lung was obscured by aerated lung. One case of pneumothorax and one case of interstitial pneumonia were also discussed as reasons for the imperfect sensitivity in that study.\textsuperscript{16}

A point to consider in the current study is that one author (TO) performed both the US and post-mortem examination. This decision to forego blinding was made \textit{a priori} based on the need for the individual conducting the post-mortem examination to know where to focus the examination of the lung in order to assess the accuracy of the ultrasound in localizing the lesion(s). Future studies incorporating blinded individuals would provide more information regarding test characteristics of ultrasound in these different settings. Furthermore, the Se and Sp estimated in the current study are applicable to dairy calves less than 12 weeks of age. As calves mature, the forelimb and thoracic musculature strengthens, limiting access to the first and second ICS. The Se of US in older animals might therefore be lower, due to inability to completely examine the cranial aspect of the cranial lung lobes.

As an alternate method of diagnosing respiratory disease, analysis of bronchoalveolar lavage fluid (BALF) detects pulmonary inflammation associated with BRD and occasionally has been used to control for the potentially confounding effect of sBRD.\textsuperscript{8} Suggested cut-points for the proportion of BALF neutrophils defining BRD are variable, ranging from 10 – 40%.\textsuperscript{4,8} Normal calves in the current study had a neutrophil proportion similar to those shown in another study\textsuperscript{26} where normal lungs were sampled at necropsy. Previously documented neutrophil proportions\textsuperscript{8} were also similar to the calves of the current study, likely because 18 of 30 clinically normal calves were excluded from the study\textsuperscript{8} due to elevated BALF neutrophil proportions prior to calculating a reference range for normal calves. Others have reported much
higher BALF neutrophil proportions in clinically normal control animals,\textsuperscript{9,10} likely reflecting the presence of sBRD in the control population. The current study was not able to distinguish a statistically significant difference in Se and Sp between BALF and US due to lack of study power, but results do suggest that BALF from truly normal calves has very few neutrophils and perhaps clinicians should consider using a lower cut-off such as 4\% when classifying respiratory disease status.

In the current study, calves with US consolidation had greater neutrophil proportions and were also significantly older than those without consolidation. The effect of age on differential cell counts in BALF has not been well characterized in dairy calves. One study did find a significant decrease in macrophage proportion and a slight numerical increase in neutrophil proportion between 2 and 4 weeks of age in Holstein calves,\textsuperscript{8} however, the increase in neutrophil proportion was minor (approximately 1 – 2\%) compared with the increase in neutrophil proportion seen in the calves of the current study. Additionally, another study in slightly older calves showed similar neutrophil proportion as the current study calves.\textsuperscript{27} The age-related changes seen in the current study likely reflect an interaction between exposure time and onset of respiratory infection rather than an inherent change specific to the lungs of the dairy calf. However, calf selection was not randomized in the current study; therefore any inferences about the effect of age and BALF characteristics at the population level should be avoided.

2.6 CONCLUSIONS

Thoracic US, when used as described in this study, can be used to provide an objective assessment of lung health and improve classification between normal calves and those affected with sBRD. While BALF total nucleated cell count should not be used to classify disease status, reasonable success can also be had when using a neutrophil proportion cut-off of $\geq 4\%$ in BALF
to diagnose sBRD if ultrasonography is unavailable. A better understanding of the effect of sBRD on future dairy calf health and performance is lacking, therefore, herd level and animal level implications of sBRD warrant further investigation in consideration of its high prevalence in dairy calves. Based on our results, it is apparent that both thoracic US and BALF using a cut-off of ≥ 4% neutrophils would present options for documenting sBRD ante mortem during such investigations.

ACKNOWLEDGMENTS

Financial support for this study was provided by the Ontario Ministry of Agriculture, Food, and Rural Affairs and Zoetis. Special thanks go to the dedicated research technicians: Jolene Cyples, Jessica Cyples, Melissa Wagner, Vivianne Bielmann, Sam Deelen, Brittany Stinson, Patrick Chung, and Sarah Stanger-Guy for their tireless work; and especially the Elora and Ponsonby Dairy Research Centre and Animal Health Laboratory staff.

2.7 REFERENCES


### 2.8 TABLES & FIGURES

Table 2.1 Landmarks for the lobes of the right lung during ultrasonographic examination.

<table>
<thead>
<tr>
<th>Lung lobe</th>
<th>Caudal</th>
<th>Middle</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>R – ICS:</td>
<td>6 – 10</td>
<td>5</td>
<td>3 – 4</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Ventral landmark(s):</td>
<td>Diaphragm</td>
<td>CCJ &amp; pleural deviation</td>
<td>Heart</td>
<td>Two blood vessels</td>
</tr>
</tbody>
</table>

R – ICS, right intercostal space(s)
CCJ, costochondral junction.
Table 2.2 Landmarks for the lobes of the left lung during ultrasonographic examination.

<table>
<thead>
<tr>
<th>Lung lobe</th>
<th>Caudal</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>L – ICS:</td>
<td>6 – 10</td>
<td>4 – 5</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Ventral landmark(s):</td>
<td>Diaphragm</td>
<td>CCJ &amp; pleural deviation</td>
<td>Heart</td>
</tr>
</tbody>
</table>

L – ICS, left intercostal space(s)
CCJ, costochondral junction.
Table 2.3 Comparison of respiratory score (RS) and bronchoalveolar lavage fluid (BALF) findings grouped by ultrasonographic (US) lesion score (LS) in Holstein dairy calves. All values represent medians (interquartile ranges). TNCC = total nucleated cell count

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal (LS 0, n = 5)</th>
<th>Comet-Tails (LS 1, n = 5)</th>
<th>Varying levels of US consolidation</th>
<th>All US consolidation (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (d)</td>
<td>28 (25 – 41)</td>
<td>32 (25 – 65)</td>
<td>68 (54 – 76)</td>
<td>65 (52 – 74)</td>
</tr>
<tr>
<td>RS</td>
<td>2 (2 – 2)</td>
<td>2 (2 – 3)</td>
<td>2 (2 – 4)</td>
<td>2 (2 – 4)</td>
</tr>
<tr>
<td>US lesion (cm)</td>
<td>0</td>
<td>0</td>
<td>0.5 (0.25 – 0.50)</td>
<td>1.5 (0.63 – 4)</td>
</tr>
<tr>
<td>TNCC (x10^9 cells/L)</td>
<td>0.59 (0.45 – 0.80)</td>
<td>0.34 (0.30 – 0.90)</td>
<td>0.51 (0.25 – 0.73)</td>
<td>0.52 (0.29 – 0.73)</td>
</tr>
<tr>
<td>Neut (%)</td>
<td>1.25 (0.75 – 1.88)^a</td>
<td>2 (2 – 11)^a</td>
<td>25 (9 – 28)^b</td>
<td>17 (12 – 20)^b</td>
</tr>
<tr>
<td>Mac (%)</td>
<td>97 (96 – 99)^a</td>
<td>91 (78 – 93)^a</td>
<td>69 (67 – 79)^b</td>
<td>79 (74 – 85)^b</td>
</tr>
<tr>
<td>Lymph (%)</td>
<td>1 (0.75 – 1.75)^a</td>
<td>7 (5 – 8)^a</td>
<td>6 (3 – 11)^a</td>
<td>4 (3 – 8)^b</td>
</tr>
</tbody>
</table>

Different superscript within a row imply a difference from the LS 0 group (alpha < 0.05) using Wilcoxon Rank Sum.

LS 0, completely normal ultrasonographic exam
LS 1, comet-tailing artifacts on ultrasonographic exam
LS 2, less than 1 cm consolidation on ultrasonographic exam
LS 3, 1 – 3 cm consolidation on ultrasonographic exam
LS 4, greater than 3 cm consolidation on ultrasonographic exam
Neut, proportion of neutrophils in BALF
Mac, proportion of macrophages in BALF
Lymph, proportion of lymphocytes in BALF
Figure 2.1 Right lung in situ. The lung is outlined in white. Ribs are labelled by number. Cranial aspect of the right cranial lobe is situated in the 1st and 2nd intercostal spaces.
Figure 2.2 Right lung with lobar consolidation (LS 4) of the cranial aspect of the cranial lobe. A) *In situ* specimen. Carcass is in left lateral recumbency. The right 2\textsuperscript{nd} rib (white star) is reflected dorsally exposing the combined 1\textsuperscript{st} and 2\textsuperscript{nd} intercostal space, revealing the cranial aspect of the right cranial lobe. B) The entire right lung removed from the carcass, showing consolidation of the cranial aspect of the cranial lobe (white star).
Figure 2.3 Ultrasonographic image of consolidated lung from Figure 2, taken in the right 1st ICS. Within the image, left = dorsal, right = ventral, top = superficial, bottom = deep. The two blood vessels are the internal thoracic artery and vein.
Figure 2.4 Histopathology results from 24 Holstein bull calves with varying degrees of ultrasonographic lung abnormalities documented within 4 hours of euthanasia and post-mortem examination. Bacterial lesions consist of neutrophilic bronchitis, bronchiolitis, and bronchopneumonia. Viral lesions consist of mononuclear bronchial and/or bronchiolar cuffing, bronchiolar necrosis, and lobular atelectasis.
Figure 2.5 Receiver operator characteristic curve for bronchoalveolar lavage fluid neutrophil proportion from Holstein dairy calves (n = 24). Curve demonstrates that a cut-off of 4% provides the highest possible sensitivity and specificity in identifying subclinical lung lesions.
CHAPTER 3. ULTRASONOGRAPHIC CHARACTERIZATION OF LUNG CONSOLIDATION AFTER EXPERIMENTAL INFECTION WITH MANNEHEIMIA HAEMOLYTICA IN HOLSTEIN BULL CALVES

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Theresa L. Ollivett, DVM, DACVIM; Joanne Hewson, DVM, PhD, ACVIM; Roland Schubotz, DVM; Jeff Caswell, DVM, PhD, ACVP

From the Departments of Population Medicine (Ollivett), Clinical Sciences (Hewson), Health Sciences Centre (Schubotz) and Pathobiology (Caswell), Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1.

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Address correspondence to Dr. Theresa Ollivett at tollivet@uoguelph.ca
3.1 ABSTRACT

Objective - Evaluate the progression of lung consolidation after experimental infection with *Mannheimia haemolytica* in dairy calves using portable ultrasonography (US)

Design – Controlled experimental study

Animals - 11 Holstein bull calves, 4 months old

Procedures - Sterile saline (Control; n = 5) or a suspension containing $10^9$ cfu/mL *M. haemolytica* (Challenged; n = 6) was endoscopically delivered into the trachea. Animals were assessed by respiratory score (RS) and portable thoracic ultrasonographic (US) examinations at time points t = -1, 2, 6, 12, 24, 48, 72, 96 and 120 h after bacterial challenge. Animals were euthanized and post-mortem examinations (PM) were performed at t = 120 h. One Challenged calf did not develop bronchopneumonia and was removed from analysis. Data were analyzed by non-parametric methods and correlation analysis.

Results - New consolidation developed 2 h post-challenge in 1 of 5 Control and 5 of 5 Infected calves ($P = 0.05$). The percentage of consolidated lung was greater in Infected calves after challenge ($P = 0.01$) at each time point except t = 6 ($P = 0.07$). Severe consolidation developed by 6 h (median; IQR 6 - 6). Maximum %US was 11.3% (median; IQR 9.9 – 14.3) which developed by 48 h (median; IQR 48 – 96). At t = 120 h, %US ranged from 5% - 46% (median %US = 7.3%; IQR = 5.9 – 9.9%) in the Infected calves. Ultrasonographic lung consolidation was more persistent than fever or a sick RS. The %US and %PM were highly correlated ($\rho = 0.92; P = 0.0002$).

Conclusions and Clinical Relevance – Ultrasonographically visible lung consolidation occurs soon after infection, progresses rapidly, and is an accurate predictor of PM findings.
Abbreviations
ICS   intercostal space
IQR   interquartile range
RS    respiratory score
PM    post-mortem
US    ultrasonography

3.2 INTRODUCTION

Thoracic ultrasonography (US) can be used to detect non-aerated or consolidated lung and is a reliable method of confirming clinical bronchopneumonia in calves.¹,² The inter-observer agreement is moderate to almost perfect (kappa = 0.6 – 1.0) depending on the experience level of the observer.³ Only one published study has assessed lung consolidation via US after an experimental bacterial infection.² In that study, there was excellent agreement between the post-mortem examination (PM) and US distribution of lesions; however, only one post-challenge US was performed at the end of the study.

Bacterial challenge models have been used successfully to establish a moderate to severe acute bronchopneumonia in calves which have developed pathologic changes similar to the gross and microscopic lesions documented in cases of naturally occurring disease.⁴ Neutrophilic infiltrates in the bronchial, bronchiolar, and alveolar compartments of the lung are the main pathological change associated with bronchopneumonia.⁵ Such changes occur as early as 2 hours and peak at 6 hours post-challenge.⁶-⁸ The infiltrates effectively displace air from the lung tissue, resulting in the lung consolidation typical of bronchopneumonia and detectable by US.
Currently, there are no published reports indicating how soon after an infectious challenge US can be used to detect lung consolidation. This knowledge would help both practicing veterinarians and researchers better understand the timing of onset and progression of lung consolidation after a bacterial challenge. The first objective of this study was to describe the progression of ultrasonographic lung consolidation after experimental infection with *Mannheimia haemolytica*. The second objective was to associate the presence and size of the ultrasonographic consolidations with PM findings.

### 3.3 MATERIALS & METHODS

**Animals** - This study was performed at the Ontario Veterinary College (OVC) at the University of Guelph, in Guelph, Ontario. Twelve Holstein bull calves, approximately 4 months of age, were randomly assigned to either Challenged (n = 6) or Control (n = 6) groups upon arrival from the University dairy. Sample size was based on previous experiences using the same challenge model of one author (JH). All calves had negative BVDV PI status after testing with the BVD antigen-capture ELISA (Animal Health Laboratory, University of Guelph) and no history of vaccination against *Mannheimia haemolytica*. Challenged calves that developed fever, depression, and lung consolidation were defined as Infected.

**Acclimation period** – All calves were allowed to acclimate to the new environment for 7 days. Calves of the same experimental group were housed together in permanent 5 m x 5 m stalls with solid concrete walls and flooring within a closed barn. All stalls had separate ventilation systems and were cleaned and bedded daily with pine shavings. Each calf was monitored daily and excluded from the study if signs of illness, such as depression, inappetence, and coughing developed during this period. Free choice grass hay, calf starter, and water were provided daily.
Calves were cared for in compliance with the Animal Care Committee of the University of Guelph (AUP #11R110).

**Study period** – The study period was defined as the hour before challenge (baseline, t = -1 h) through until 120 h following challenge. All calves remained in the same stalls as during the acclimation period. No equipment was shared between stalls except for the US unit which was thoroughly wiped with disinfectant between stalls.

**Challenge model and procedures** - All calves that remained free from illness during the acclimation period (Control, n = 5; Challenge, n = 6) were assessed for respiratory score (RS) at t = -1 h. The RS included an assessment of body temperature, nasal and ocular discharge, ear position, and cough⁹ (Appendix 1). A RS ≥ 5 was used to indicate a sick calf. Following RS, both hemithoraces were scanned using a portable linear rectal transducer set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 16 dB (Near 13 dB; Far 36 dB; Ibex Pro, E. I. Medical). Approximately 300 mL of 70% isopropyl alcohol was applied to the hair as the transducing agent. The hair of the thorax was not shaved or clipped.

Systematic scanning of right and left caudal lobes, the right middle lung lobe, and the cranial and caudal aspects of both cranial lobes, started dorsally at the level of the epaxial muscles in the right/left 10th intercostal space (ICS), and moved cranially to the right 1st or left 2nd ICS. The scapula marked the dorsal margin of the examination cranial to the 7th ICS bilaterally. The lung adjacent to the right 4th through 1st ICS and left 4th through 2nd ICS was examined with the US transducer between the forelimb and the cranial ventral thoracic body wall. Within each space, the probe was positioned parallel to the ribs and moved ventrally towards the sternum until specified US landmarks were visualized (Tables 3.1 and 3.2).
Peripheral lung tissue was considered normal when a hyperechoic line with reverberation artifact was present, signifying the interface between the high impedance tissue of the thorax and the low impedance tissue of the lung.\textsuperscript{10} Non-aerated or consolidated lung appeared hypoechoic and lacked both the bright white band at the pleural interface and reverberation artifact. The dorsal-to-ventral extent of consolidated lung was measured within each space using the 1 cm grid lines on the US screen. The total amount of US consolidation was calculated as the sum of all superficial consolidations. Percentage of consolidated lung (%US) was then determined by dividing the total amount of consolidation by the total amount of lung available to scan. The total amount of lung available to scan was determined by measuring and summing the US distance between the dorsal-most extent of the lung to the ventral image landmark within each ICS (Tables 3.1 and 3.2) in 4 non-study Holstein calves of equivalent age and weight (Table 3.3). Specific information regarding the non-study calves is available in Appendix 3.

After the baseline examination, calves were sedated with xylazine hydrochloride (0.05 mg/kg, IV) and challenged with 25 ml solution containing $10^9$ cfu of \textit{M. haemolytica} at log phase (Challenged) or 25 ml of sterile saline (Control). Inocula were endoscopically delivered into the distal trachea.\textsuperscript{11} Animals were reassessed by RS and US at 2, 6, 12, 24, 48, 72, 96 and 120 h post-challenge. Animals were euthanized with an overdose of barbiturate intravenously following the final assessment. Post-mortem examinations were performed by a blinded board certified pathologist (JC) and pulmonary consolidation was subjectively categorized as present or absent for each calf. Digital images were captured from the lateral perspective of the right and left lung for later morphometric assessment. Tissue samples for histopathology were taken from the border zone between normal and consolidated lung. In unaffected lungs, histopathology samples were taken from each lobe. Routine hematoxylin and eosin stains were made of
prepared histopathology sections after at least 24 hours of fixation within 10% buffered formalin.

Morphometric analysis was used to quantify the percent surface area of consolidated lung on gross examination. In short, each lung was outlined to ascertain the total surface area of that particular lung. Then, each area of consolidation was outlined and summed to ascertain the total surface area of consolidation. The combined surface area of consolidation for both lungs was divided by the total surface area of both lungs to determine the overall percent consolidation (%PM).

**Statistical Analysis** - Continuous outcomes including %US at each time point, time to severe US consolidation, maximum overall %US, time to maximum overall %US, and maximum %US within an intercostal space were described using medians and interquartile ranges. Comparisons of %US at each time point were made using Wilcoxon rank sum tests and the correlation between %US (t = 120 h) and %PM (t = 120 h) was made using Spearman correlation coefficient because these data were generally not normally distributed. Fisher’s exact test was used to compare proportion positive for US consolidation at 2 h between treatment groups. Agreement between US and PM diagnosis using consolidation as a dichotomous outcome (yes = any amount of consolidation; no = no consolidation) was assessed with Cohen’s kappa statistic. Severe US consolidation was defined as %US ≥ 3%. Fever was defined as T > 39.4°C. Data were analyzed with commercially available software (SAS version 9.4, SAS Institute Inc., Cary, NC). Alpha ≤ 0.05 was considered statistically significant. One Challenged calf did not develop bronchopneumonia and was removed from the analysis.
3.4 RESULTS

All RS, rectal temperatures, and percentage of consolidation lung (both %US and %PM) for each calf are presented in Tables 3.4 – 3.6. Ultrasonographic images were normal in 4 of 5 Control and 3 of 5 Infected animals prior to challenge. New consolidation was noted 2 h post-challenge in 1 of 5 Control and 5 of 5 Infected animals (P = 0.05). Compared to Controls, %US was greater in Infected calves after challenge (P = 0.01) at each time point except t = 6 (P = 0.07). In the Infected calves, severe consolidation developed by 6 h (median; IQR 6 - 6). Maximum %US was 11.3% (median; IQR 9.9 – 14.3) which developed by 48 h (median; IQR 48 – 96). Within an ICS, the largest lesion developed by 24 h (median; IQR 12 – 48). At t = 120 h, %US ranged from 5% - 46% (median %US = 7.3%; IQR = 5.9 – 9.9%) in the Infected calves. There was minimal or no consolidation in the Control calves (median %US = 0%; IQR = 0 - 0.1%) at t = 120 h. Ultrasonographic lung consolidation was more persistent than fever or a sick RS (Figure 3.2).

Fair agreement existed between the US and the pathologist’s diagnosis of consolidation (κ = 0.38; 95% CI, - 0.32 to 1) when both were categorized as a dichotomous outcomes. The discordant outcomes resulted from two calves identified as either normal by the pathologist and consolidated by the ultrasonographer or vice versa. The US consolidation was less than 1% in both cases. Histologic changes consistent with M. haemolytica infection, including coagulation necrosis, were present in lung tissue from all Infected calves and none of the Control calves regardless of clinical signs (Figure 3.3). Interestingly, all of the Control calves had histologic evidence of mild viral airway injury including peribronchiolar mononuclear cuffing and/or bronchial necrosis. One Control calf (Calf 5) also had a small, focal area of bronchopneumonia. The %US and %PM were highly correlated (ρ = 0.92; P = 0.0002).
None of the Control calves had fevers at the time of US before or after challenge (Table 3.4). All Infected calves developed fever 6 h post-challenge. Respiratory scores were normal throughout the study in all Control calves except for the baseline score in one calf (Table 3.5). Respiratory scores changed over time but were normal in 4 of 5 Infected calves at the final examination (Table 3.5). No calves required veterinary intervention during the study.

3.5 DISCUSSION

Using portable equipment, this study successfully documented the progressive changes in US lung consolidation after experimental *M. haemolytica* infection in bull calves. This challenge model was suitable for the purposes of this study as the infection was severe enough to produce significant clinical, US, gross PM, and histologic abnormalities in the designated time frame without incurring the need for veterinary intervention. Ultrasonographic lung consolidation developed within hours of challenge and progressed rapidly within a 48 h time period. These US changes were more reliable than clinical abnormalities in identifying Infected calves by the end of the study period and were highly correlated to the size of post-mortem (PM) lesions.

Regarding the progression of disease, the development of US lung consolidation paralleled the development of bronchopneumonia as demonstrated by others.\textsuperscript{7,8,12,13} One study reported that histologic changes occur as soon as 2 hours after infection,\textsuperscript{7} which coincides with the development of new US consolidation post-challenge in the current study. Our results are also consistent with another report\textsuperscript{13} in which the percent of gross consolidation ranged from 1.2\% - 23.9\% with a median 10\%, indicating a similar level of challenge. In the aforementioned study,\textsuperscript{13} histologic signs of resolution were reported to begin as early as 5 – 9 days after infection. In the current study, 4 of 5 Infected calves had numerical reductions in the amount of consolidated lung at the last examination. A longer follow up period would be necessary to
understand how US consolidation changes as time progresses considering that microscopic focal areas of coagulation necrosis will undoubtedly undergo fibrosis and the remaining areas of bronchopneumonia have the potential to resolve, re-aerate, and appear US normal.

In this study, Infected calves developed a “sick” RS soon after infection due to cough and fever at the 2 and 6 h time points, respectively. The presence of the Gram negative bacteria, *M. haemolytica*, in the lower airways stimulates an acute phase response, resulting in fever, as well as acute bronchial inflammation and alveolar edema, resulting in cough. Since RS returned to normal in nearly all Infected calves by 24 h, it appears that the animals were able to cope with this initial endotoxic challenge. Unfortunately, resolution of fever and the abnormal RS did not represent resolution of lung lesions as evident by %US, %PM, and histologic analysis. This disconnect between systemic and pulmonary manifestations of bacterial respiratory disease contributes to the presence of inapparent or variable clinical signs as well as the poor estimation of disease progression that is documented in other studies.\textsuperscript{13-18}

Unique aspects of this study include the development and description of the systematic US examination technique and method of calculating %US. Previously, US studies did not evaluate the cranial-most portion of lung, nor did they specify ventral image landmarks.\textsuperscript{1,2,19-23} In one report,\textsuperscript{20} bronchopneumonia was described as occurring predominantly in the cranioventral portions of the lung; however, their technique evaluated only the caudal lung lobes as the examination did not extend cranial to the 7th ICS, thereby excluding the entire left cranial lobe, the right middle lung lobe, and the entire right cranial lobe. It is likely that the thorough technique used in the current study allowed for such a high degree of correlation between %US and %PM, because of this tendency for bronchopneumonia to effect the cranial and middle lung lobes.
A previously published scoring system was used to demonstrate that calves with higher US scores had more gross consolidation, but an estimate of the percent consolidation was not determined. The current study compared the amount of superficial lung consolidation to the amount of superficial lung available to the US, in order to estimate a percentage of lung consolidation. The standard measurements of lung size were derived from animals of similar breed, age, and weight. Calculating the percentage of lung using this standard removed the effect of animal size which would occur if using absolute measures. Measuring the superficial surface of the lung does give the cranial lobes disproportionate weight, since the depth of the caudal lung lobes and the accessory lung lobe cannot be accounted for using US. However, the contributions from each lung lobe are comparable to a previous description in which the mass of each lobe was compared to the mass of the total lung.

This challenge model failed to create bronchopneumonia in one of the challenged calves. Although it was not specifically documented in this case, it is possible that the calf coughed and swallowed a substantial portion of the inoculum resulting in a challenge less than that of the other calves. Although an unintended outcome, US was useful in confirming this challenge failure ante-mortem.

A limitation of this study was the short follow-up period. Extending the duration of the follow-up would have provided an opportunity to determine how RS and US consolidation fluctuate over a prolonged period of time. Associations between RS, US findings, and the need for veterinary intervention might also have been identified if the study was extended and clinical diseased progressed over time.
3.6 CONCLUSION

Ultrasonographic lung consolidation developed soon after infection, progressed rapidly, and persisted longer than the clinical signs associated with bronchopneumonia in post-weaned Holstein bull calves. The %US was significantly different between Control and Infected calves and was highly correlated with the amount of lung consolidation present at post-mortem examination. Although more work is needed to assess the value of identifying lung consolidation in the absence of clinical signs, thoracic US might prove to be a useful diagnostic tool in future BRD research as well as for monitoring respiratory health on dairy farms.

3.7 REFERENCES


### Table 3.1 Ultrasonographic landmarks for the lobes of the right lung in dairy calves.

<table>
<thead>
<tr>
<th>Lung lobe</th>
<th>Caudal</th>
<th>Middle</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>R – ICS*:</td>
<td>6 – 10</td>
<td>5</td>
<td>3 – 4</td>
<td>1 - 2</td>
</tr>
<tr>
<td>Ventral landmark(s):</td>
<td>Diaphragm</td>
<td>CCJ† &amp; pleural deviation</td>
<td>Heart</td>
<td>Two blood vessels</td>
</tr>
</tbody>
</table>

---

* right intercostal space(s)
† costochondral junction.
Table 3.2 Ultrasonographic landmarks for the lobes of the left lung in dairy calves.

<table>
<thead>
<tr>
<th>Lung lobe:</th>
<th>Caudal</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>L – ICS(^\ast):</td>
<td>6 – 10</td>
<td>4 – 5</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Ventral landmark(s):</td>
<td>Diaphragm</td>
<td>CCJ(^\dagger) &amp; pleural deviation</td>
<td>Heart</td>
</tr>
</tbody>
</table>

\(^\ast\) left intercostal space(s)
\(^\dagger\) costochondral junction.
Table 3.3 Contributions of each lung lobe to the total lung based on ultrasound (US) assessment of 4 non-study Holstein calves of similar weight and age.

<table>
<thead>
<tr>
<th>Lobe</th>
<th>Intercostal Spaces</th>
<th>Linear length (cm)*</th>
<th>% of Total lung†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right / Left</td>
<td>Right / Left</td>
<td>Right / Left</td>
</tr>
<tr>
<td>Caudal</td>
<td>10 – 6 / 10 – 6</td>
<td>61 / 60</td>
<td>30 / 30</td>
</tr>
<tr>
<td>Middle</td>
<td>5 / NA</td>
<td>13 / NA</td>
<td>6 / NA</td>
</tr>
<tr>
<td>Caudal aspect of cranial lobe</td>
<td>4 – 3 / 5 – 4</td>
<td>15 / 21</td>
<td>7 / 5</td>
</tr>
<tr>
<td>Cranial aspect of cranial lobe</td>
<td>2 - 1 / 3 – 2</td>
<td>18 / 16</td>
<td>9 / 8</td>
</tr>
</tbody>
</table>

*Linear length: Sum of the length of intercostal spaces corresponding to each lung lobe. For example, the combined length of intercostal spaces 6-10 overlying the right caudal lung lobe is 61 cm.

†% of Total lung: total linear length of US scan, as a percentage of the total length of lung. For example, the combined length of intercostal spaces 6-10 overlying the right caudal lung lobe is 30% of the total length of the intercostal spaces overlying the entire lung.
Table 3.4 Body temperature (°C) for each calf at the time of each US examination. C = Control calves (n = 5), I = Infected calves (n = 5).

<table>
<thead>
<tr>
<th>ID</th>
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<th>Hours post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>39</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>38.4</td>
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<tr>
<td>3</td>
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<td>39.2</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td>38.2</td>
</tr>
</tbody>
</table>
Table 3.5 Respiratory Scores (McGuirk, 2008) for each calf at the time of each ultrasound examination. C = Control calves (n = 5), I = Infected calves (n = 5).

<table>
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</tr>
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<td>I</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>I</td>
<td>0</td>
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</table>
Table 3.6 Percent of ultrasonographic (US) consolidation for each calf at each US examination. Measurements taken post-challenge are new areas of consolidation. C = Control calves (n = 5). I = Infected calves (n = 5). 120* = percentage of post-mortem lung consolidation based on morphometric analysis.

<table>
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<th>ID</th>
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<th>-1</th>
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<th>24</th>
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<th>96</th>
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</tr>
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<td>C</td>
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<tr>
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<td>5.9</td>
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<td>3.0</td>
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<tr>
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<td>0.4</td>
<td>0.0</td>
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<td>7.9</td>
<td>9.9</td>
<td>11.3</td>
<td>9.9</td>
<td>12.3</td>
</tr>
</tbody>
</table>
Figure 3.1 Comparison of the percentage of consolidated lung in Control (solid line, n = 5) and Infected (dashed line, n = 5) calves.
Figure 3.2 Proportion of Infected calves (n=5) with severe consolidation (black bar), fever (striped bar), and a sick respiratory score (RS > 4; grey bar).
Figure 3.3 Histologic lesions of bronchopneumonia caused by *Mannheimia haemolytica* infection in an infected calf with normal rectal temperature (T = 39°C) and respiratory score (RS = 3) at t = 120 h post-challenge. (A) Low magnification, 4X, showing coagulation necrosis (black star). (B) High magnification (40X) showing neutrophilic exudate within the bronchiolar lumen (black arrow).
CHAPTER 4. A RANDOMIZED CONTROLLED CLINICAL TRIAL TO EVALUATE THE EFFECT OF AN INTRANASAL RESPIRATORY VACCINE ON CALF HEALTH, ULTRASONOGRAPHIC LUNG CONSOLIDATION, AND GROWTH IN HOLSTEIN DAIRY CALVES

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*Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph N1G 2W1

†Department of Population Medicine and Diagnostic Sciences, Cornell University, College of Veterinary Medicine, Ithaca, NY 14850

‡Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1

§Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1

Corresponding author: David Kelton, Department of Population Medicine, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1; Phone: (519) 824 - 4120 ext. 54808 Fax: (519)763 - 8621; Email: dkelton@uoguelph.ca
4.1 ABSTRACT

Although passive transfer of antibodies to the newborn calf provides many great benefits, high levels of maternal antibodies may limit the ability of the calf to respond to injectable vaccines against respiratory viruses. Experimental challenge studies have demonstrated varying efficacies of vaccines administered via the intranasal route. The objective of this randomized controlled clinical trial was to evaluate the effect of a commercially available trivalent vaccine designed for intranasal use. A total of 468 calves from 3 herds were enrolled and randomized into 3 treatment groups (positive control, PC, n = 211; intranasal vaccine, IN, n = 215; negative control, NC, n = 42) for an 8 – 12 week follow up period. The PC consisted of one dose of commercially available multivalent injectable vaccine against BRSV, IBR, PI3, and BVD administered subcutaneously at 6 weeks of age. The IN was administered twice (first dose: 3 – 6 d of age; second dose: 6 weeks of age) and contained antigen against BRSV, IBR, and PI3. The NC, sterile saline, was administered intranasally and subcutaneously twice (first dose: 3 – 6 d of age; second dose: 6 weeks of age). Herds were visited weekly. Clinical illness associated with bovine respiratory disease was assessed using systematic respiratory scoring; and thoracic ultrasonography (US) was used to identify calves with lung lesions. Rib fractures were identified in 6% of calves. There was a significant association between rib fractures and calving ease. Overall, 54% of the calves had at least one episode of abnormal respiratory score (ILL). After controlling for the effects of herd, sex, and scours using multivariable logistic regression, vaccination protocol did not affect the occurrence of ILL. Similarly, 54% of the calves had at least one episode of lung consolidation ≥ 3 cm (CON). After controlling for herd, dystocia, and rib fractures using multivariable logistic regression, vaccine protocol significantly affected the odds of CON. The odds of CON in PC were 1.63 (95%CI: 1.04 – 2.56) times the odds of CON.
in IN, and 0.38 (95% CI: 0.16 – 0.93) times the odds of CON in NC. The odds of CON in IN were 0.23 (95% CI: 0.09 – 0.59) times the odds of CON in NC. The outcomes ILL and CON were positively associated; however the measure of agreement was only fair (kappa = 0.38). Multivariable linear regression revealed a significant interaction between vaccine protocol and Herd on average daily gain (ADG). In Herd 2, IN increased ADG as compared to PC. In contrast, in Herd 1, IN decreased ADG. None of the protocols affected ADG at Herd 3. There was no difference in overall mortality rates between protocols and no adverse events were associated with any of the protocols. In conclusion, a commercially available trivalent IN vaccine protocol reduces the prevalence of lung lesions and has the potential to improve calf growth in commercially raised dairy calves.

**Key words:** bovine respiratory disease, intranasal vaccine, ultrasonography

### 4.2 INTRODUCTION

Calves are born agammaglobulinemic and must ingest maternal colostrum for immune support until their own immune system is sufficiently developed. Although maternal transfer of antibody to the newborn calf provides many great benefits (Faber et al., 2005), high levels of maternal antibodies are associated with a delayed antibody production by the neonate and as well as selective inhibition of lymphocyte responses (Tizard, 2013). The potential for maternal blockade has caused concern regarding the practice of early life vaccination to prevent bovine respiratory disease (BRD), as maternal antibodies can be present for up to 6 months of age (Menanteau-Horta et al., 1985).

It has been established that 3 - 8 day old Holstein calves are capable of mounting a mucosal immune response in the face of maternal antibodies (Hill et al., 2012); however, over
the last 10 years, reports regarding the potential for intranasal vaccination to protect young dairy calves with and without maternal antibodies from infection with BRSV and PI3 have been inconsistent. One report describes 2 controlled experiments in which seropositive and seronegative Holstein calves were vaccinated intranasally with a product intended for subcutaneous injection at 3 – 8 days of age followed by challenge 21 d later or at 4.5 months of age (Ellis et al., 2010). After delayed challenge at 4.5 months, seropositive calves had wider temperature fluctuations compared to seronegative calves; however, there was no difference in maximum mean clinical score, the number of days with an elevated clinical score, the partial pressure of arterial oxygen, lung lesion score, or mortality. In that same report, when calves were challenged 21 d after IN vaccination with a similar product containing a lower dose of BRSV antigen, seronegative vaccinated calves had less extensive lung lesions at post-mortem examination, however no differences in clinical score or mortality were observed. Ellis (2013) demonstrated that IN vaccination between 3 – 8 days of age resulted in improved partial pressure of oxygen (PaO2), fewer lung lesions and lower mortality rate than unvaccinated calves following a BRSV challenge 9 weeks after vaccination but not after 14 weeks after vaccination, suggesting that the duration of immunity to BRSV is short lived. Additionally, two doses of monovalent injectable BRSV product used intranasally completely protected calves from clinical disease; and calves receiving just one dose had minimal signs of respiratory disease whereas all control calves required euthanasia due to severe respiratory disease (Ellis et al., 2007). Most studies used either laboratory designed attenuated virus (Wolums et al., 2004) or commercially available products designed for injection (Ellis et al., 2007, 2010; Vangeel et al., 2007). Only one report studied a commercially available product designed for intranasal use (Ellis et al., 2013). None of these evaluated vaccine efficacy in a field trial setting.
The previously mentioned challenge studies often found that IN calves had less extensive lung lesions than control calves despite the lack of observable clinical changes. In the beef industry, several reports suggest that evaluation of lung lesions at harvest may be a more accurate means of documenting BRD than clinical observations (Wittum et al., 1996; Thompson et al., 2006; White et al., 2012). Unfortunately, documenting lung lesions directly requires euthanasia and often limits the size of study populations. As an alternative, thoracic ultrasonography (US) can be performed quickly and provides an accurate ante-mortem assessment of lung health (Rabeling et al, 1998, Ollivett PhD Thesis Chapter 2, Ollivett PhD Thesis Chapter 3). Therefore, the primary objective of this randomized controlled clinical trial was to evaluate the effect of an intranasal vaccine on the health of young Holstein dairy calves. The secondary objectives were to evaluate the effect of this vaccine on US lung lesions and growth. Results from this study will help dairy producers and bovine practitioners in the decision making process regarding vaccination of young dairy calves.

4.3 MATERIALS AND METHODS

Animals and Facilities

This study was carried out on 3 dairies in southwestern Ontario, Canada, between January and December 2012. Two of these herds were the Elora (Herd 1) and Ponsonby (Herd 3) Dairy Research Centres associated with the University of Guelph. The third dairy was a privately owned commercial herd (Herd 2). Each herd was visited twice a week (all herds) or three times a week during periods of high enrolment (Herd 2 only) in order to enrol calves twice a week. Holstein calves, male and female, were enrolled between 3 – 6 days of age into 3 groups according to vaccine protocol (positive control, PC; intranasal vaccine, IN; and negative control, NC) for an 8 – 12 week follow up period. A birth record was filled out by the dairy producer
after the birth of each calf (Appendix 2). The PC (Bovi-Shield Gold 5, Zoetis) consisted of one dose of commercially available multivalent injectable vaccine against BRSV, IBR, PI3, and BVD by subcutaneous administration at 6 weeks of age. The IN (Inforce 3, Zoetis) was administered twice (first dose: 3 – 6 d of age; second dose: 6 weeks of age) and contained antigen against BRSV, IBR, and PI3. The NC, sterile saline, was administered both intranasally and subcutaneously twice (first dose: 3 – 6 d of age; second dose: 6 weeks of age). All doses of IN were administered via a single-use plastic nasal cannula (Zoetis, New York City, NY) into one nostril. All injections were administered subcutaneously in the neck. Treatments were administered by members of the research team not involved in respiratory scoring or US. Different methods of randomization of calves into the three treatment groups were performed for each herd due to differences in barn design in order to prevent contamination of PC and NC from nasal shedding of virus by IN. Weight, RS, and US observations were performed by the principle investigator who was blinded to treatment throughout all data collection. A birth record was filled out by the dairy producer after the birth of each calf (Appendix 2). This study was conducted with the approval of the University of Guelph’s Animal Care Committee (AUP #11R110).

**Herd specific calf management.**

In Herd 1 (Elora; lactating cows, n = 150), calves were fed 4 L of single source colostrum within 24 h of birth. Calves were housed in either individual stalls within an enclosed room with an active ventilation system, or outside tethered to individual plastic hutches. Calves were fed 6 L of whole unpasteurized milk per day until approximately 6 weeks of age, at which point they were gradually weaned and moved to group housing by 8 weeks of age. Free choice water and calf starter were available beginning at 3 days of age. Prior to the start of the study, rotating
treatment groups were assigned to each room (enclosed nurseries, n = 3; outdoor “hutch room”, n = 1) by drawing the protocol name from a hat with replacement. Four cycles were drawn for each room to accommodate the number of calves for the anticipated duration of the study. Each room was filled with 8 - 10 calves over a 2 week period. Calves were housed together according to birth order regardless of sex. Each room was cleaned, disinfected, and allowed to sit empty for approximately 1 week before new calves were added.

In Herd 2 (commercial; lactating cows, n = 650), single source colostrum (2 L) was offered by bottle within 30 minutes of birth. An additional 4 L was offered in 2 separate feedings over the 24 h following birth. Calves were fed 6 L unpasteurized whole milk twice daily while housed in individual pens until approximately 3 weeks of age. Calves were then moved as a group of 20 animals per pen. Eight liters unpasteurized whole milk was offered via an automated system in the group pens (Forster Technik, De Laval, Peterborough, ON). Calves were allowed 3 L per feeding within a 3 h period. Free choice starter was offered within the first 3 days of life. Water was not available until the calves reached the group pen. Individual and group pens were bedded with a sawdust base covered by a top layer of straw. Calves were removed from the group pen at approximately 8 weeks of age. As of April, 2012, tulathromycin (2.5 mg/kg; 1.1 mL/100 lb), subcutaneously once; Zoetis) was administered to all calves at movement from the single pen to the group pen. Calves spent the first 24 – 36 h of life in a straw-bedded room adjacent to the maternity area before moving to one of two identical recently built barns. Each barn held 40 calves in individual stalls and 80 calves split between 4 group pens for a total of 120 calves per barn. Each barn was curtain sided and used both natural and positive pressure ventilation systems. As calves were born, individual stalls were filled in one barn, followed by the second barn. The owners of this commercial facility were not willing to have a NC group;
therefore calves were only enrolled into PC and IN groups. Treatment groups were randomly assigned at the barn level prior to the start of the study by picking the protocol from a hat.

In Herd 3 (Ponsonby; lactating cows, n = 55), calves were fed 4 L of single source colostrum within 24 h of birth followed by 2 L unpasteurized whole milk three times daily until abrupt weaning at approximately 8 weeks of age. All calves were housed outside tethered to individual plastic hutches until weaning at which point they were moved to group housing by 8 weeks of age. Free choice water and calf starter were available beginning at 3 days of age. Calves were blocked by sex and treatment was assigned to each hutch by drawing protocol names from a hat in sets of three without replacement.

**Blood collection, weighing, health scoring, and ultrasonographic data collection**

For assessing passive transfer of maternal antibodies, whole blood was collected from 3 to 6 day old calves by jugular venipuncture, using a 20-gauge, 1-inch hypodermic needle (BD Vacutainer Precision Glide, Becton, Dickinson and Co., Franklin Lakes, NJ), into a sterile, plastic, commercial blood collection tubes without anticoagulant. Blood tubes were stored on ice and within 4 to 6 hours of collection, serum was separated by centrifugation at 3000 rpm for 15 min at ~20°C. Analysis of serum total protein (STP) was performed by a research assistant using a digital refractometer (Misco PA202X-003-105, Cleveland, Ohio, USA).

At each examination, calves were weighed 3 times using a weigh tape (Coburn Company, Whitewater, WI) and these weights were averaged to provide the weight for each examination. Average daily gain between enrolment and 56 days after enrolment was calculated by dividing the difference in the weight recorded 56 days after enrolment and enrolment by 56. Calves that died before 56 d of age were excluded from the ADG analysis.
Respiratory scoring (RS; Appendix 1; Lago et al., 2006; McGuirk, 2008) and US were performed at each visit. Briefly, the RS assigned 0 – 3 points for each of the following categories: rectal temperature, nasal discharge, cough, and ocular discharge or ear position. Respiratory scores could range from 0 to 12 and any calf with RS > 4 was considered sick (McGuirk, 2008). Fecal scores (FS) were obtained after each RS by direct visualization of fresh manure. Digital examination of the rectum was used to stimulate defecation. Fecal scores were based on a 3 -point scale; 1 = sample is in “patty” form; minimal water content, does not flow across or down a surface; 2 = sample is more of a puddle, some water content, flows slowly across or down a surface; 3 = sample is watery, flows across or down a surface while leaving some to no adherent material (Moore et al., 2003). Regarding US, both hemithoraces were scanned using a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 16 dB (Near 13 dB; Far 36 dB; Ibex Pro, E. I. Medical, Loveland, CO). Approximately 300 mL of 70% isopropyl alcohol was applied to the hair as a transducing agent. The hair of the thorax was not shaved or clipped. Systematic scanning of the lung started dorsally at the level of the scapula in the right/left 10th intercostal space (ICS), and moved cranially to the right 1st or left 2nd ICS. The lung adjacent to the right 4th through 1st ICS and left 4th through 2nd ICS was examined with the US transducer between the forelimb and the cranial ventral thoracic body wall. Within each ICS, the probe was held parallel to the rib and advanced ventrally towards the sternum until specific US landmarks were visualized, before moving to the next cranial ICS.

Peripheral lung tissue was considered normal when a hyperechoic line with reverberation artifact was present, signifying the interface between the high impedance tissue of the thorax and the low impedance tissue of the lung (Blond et al., 2009). Consolidated lung appeared
hypoechoic and lacked both the bright white band at the pleural interface and reverberation artifact. The depth and dorsal to ventral extent of consolidated lung were measured within each ICS using the 1cm grid lines on the US screen. All observations were spoken and recorded using a digital voice recorder and later manually transcribed into a database (Microsoft Access 2010, Redmond, WA).

Statistical Analyses

Continuous variables included EAGE (age at enrolment, d), AGE1 (age at first ultrasound examination, d), and W1 (weight at first ultrasound, kg). Non-dichotomous categorical variables included TREATMENT (1 = PC; 2 = IN; 3 = NC), HERD (1 = Herd 1; 2 = Herd 2; 3 = Herd 3), and SEASON of enrolment (WINTER = January through March; SPRING = April through June; SUMMER = July through September), Birth Weight Category (0 = W1 < 40 kg; 1 = 40 kg ≤ W1 < 46 kg; 2 = W1 ≥ 46 kg) and EXAM (chronologically ordered number of ultrasound exam). Dichotomous variables included ILL (all RS ≤ 4 = 0; 1 or more RS > 4 = 1); CON (less than 3 cm US lung consolidation on at all examinations = 0; 1 or more US examinations with ≥ 3 cm US lung consolidation present = 1); SEX (male = 0; female = 1); TWIN (no = 0; yes = 1); DYST (no assistance or easy pull = 0, hard pull or surgical delivery = 1), FPT (STP ≥ 5.2 g/dL = 0; STP < 5.2 g/dL = 1, Tyler et al., 1996); HOUSING (outdoors = 0; indoors = 1); SCOUR (FS < 3 within 21 days of enrolment = 0; FS = 3 during at least one exam within 21 days of enrolment = 1) and RIB (rib fracture not palpable or visible on US = 0, rib fracture palpable and visible on US = 1). The 3 cm cut-off value for CON was determined by selecting the value between the 90th and 95th percentiles (2.25 cm and 3.5 cm, respectively) of US lung consolidation in those calves with any amount of lung consolidation during their first US.
SCOUR was intended to capture those calves having at least one bout of severe diarrhea during the high risk period of the first 21 days of life.

A standard statistical package was used for all analyses (SAS version 9.4, SAS Institute Inc., Cary, NC) except for the sample size calculation (Stata 12.1, Stata Corp LP. College Station, TX). Sample size of 270 calves per treatment was estimated initially to provide a power of 80% and detect a 10% difference in clinical disease with $\alpha = 0.05$. Sample size was re-estimated and adjusted down 8 weeks into the study. All continuous variables were assessed for normality using the Shapiro-Wilk test. Measures of central tendency are presented as mean (standard deviation, SD) for ADG, and median (interquartile range, IQR) for EAGE. Raw ADG were compared using the t-test. Enrolment ages were compared by Wilcoxon rank sum test. Categorical variables (birth weight, DYST, TWIN, SEX, first RS, first US, FPT, RIB, ILL, CON) were assessed with contingency tables and Chi square analysis, or Fisher’s exact test when expected frequencies in individual cell counts were < 5. Pearson’s correlation coefficient was estimated to determine the association between RIB and DYST as well as ILL and CON. When a calf died or was euthanized due to severe disease, a field based post-mortem examination was performed by a veterinarian. Overall mortality risk and the risk of death from BRD were calculated.

Logistic regression models were fit for the outcomes ILL and CON using the GLIMMIX procedure. The predictor of interest was TREATMENT. All variables offered to the univariable model that resulted in P < 0.20 were entered into the multivariable model as fixed effects, including HERD. TREATMENT was forced into the model even if not significant in the univariable analysis. All two-way interactions between TREATMENT and fixed effects were tested for significance. Manual backwards stepwise elimination was used to refine the model to
include variables that were significant at \( \alpha \leq 0.05 \) level. Additionally, a change in estimate criterion of \( \geq 25\% \) was used to assess for confounding prior to the final elimination of a variable. Predicted means for CON and ADG were assessed using the LSMEANS statement and these were adjusted using the DIFF option. Type 3 tests of fixed effects were used to determine significance which was set at \( \alpha < 0.05 \). Collinearity (Type II Tolerance \( < 0.10 \)) was assessed with the GLM procedure.

Average daily gain was modeled using the MIXED procedure. The primary predictor of interest was TREATMENT. Variables were offered similar to the logistic regression model. Knowing that male calves typically grow faster than heifer calves (Koch et al., 1959), SEX was forced into the model even though it was not significant in the univariable analysis. Predictions based on categorical variables were assessed using the LSMEANS statement. Pearson’s correlation coefficients were calculated to assess the correlations between model predictions and actual observations. Each model was assessed graphically for outliers and the normality of residuals was tested using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov, and Cramer-von Mises tests. Alpha was \( P < 0.05 \).

### 4.4 RESULTS

A total of 468 calves from 3 herds were enrolled and randomized into 3 treatment groups (PC, \( n = 211 \); IN, \( n = 215 \); NC, \( n = 42 \)). Distributions of variables potentially affected by incomplete randomization are summarized in Table 4.1. There was a difference in TREATMENT group size between herds (\( P < 0.0001 \)) due to the lack of NC at Herd 2. However, there was no difference (\( P = 0.48 \)) in the proportions of calves enrolled in PC and IN. Calves were younger at enrolment in IN (median age, IQR: 4, 3 – 5 d) compared to PC (median age, IQR: 4, 4 – 5 d; \( P = 0.02 \)) and NC (median age, IQR: 5, 4 – 6 d; \( P = 0.02 \)). However, there was
no difference \((P = 0.22)\) in EAGE between PC and NC. Serum protein data were missing from PC \(n = 3\), IN \(n = 5\), and NC \(n = 2\). The risk of FPT was greater for NC compared to PC and IN \(P = 0.02\) and \(P = 0.08\), respectively. The risk of FPT was not different between PC and IN \(P = 0.39\). Rib fractures were observed on 6\% \((28/468)\) of calves. Rib fractures and calving ease were associated \((r = 0.17, P = 0.0002)\) but there was no association between rib fractures and TREATMENT \(P = 1.0\). Overall, 54\% \((251/468)\) calves were ILL positive. Before controlling for fixed effects, NC had a lower proportion of ILL positive calves than PC \(NC = 33\%\) vs. PC = 59\%, \(P = 0.004\), and IN \(IN = 53\%, P = 0.03\). There was no difference in proportion of ILL calves between PC and IN \(P = 0.21\). After controlling for the effects of HERD, SEX, and SCOUR, TREATMENT was not associated with the odds of ILL \((Table 4.2; P = 0.32)\). Overall, 54\% \((253/468)\) of the calves were CON positive. Before controlling for fixed effects, NC had a lower proportion of CON positive calves than PC \(NC = 33\%\) vs. PC = 60\%, \(P = 0.002\), and IN \(IN = 53\%, P = 0.02\). There was no difference in proportion of CON positive calves between PC and IN \(P = 0.14\). However, after controlling for HERD, DYST, and RIB, TREATMENT was significantly associated with the odds of CON \((Table 4.3; P = 0.005)\). The predicted probabilities of CON for each TREATMENT are shown in Figure 4.1. The odds of CON in PC were 1.63 \((95\%CI: 1.04 – 2.56)\) times the odds of CON in IN \((P = 0.03)\) and 0.38 \((95\%CI: 0.16 – 0.93)\) times the odds of CON in NC \((P = 0.03)\). The odds of CON in IN were 0.23 \((95\%CI: 0.09 – 0.59)\) times the odds of CON in NC \((0.002)\). A 2 x 2 contingency table compares the overall distribution of CON and ILL \((Table 4.4)\). The outcomes CON and ILL were positively associated \((r = 0.39, P < 0.0001)\) but their agreement was only fair \((kappa = 0.38)\). The ADG \((mean (SD))\) for all calves was 0.56 kg/d \((SD = 0.17)\). Before controlling for the fixed effects, the ADG for NC was 1.53 lb/d \((SD = 0.31)\), which was greater \((P < 0.0001)\) than both PC and IN;
however, PC and IN were not different (PC: 0.55 kg/d (SD = 0.17) vs. IN: 0.55 kg/d (SD = 0.17; \( P = 0.56 \)). Multivariable linear regression revealed a significant interaction between TREATMENT and HERD on the outcome ADG (\( P < 0.01 \); Table 4.5). In Herd 1, IN decreased ADG by 0.10 kg/d (SE = 0.03) compared to PC (\( P < 0.01 \)). In contrast, at Herd 2, IN increased ADG by 0.03 kg/d (SE = 0.02; \( P = 0.04 \)). TREATMENT did not affect ADG at Herd 3 (\( P > 0.25 \)). Thirty-nine (8%) calves died or were euthanized during the study. The risk of death from respiratory disease was 1.9% (9/468). There was no difference in overall mortality rates between protocols (\( P = 0.58 \)).

4.5 DISCUSSION

The purpose of this study was to compare the effect of an intranasal vaccination protocol on bovine respiratory disease and growth in Holstein dairy calves. Results from two different classification methods, respiratory score (RS) and thoracic ultrasonography (US), were used to identify diseased calves; and growth was assessed during the first 56 d of life. There was no effect of 2 doses of a commercially available trivalent IN respiratory vaccine (experimental treatment, IN, first dose: 3 – 6 d of age; second dose: 6 weeks of age) by 6 weeks of age on the odds of having at least one episode of clinical illness (ILL) during the study period as compared to a commercially available SC product (positive control, PC, only dose: 6 weeks of age) or sterile saline (negative control, NC) after accounting for confounding variables. In contrast, IN significantly reduced the odds of having at least one episode of 3 cm or more of ultrasonographic lung consolidation (CON) compared to both positive and negative controls. The effect of IN on calf growth was herd dependent. This interaction resulted in greater ADG in Herd 2 in IN treated calves as compared to PC. In Herd 1, IN was associated with either a lower ADG or no change in
ADG as compared to the PC and NC, respectively. No differences in ADG were observed in Herd 3.

Clinical observation is used commonly to detect respiratory disease (Amrine et al., 2013); however, compared to US, respiratory scoring did not detect the disease sparing effects of IN in the current study. As previously mentioned, lung lesions typically affect more calves at slaughter than indicated by observation based treatment records (Wittum et al., 1996; Thompson et al., 2006) suggesting the presence of subclinical disease. Although most of this work was performed in feedlot cattle, the current study as well as Buczinski (2014), supports the concept that similar patterns are present in the dairy calf populations. Additionally, the fact that agreement between diagnostic methods was just “fair” indicates that different populations of calves were identified by each predictor. It is possible that a clinical difference did not exist, the cut-points used to create ILL and CON were inappropriate, or the subjective nature and variability inherent to clinical scoring precluded finding small differences in a limited study population. The disagreement between the two variables rested evenly between CON positive calves that were ILL negative and ILL positive calves that were CON negative. This could be the result of clinical scoring systems that are not highly specific to the disease to which they are supposed to be detecting. As can be seen in the logistic regression model, the significant association between SCOUR and ILL highlights the fact diseases other than BRD can evoke a positive test when respiratory scoring. Parceling out the effect of these variables on objective measures such as calf growth will help researchers understand which variable, ILL, CON, or both, most accurately identify the population of calves affected with BRD.

Rib fractures are common in neonatal foals (Jean et al., 1999); however their presence and effect on ADG was an unexpected finding in this study in dairy calves. Rib fractures were
typically located near the costochondral junction of the cranial thorax, similar to previous reports (Mee, 1993), and were easily imaged via US. Six-percent of calves in the current study had obvious rib fractures, which is much lower than previous reports of 23% in calves that die during the perinatal period (Schuijt et al., 1990) and 40% of calves born with veterinary assistance (Mee, 1993). This likely reflects different study populations, although an equine study reported a 20% prevalence of rib fractures in foals on one breeding herd; twice as many resulting from dystocia than normal parturitions (Jean et al., 1999). Despite the fact that none of the observed fractures were severely displaced or causing internal thoracic abnormalities such as hemithorax or pneumothorax, rib fractures did contribute significantly to the variation in ADG. Interestingly, recent work has shown that anti-inflammatory doses of meloxicam given immediately after a difficult birth results in greater calf growth during the early weeks of life (C. Murray, PhD Thesis, University of Guelph). One reason for this finding could be treatment of the pain and inflammation associated with rib fractures which allows for improved utilization of liquid feed and greater growth. In the current study, there was an association between dystocia and rib fractures, however the correlation was very low and highlights the fact that a particularly difficult calving is not a prerequisite for rib fractures.

Although herd differences were noted in this study, the fact that only 3 herds were included is a limitation to the ability to draw inferences about herd specific factors regarding the efficacy of IN. Inclusion of a large number of herds varying in locale and management practices would be necessary to determine exactly which situations IN would be most effective. An additional limitation is the potential confounding effect of barn on the relationship between the measured outcomes and treatment at Herd 2. This design was intentional as IN and PC calves needed to be housed separately to prevent exposure of PC to IN vaccine virus. The authors do
acknowledge that switching the treatment status of each barn would have been ideal. However, the feasibility and additional time requirements necessary for incorporating washout periods when transitioning between groups were considered not practical on this commercial farm. The two barns were close in proximity to each other, being separate by a milk preparation room; and barn age, design, flow of animals, cleaning, and labor were identical. These factors should have helped to reduce the risk of a barn effect on calf health and performance.

This study differed from previous work on the efficacy of intranasal vaccination in several ways. The calves in the current study were conventionally raised and likely exposed to chronic, low level natural challenges from the whole spectrum of respiratory pathogens (Gorden and Plummer, 2010). In contrast, previous studies incorporated controlled experimental BRSV challenges (Woolums et al., 2004; Vangeel et al., 2007; Ellis et al., 2007, 2010, 2013) and PI3 (Vangeel et al., 2007) intended to replicate natural disease in small group of animals. Despite the high prevalence of disease in the study population, the risk of death from respiratory disease was low, suggesting that the natural challenge in this study might not be as acutely aggressive as those demonstrated by single pathogen challenge models as several calves from each BRSV challenge died or required euthanasia due to the severity of disease (Woolums et al., 2004; Ellis et al., 2007, 2013). Acknowledging that previous study designs were in part due to federal regulations regarding licensing procedures for new vaccines (Ellis et al., 2013), data from this field study might be more relevant to dairy producers and bovine practitioners.

4.6 CONCLUSIONS

A commercially available trivalent IN vaccine has the potential to reduce the lung lesions associated with BRD and improve growth in young dairy cattle. Although these findings were significant, herd factors play a role in determining whether or not significant changes in average
daily gain will be seen. Also, IN vaccination did not eliminate the risk of disease in the current study; therefore this practice should not be viewed as a “magic bullet”. Best management practices regarding calf nutrition, housing strategies, ventilation, and appropriate vaccination protocols should be integrated to provide the optimal environment for the growing dairy calf.

ACKNOWLEDGMENTS

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4.7 REFERENCES


### 4.8 TABLES & FIGURES

Table 4.1 Distribution of variables on 468 calves enrolled in a randomized blinded field study by calf characteristic and treatment group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>Treatment Group, no. (% across category)</th>
<th>PC</th>
<th>IN</th>
<th>NC</th>
<th>P - value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herd</td>
<td>Herd 1</td>
<td></td>
<td>46 (40.4)</td>
<td>37 (32.5)</td>
<td>31 (27.2)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Herd 2</td>
<td></td>
<td>156 (48.0)</td>
<td>169 (52.0)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Herd 3</td>
<td></td>
<td>9 (31.0)</td>
<td>9 (31.0)</td>
<td>11 (37.9)</td>
<td></td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>&lt; 40 kg</td>
<td></td>
<td>50 (46.7)</td>
<td>49 (45.8)</td>
<td>8 (7.5)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>40 – 46 kg</td>
<td></td>
<td>117 (48.0)</td>
<td>106 (43.4)</td>
<td>21 (8.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 46 kg</td>
<td></td>
<td>44 (37.6)</td>
<td>60 (51.3)</td>
<td>13 (11.1)</td>
<td></td>
</tr>
<tr>
<td>Calving ease</td>
<td>hard pull, no</td>
<td></td>
<td>183 (45.6)</td>
<td>182 (45.4)</td>
<td>36 (9.0)</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>hard pull, yes</td>
<td></td>
<td>25 (42.4)</td>
<td>30 (50.9)</td>
<td>4 (6.8)</td>
<td></td>
</tr>
<tr>
<td>Twin</td>
<td>No</td>
<td></td>
<td>184 (44.1)</td>
<td>193 (46.3)</td>
<td>40 (9.6)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td></td>
<td>21 (50.0)</td>
<td>19 (45.2)</td>
<td>2 (4.8)</td>
<td></td>
</tr>
<tr>
<td>FPT</td>
<td>No</td>
<td></td>
<td>200 (46.5)</td>
<td>196 (45.6)</td>
<td>34 (7.9)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td></td>
<td>9 (31.0)</td>
<td>14 (48.3)</td>
<td>6 (20.7)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td></td>
<td>112 (44.3)</td>
<td>124 (42.3)</td>
<td>17 (6.7)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td>99 (46.1)</td>
<td>91 (42.3)</td>
<td>25 (11.6)</td>
<td></td>
</tr>
<tr>
<td>First RS</td>
<td>&lt; 5</td>
<td></td>
<td>208 (45.3)</td>
<td>211 (46.0)</td>
<td>40 (8.7)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>≥ 5</td>
<td></td>
<td>3 (33.3)</td>
<td>4 (44.4)</td>
<td>2 (22.2)</td>
<td></td>
</tr>
<tr>
<td>First US</td>
<td>&lt; 3 cm</td>
<td></td>
<td>207 (45.0)</td>
<td>211 (45.9)</td>
<td>42 (9.1)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>≥ 3 cm</td>
<td></td>
<td>4 (50.0)</td>
<td>4 (50.0)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

PC – positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI3, and BVD administered subcutaneously at 6 weeks of age.

IN – intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI3 administered intranasally at 3 – 6 days of age.

NC – negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.

NA – not applicable

FPT – failure of passive transfer; cut-off serum Total Protein < 5.2 mg/dL

RS – respiratory score, Wisconsin Calf Scoring Chart

US – ultrasound examination
Table 4.2 Multivariable logistic regression model for the predictions of clinical illness (ILL) in 468 Holstein dairy calves from 3 herds in southwestern Ontario randomly assigned to receive one of three pre-weaning vaccination protocols. ILL calves had a respiratory score > 4 on at least one occasion during the study period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>P – value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>- 0.35</td>
<td>0.51</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.15</td>
<td>0.41</td>
<td>0.71</td>
<td>1.16 (0.52-2.61)</td>
</tr>
<tr>
<td>IN</td>
<td>- 0.16</td>
<td>0.42</td>
<td>0.70</td>
<td>0.85 (0.38-1.93)</td>
</tr>
<tr>
<td>NC</td>
<td>referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.23</td>
<td>0.47</td>
<td>0.62</td>
<td>1.26 (0.50-3.16)</td>
</tr>
<tr>
<td>2</td>
<td>1.58</td>
<td>0.46</td>
<td>&lt; 0.001</td>
<td>4.85 (1.95-12.06)</td>
</tr>
<tr>
<td>3</td>
<td>referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>- 0.41</td>
<td>0.20</td>
<td>0.04</td>
<td>0.66 (0.46-0.98)</td>
</tr>
<tr>
<td>Female</td>
<td>referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Scours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>- 0.65</td>
<td>0.22</td>
<td>&lt; 0.01</td>
<td>0.52 (0.89-0.80)</td>
</tr>
<tr>
<td>Yes</td>
<td>referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

PC – positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI3, and BVD administered subcutaneously at 6 weeks of age.
IN – intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI3 administered intranasally at 3 – 6 days of age.
NC – negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.
Table 4.3 Multivariable logistic regression model for the prediction of CON in 451 Holstein dairy calves from 3 herds in southwestern Ontario randomly assigned to receive one of three pre-weaning vaccination protocols. CON = 3cm or more ultrasonographic lung consolidation on at least one occasion throughout the study period.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>P – value</th>
<th>Odds ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1.25</td>
<td>0.79</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>-0.96</td>
<td>0.46</td>
<td>0.03</td>
<td>0.38 (0.16-0.93)</td>
</tr>
<tr>
<td>IN</td>
<td>-1.45</td>
<td>0.47</td>
<td>&lt;0.01</td>
<td>0.23 (0.09-0.59)</td>
</tr>
<tr>
<td>NC referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.16</td>
<td>0.57</td>
<td>0.77</td>
<td>1.17 (0.39-3.56)</td>
</tr>
<tr>
<td>2</td>
<td>2.64</td>
<td>0.58</td>
<td>&lt;0.0001</td>
<td>14 (4.4-43.82)</td>
</tr>
<tr>
<td>3 referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Dystocia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-0.73</td>
<td>0.35</td>
<td>0.04</td>
<td>0.48 (0.25-0.95)</td>
</tr>
<tr>
<td>Yes referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Rib fracture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>-1.31</td>
<td>0.56</td>
<td>0.02</td>
<td>0.27 (0.09-0.81)</td>
</tr>
<tr>
<td>Yes referent</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

PC – positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI3, and BVD administered subcutaneously at 6 weeks of age.
IN – intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI3 administered intranasally at 3 – 6 days of age.
NC – negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.
Table 4.4 A 2 x 2 contingency table comparing the distribution of CON and ILL in Holstein dairy calves (n = 468).

<table>
<thead>
<tr>
<th>ILL</th>
<th>CON</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>181</td>
<td>70</td>
</tr>
<tr>
<td>0</td>
<td>72</td>
<td>145</td>
</tr>
</tbody>
</table>

ILL – At least one respiratory score > 4
CON – At least one ultrasound exam with 3 cm lung consolidation
Table 4.5 Multivariable linear regression model for ADG on 421 Holstein dairy calves from 3 herds in south-western Ontario randomly assigned to receive one of three pre-weaning vaccination protocols. Calves dying before 56 d of age were not included in the analysis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>SE</th>
<th>95% CI</th>
<th>P – value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.513</td>
<td>0.0761</td>
<td>0.364, 0.663</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.00439</td>
<td>0.0638</td>
<td>−0.121, 0.130</td>
<td>0.95</td>
</tr>
<tr>
<td>IN</td>
<td>−0.0426</td>
<td>0.0655</td>
<td>−0.171, 0.0862</td>
<td>0.52</td>
</tr>
<tr>
<td>NC</td>
<td>referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Herd</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>−0.0549</td>
<td>0.0606</td>
<td>−0.174, 0.0642</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>−0.173</td>
<td>0.0625</td>
<td>−0.295, −0.0497</td>
<td>0.006</td>
</tr>
<tr>
<td>3</td>
<td>referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Twin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.138</td>
<td>0.0253</td>
<td>0.0882, 0.188</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Yes</td>
<td>referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dystocia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.0467</td>
<td>0.0212</td>
<td>0.00497, 0.0884</td>
<td>0.028</td>
</tr>
<tr>
<td>Yes</td>
<td>referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>House</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outdoors</td>
<td>−0.0285</td>
<td>0.0378</td>
<td>−0.103, 0.0459</td>
<td>0.45</td>
</tr>
<tr>
<td>Indoors</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rib fracture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.0565</td>
<td>0.0285</td>
<td>−0.000616, 0.113</td>
<td>0.048</td>
</tr>
<tr>
<td>Yes</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.0447</td>
<td>0.0143</td>
<td>0.0166, 0.0728</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Female</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Birth weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 40 kg</td>
<td>−0.0645</td>
<td>0.0212</td>
<td>−0.106, −0.0228</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>40 – 46 kg</td>
<td>−0.0217</td>
<td>0.0173</td>
<td>−0.0557, 0.0123</td>
<td>0.21</td>
</tr>
<tr>
<td>&gt; 46 kg</td>
<td>Referent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrolment age, d</td>
<td>0.00332</td>
<td>0.0060</td>
<td>−0.0086, 0.0152</td>
<td>0.58</td>
</tr>
<tr>
<td>Protocol * Herd Interaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC * 1</td>
<td>0.0494</td>
<td>0.0733</td>
<td>−0.0947, 0.193</td>
<td>0.50</td>
</tr>
<tr>
<td>PC * 2</td>
<td>−0.0804</td>
<td>0.0687</td>
<td>−0.216, 0.0547</td>
<td>0.4</td>
</tr>
<tr>
<td>IN * 1</td>
<td>0.00023</td>
<td>0.0754</td>
<td>−0.149, 0.148</td>
<td>0.99</td>
</tr>
</tbody>
</table>

PC – positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI₃, and BVD administered subcutaneously at 6 weeks of age.
IN – intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI₃ administered intranasally at 3 – 6 days of age.
NC – negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.
Figure 4.1 Predicted probability of CON by protocol adjusted over all other model variables. PC = white; IN = dark grey; NC = light grey. N = 451. Error bars represent standard error of the mean. CON = occurrence of 3 cm lung consolidation or more during at least one ultrasound examination. PC = positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI₃, and BVD administered subcutaneously at 6 weeks of age. IN = intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI₃ administered intranasally at 3 – 6 days of age. NC = negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.
Figure 4.2 Differences in least squares mean average daily gain (ADG; kg/d) showing the interaction between TREATMENT and HERD. PC = white; IN = dark grey; NC = light grey. The linear mixed model controlled for protocol, herd, twin, housing, dystocia, rib fractures, birth weight, and age at enrollment. Different letters within a herd category are different (P < 0.05). PC = positive control: 2 mL commercially available multivalent injectable vaccine against BRSV, IBR, PI₃, and BVD administered subcutaneously at 6 weeks of age. IN = intranasal treatment: 2 mL commercially available trivalent injectable vaccine against BRSV, IBR, and PI₃ administered intranasally at 3 – 6 days of age. NC = negative control: 2 mL sterile saline administered both intranasally and subcutaneously at 3 – 6 days of age and 6 weeks of age.
CHAPTER 5. THE EFFECT OF RESPIRATORY DISEASE ON LYING BEHAVIOR IN HOLSTEIN DAIRY CALVES

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T. L. Ollivett,*, D. V. Nydam†, T. Duffield*, K. E. Leslie,*, G. Zobel‡, J. Hewson§, J. Caswell#, D. Kelton*†

*Department of Population Medicine, Ontario Veterinary College, University of Guelph, Guelph N1G 2W1

†Department of Population Medicine and Diagnostic Sciences, Cornell University, College of Veterinary Medicine, Ithaca, NY 14850

‡Faculty of Land and Food System, University of British Columbia, Vancouver, BC, V6T 1Z4

§Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1

#Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, ON N1G 2W1

Corresponding author: David Kelton, Department of Population Medicine, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1; Phone: (519) 824 - 4120 ext. 54808 Fax: (519)763 - 8621; Email: dkelton@uoguelph.ca
5.1 ABSTRACT

The objective of this cohort study was to determine the effect of naturally occurring bovine respiratory disease (BRD) on lying behavior in preweaned Holstein dairy calves. This study was carried out on one commercial dairy in south-western Ontario, Canada during November and December 2012. Calves were enrolled at 10 days of age and were grouped according to vaccination status (n = 19 vaccinated and n = 20 unvaccinated). Each calf was examined at weekly intervals for signs of BRD before and during the peak 4 week period when calves are at risk for developing disease. Respiratory scoring (RS) and thoracic ultrasonography (US) were performed at each of the five visits. Individual electronic accelerometers recorded lying behavior throughout the 4 week period. Descriptive statistics and repeated measures linear models were fit using commercially available software. Fever, sickness, and lung consolidation increased from 11%, 3%, and 0%, respectively, at the first examination to 33%, 33%, and 58%, respectively, at the last examination (P < 0.05). Overall, calves spent 20.6 ± 0.7 h/d (mean ± SE) or 86% of the day lying down. Lying time (LT) decreased by 4 ± 1 min/d for each successive day of age. Fever was associated with 44 ± 14 min/d additional LT. Calves housed in group pens had lower but not statistically significant LT than those housed in single pens. Also, housing type confounded the LT estimate for age. Ultrasonographic lung consolidation and health status (RS > 4) were not significantly associated with LT. Lying bout duration (BD) was 72 (61 – 85) min/d (median; IQR), and the lying bout frequency (BF) was 16 bouts per day (13 – 18) (median; IQR). Health status and lung consolidation were not significantly associated with BD or BF. Fever numerically increased BD but was not associated with BF. Monitoring LT in preweaned dairy calves might have a place in identifying febrile animals requiring individual examination and possible intervention. Further studies are needed to determine if early identification and
treatment of animals experiencing fever improves future health and performance as compared to traditional methods of disease identification. Lastly, researchers should consider monitoring rectal temperature during behavioral studies to assess for bias due to undetected fevers.

Key words: dairy calf pneumonia, accelerometer, ultrasound

5.2 INTRODUCTION

Bovine respiratory disease (BRD) is the second most common cause of illness in dairy calves, affecting approximately 12 – 16% of all pre-weaned calves annually (Stanton et al., 2010; USDA, 2010). Variability in the within herd prevalence exists and can reach as high as 90% (Heins et al., 2014). Moreover, in North America, 22% of all pre-weaned calf deaths result from this disease process (USDA, 2010). This mortality has not improved over the last twenty years despite advances in preventative and therapeutic strategies (Gorden and Plummer, 2010).

The short-term costs associated with managing BRD are approximately $10 to $16 per calf (as reviewed by Gorden and Plummer, 2010). Long term effects might increase such estimates as a result of reductions in post-weaning growth rates, longevity and future production (Bach, 2011; Stanton et al., 2012). Early diagnosis and treatment is needed to reduce treatment failures and the subsequent negative outcomes and economic impacts associated with chronic disease (McGuirk, 2008).

Early diagnosis and management of sick calves falls primarily under the responsibility of on-farm personnel, with the guidance of the herd veterinarian (Gorden and Plummer, 2010). Unfortunately, the diagnostic sensitivity of on-farm personnel in identifying BRD as the cause of death can be problematic. For instance, Sivula et al. (1996) found that only 56% of ill calves were identified, with the remaining calves being left untreated. As such, systematic respiratory
scoring (RS) and thoracic ultrasonography (US) protocols have been developed to improve diagnostic accuracy and ameliorate the economic and welfare implications of BRD (McGuirk, 2008; Buczinski et al., 2013; Ollivett PhD Thesis Ch 2 and Ch 3). Respiratory scoring systems are useful; however, subclinical disease and the subjective nature of scoring systems result in both false negative and false positive diagnoses. Conversely, US has excellent diagnostic sensitivity and specificity for both clinical (Rabeling et al., 1998) and subclinical bronchopneumonia (Ollivett PhD Thesis Ch 2.) The severity of ultrasonographic lung consolidation is highly associated with the amount of lung consolidation at gross post-mortem examination (Ollivett PhD thesis Ch. 2 and Ch. 3). In addition, inter and intra-observer reliability for US is good to excellent (Buczinski et al., 2013). A major drawback of US is that it requires individual animal handling. Therefore, an automated tool able to detect behavioral changes during the early stage of disease would be a more practical approach to identifying and managing disease.

Sickness behavior, a coordinated set of responses to an infectious or inflammatory condition, (Johnson, 2002) can be assessed, in part, by analyzing lying behavior of affected animals (Borderas et al., 2008; von Keyserlingk et al., 2009). Changes in behaviour have been used successfully to identify illness in adult dairy cattle (Huzzey et al., 2007). Previous work addressing lying behavior often used visual observation from video recorders (Hänninen et al., 2005; Borderas et al., 2008) whereas technology now exists to objectively evaluate specific aspects of lying patterns without the labor of analyzing large quantities of video (Müller and Schrader, 2003; Bonk et al., 2013). Automated measures of lying behavior that have been validated in the dairy calf consist of total lying time (LT), total standing time, number and
duration of individual lying and standing bouts, and the laterality of the lying position (Bonk et al., 2013).

The effect of specific diseases on lying behavior in dairy calves has not been extensively studied. However, a low dose injection of bacterial endotoxin does appear to alter lying patterns (Borderas et al., 2008); this is relevant to the current study as endotoxin is a cell wall component of gram negative bacteria, including the common respiratory pathogens *M. haemolytica* and *P. multocida*. Therefore, the objective of this study was to determine the effect of naturally occurring BRD on lying behavior in preweaned Holstein dairy calves. Information gained from this study might help improve the timeliness and accuracy of BRD diagnosis on commercial dairy farms. Additionally, this analysis will provide baseline lying indices for researchers studying lying patterns in dairy calves in the future.

### 5.3 MATERIALS AND METHODS

#### Animals and Facility

This study was carried out on one commercial dairy farm in south-western Ontario, Canada, during November and December 2012. A convenience samples of 39 Holstein calves were enrolled at 10 days of age and were grouped according to vaccination status (unvaccinated, n = 19: male = 9, female = 10; vaccinated, n = 20: male = 10, female =10). Each group was housed in identical but separate barns. Vaccine treatments consisted of a commercially available product designed for intranasal use (2 mL Inforce 3, intranasal (IN), Zoetis, NY, NY) or placebo (2 mL sterile saline, IN) and were administered via a single-use plastic nasal cannula (Zoetis, NY, NY) into one nostril at 3 – 6 d of age. Vaccination status of the barn was randomly assigned prior to the start of the study as part of a separate research project (Ollivett PhD Thesis Ch 4).
For the purposes of the previous study, treatments were assigned at the barn level to prevent contamination of control calves from nasal shedding of virus by the vaccine treated calves. Respiratory scoring (McGuirk, 2008) and US were performed by a study author (TO) who was blinded to vaccination status (VS). Treatments were administered by a member of the research team not involved in any of the examinations.

**Farm specific calf management.**

Single source colostrum (2 L) was offered by bottle within 30 minutes of birth. An additional 4 L was offered in 2 separate feedings over the 24 h following birth. Esophageal tube feeding was used only when a calf completely refused to suck. Calves were fed 6 L of unpasteurized whole milk daily while housed in individual 1.16 m x 2 m pens until approximately 3 weeks of age. Calves were then moved as a group of 20 animals to a 10 m x 20 m pen. Eight liters of unpasteurized whole milk was offered via an automated system in the group pens (Forster Technik, De Laval, Peterborough, ON). Calves were allowed 3 L per feeding bout within a 3 h period. There was 1 nipple available per group. Free choice starter was offered within the first 3 days of life. Water was not available until the calves reached the group pen. Individual and group pens were bedded with a sawdust base and a top layer of straw. Tulathromycin (2.5 mg/kg; 1.1 mL/100 lb, subcutaneously once) was administered to all calves at movement from the single pen to group pen. This study was conducted with the approval of the University of Guelph’s Animal Care Committee (AUP #11R110).

**Respiratory scoring and ultrasonography data collection**

Respiratory scoring (RS; Appendix 1; Lago et al., 2006; McGuirk, 2008) and US were performed at weekly for 4 weeks. Briefly, the RS assigned 0 – 3 points for each the following
categories: rectal temperature, nasal discharge, cough, and ocular discharge or ear position. Respiratory scores could range from 0 to 12 and any calf with RS > 4 was considered sick (McGuirk, 2008). Regarding US, both hemithoraces were scanned using a portable linear rectal ultrasound set at a depth of 9 cm, frequency of 6.2 MHz, and gain of 16 dB (Near 13 dB; Far 36 dB; Ibex Pro, E. I. Medical, Loveland, CO). Approximately 300 mL of 70% isopropyl alcohol was applied to the hair as a transducing agent. The hair of the thorax was not shaved or clipped. Systematic scanning of the cranial ventral lung started dorsally at the level of the scapula in the right/left 6th intercostal space (ICS), and moved cranially to the right 1st or left 2nd ICS. The lung adjacent to the right 4th through 1st ICS and left 4th through 2nd ICS was examined with the US transducer between the forelimb and the cranial ventral thoracic body wall. Within each ICS, the probe was parallel to the rib and advanced ventrally towards the sternum until specified US landmarks were visualized before moving to the next cranial ICS (Tables 5.1 and 5.2).

Peripheral lung tissue was considered normal when a hyperechoic line with reverberation artifact was present, signifying the interface between the high impedance tissue of the thorax and the low impedance tissue of the lung (Blond et al., 2009). Consolidated lung appeared hypoechoic and lacked both the bright white band at the pleural interface and reverberation artifact. The depth and dorsal to ventral extent of consolidated lung were measured within each ICS using the 1cm grid lines on the US screen. The total amount of US consolidation was calculated as the sum of all superficial consolidations. Percentage of consolidated lung (PC) was calculated by dividing the total amount of consolidation by the total amount of lung available to scan. The amount of lung available to scan was measured as the US distance between the dorsal most extent of the lung to the ventral image landmark (Tables 5.1 and 5.2) in 32 non-study
animals of equivalent breed, age and weight. The non-study animals were apparently healthy and scanned by one study author (TO). All observations were spoken and recorded using a digital voice recorder and later manually transcribed into a database (Microsoft Access 2010, Redmond, WA).

**Lying Time Analyses**

Accelerometers recorded lying behavior for each calf (HOBO Pendant G Acceleration Data Logger, Onset Computer Corporation, Pocasset, MA). The accelerometers were attached according to methods as outlined in UBC AWP (2013). Felt cut to a 25 cm square was wrapped around the metatarsus to prevent rubbing and the accelerometer was secured in place with half of a standard 10 cm roll of vet wrap (Co-Flex, Andover Coated Products, Salisbury, MA). Prior to application, the accelerometer was placed into a small sealed plastic bag to prevent water damage. Accelerometers were applied during the first exam, replaced approximately 14 d later, and removed at the last examination. Data recording started at noon of the first study day, 4 hours after application. Lying time, lying bouts, and bout duration were compiled using SAS (UBC AWP, 2013).

**Statistical Analyses**

Data for PC, temperature, and RS were dichotomized to create the new variable ultrasound status (USS), fever status (FS), and health status (HS) for each calf at each examination. Percent consolidation $\geq 3\%$ was used to categorize calves into affected (USS = 1) and unaffected (USS = 0) groups based on the amount of consolidation present when 1/3 of the cranial aspect of the cranial lobe or the right middle lung lobe is affected with lobar pneumonia. A temperature of 39.4°C (103.0 °F) was used to categorize calves into affected (FS = 1) and
unaffected (FS = 0) groups (McGuirk, 2008). Respiratory score of ≥ 5 was used to categorize calves into sick (HS = 1) and non-sick (HS = 0) groups (McGuirk 2008). Vaccination status (VS) was also coded as vaccinated (VS = 1) and non-vaccinated (VS = 0) groups. Fisher’s exact and Pearson’s chi-square tests were used to screen for differences in USS, HS, and FS proportions at each examination (Stata 12.1, Stata Corp LP. College Station, TX). Continuous data were analyzed with Wilcoxon rank sum due to the lack of normality for some variables (Kaleidograph 4.1.1, Synergy Software, Reading, PA). Pearson’s correlation coefficients were estimated to assess the correlations between model predictions and actual observations (SAS version 9.4, SAS Institute Inc., Cary, NC).

A repeated measures analysis was performed using the MIXED procedure (SAS version 9.4, SAS Institute Inc., Cary, NC). Models were fit for total lying times (LT), lying bout duration (BD), and lying bout frequency (BF). Total LT, BD, and BF were calculated for the 24 hours prior to each US and RS for each time point except Exam 1 when the 24 h after the exam was used. Fixed effects of interest were VS, USS (prevalent cases), FS (prevalent cases), and HS (prevalent cases). Repeated measures were obtained from calves nested within VS. Sex (0 = male; 1 = female), age (days; continuous variable), and housing type (group pen = 1; single pen = 0) were included as potentially confounding variables (CFV). Separate models were created without (preliminary models) and with FS, USS, and HS (secondary models) to assess both the full and confounding effect of VS on each outcome; as FS, USS, and HS are intervening variables between VS and LT (Figure 5.1). In each case, all variables including all biologically plausible two-way interactions were initially included in the model. The correlation structure was selected based on the lowest Akaike’s Information Criterion. Manual backwards stepwise elimination was used to refine the model to include variables that were significant at the P ≤ 0.05
level. Additionally, a change in estimate criterion of ≥ 25% was used to assess for confounding prior to the final elimination of a variable. Ultrasound score and HS were forced into the model when necessary as specific predictors of interest. Each model was assessed graphically for outliers and the normality of residuals was tested using the Shapiro-Wilk, Anderson-Darling, Kolmogorov-Smirnov, and Cramer-von Mises tests. The residuals resulting from the BD and BF repeated measures model were not normally distributed according and were therefore log-transformed. Alpha was P < 0.05.

5.4 RESULTS

Thirty-nine calves were originally enrolled into the study. Three calves were removed because of failure of the accelerometer (n = 2) and euthanasia due to cerebellar ataxia (n = 1). Descriptive characteristics for age, PC, LT, BD, and BF at each examination are summarized in Table 5.3. The prevalence and incidence of fever, sickness, and moderate to severe lung consolidation for all calves at each examination are summarized graphically in Figure 5.2.

In the preliminary model, age was associated with LT (P = 0.03; \( R^2 = 0.18 \)) but VS was not (P = 0.68; model not shown). In the secondary model in which USS, HS and FS were forced, calves spent 20.6 ± 0.7 h/d (mean ± SE) or 80% of the day lying down when all variables were set to the referent. Lying time decreased by 4 ± 1 min/d for each successive day of age (P < 0.001). Fever was associated with 44 ± 14 min/d additional LT (P < 0.01). The difference in least square means for LT between febrile and afebrile calves is shown graphically at specified ages in Figure 5.3. Calves housed in group pens had numerically lower LT than those housed in single pens (P = 0.11). However, the removal of housing type from the model resulted in a 39% change in the age estimate suggesting that housing type confounds the effect of age on LT. Moderate to severe lung consolidation and HS were not significantly associated with lying time (P > 0.20).
Throughout the entire study period, the BF averaged 16 (13 – 18) (median; IQR), and the BD averaged 72 (61 – 85) min/d (median; IQR). Health status and USS were not significantly associated with BD or BF ($P > 0.30$). Fever numerically increased BD ($P = 0.07$) but was not associated with BF ($P = 0.30$). The type 3 tests of fixed effects for the secondary LT, BD, and BF models are summarized in Table 5.4.

### 5.5 DISCUSSION

In this study, we successfully documented the development of naturally occurring respiratory disease in pre-weaned dairy calves using clinical examination and diagnostic ultrasonography. As per the main objective, we determined the impact of such disease on lying behavior and found that age and fever were significant predictors of LT but not BF or BD. Intranasal vaccination did not directly affect LT or BD and did not confound any variables within the models. There was a significant interaction between VS and sex where vaccinated bull calves had significantly higher BF than unvaccinated males or females of either VS. Although statistically significant, one additional lying bout per day is likely biologically unimportant, especially considering that the model accounts for relatively little of the variation in BF. Although it was necessary to incorporate VS into the model because of the potential impacts on disease status, this study was not designed to fully investigate the benefits of a specific vaccine protocol, and as such VS will not be discussed further.

The lying behavior of pre-weaned dairy calves has previously been studied (Webster et al., 1985; Chua et al., 2002; Panivivat et al., 2004; Hänninen et al., 2005). The general purpose of this older body of literature was to compare the effect of different management factors, such as type of rearing system (Webster et al., 1985), bedding material (Panivivat et al., 2004), or social groupings (Hänninen et al., 2005), on lying patterns. Very little consideration has been given in
the literature regarding how disease affects lying behaviours such as time, bout duration and bout frequency. Since the effect of respiratory disease was the focus of current study, precise methodology was utilized to differentiate the effects of the disease including fever, abnormal respiratory score, and ultrasonographic consolidation. The only predictor that affected lying behaviour, specifically lying time, was fever. These results suggest that automated technologies, such as accelerometers, while potentially useful in detecting febrile calves, will not be useful in detecting cases of respiratory disease in which fever is absent.

Fever is one component of the body’s response to infectious disease and results from pro-inflammatory cytokines acting on the hypothalamus to increase the thermoregulatory set-point (Johnson, 2002). Metabolism increases to generate additional heat, and movement is restricted to prevent radiant heat loss (Johnson, 2002). This latter phenomenon manifested in our study as significantly longer LT, and suggests that the circulating cytokine profile is different between febrile calves with and without lung consolidation or other clinical signs associated with respiratory disease. This finding is in contrast with Borderas et al. (2008) who found that the administration of endotoxin, which caused fever, did not affect total LT. It is possible that the low dose challenge and short duration of fever in Borderas et al., (2008) as well as animal husbandry and period of analysis could contribute to such differences.

In the current study, calves spent 86% of the day lying down, slightly more than the 70 - 80% described in previous reports (Chua et al., 2002; Panivivat et al., 2004; Hänninen et al., 2005). Furthermore, age related reductions in lying time of 10 – 20% have been documented between the first and fourteenth weeks of age, similar to our findings (Webster et al., 1985; Panivivat et al., 2004). The association between age and LT is likely due to the transition from a functional monogastric animal to a functional ruminant. Additional standing time allows for the
dry matter consumption that is required for normal rumen development. Although housing type was not a significant variable in the model, it did confound the estimates for age in the LT model. This means that one will overestimate the effect of age on LT if housing type is not included in the analysis.

This study was limited in its ability to evaluate subtle changes associated with the nested structure of behavioral activities as described by Magnusson (2000). Accelerometers only measure the time spent with the body in a certain position relative to the ground (Bonk et al., 2013). Examples of the nested structure of behavior include body position while lying down, i.e. sternal vs. lateral recumbency, lying awake vs. lying asleep, or body position while standing, i.e. standing with head up and engaged vs. standing with head down (Borderas et al., 2008). A more sensitive analysis of intra-period behavioral changes (Trénel et al., 2009) using ethograms accompanied by video monitoring might have shown associations between these behavioral patterns and USS or HS that were not otherwise discernible.

The prevalence of significant lung consolidation in this herd increased progressively between the first and fifth examination. Interestingly, the incidence increased initially but remained stable from the third examination until the end of the study. This pattern suggests that the duration of lung consolidation is contributing to the overall prevalence of disease based on the epidemiological principle “prevalence ≈ incidence x duration of disease.” When comparing these numbers with the prevalence and incidence of respiratory score or fever, one can appreciate that the biology of enzootic pneumonia is more complicated than clinical signs might suggest.
5.6 CONCLUSIONS

This study documented the relationships between clinical parameters, diagnostic ultrasonography, and lying behaviors in pre-weaned dairy calves from a herd with a high prevalence of endemic respiratory disease. Total LT was significantly affected by fever and age. Monitoring LT in preweaned dairy calves might have a place in identifying febrile animals requiring individual examination and possible intervention. Further studies are needed to determine if identification of such animals results in better outcomes as compared to traditional methods of disease identification. Additionally, researchers should consider monitoring body temperature during behavioral studies to assess for biased distributions of fever. This is especially important since clinical signs do not always predict fever and will not always indicate which animal should be tested. Lastly, since quantity and quality of liquid feed, facility design, and animal husbandry practices could influence lying patterns of dairy calves, inferences should only be extrapolated to similarly grown dairy calves.

ACKNOWLEDGMENTS

Financial support for this study was provided by the Ontario Ministry of Agriculture, Food, and Rural Affairs and Zoetis. Special thanks go to the dedicated research technicians: Vivianne Bielmann, Sam Deelen, Brittany Stinson, Patrick Chung, and Sarah Stanger-Guy for their tireless work; and especially the dairy producer for all of the kindness shown to us during the study period.
5.7 REFERENCES


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Table 5.1 Landmarks for the lobes of the right lung in dairy calves during an abbreviated ultrasonographic examination focusing on the cranioventral lung.

<table>
<thead>
<tr>
<th>R – ICS¹</th>
<th>Lung lobe</th>
<th>Caudal</th>
<th>Middle</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral landmark(s)</td>
<td>Diaphragm</td>
<td>6</td>
<td>5</td>
<td>3 - 4</td>
<td>1 - 2</td>
</tr>
<tr>
<td></td>
<td>CCJ² &amp; pleural deviation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Two blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ right intercostal space(s)
² costochondral junction.
Table 5.2 Landmarks for the lobes of the left lung in dairy calves during an abbreviated ultrasonographic examination focusing on the cranioventral lung.

<table>
<thead>
<tr>
<th>Lung lobe</th>
<th>Caudal</th>
<th>Caudal aspect of cranial lobe</th>
<th>Cranial aspect of cranial lobe</th>
</tr>
</thead>
<tbody>
<tr>
<td>L – ICS(^1)</td>
<td>6</td>
<td>4 - 5</td>
<td>2 - 3</td>
</tr>
<tr>
<td>Ventral landmark(s)</td>
<td>Diaphragm</td>
<td>CCl(^2) &amp; pleural deviation</td>
<td>Heart</td>
</tr>
</tbody>
</table>

\(^1\) left intercostal space(s)

\(^2\) costochondral junction
Table 5.3 Descriptive characteristics (median, interquartile range) for age, percentage of consolidated lung (PC), lying time (LT), lying bout duration (BD), and lying bout frequency (BF) in 36 Holstein calves at each examination.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Exam 1</th>
<th>Exam 2</th>
<th>Exam 3</th>
<th>Exam 4</th>
<th>Exam 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, d(^1)</td>
<td>10 (8 – 11)(^c)</td>
<td>16 (14 – 18)(^d)</td>
<td>21 (20 – 25)(^c)</td>
<td>29 (27 – 31)(^b)</td>
<td>35 (33 – 36)(^a)</td>
</tr>
<tr>
<td>PC(^2)</td>
<td>0.7(0 – 0.7)(^b)</td>
<td>1.5(0 – 4.0)(^bc)</td>
<td>4.1(1 – 10)(^ac)</td>
<td>5.3(2 – 8.9)(^a)</td>
<td>6.9(4 – 10)(^a)</td>
</tr>
<tr>
<td>LT, min/d</td>
<td>1152(1078 – 1220)(^a)</td>
<td>1141(1085 – 1188)(^a)</td>
<td>1104(1034 – 1139)(^b)</td>
<td>1106(1027 – 1169)(^b)</td>
<td>1037(975 – 1081)(^bc)</td>
</tr>
<tr>
<td>BD, min/bout</td>
<td>72(62 – 82)(^bc)</td>
<td>82(65 – 90)(^a)</td>
<td>69(53 – 84)(^bd)</td>
<td>69(60 – 78)(^bd)</td>
<td>70(71 – 84)(^acd)</td>
</tr>
<tr>
<td>BF, no./d</td>
<td>16(15 – 18)(^a)</td>
<td>15(13 – 17)(^bc)</td>
<td>16(14 – 19)(^a)</td>
<td>16(14 – 18)(^ac)</td>
<td>15(13 – 17)(^bc)</td>
</tr>
</tbody>
</table>

\(^a\)–\(^d\) medians without a common superscript within a row differ (P ≤ 0.05)

\(^1\) day

\(^2\) the median amount of all ultrasonographic consolidations
Table 5.4 Type 3 tests of fixed effects from the secondary models for lying time (LT), lying bout duration (BD), and lying bout frequency (BF). Fever, USS, and HS were forced into each model (MIXED procedure; SAS version 9.4, SAS Institute Inc., Cary, NC).

<table>
<thead>
<tr>
<th>Variable</th>
<th>NDF</th>
<th>DDF</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lying time model, $R^2 = 0.27$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>139</td>
<td>12.23</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
<td>29</td>
<td>9.85</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Housing type</td>
<td>1</td>
<td>35</td>
<td>2.67</td>
<td>0.11</td>
</tr>
<tr>
<td>USS</td>
<td>1</td>
<td>25</td>
<td>1.51</td>
<td>0.23</td>
</tr>
<tr>
<td>HS</td>
<td>1</td>
<td>25</td>
<td>0.59</td>
<td>0.45</td>
</tr>
<tr>
<td><strong>Bout duration model, $R^2 = 0.04$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
<td>29</td>
<td>3.51</td>
<td>0.07</td>
</tr>
<tr>
<td>HS</td>
<td>1</td>
<td>25</td>
<td>0.57</td>
<td>0.46</td>
</tr>
<tr>
<td>USS</td>
<td>1</td>
<td>25</td>
<td>0.31</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Bout frequency model, $R^2 = 0.16$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>32</td>
<td>5.49</td>
<td>0.03</td>
</tr>
<tr>
<td>VS*sex</td>
<td>1</td>
<td>32</td>
<td>5.36</td>
<td>0.03</td>
</tr>
<tr>
<td>VS</td>
<td>1</td>
<td>32</td>
<td>3.20</td>
<td>0.08</td>
</tr>
<tr>
<td>Fever</td>
<td>1</td>
<td>29</td>
<td>1.10</td>
<td>0.30</td>
</tr>
<tr>
<td>HS</td>
<td>1</td>
<td>25</td>
<td>0.89</td>
<td>0.35</td>
</tr>
<tr>
<td>USS</td>
<td>1</td>
<td>25</td>
<td>0.01</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Figure 5.1 Causal diagram illustrating the effect of vaccination status (VS), fever, health status (HS), ultrasound score (USS), sex, age, and housing on lying time (LT) demonstrating that HS, Fever, and USS are intervening variables between VS and LT. Potential confounding effects have been left out for the sake of simplicity of the illustration.
Figure 5.2 Prevalence and incidence of (A) moderate to severe ultrasonographic lung consolidation, (B) fever, and (C) sick RS in 36 pre-weaned Holstein dairy calves. Dash line (--) is prevalence. Solid line (—) is incidence risk.
Figure 5.3 Differences of least squares means (± SE) in lying time between calves with and without fever at the following ages: 7 d (minimum), 14 d (10th percentile), 21 d (median), 30 d (75th percentile), and 43 d (maximum age) in 36 Holstein calves (n = 180 observations). The linear mixed model included vaccination status, housing type, health status, and ultrasound status.
CHAPTER 6. CONCLUSIONS AND FUTURE RESEARCH

6.1 GENERAL DISCUSSION AND LIMITATIONS

In general, inconsistent clinical signs hinder the appropriate diagnosis of BRD in young dairy calves. Portable thoracic US with a linear probe designed for transrectal US can be used by a veterinarian as a valid method of diagnosing lung lesions regardless of the clinical state of the calf.

The work presented in Chapter 2 demonstrated that US has excellent sensitivity (94%; 95% CI: 69 - 100%) and specificity (100%; 95% CI: 64 - 100%) when used to diagnose lung lesions in calves affected with subclinical BRD. While BALF total nucleated cell count should not be used to classify disease status, reasonable success can be had when using a neutrophil proportion cut-off of 4% if ultrasonography is unavailable. A better understanding of the effect of subclinical BRD on future dairy calf health and performance is lacking, therefore, herd level and animal level implications of subclinical BRD warrant further investigation. In this study, the significance of comet-tailing could not be fully elucidated. One calf with severe comet-tails did have an area of consolidation hidden by aerated lung; however, all other cases were grossly normal. In the human literature, comet-tails are often referred to as a sign of edema, however, they are so commonly seen in dairy cattle as to be considered a variation of normal (Scott, 2013). It is possible that with a larger sample size a difference in cell counts could have been detected between completely normal lungs, comet-tailing and the varying degrees of consolidation. Also, the population of calves used in this study were selected based on convenience and the need to fulfill a quota of specifically sized lesions. Changing this study to a randomly selected
population of calves with varying degrees of clinical abnormalities might change the BALF findings as well as the sensitivity and specificity of US.

In Chapter 3, US lung consolidation developed soon after infection, progressed rapidly, and persisted longer than the clinical signs associated with BP in post-weaned Holstein bull calves. The %US was significantly different between Control and Infected calves and was highly correlated with the amount of lung consolidation present at PM. Although more work is needed to assess the value of identifying lung consolidation in the absence of clinical signs, thoracic US might prove to be a useful diagnostic tool in future BRD research. It is interesting that despite the lack of supportive care or antimicrobial treatment, all of the calves in this study had significant clinical improvement by the end of the study despite severe bronchopneumonia at post-mortem examination. It is possible that the initial fever and abnormal respiratory score were simply the result of inflammation responding to a large amount of gram negative bacterial endotoxin present within the airways, and not infection, per se. Then, following neutralization of endotoxin, the body temperature and respiratory scores returned to normal. In many clinical situations, antimicrobials ± anti-inflammatories would have been administered followed by a feeling of success 24 – 48 hours later when the animal appeared improved. How often do we inappropriately credit an antimicrobial for “fixing” an infection when in reality, the underlying problem wasn’t resolved? Future priorities should include antimicrobial efficacy studies in which specific outcomes including onset, duration, and resolution of US lung lesions are assessed. By doing so, true antimicrobial effects will not be obscured by temporary improvements in clinical conditions associated with the animals’ own immune system.

Chapter 4 demonstrated that a commercially available trivalent IN vaccine has the potential to reduce the lung lesions associated with BRD and improve growth in young dairy
cattle. Although these findings were significant, herd factors play a role in determining whether or not changes in average daily gain will be appreciated. Also, IN vaccination did not eliminate the risk of disease in the current study; therefore this practice should not be viewed as a “magic bullet”. This study was limited by the small number of farms enrolled as well as the significant differences in size and disease challenges between the two research centres and the commercial herd. Our inability to see an effect on growth from the IN vaccine in the two smaller herds was likely due to the significant herd effect on growth and small herd size. Running a similar study on several dozen farms with various management styles and disease challenges would enhance our ability to predict how the IN vaccine will work in different management systems.

Chapter 5 documented the relationships between clinical parameters, diagnostic ultrasonography, and lying behaviors in pre-weaned dairy calves from a herd with a high prevalence of endemic respiratory disease. Total LT was significantly affected by fever and age but not US lung lesions. Although it was a small study, these results highlight the inconspicuous nature of BRD and the lack of obvious behavioural changes in some calves despite extensive lung lesions. It would have been of interest to carry this study out for a longer period of time to evaluate the effect of chronicity of US lung lesions on behavioural patterns.

6.2 FUTURE RESEARCH

This body of work has demonstrated the usefulness of US to quickly and reliably detect lung lesions in young dairy calves affected by BRD. With appropriate extension efforts, teaching practitioners and researchers how to scan and interpret images would give them a valid tool with which they can systematically monitor calf lung health. However, there is a learning curve to US and data collected by improperly trained individuals could result in faulty information. For example, in Chapter 2, 4/5 of the most severely affected calves would have been classified as
normal if the first and second intercostal spaces were not examined. Technique is imperative when investigating subclinical BRD and a certification process might be necessary to ensure the collection of accurate, high quality data.

Table 4 in Chapter 4 shows the breakdown of calves by CON and ILL in a 2 x 2 table highlighting the four populations of calves on dairies: 1) normal calves, 2) calves with lung lesions and high RS, 3) calves with lung lesions and low RS, and lastly 3) calves with no lung lesions and high RS. Initially, future studies should focus on identifying how CON and ILL affect future performance outcomes such as growth, age at first calving, milk production, and longevity in the aforementioned populations of calves, as these are the outcomes of interest to bovine practitioners and dairy producers. Once these relationships are understood, and if relevant, the next step would be to measure the effect of specific preventative and therapeutic interventions on CON and ILL. Eventually, research should focus on identifying prevalence goals, how to implement US, and how to measure its impact at the farm level.

In addition to epidemiological studies, viral challenges modeled after Chapter 3 might provide significant information regarding the US changes associated with viral infection. If practitioners are able to distinguish between viral and bacterial respiratory disease via US, perhaps there is room for more selective use of antimicrobials. Lastly, comparing genetic profiles and immune responses between clinical, subclinical, and unaffected calves might help determine why some appear resistant to the effects of lung lesions and allow us the opportunity to select for these animals.

Thoracic US in dairy calves is a valuable technique and should become the Gold Standard ante mortem test for BRD detection if future studies are able to identify production
implications of subclinical BRD and successful on-farm implementation strategies. Then, by focusing future research on the extension and training of bovine practitioners and dairy researchers in systematic US and respiratory scoring, we will further enhance our ability to identify, prevent, and manage BRD.

6.3 REFERENCES

**APPENDIX**

Appendix 1. The Wisconsin Calf Scoring Chart. Calves scoring > 4 are considered sick and treatment is recommended.

<table>
<thead>
<tr>
<th>Calf Health Scoring Criteria</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal temperature</td>
<td></td>
<td>101-101.9</td>
<td>102-102.9</td>
<td>≥103</td>
</tr>
<tr>
<td>Cough</td>
<td>None</td>
<td>Induce single cough</td>
<td>Induced repeated coughs or occasional spontaneous cough</td>
<td>Repeated spontaneous coughs</td>
</tr>
<tr>
<td>Nasal discharge</td>
<td>Normal serous discharge</td>
<td>Small amount of unilateral cloudy discharge</td>
<td>Bilateral, cloudy or excessive mucus discharge</td>
<td>Copious bilateral mucopurulent discharge</td>
</tr>
<tr>
<td>Eye scores</td>
<td>Normal</td>
<td>Small amount of ocular discharge</td>
<td>Moderate amount of bilateral discharge</td>
<td>Heavy ocular discharge</td>
</tr>
<tr>
<td>Ear scores</td>
<td>Normal</td>
<td>Ear flick or head shake</td>
<td>Slight unilateral droop</td>
<td>Head tilt or bilateral droop</td>
</tr>
</tbody>
</table>
Appendix 2. Birth Record filled out by the dairy producer for each calf born during the study period.

**Birth Record**

**Calf ID:**

**Dam ID:**

**Date of Birth (dd/MON/yyyy):**

---

**Calving Information:** *(circle answer)*

1) **Assistance:**
   - Unassisted
   - Easy pull
   - Hard pull
   - Surgery

2) **Stillbirth:**
   - Born dead
   - Died <24hrs
   - Alive at 24hrs

3) **Single**
   **Twin**
   **Triplet**

*Please provide ID of twin/triplet calves:*

---

**Health Problems within first 24 hours of life** *(check all that apply):*

- □ aspiration/respiratory distress
- □ milk drains from nose when drinking
- □ other *(specify):*
Appendix 3. Animal level information regarding the 4 non-study Holstein heifer calves that were used to compare the contributions of each lung lobe to the total lung based on ultrasonographic assessment. LL = linear length of lung – sum of the ultrasonographic lengths of lung within intercostal spaces 1 - 10 for the right lung, or 2 – 10 for the left lung. % of Total lung = total linear length of US scan, as a percentage of the total length of both lungs. NA = not applicable. Weights were obtained via weight tape (Coburn Company, Whitewater, WI).

<table>
<thead>
<tr>
<th>ID</th>
<th>Age, d</th>
<th>Weight, kg</th>
<th>Lung lobe</th>
<th>Intercostal Space(s)</th>
<th>Linear length, cm</th>
<th>% of Total lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>140</td>
<td>159</td>
<td>Caudal</td>
<td>10 – 6 / 10 – 6</td>
<td>64 / 58</td>
<td>29 / 27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Middle</td>
<td>5 / NA</td>
<td>15 / NA</td>
<td>7 / NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Caudal aspect of cranial lobe</td>
<td>4 – 3 / 5 – 4</td>
<td>24 / 23</td>
<td>11 / 11</td>
</tr>
<tr>
<td></td>
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