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Bronchoalveolar Lavage and Respiratory System Scoring of Normal Holstein Calves and Calves with Respiratory Disease

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Abstract

Bronchoalveolar lavages (BALs) were performed on Holstein calves with (n=63) and without (n=63) clinical signs of respiratory disease on commercial dairy herds (n=24) in Wisconsin. Evaluation of respiratory disease was performed on farm for each calf using a scoring system that attributed cumulative number values to clinical parameters that are easily assessable by producers or veterinarians (fever, nasal and ocular discharge, presence or absence of a spontaneous or inducible cough). Cytologic evaluation, aerobic bacterial culture and culture specific for Mycoplasma were performed on aliquots of BAL fluid from each calf. Statistical analyses comparing the proportion of different white blood cell populations in BAL fluid from calves with or without a high respiratory disease score as well as those with and without positive bacterial or Mycoplasma cultures were performed. The respiratory scoring system was evaluated for its accuracy in predicting respiratory disease as determined by positive BAL culture. Neutrophil percentages were higher and macrophages lower in calves with a respiratory score > 4 or a positive BAL culture. The sensitivity and specificity of a respiratory score > 4 compared to a positive BAL culture were 0.59 and 0.4 respectively. Calves with a respiratory score > 4 were significantly older than low scoring calves. Early detection of respiratory disease in young dairy calves was facilitated using a respiratory scoring system and BAL fluid results.

Keywords: Calf; Respiratory Disease; Bronchoalveolar Lavage

Abbreviations

BAL: Bronchoalveolar Lavage;
ROC: Receiver Operator Curve;
BRSV: Bovine Respiratory Syncytial Virus;
BHV-1: Bovine Herpes Virus -1

Introduction

Pneumonia is responsible for 21.3% and 50.4% of pre-weaned and weaned dairy heifer deaths, respectively at an estimated cost of $14.71 per calf year in the United States [1,2]. The relevance of respiratory disease worldwide in Holstein dairy calves has been established by numerous other studies in the US [3], Europe [4,5] and Canada [6,7]. Despite the importance of respiratory disease, its detection is problematic and delayed diagnosis may result in prolonged use of antibiotics, a high recurrence rate, and the development of refractory sequelae such as chronic lung injury, pulmonary abscessation and ear infections [8]. Subclinical, clinical and chronic pneumonia of calves has a negative impact on growth, reproductive performance, milk production and longevity [9,10].

Physical examination criteria that can be used on farm for the diagnosis of respiratory disease include fever, nasal and/or ocular discharge, presence of a cough and increased respiratory rate and effort. On modern larger dairies in the United States specific veterinary input is not always sought but may provide additional

information via thoracic auscultation and rarely, radiographic and ultrasonographic examination [8]. Specific elucidation of an etiologic cause may be pursued via nasal or pharyngeal swab, transtracheal fluid aspirate, bronchoalveolar lavage (BAL), serology or autopsy derived specimens of lung tissue. It is evident that early detection of respiratory disease in calves in modern dairying, at least within the United States, will rely heavily on producer driven recognition of disease. It would therefore be valuable to establish diagnostic tools that can be utilized on farm and that have been validated as sensitive and specific for calfhood respiratory disease. The objectives of this study were therefore to document the effectiveness of a respiratory scoring system in the detection of pneumonia and to compare BAL cytology in healthy calves and those with bacterial and clinical evidence of pneumonia.

Materials and Methods

Animals

Holstein heifer calves from 24 commercial dairy farms in Wisconsin provided the subject material for this study. Calves were enrolled during visits to dairy farms for teaching purposes (controls) as well as during clinical investigations for respiratory disease outbreaks (controls and calves with respiratory disease) performed by the food animal production medicine service of the Veterinary Medical Teaching Hospital of the University of Wisconsin. Procedures were performed under approval by the University of Wisconsin Institutional Animal Care and Use Committee. A total of 126 calves were enrolled in the study over a continuous, approximate 18 month time period, 63 calves were controls and 63 calves were categorized as having respiratory disease.

Evaluation of Respiratory Disease Using a Numerical Scoring System

Each calf enrolled in the study underwent physical examination by one of the investigators and was scored according to a previously described numerical scoring system [11] that is detailed in Table 1. This scoring system calculates a respiratory disease score representing the sum of the four individual clinical sign categories. A score of 5 or greater was used as the cut-off point for categorizing a calf as having clinical evidence of respiratory disease.

Table 1. Respiratory Scoring System. The score from each of the 4 clinical parameters (temperature, cough, nasal discharge, eye or ear) were summed for the total respiratory score for each calf.

	Points allocated for signs below			
Clinical sign	0	1	2	3
Rectal temperature, ˚C *(˚F)*	37.8-38.2 *(100-100.9)*	38.3-38.8 ˚C *(101-101.9)*	38.9-39.3 ˚C *(102-102.9)*	≥ 39.4 ˚C *(>103.0)*
Cough	None	Induce single	Induce repeated or occasional spontaneous cough	Repeated spontaneous coughing
Nasal discharge	Normal serous	Small amount of unilateral, cloudy	Bilateral, cloudy or excessive mucus	Copious, bilateral, mucopurulent nasal discharge
Eye or ear	Normal	Mild ocular discharge	Bilateral purulent ocular discharge or unilateral ear drop	Head tilt or both ears dropped

BAL Technique

All study calves underwent a BAL procedure under sedation using a sterilized, flexible 10 French X 36 inch catheter with a 3-cc balloon cuff (Mila International, Inc. Medical Instrumentation for Animals, Florence KY). Calves were sedated by intramuscular injection with 0.1 mg/kg xylazine, positioned in sternal recumbency and the nostrils cleaned with a dry gauze sponge. The head and neck of the calf were extended to facilitate passage of the sterile BAL catheter by an investigator wearing surgical gloves. Prior to catheter introduction into the nostril, sterile saline was dripped into the catheter to lubricate the guide-wire stylette. The BAL catheter was then introduced into the ventral meatus of the nose through which it was advanced until it encountered resistance in the caudal pharynx. The calf's head was then ventroflexed while simultaneously elevating the ventral mandible and the catheter advanced down the trachea during the inspiratory phase of the respiratory cycle. The catheter was rapidly advanced until resistance occurred as it wedged in a lung bronchus. The guide-wire stylette was removed, the balloon cuff inflated with 3 cc of air and 120-ml of sterile saline was infused using 60-ml syringes with a stopcock and catheter tipped adapter attached. Immediately after the 120 ml infusion, negative pressure was applied to aspirate BAL fluid. A second 120-ml infusion was introduced and aspirated as described and the pooled fluid sealed in a specimen cup and preserved in a cooler until processing. BAL fluid was refrigerated until analyzed within 12 hours of collection according to farm distance from the laboratory.

Bacteriologic Investigation of BAL Fluid

A 5-ml aliquot of the pooled sample from each calf was

divided and used for aerobic bacterial and Mycoplasma cultures. Samples for aerobic culture were inoculated onto trypticase soy agar supplemented with 5% sheep blood (Remel, Lenexa, KS), chocolate agar (Remel, Lenexa, KS), and Levine eosin methylene blue agar (Remel, Lenexa, KS). Samples for Mycoplasma culture were inoculated onto Mycoplasma agar (University of California at Davis Biological Media Services, Davis, CA) and into a tube of Mycoplasma broth (University of California at Davis Biological Media Services, Davis, CA). Mycoplasma broth was subcultured after 48 hours of incubation onto a Mycoplasma agar plate. All cultures were incubated at 37°C in 5% CO_2. Aerobic cultures were examined for growth after 24, 48, and 72 hours of incubation. Microorganisms were identified using standard identification procedures [12]. Mycoplasma cultures were examined microscopically for growth consistent with Mycoplasma spp. at 24 hour intervals for up to 14 days. Any Mycoplasma spp. growth was considered significant.

Immunobinding assay for speciation of Mycoplasma

The assay followed the procedure of Kotani and McGarrity [13], with minor modifications. The assay was performed at room temperature. Nitrocellulose membranes (NC) (Bio-Rad Laboratories, Inc. Hercules, CA, USA) were cut into 2.5 cm^2 sheets, placed in 60 x 15 mm plastic culture dishes and washed for 5 min. in distilled H_2O (dH_2O) with gentle agitation. The dH_2O was aspirated and the NC sheets allowed to air dry. The washed and dried NC sheets were placed on Mycoplasma colonies, pressed lightly with a spatula, removed with a forceps and returned to the culture dishes. Eight impressions were made for each Mycoplasma culture being assayed. Ten percent buffered formalin (Sigma, St. Louis, MO, USA) was added to the NC sheets for 10 min. to fix and was then aspirated. Subsequent steps were all performed with agitation and fluids were aspirated after each incubation was complete. The NC sheets were washed for 10 min. in Tris-buffered saline (TBS, pH 7.4) containing 0.3% (v/v) H_2O_2 to inactivate any endogenous peroxidase. NC sheets were then blocked for 30 min. with TBS containing 10% (v/v) horse or swine serum (Sigma) and 0.2% (v/v) Tween 20 (Sigma) (blocking solution). Rabbit polyclonal antisera to 8 species of bovine Mollicutes (described below) were added (1 antiserum per culture dish) diluted in blocking solution to a predetermined optimum concentration for 30 min. NC sheets were then washed 3 times with TBS. This was followed by 30 min. incubation with goat anti-rabbit IgG HRP (Cappel Research Reagents, ICN Biomedicals, Inc., Aurora, OH, USA) diluted 1:1000 in blocking solution, followed by washing as above. Developing solution was then added. The developing solution was made by dissolving 15 mg. of 4-chloro-1-naphthol (Sigma) in 5 ml. methanol and adding that to 0.5 ml. 3% H_2O_2 in 25 ml. TBS just prior to use. NC sheets were observed for the development of purple color. Once color development was apparent, the NC sheets were washed 3 times with dH_2O and allowed to air dry.

Preparation of polyclonal rabbit antisera

Rabbit antisera to 8 species of bovine Mollicutes, specifically Mycoplasma bovis, M. bovirhinis, M. bovigenitalium, M. alkalescens, M. arginini, M. californicum, M. canadense, and Acholeplasma laidlawii were produced in a manner similar to that previously described for avian Mycoplasmas [14], with the exception that the mycoplasmas for use as the immunogen were grown in Modified Hayflick's medium supplemented with rabbit serum.

Cytological Evaluation of BAL Fluid

An approximately 20 ml BAL fluid aliquot was submitted from each calf for cytological interpretation, specifically involving routine staining of cytospin and direct smear preparations. The proportion of neutrophils, lymphocytes, macrophages, eosinophils, and basophils was calculated in each sample based upon a 200 cell differential count performed by a board certified clinical pathologist. BAL evaluations were performed within 24 hours of collection.

Statistical Analyses

The age distribution of calves in the two groups was determined to be normally distributed and compared for differences using a paired T test. Descriptive statistics were created for the proportions of the different subpopulations of leucocytes in BAL fluid for controls (respiratory score < 4), for calves with clinical evidence of respiratory disease (respiratory score > 4), calves with positive aerobic bacterial or Mycoplasma cultures and those with negative cultures. These data were first screened for normal distribution, which was not present, and descriptive statistics appropriate for non-Gaussian variables calculated (median, maximum, minimum and quartile ranges). Comparisons in leukocyte subpopulations between calves with respiratory scores > 4 and those with respiratory scores < 4, as well as those with and without positive cultures were made using the Mann Whitney test. Significance was set at $p < 0.05$. To examine any potential influence of housing, comparisons were also made for respiratory score and culture between calves that were housed outdoors or indoors. Age of calf at sampling was also examined to determine whether the proportion of leucocyte sub-populations in BAL fluid varied according to this variable in calves with a total respiratory score of < 4. Significance was set at $p < 0.05$.

The accuracy of the respiratory scoring system was further described using receiver operator (ROC) curves. Specifically, ROC curves were created comparing the total respiratory score, as well as each of its components, with

a gold standard of a positive culture. In addition ROCs were also plotted comparing each of the leukocyte subpopulations versus a gold standard of a positive culture. Sensitivity and specificity values were calculated for each parameter described by ROC.

Results

Animals

126 Holstein heifer calves were enrolled in the study. The control group (n=63) had a mean age at sampling of 33 days (s.d. +/- 22) compared to a mean age of 41 days (s.d +/- 16) in the group with a total respiratory score of > 4 (n=63). The age of the group with the high respiratory score was significantly higher (p=0.03) than the control group. Of the 63 control calves 5 were housed outdoors in hutches and 58 were housed indoors, compared to the group with a respiratory score > 4 in which 54 were housed indoors and 9 were housed outdoors in hutches.

BAL Cytology

Descriptive statistics for the different leukocyte sub-populations are given in Tables 2-5. The median macrophage and neutrophil percentages in BAL fluid for calves with a respiratory score < 4 were 73% and 19% respectively compared to median values of 66% and 26% for calves with a respiratory score > 4. When the data set was classified according to a negative or positive BAL culture the median values for macrophage and neutrophil percentages were 70% and 20% for culture negative individuals and 51% and 40% for culture positive calves respectively. Comparisons of leukocyte subpopulation percentages between calves with a respiratory score of < 4 compared to those with a score of > 4 showed that the percentage of neutrophils was significantly higher in calves with a score > 4 compared to those with a score < 4 (p = 0.02) as well as being higher in calves with a positive culture compared to those with a negative BAL culture (p = 0.006). Correspondingly, the percentage of macrophages was significantly lower in BAL fluid from calves with a respiratory score of > 4 compared to those with a score of < 4 (p=0.04), and was also lower in calves with a positive culture compared to those with a negative BAL culture (p = 0.05). There were no differences in the proportion of leucocyte sub-populations in calves with a respiratory score of < 4 according to age at sampling.

Table 2. Descriptive Statistics For Calves With Respiratory Score <4 (n=63).

	Neutrophils	Macrophages	Lymphocytes	Eosinophils
Median	19	73	3	0
Min	1	3	0	0
Max	95	99	64	30
25%	6	48	0	0
75%	37.5	88	8	0

Table 3. Descriptive Statistics For Calves With Respiratory Disease Score > 4 (n=63).

	Neutrophils	Macrophages	Lymphocytes	Eosinophils
Median	26	66	2	0
Min	2	3	0	0
Max	97	97	57	3
25%	16	35	0	0
75%	52	76.5	7	0

Table 4. Descriptive Statistics For Calves With Negative Bacterial Cultures (n=85).

	Neutrophils	Macrophages	Lymphocytes	Eosinophils
Median	20	70	3	0
Min	1	3	0	0
Max	96	99	64	30
25%	8	45	1	0
75%	32	85	9	0

Table 5. Descriptive Statistics For Calves With Positive Bacterial Cultures (n=41).

	Neutrophils	Macrophages	Lymphocytes	Eosinophils
Median	40	51	3	0
Min	2	3	0	0
Max	97	97	30	1
25%	16	30	0	0
75%	63	79	7	0

BAL Microbiology

Of the 63 control calves there were 17 calves from which significant positive BAL aerobic bacterial cultures were obtained. There were 15 calves from which Pasteurella multocida was isolated, 2 from which Trueperella pyogenes was isolated and 1 calf from which both Pasteurella multocida and Trueperella pyogenes were isolated. No positive Mycoplasma cultures were obtained from control calves judged to be clinically free of respiratory disease (respiratory score < 4). Of the 63 calves with clinical evidence of respiratory disease (respiratory score > 4) there were 24 individuals with positive cultures with either aerobic bacteria or Mycoplasma spp. The 24 positive cultures were obtained from 14 different farms. Fifteen calves had single cultures with Pasteurella multocida originating from 8 different farms, whilst 2 had mixed cultures with Pasteurella multocida and Mycoplasma bovis or Pasteurella multocida and Mannheimia haemolytica respectively. A further 3 calves in the respiratory disease group had single cultures with Trueperella pyogenes, and 4 calves had significant single species growth with Mycoplasma

bovis, Mycoplasma bovirhinis, Pseudomonas aeruginosa and Mannheimia haemolytica respectively. There was no significant difference in the numbers of positive cultures (aerobic or Mycoplasma) obtained in those calves that were housed indoors or outdoors.

Evaluation of BAL Scoring System

The ROC depicting the accuracy of the respiratory scoring system compared to a positive BAL culture is given in Figure 1. The AUC was 0.65 which was the same AUC obtained for the ROC depicting the accuracy of neutrophil percentage in BAL fluid compared to a positive culture (Figure 2). Of all the other variables examined (individual components of the scoring system or leukocyte subpopulations) only the presence and severity of a cough demonstrated an AUC of > 0.60 when compared to a gold standard of a positive culture (Figure 3), with an AUC of 0.63 when a cut off score of 2 was used from the cough component of the scoring system (Table 1). The sensitivity and specificity of a cough score of 2 (denoting an occasional spontaneous cough or inducible repeated cough) or greater were 0.46 and 0.66 respectively. The sensitivity and specificity of a total respiratory score > 4 compared to a positive BAL culture were 0.59 and 0.4 respectively. By using a cut-off point of > 40% neutrophils in the BAL fluid a sensitivity of 0.54 and a specificity of 0.8 for a positive BAL culture were obtained. There was no difference in total respiratory score between calves that were housed indoors or outdoors.

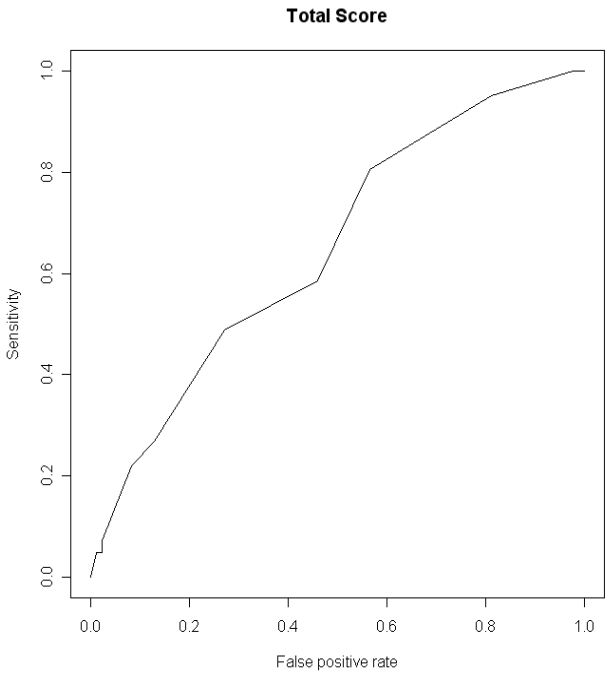

Figure 1. ROC Plot Depicting Accuracy of the Total Respiratory Score Compared to a Positive BAL Culture.

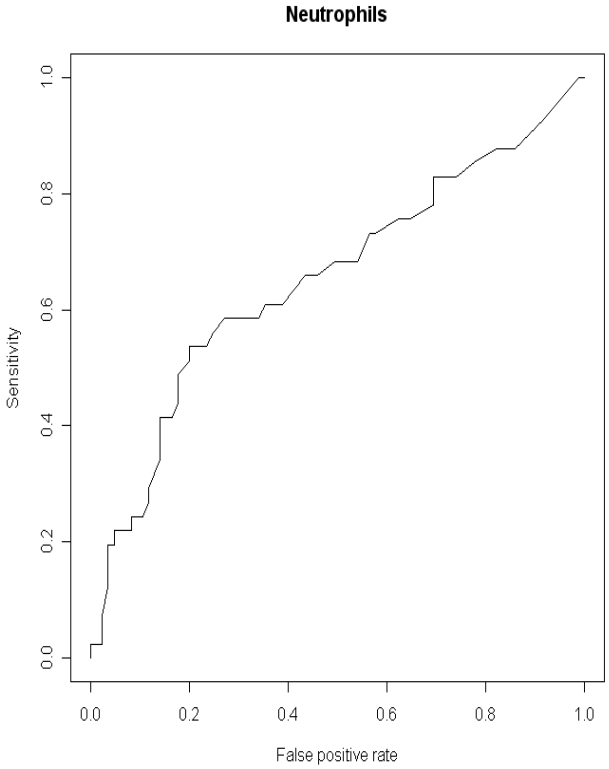

Figure 2. ROC Plot Depicting Accuracy of the Neutrophil Percentage in BAL Fluid Compared to a Positive BAL Culture.

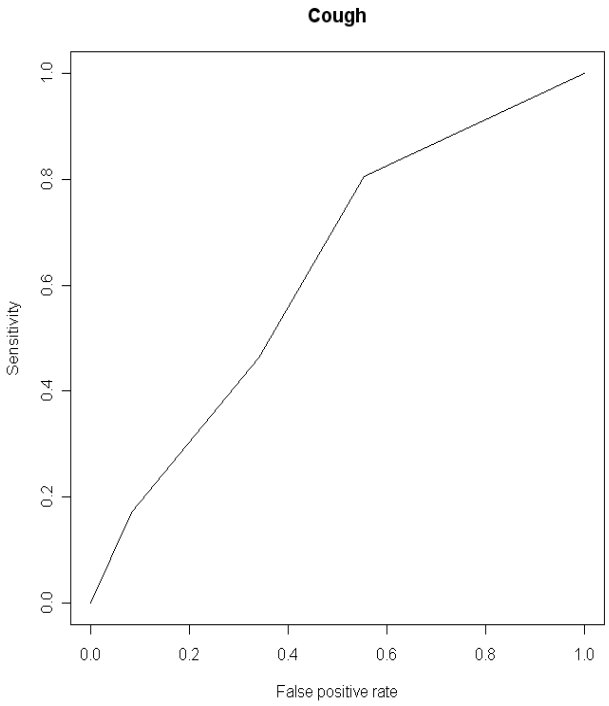

Figure 3. ROC Plot Depicting Accuracy of the Presence of a Cough Compared to A Positive BAL Culture.

Discussion

This study describes a simple to perform and easy to instigate scoring system for the evaluation of respiratory disease in calves. The BAL procedure was well tolerated by calves in this study with no identifiable side effects at the time of the procedure or negative feedback from producers with respect to deleterious effects in the ensuing hours or days. Undoubtedly the BAL procedure should only be performed by veterinarians but the authors have extensive experience of its utility in both calf and adult respiratory disease investigations and view it as a legitimate alternative to other diagnostic procedures currently used to pursue an etiologic cause for bovine pneumonia such as transtracheal aspirate, nasopharyngeal swabs and serology. This is particularly true of calves where the traditional transtracheal aspirate performed percutaneously is not always simple to perform or well tolerated. Several previous studies examining the utility of the BAL procedure in the diagnostic evaluation of lower airway disease in 6-8 month old beef feedlot calves have been published [15-18]. It has also been a readily performed and repeatable research technique utilized in many infectious disease studies involving common bovine viral [19-21] and bacterial pathogens [22,23]. The normal distribution of leukocyte sub-populations in healthy 6-8 month old feedlot calves has been described as up to 90% alveolar macrophages [24,25] with the remaining cells being predominantly neutrophils. A study by Allen et al [16] examining BAL cytology in healthy and pneumonic feed lot calves attributed mean macrophage and neutrophil proportions of 64.1% and 28.4% to healthy calves which compares closely to our median values of 73% and 19% and 70% and 20% for Holstein dairy calves with respiratory scores < 4 and negative BAL cultures respectively. In that same study by Allen et al [16] calves with clinical evidence of respiratory disease (fever plus at least two of poor appetite, lack of rumen fill, copious nasal discharge, spontaneous cough and a respiratory rate > 40) had mean macrophage and neutrophil proportions of 50.6% and 44.6% respectively compared to our median values of 66% and 26%, and 51% and 40% for Holstein dairy calves with respiratory scores >4 and positive cultures respectively. From this it would appear that the culture positive calves from our study most closely resemble the clinically ill calves from the study by Allen et al [16] with respect to BAL cytology. In the calves of our study the proportions of leucocyte subpopulations in BAL fluid were not observed to change with age of the calf in the control group. The authors can only find one other comparable study in which mononuclear subpopulations were evaluated over time in a normal population of calves [26] in which the authors did demonstrate a reduction in neutrophil proportions and an increase in alveolar macrophage population with increasing age. However, the calves in that study were older than the calves in our study with the changes in subpopulations being noted between 2 and 4 months of age, compared to the mean age of calves in our study being 33 and 41 days for low and high respiratory score groups respectively.

Because dairy calf pneumonia occurs as early as 2-weeks of age, is an endemic problem in many dairy herds, is under-diagnosed and is economically important [2] we felt it important to evaluate BAL cytology and the accuracy of the respiratory scoring system in dairy calves as opposed to relying on data extrapolated from studies in older beef calves. A time-efficient, cost-effective method that can be used to obtain multiple diagnostic samples from young calves in a herd investigation of dairy calf pneumonia is needed, however, and is also described in this study. Similar to a previous study that used a specialized BAL catheter with a balloon cuff [26], we describe the adaptation of commercially available foley catheter, which can be sterilized and re-used, to obtain BAL fluid in pre-weaned dairy calves. As stated BAL fluid collection from young calves has been described but a comparison of the cytologic and microbial characteristics of BAL fluid from normal and diseased young, individually housed dairy calves has not.

To these ends we used a scoring system suitable for farm use by trained personnel using respiratory disease definitions previously described [27-29]. The described scoring system utilizes a graded scale for characterizing rectal temperature, nasal discharge, ocular discharge, ear carriage, and inducible cough as being normal (0), mild (1), moderate (2) or more severely (3) abnormal. Given individual animal variation, clinical signs characterized with a numerical score of 0 or 1 could be normal. Abnormal clinical signs receive a score of 2 or 3. In the described respiratory disease scoring system, a calf must be abnormal in at least 2 categories to reach a total score of > 4, which served as the basis of classifying calves as diseased. Rectal temperature elevation is a non-specific clinical sign that is usually present in calves with early respiratory disease. A febrile response, generally considered to be > 39.5 C or 103 F, may not be exhibited by very young calves with respiratory disease, particularly if they are septic. To address this, some respiratory disease studies in young calves have reduced the weight of fever in defining clinical cases [27,28]. Using the scoring system described in our study, incremental elevation in rectal temperature is weighted on a 0 to 3 scoring system. A classical febrile response of 103 F or greater is given a score of 3. The calves in this study could have been normal, have early, chronic or resolving respiratory disease on the day that they were examined. The rectal temperature score cannot stand alone to classify a calf as having respiratory disease. A re-analysis of our data, eliminating temperature from the total respiratory score and using an optimal cut point

for classifying a calf as having a positive BAL culture with a score of 3 or higher, gives a sensitivity of 0.59 and specificity of 0.56, compared to 0.59 and 0.4 when temperature was included and a score of 5 or higher was used. This suggests that the removal of temperature and an adjustment of the numerical score to 3 or higher may slightly improve specificity. However, neither versions of the scoring system had specificity values that exceeded the use of a 40% or greater neutrophil proportion in BAL fluid for prediction of a positive BAL culture.

The issue of age at first onset of respiratory disease in heifer calves has been the subject of several studies and there is evidence that many calves with respiratory disease have their first episode of pneumonia pre-weaning [2,9]. Indeed much of the impetus behind performing this study was to improve early identification of calves with respiratory disease in order to improve therapy and reduce morbidity and mortality. We were able to demonstrate in this study that the mean age of calves with a high respiratory score was significantly higher than that of the control group at 41 days of age. For most commercial dairy calves this would be towards the end of the pre-weaning period but one should demonstrate caution in interpreting this as an average age at which respiratory disease first occurred in these calves. The design of the study meant that the farms were visited and calves sampled at one single time point so it is unknown for how long high respiratory score calves may have had disease prior to sampling. It might be more accurate to view the mean age at sampling (41 days) as a rather conservative number with many of the affected individuals likely being abnormal for some time before sampling.

Our detailed statistical evaluation of the scoring system gave us a sensitivity of 59% and a specificity of 46% compared to a gold standard of a positive BAL culture when a cut-off point of 5 or greater was used. The disadvantage of choosing a higher numerical value for the scoring system to identify calves at risk for respiratory disease would of course be that whilst a higher specificity could be achieved sensitivity would decline. Admittedly our selection of a positive BAL culture as the gold standard was somewhat arbitrary and has the potential itself to be undermined by sensitivity and specificity issues. The authors readily acknowledge that a BAL only samples one portion of a lung and relies on a unilateral lobar sample as being reflective of the cytology and microbiology in the remainder of the lung fields, and as such we may have failed to pick up more localized infectious disease in some individuals. In addition it is possible that the passage of an endobronchial tube nasally risked contamination from the nares, pharynx or trachea, potentially contributing false positives with respect to culture of lower airway pathogens. This modest value for specificity was surpassed when a cut-off point of 40% for the proportion of neutrophils in BAL fluid was used instead (80% specificity) although the sensitivity of this was similar at 54%. These values for sensitivity and specificity underscore the challenges that veterinarians and producers face in the accurate clinical and diagnostic identification of calves with true respiratory disease.

Both control calves and those with respiratory disease enrolled in this study came from multiple farms and were housed predominantly in indoor, naturally ventilated calf barns. Only a minority of calves (5 in the low score group versus 9 in the high score group) were housed outdoors. As a consequence this study has limited power to examine the effect of housing systems and quality of ventilation on BAL cytology in healthy or pneumonic calves. The statistical analyses performed did not demonstrate any differences in the numbers of positive aerobic or Mycoplasmal cultures obtained in calves housed either indoors or outdoors. Similarly there were no statistically significant differences noted in the respiratory score obtained from calves according to the housing system under which they were managed. It has been shown that merely moving healthy adult horses indoors influences BAL cytology in favor of an increased proportion of neutrophils [30] but the extent to which different housing and ventilation systems may influence BAL leucocyte subpopulations in calves was not specifically addressed in our study and remains a potential worthy area of future investigation.

The microbial flora cultured from BAL aliquots in this study were typical lower airway pathogens that are commonly encountered in pneumonic calves, and were consistent with previous studies culturing BAL fluid in calves [15]. In some instances the bacterial species obtained are considered upper airway commensals and their presence in BAL fluid may reflect lung colonization due to primary infection, impaired mucociliary clearance or co-existent or antecedent viral infection. Although we did not pursue specific viral etiologies in any calf in this study, the BAL procedure can be used to obtain diagnostic samples to pursue viral causes of respiratory disease such as BRSV and BHV-1. Of interest was our finding of positive bacterial cultures in 17 of the 63 control calves with low respiratory scores which further emphasizes how clinically occult respiratory disease may be in young dairy calves. The metabolic, growth and economic ramifications of lower airway bacterial contamination in the absence of obvious clinical evidence of pneumonia is unknown but it is reasonable to assume some deleterious effect.

In summary we have established normal ranges for BAL cytology in dairy heifer calves using a large control population that are comparable to previous historical literature drawn from outdoor feedlot cattle. Furthermore

we confirmed that calves with clinical and bacteriologic evidence of respiratory disease are characterized by a significantly increased neutrophilic response in BAL fluid, with a concomitant reduction in the normal preponderance of alveolar macrophages. The respiratory scoring system that was examined showed modest sensitivity and specificity for identifying calves with culture positive BAL samples when a cutoff point of 5 or greater was used. Greater sensitivity was achieved when the differentiating test was a cut off of greater than 40% neutrophils within the BAL fluid. Further studies are indicated to improve the sensitivity of practical, on-farm identification, of incipient respiratory disease in replacement heifers to curtail the well-established economic and health implications of a missed diagnosis. It is also important that poorly specific on-farm observations do not result in the overuse of antibiotics.

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