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A multimodal clinical and diagnostic approach to bovine respiratory disease complex (BRDC) in dairy calves

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Abbreviations list

α1-AT	α1-antitripsin
AGP	α1-acid glycoprotein
APPs	acute phase proteins
APR	acute phase response
AUS	thoracic auscultation
BALf	Bronco Alveolar Lavage fluid
BCoV	Bovine Corona Virus
BHV-1	bovine herpes virus-1
BP	Bronchopneumonia
BRDC	Bovine Respiratory Disease Complex
BRSV	bovine respiratory syncytial virus
BVDV	bovine viral diarrhoea virus
CHSC	Calf health scoring chart
CIS	clinical illness scores
CI	confidence interval
CNS	Central nervous system
Cp	ceruloplasmin
D.A.R.T	depression, appetite, respiration, temperature
DNS	deep nasal swab
ELISA	enzyme-linked immunosorbent system
FPT	failure of passive transfer
Hp	Haptoglobin
IBR	infectious bovine rhinotracheitis
ICS	intercostall space
Ig	immunoglobulins
IgG	immunoglobulin G
IL-1	Interleukin-1
IN	intranasal
IV	intravenous
LBP	lipopolysaccharide binding protein
LKT	leukotoxin
LPS	lipopolysaccharide
nBAL	non-endoscopic bronco alveolar lavage
NPT	Neopterin
NSAID	Non-steroidal anti-inflammatory drugs
OR	odds ratio
P	p value
PCR	Polimerase Chain Reaction
PCT	Procalcitonin
PI-3 V	parainfluenza type 3 virus
PON1	Peroxonase 1
Ppm	parts per million
Q	perfusion
RNA	RiboNucleic Acid
RTX	Repeats in toxin
SAA	Serum Amyloid A
Se	sensitivity
SID	strong ion difference
SIG	strong ion gap
Sm	Seromucoid
Sp	specificity
Spp	species
TAT	turn around time
Tf	transferrin
TNF	Tumor necrosis factor
TTW	transtracheal wash
TUS	Thoracic Ultrasonography
US	Ultrasonography
Va	alveolar ventilation
VNT	virus neutralization test
WCRSC	Wisconsin calf respiratory scoring chart

Abstract

Calf bronchopneumonia (BP), or Bovine Respiratory Disease Complex (BRDC), to date is still difficult to diagnose due to the multifactorial nature of its etiopathogenesis and aspecificity of clinical symptomatology.

In this research project, four studies were conducted to investigate various aspects of BRDC, the objectives were:
1) To study the prevalence of BRDC-related pathogens in the territory of northern Italy;
2) To develop an accurate ultrasound diagnosis method that can be used under field conditions;
3) To standardize lung auscultation in order to increase its diagnostic accuracy;
4) To analyze hemogas-analytical and metabolic changes during BRDC by correlating them with clinical and ultrasonographic diagnosis.
For this purpose, pre-weaning Italian Friesian calves from herds in northern Italy were included, and observations were performed under field conditions.

In our first study, an epidemiological investigation of pathogens in the lower airways of calves that showed respiratory symptoms for less than three days and had not received antibiotic treatment in the previous two weeks was conducted. Ten farms in northern Italy were included, and trans-tracheal washes (TTW) were performed on 6 calves from each farm. Culture and molecular tests were performed on the samples, the results were analyzed obtaining the total and farm prevalence, and the prevalence of coinfections were evaluated. *Pasteurella multocida* and *Mannheimia haemolytica* were found in 37% and 12% of the samples, respectively, with a prevalence of 70% and 40% of the farms examined. The presence of *Mycoplasma bovis* was detected in 20% of the farms with a prevalence of 13% of the tested population. The viral pathogens detected were Bovine Coronavirus and Bovine Respiratory Syncytial Virus in 15% and 7% of cases, respectively. Only coinfections determined by two pathogens were evidenced: for *P. multocida* in 36% of the isolations, *M. haemolytica* (86%), *M. bovis* (50%), BCoV (56%), BRSV and others in 100% of the isolations. We observed that the prevalence of *Mycoplasma bovis*, although low in the population, reaches very high values at the farm level. In our population, the etiological agents with higher occurrence were *Pasteurella multocida* and BCoV. In order to reinforce the prudent use of antimicrobials, diagnoses should also be investigated from the etiological aspect so as to set up treatment choices in the most effective way.
In the second study, the accuracy of a rapid ultrasound technique: Focused Lung Ultrasound (FLUS) was evaluated by comparing it with systematic Thoracic Ultrasonography (TUS) nowadays considered an imperfect gold standard for the diagnosis of BRDC-associated lung lesions. For this study 135 animals were subjected to both ultrasound investigations, and the McNemar test was used to evaluate statistical differences and agreement was assessed by weighted Kappa. A total of 76 calves out of 135 had a TUS score ≥ 2 (focal lung lesion > 1 cm) and were therefore considered to be affected by BRDC. The FLUS had sensitivity of 81.6% (95% CI = 71.0-89.5%), specificity = 100% (95% CI = 93.9-100%), positive predictive value of 100%, negative predictive value of 96.6% (95% CI = 94.7-97.9%) and accuracy of 97% (95% CI = 92.6-99.2%). McNemar's test showed a difference of 10.3% between FLUS and TUS. The agreement between TUS and FLUS was substantial (weighted kappa test 0.78). This study shows that the focused method could be used as an additional tool for evaluating consolidation, especially when examining a large number of postweaned dairy calves.
In our third study, we investigated the accuracy of lung auscultation. This method suffers from non-unique definition of lung sounds, resulting in poor/moderate accuracy. To evaluate if standardization of nomenclature and method could increase accuracy in diagnosis, 330 calves were examined. Each calf received: clinical evaluation with Wisconsin Score (sick if > 4), TUS ultrasound (sick if ≥ 2) and lung auscultation. Thoracic auscultation was classified as follows: AUSC1 (all pathological sounds), AUSC2 in which hisses, crackles, increased bronchial sounds and pleural rubs were considered, and AUSC3 when only increased bronchial sounds or pleural rubs were evaluated. The accuracy of AUSC

categorizations was determined using three imperfect diagnostic tests by a Bayesian latent class model and sensitivity analysis (informative vs. weakly informative vs. non-informative priors and with vs. without covariance between ultrasound and clinical scoring). Based on the priors used, sensitivity (95%BCI) of AUSC1 ranged from 0.89 (0.80-0.97) to 0.95 (0.86-0.99), with specificity (95%BCI) from 0.54 (0.45-0.71) to 0.60 (0.47-0.94). Removing the increased breath sound from the AUSC categorizations results in a pronounced increase in specificity (for AUSC3, specificity ranged from 0.97 [0.93-0.99] to 0.98 [0.94-0.99]), but at the cost of a decrease in sensitivity (from 0.66 [0.54-0.78] to 0.81 [0.65-0.97]). The impact of priors on posterior densities of AUSC was limited. We could conclude that a standardized definition of lung sounds improved the accuracy of AUSC for BP diagnosis in calves.
The last study in this research project aimed to determine what is the best diagnostic approach to identify metabolic abnormalities in sick calves. A total of 231 animals were enrolled in which were performed: clinical and ultrasonographic evaluation, blood gas examination and serum titration of paraoxonase-1 (PON-1) as a biomarker of inflammation. Calves were grouped as "healthy" or "calves with BP" based on clinical scores (Wisconsin [WISC5], California [CALIF], an alternative Wisconsin approach [WISC2points]) and two TUS cut-offs (≥ 1 cm; ≥ 3 cm); and as "healthy," "upper respiratory tract infection," "subclinical BP," and "clinical BP" based on the combination of clinical and TUS scores. Abnormalities were more extensive in calves diagnosed with TUS. Glucose concentrations were significantly lower in "calves with BP" (median TUS1cm = 5.2 mmol/L, 25-75% interquartile range [IQR] 5.5-6.6; median TUS3cm = 5.1 mmol/L, IQR 4.4-6.1) than in "healthy" (median TUS1cm = 5.8 mmol/L, 5.5-6.6; median TUS3cm = 5.7 mmol/L, IQR 5.2-6.3; $P < 0.001$). The concentration of unmeasured strong ions was significantly lower in "BP calves" diagnosed by TUS1cm (median = 0.8 mmol/L, IQR -0.9-3) than in "healthy" (median = 2.8, IQR 1.1-4.9; $P < 0.001$). PON-1 was significantly lower in "BP calves" (median TUS1cm = 64.1 U/mL, IQR 40.8-78; median TUS3cm = 59.2 U/mL, IQR 37.7-72.4) than in "healthy" (median TUS1cm = 77.3 U/mL, IQR 61.9-96.5; median TUS3cm = 72.9 U/mL, IQR 53.4-95.4; $P < 0.001$). None of the parameters showed clear distinctions between calves with clinical and subclinical BP. In conclusion, calves diagnosed with TUS identified more significant metabolic abnormalities.

The present study provided new knowledge on BRDC, and in particular on aspects related to epidemiology, the tools we possess to improve the diagnosis of BP in the field, and the evaluation of metabolic changes in animals affected by respiratory disease. Thanks to the results obtained, we can assert that to date, thoracic ultrasonography remains the best in vivo method for identifying sick animals, and related to clinical scores, it discriminates with greater accuracy those animals manifesting metabolic and hemogasanalytic alterations. In addition, the development of rapid ultrasound techniques such as FLUS are shown to be very applicable under field conditions. Diagnostic methods considered inaccurate, such as auscultation, can be a valuable tool by providing unambiguous definition and interpretation of sounds. Finally, in a One Health perspective, it is necessary to perform etiologic diagnosis aimed at setting up a proper vaccination and treatment plan.

Introduction

Bovine Respiratory Disease Complex (BRDC)

Bovine respiratory disease complex (BRDC) is a multifactorial disease determined by a combination of microbial pathogens, compromised host immunity, environmental factors, and inadequate housing conditions (Lima et al., 2016). BRDC has great economic relevance in the global cattle industry (Edwards, 2010) as well as in dairy industry, where it represents a major issue in replacement dairy calves (McGuirk, 2008). Despite advances in veterinary medicine and technology to control the BRDC, it remains a huge economic burden for both dairy and beef industries, due to calf mortality, treatment costs and additional labor incurred (Lima et al., 2016). In the United States, about 16% of calves are affected by BRDC and 90.2% of sick animals are treated with antibiotics (NAHMS, 2012); however, survival and future reproductive performance of many calves treated with antibiotics are compromised (Stanton et al., 2012). For this reason, BRDC is becoming increasingly important to the high economic and welfare costs associated with this disease (Stanton et al., 2012).

The upper respiratory tract of healthy calves contains many opportunistic bacteria including pathogens such as *Mannheimia haemolytica, Pasteurella multocida* and *Histophilus somni* (Lima et al., 2016). Initial infection by viruses, such as parainfluenza type 3 virus (PI-3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), bovine herpes virus-1 (BHV-1), or bacteria like *Mycoplasma bovis*, has been suggested to impair clearance of the above-mentioned infecting bacteria, resulting in lesions of the respiratory tract. Other microorganisms are isolated from bovine pneumonic lung tissues. These include *Trueperella pyogenes*, which may be an opportunistic pathogen or could be associated with chronic pneumonia cases after prolonged antimicrobial therapy (Griffin et al., 2010). Early detection of bronchopneumonia and effective treatments are fundamental to reducing mortality in affected calves, however without the presence of a gold standard to diagnose BRDC, and the presence of many subclinical animals, this task turns out to be difficult (McGuirk, 2008). In a recent study, Buczinski and Pardon (2020) suggest that the term BRD, which summarize the complexity of the interaction involved in this disease, does not help to identify the individual case definition to initiate therapy, which has always remained poorly defined.

For these reasons, BRDC is one of the most extensively studied diseases in cattle, with research beginning in the late 1800s and continuing today (Taylor et al., 2010).

Epidemiology and Risk factors

BRDC is a global issue. Studies conducted in the United States found a morbidity of 22.8% in dairy calves by evaluating more than 11,000 (Dubrovsky et al., 2019) similar result to other U.S. studies such as that of Windeyer et al. (2014), in which 21.9% of all calves were diagnosed with BRD. To date, there is no gold standard for the diagnosis of BRDC, and in some research clinical parameters, or ultrasound scores, or even etiologic investigations have been used to define the status of disease. In a study conducted in Canada by Francoz et al. (2015) 22 out of 95 calves (23%) were considered to have clinical pneumonia in this study. Overall, 49 calves (52%) had ultrasonographic evidence of consolidation (Depth≥1 cm). Among these 49 calves, 32 (65%) had a CRSC score<7. On the other hand, among the 22 calves with a score≥7, five (23%) had no evidence of lung consolidation on ultrasonography. Many studies conducted to investigate the prevalence of respiratory pathogens in dairy cattle have been performed collecting sample from upper airways from nasal or deep nasopharyngeal swabs (Lee et al., 2022; Aly et al., 2021; Studer et al., 2021; Francoz et al., 2015). Due to the increasing complexity of execution, fewer studies have used BAL and even less have performed sampling with TTW of which some were conducted in Italy for example, the study by Bottinelli et al., (2017) in which they were focused on investigating only the genus *Mycoplasma*. Due to the complexity of this disease, and the extreme variability resulting from seasons and managerial factors, it is not possible to accurately define the prevalence of BRDC in dairy herds worldwide as well as in Europe and Italy, and further studies are needed.

BRDC is a multifactorial disease arising from a combination of environmental, host, management, viral, and bacterial factors (Boileau & Kapil, 2010). There are animal related predisposing factors such as: animal age, decreased immune responsiveness due to animal stress, lack of previous viral exposure or vaccination, inadequate passive immunoglobulin transfer in newborn calves, nutritional deficiencies, and dehydration (Callan & Garry, 2002). Another host-related factor is anatomic and functional features of bovine lung. Cattle have a relatively long tracheobronchial tree, which increases the volume of dead space, which reduces the amount of fresh oxygen reaching the lung, thus increasing the risk of alveolar hypoventilation with partial obstruction. The increased dead space in cattle may not affect respiratory tract immunity *per se*; however, it increases the surface area for particulate deposition and increases the transit time of inhaled vapors, gases, and particulate matter (Ackermann et al., 2010). Environmental risk factors include high air humidity or dust content, speedy changing of environmental temperature, extreme heat or cold, and high concentrations of noxious gases such as ammonia (Callan & Garry, 2002). On one side, high temperature and air humidity are risk factors for increased bacteria counts and they allow a better diffusion of airborne

pathogens (Gorden & Plummer, 2010; Stöber, 2004). On the other hand, a high humidity reduces the alveolar macrophages activity as the immunoglobulin concentration in the upper airways (Stöber, 2004), cold temperatures and perhaps temperature fluctuations can decrease mucociliary clearance, predisposing animals to respiratory disease (Diesel et al., 1991). Moreover, the lower critical temperature, at which additional energy is needed for heat production, lies in the range of 10-15°C for calves in the first two weeks of life, declining with age to approximately 6-10 °C in older calves, and is highly dependent on air speed (Davis & Drackley, 1998; Webster, 1984). In fact, ventilation is one critical environmental aspect, which plays an important role in minimizing airborne contaminants that can impair respiratory function and defense mechanisms. Appropriate ventilation is also important in maintaining acceptable humidity and ambient temperature levels (Callan & Garry, 2002). To prevent adverse conditions, at least 4 air changes per hour are needed in winter and up to 40 in summer (Lorenz et al., 2011). With an inadequate ventilation, build-up of toxic gases, such as ammonia, can contribute to a respiratory damage, a decreased mucociliary clearance, a decreased alveolar macrophage activity (Callan & Garry, 2002).

Above-average temperatures (14.2 °C) were correlated with an increased prevalence of ultrasound-detected lung consolidation lesions of ≥1 cm ($P = 0.005$), ≥3 cm ($P = 0.002$), and ≥6 cm ($P < 0.01$) (Van Leenen et al., 2020). Lung consolidation and alterations in cytology were also associated with increased ammonia concentrations above 4 ppm in 24-h measurements, more in detail, consolidations ≥1 cm (odds ratio (OR) = 1.73; confidence interval (CI) = 1.02-3.07; $P = 0.04$) and also with the percentage of Bronco Alveolar Lavage fluid (BALf) epithelial cells ($P = 0.01$). Air velocity > 0.8 m/s was associated with higher odds of lung consolidation of ≥3 cm (OR = 6.8; CI = 1.2-38.5; $P = 0.04$) and ≥6 cm (OR = 15.9; CI = 1.2-200.0; $P = 0.04$) (Van Leenen et al., 2020).

Dust particles, from organic and inorganic sources, contribute to the impairment of respiratory defence mechanisms. Particles greater than 5 µm are filtered out by the nasal passages; most particles from 2 to 5 µm are removed by the mucociliary clearance of the trachea and bronchi, and particles less than 2 µm can penetrate to the alveolar spaces (Callan & Garry, 2002). Finally, inhaled endotoxin can contribute to pulmonary damage by initiating inflammatory reactions within the alveoli and alveolar vascular endothelium (Callan & Garry, 2002). In addition to environmental factors, there are many managerial issues that can lower respiratory tract immunity. These include transportation, weaning, overcrowding, changes in social structure, changes in feedstuffs (Ackermann et al., 2010). Another managerial issue that increases the risk to be affected by infectious diseases is failure of passive transfer (FPT). In fact, placenta of the cow does not allow the transmission of protective immunoglobulins (Ig) during gestation. The transfer of Ig depends on the absorption of maternal Ig from colostrum after birth. If after 24 and 48 hours of age the serum IgG concentration is less than

10 g/L, calves have FPT. Achieving early and adequate intake of high quality colostrum is widely recognized as the single most important management factor in determining health and survival of the neonatal calf (McGuirk & Collins, 2004; Weaver et al., 2005). In conclusion when a calf succumbs to BRDC it is most likely due to an accumulation of errors earlier in life. One or numerous 'stressors' listed above results in a compromised immune system that may or may not have been adequately protected from viral and/or bacterial challenge by vaccination and/or natural challenge. The immune system is overwhelmed, the resident bacterial normal flora invades the lung and sickness occurs (Hilton, 2014).

Etiology and pathogenesis of BRDC

The main cause of respiratory infectious diseases are viruses and bacteria that often interact with each other (Bosch et al., 2013). Although infectious bronchopneumonia of ruminants is usually caused by two or more infectious agents acting together, some agents can also cause significant disease alone (Woolums et al., 2009). Remembering that local and systemic defence systems are weakened by environmental and managerial factors, viruses can act as door openers for bacteria that normally reside in the upper airways, thus allowing pulmonary colonization and subsequent infection.

Typical viral pathogens are: infectious bovine rhinotracheitis virus (BHV-1), bovine viral diarrhea virus (BVDV), parainfluenza type 3 virus (PI-3), and bovine respiratory syncytial virus (BRSV) (Hilton, 2014). The most common bacterial pathogens involved with the BRDC complex include: *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*); *Pasteurella multocida*; *Histophilus somni* (formerly *Haemophilus somnus*); and *Mycoplasma bovis* (Edwards, 2010). These bacteria are commensals in the nasopharynx that may establish infection in the lungs of cattle that are subjected to a variety of stresses (Confer, 2009). There are bacteria uncommonly isolated, whom importance in BRD is uncertain or minor (Woolums et al., 2009), including *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas aeruginosa*, and *Chlamydia* spp., are also occasionally identified in young calves (Callan & Garry, 2002). Pathogens that are considered important in BRDC can be isolated from the upper respiratory tract of healthy cattle and calves. If none of other predisposing causes of disease is present, it seems that their presence is not of major significance (Callan & Garry, 2002).

Viruses

Many viruses, including BHV-1, BRSV, PI-3, bovine coronavirus (BCoV), BVDV and bovine reovirus, have been etiologically associated with respiratory disease in cattle (Ellis, 2009). Viruses are believed to predispose the host, allowing for severe bacterial pneumonias, in four ways. First, viruses cause damage to nasal mucosal epithelial cells and altered mucociliary clearance, allowing bacterial attachment, growth, and colonization. Second, they cause tracheal mucosal epithelial cell damage reducing effectiveness of the mucociliary apparatus: this results in bacterial attachment, growth, and colonization. Third, innate defenses of the airways and lung are suppressed by viral infections through damage or depletion of macrophages and neutrophils (major phagocytic cells in host defense). Finally, viruses cause suppression of immune system effectors such as T-cell (cell mediated) and B-cell (humoral). These immunosuppression effects on T- and B- cell systems are major risk factors caused by selected viruses such as BVDV (Taylor et al., 2010). Moreover, viruses are believed to predispose to bacterial infection in 2 distinct ways. Firstly, viral agents can cause

direct damage to respiratory clearance mechanisms and lung parenchyma, facilitating translocation of bacteria from the upper respiratory tract and establishment of infection in compromised lungs. Secondly, viral infection can interfere with the immune system's ability to respond to bacterial infection (Taylor et al., 2010). These viral pathogens primarily infect the upper respiratory tract, resulting in rhinitis, tracheitis, and bronchitis. Their ability to cause direct pulmonary disease is generally limited, except for the bovine respiratory syncytial virus, which can also cause severe lung damage as a primary agent (Callan & Garry, 2002).

Bovine herpesvirus-1 (BHV-1)

Transmission of BHV-1 is usually transmitted by a direct nose to nose contact, but it could be also transmitted over short distances by aerosol. Once in the upper airways, BHV-1 enters epithelial cells (Ellis, 2009). BHV infection of susceptible animals can result in severe disease with coalescing erosions and ulceration of the upper respiratory tract mucous membranes (Caldow et al., 2018), compromising the normal surface defenses to the extent that fatal bacterial pneumonia results (Yates, 1982). Erosions and ulcers in the upper respiratory tract are the pathological hallmarks of infectious bovine rhinotracheitis (IBR), in fact, BHV-1 infection causes lysis of infected cells (Ellis, 2009). Widespread lysis of ciliated epithelium in the trachea disrupts the housekeeping functions of the mucociliary escalator, and results in a failure to clear bacteria from the upper airways, leading to bacterial deposition in the alveoli (Ellis, 2009). Secondarily BHV-1 causes immunosuppression, resulting from dysfunction of neutrophils, lymphocytes and macrophages (Woolums et al., 2009; Fratrić, 2013). In many, if not most BHV-1-associated BRDC cases there is a mixed infection with bacteria, that results in severe lower respiratory tract disease (Ellis, 2009).

Bovine viral diarrhea virus (BVDV)

Transmission mainly occurs through per orally or nasopharingeally, through excretions o secretions becoming from infected animals, mainly persistently infected animals (Stöber, 2004). BVDV has a main role in predisposing cattle to BRDC, through its immunosuppressive effect on the host (Baker, 1995) (Fulton and Burge, 2000). Due to its immunosuppressive effect, it compromises cellular specific immune system defenses, and it act synergically with BHV-1, BRSV, *Mycoplasma* spp. and *Pasteurella* spp. (Stöber, 2004).

Parainfluenza virus type 3 (PI-3)

PI-3 is a long-recognized, endemic infection in dairy and beef cattle populations. Virus is spread primarily by large droplet transmission, and its cytopathic effects on the structural components of the

mucociliary apparatus, together with the effect on local and systemic immune responses, contribute to the establishment of secondary bacterial infections that are a common feature in BRDC. When not complicated, symptomatology is usually mild, consisting of fever, nasal discharge and dry cough (Ellis, 2010).

Bovine Respiratory Syncytial virus (BRSV)

BRSV is a major cause of respiratory disease and a major contributor to the bovine respiratory BRDC. BRSV is shed in nasal secretions and transmitted by contact with nasal secretions or aerosol over short distances. Following transmission, BRSV can be found in a variety of ciliated and non-ciliated epithelial cells in the respiratory tract, including the airways and pulmonary parenchyma (Brodersen, 2010; Ellis, 2009). Calves and adults are susceptible (Brodersen, 2010). There are different opinions about recurrent infections; according to Brodersen (2010), the most likely explanation for recurrent infections is that BRSV was reintroduced into the herd before new outbreaks. Continuous generation of mutant viruses is believed to be a key adaptive strategy of RNA viruses. On the other hand, based on Ellis article (2009), outbreaks without acute infection nor introduction of new animals may be due to long-term survival of the virus in the lymphoid or other tissue in some carrier animals. In BRSV-infected airways, the normal non-specific defense mechanism of mucocilliary escalator is disrupted. This results in deposition of bacteria in the lower respiratory tract, and alone, could contribute significantly to secondary bacterial bronchopneumonia (Ellis, 2009). If bacteria (eg. *M. haemolytica*) complicate the primary infection, a second phase characterized by emphysema will manifest.

Bovine Corona Virus (BCoV)

BCoV has recently been associated to BRDC, but its role is still unknown (Boileau & Kapil, 2010). BCoV is a pneumoenteric virus that infects the whole respiratory tract and intestine (Saif, 2010). Infection is transmitted primarily via fecal-oral and secondarily respiratory through aerosol. This virus can persist in adults animals as subclinical infection, and under stressful conditions it can be shed (Boileau & Kapil, 2010). Underlying disease, respiratory coinfections, dose, route of infection, and immunosuppression (corticosteroids) related to shipping or immunosuppressive viral coinfections are potential cofactors that could exacerbate the severity of BCoV infections and the BRDC (Saif, 2010). It is still unclear whether the coronavirus causing the enteric form and the one causing the respiratory form are antigenically different (Boileau & Kapil, 2010).

Bacteria and bacteria like agents

Most of the following infectious bacteria are not capable of inducing significant disease without the presence of other predisposing environmental factors, physiologic stressors, or concurrent infections (Griffin et al., 2010).

Mannheimia haemolytica

M. haemolytica is considered the most important among bacteria (Confer, 2009). In healthy cattle, *M. haemolytica* is a natural inhabitant of the upper respiratory tract (Confer & Ayalew, 2018). According to Rice and colleagues (2007) *M. haemolytica* maintains a commensal relationship with the host until homeostatic conditions change as a result of stress or coinfection (Griffin et al., 2010). Stress caused by environmental changes, shipping, weaning, comingling, and viral infections consents the bacterium to proliferate, to be released from biofilm on the upper respiratory surface, and to be inhaled into the lower respiratory tract (Confer & Ayalew, 2018). *Mannheimia haemolytica* has many virulence factors associated with pathogenesis (Griffin et al, 2010), including protein adhesins, lipopolysaccharide (LPS), secreted enzymes and a ruminant specific RTX toxin – leukotoxin (LKT). The result of these virulence factors is alveolar and vascular damage causing pulmonary inflammation (Confer, 2009). Moreover, these virulence factors play an essential role in the ability of *M. haemolytica* to evade clearance and avoid host defenses while rapidly reproducing in the lower respiratory tract (Griffin et al., 2010). Histologically, *M. haemolytica* is associated to the presence of fibrinous-purulent bronchopneumonia and coagulative multifocal necrosis (Stöber, 2004).

Pasteurella multocida

P. multocida is clearly one of the primary bacterial pathogens associated with the clinical syndromes defining BRDC, although its mere presence in the upper respiratory tract is not diagnostic of the disease (Dabo et al., 2008). This Gram-negative bacterium is acquired by calves at an early age from dam and is a common nasopharyngeal commensal (Confer, 2009). Additional predisposing factors are required for the development *P multocida*. associated pneumonia (Griffin et al., 2010). As mentioned by Griffin et al. (2010), *P. multocida* isolation from nasal secretion of calves suffering from clinical respiratory disease is about twice as high as the isolation rate in clinically normal calves. Histologically, *P. multocida* is associated to the presence of lobar fibrinous pneumonia (Stöber, 2004).

Histophilus somni

Like other discussed BRDC bacteria, *H. somni* (formerly *Haemophilus somnus*) in calves and feeder cattle is a commensal gram-negative bacterium residing in the nasopharyngeal region but may colonize the lower respiratory tract (Griffin et al., 2010). *H. somni* is an opportunistic normal habitant of the upper and lower reproductive tract of cattle and other ruminants, and it acts as a pathogen under certain circumstances via mechanisms that remain poorly understood (Guzmán-Brambila et al., 2012). Clinical signs are compatible with an upper airway infection or with severe fibrinous pleuro-broncho-pneumonia, without pathognomonic aspects (Stöber, 2004). The bacterium has been associated with several clinical manifestations including meningoencephalitis (Griffin et al., 2010).

Mycoplasma bovis

Young calves can acquire *M. bovis* by ingestion of contaminated milk, and this might be a basis for the few calves that are infected with this pathogen on arrival in feedlots. However, most *M. bovis* infections in feedlot cattle are thought to be acquired by contact with the nasal secretions of infected calves (Caswell et al., 2010). Often, *Mycoplasma* spp. are present in healthy-calf airways, but especially in those of calves with bronchopneumonia (Stöber, 2004). *M. bovis* may act as a primary pathogen, yet many cases are coinfected with other bacteria or viruses, and evidence suggests that *M. bovis* colonizes and perpetuates lung lesions that were initiated by other bacteria, such as *M. haemolytica* (Caswell et al., 2010). Therefore, clinical severity can variate from mild to severe (Stöber, 2004). Even if clinical features do not differentiate *M. bovis* pneumonia from other bacterial causes, chronic respiratory disease, recurrent treatment failure, and lameness caused by arthritis or tenosynovitis are suggestive (Caswell et al., 2010). Moreover, *Mycoplasma* spp. has been associated with severe otitis media in calves, based on isolation from the external ear canal and nares (Foster et al., 2009). Unfortunately, even if *M. bovis* elicits a robust humoral immune response, the resulting antibodies are not protective because of the variable surface proteins (Caswell et al., 2010).

Other bacterial pathogens associated with BRDC

Trueperella pyogenes

Transmission of *T. pyogenes* occurs during young age: calves acquire the bacterium from their dams. Pulmonary lesions ascribed to *T. pyogenes* are primarily severe abscesses within areas of chronic bronchopneumonia or chronic pleuropneumonia (Lopez, 2007; Confer, 2009) *Trueperella pyogenes* (previously *Arcanobacterium pyogenes*, formerly *Actinomyces pyogenes*, and before that, *Corynebacterium pyogenes*) is a gram-positive, that is a common cause of internal or subcutaneous abscesses in ruminants. *Trueperella pyogenes* is occasionally isolated from the lungs of ruminants with pneumonia, typically from animals that have chronic pneumonia, possibly from grossly visible abscesses. This species is viewed as an opportunist that contributes to respiratory disease as a secondary or possibly "tertiary" invader after viral pathogens and other bacterial respiratory pathogens have become established. *Trueperella pyogenes* has a variety of virulence factors including a cytolytic toxin, as well as several molecules that aid adherence to host cells. Diagnosis of this pathogen is usually made as an incidental finding at necropsy of an animal with significant respiratory disease from other primary causes. Finding *Trueperella pyogenes* during a respiratory necropsy should not induce efforts to directly treat or prevent this pathogen, but rather should induce efforts to prevent other primary or secondary causes of pneumonia and to treat animals in a timely manner with appropriate therapy for an adequate duration in order to prevent chronic pneumonia (Woolums, 2008).

Bibersteinia trehalosi

Most recently, *B. trehalosi*-associated pneumonia in young dairy calves has been described (Confer, 2009), formerly *P. haemolytica* biotype T has been infrequently isolated from young dairy calves and adult cows with respiratory disease. It is currently uncertain whether *B. trahalosi* acts as a primary respiratory pathogen in bovine respiratory disease or plays a secondary and opportunistic role. However, the fact that were also associated with granulomas and was obtained from a joint suggests at least the possibility that these bovine strains may have a role in pathogenic processes (Blackall et al., 2007).

Clinical symptomatology

The symptomatology of BRDC can be very different between different subjects. This variety is due to host and pathogen features, and the onset of the pathology (Stöber, 2004).

In the study performed by Baruch et al. (2019) clinical illness scores (CIS) during the viral phase corresponded to "normal", "mild" and "moderate". Later in the study, during the bacterial phase, CIS of "severe" and "moribund" were observed.

Clinical signs commonly observed include high fever (about 40-41.5°C), depression, decreased appetite, nasal and ocular discharge, coughing and varying degrees of dyspnea (Urban-Chmiel & Grooms, 2012).

Initially it is possible to observe a mild symptomatology and slightly compromised general conditions. At this stage calves my present fever, inappetence, cough, sometimes serum-mucous nasal discharge, sialorrhea and epiphora with conjunctiva inflammation (Stöber, 2004).

In more severe cases symptoms are influenced by bacteria or mycoplasma. At these stages, general condition is compromised, and severe dyspnea and cough are observed. Air hunger attitude consist in standing with elbows abducted, head extended on the neck and flared nostrils. Moreover, anorexia, asthenia, mucopurulent nasal discharge, fever, injected capillaries associated to cyanosis of the mucous membranes and frequent and pounding cardiac activity (Stöber, 2004).

In advanced stages with complications or relapses, calves are dull, depressed, they lay down, extending the head on the neck. Additionally, anorexia, weight loss, nasal discharge, bad fur conditions and arthritis may be present (Stöber, 2004). Animals that approach death, with greater consolidation percentages, have lower temperatures (Baruch et al., 2019).

Diagnostic methods

Calf respiratory scoring chart

Diagnosis of BRDC in cattle placed in feedlots is based on clinical illness (CI) detected by pencheckers. Unfortunately, this diagnostic approach is not always accurate. Cattle are prey animals and consequently will often mask signs of sickness, especially in presence of humans (Timsit et al., 2016). A scoring system is a standardized method for evaluating the status of a disease, a laboratory specimen, or a radiologic image. Measurable elements of the object under study are rated according to their severity or stage, and the sum of the scores for each rated element is tallied (Farlex, 2009).

Clinical scoring systems use information that can be rapidly collected from patients to assess patient health and prognosis and have been used in a variety of human and veterinary applications. Scoring systems assign values to clinical signs, which are used to determine a total score. The patient's total

score, in turn, should correspond to their risk or likelihood of disease. Clinical signs that are difficult to measure with adequate precision or that require expensive or time-consuming methods to measure should not be included (Love et al., 2014). A major issue in the BRDC control is that there is no gold standard to detect this syndrome (McGuirk & Peek, 2014).

As McGuirk (2008) emphasize, delayed diagnosis results in prolonged use of antibiotics, a high recurrence rate, the development of refractory sequelae, such as pulmonary abscessation, ear infections, and endemic herd problems. Early recognition and effective treatment of sick calves may reduce mortality (McGuirk, 2008).

The first score published was developed by Thomas et al. (1977), as a research tool to quantitatively classify the severity of BRDC in calves experimentally inoculated with BRSV or BVDV (Thomas et al., 1977). Another system, used in beef cattle, is called D.A.R.T (depression, appetite, respiration, temperature). As mentioned by Griffin (2014), checking on these key signs has gained wide acceptance as a training tool for helping caregivers learn to identify cattle during the early stages of BRDC.

More recently, a scoring system was developed by veterinarians at the University of Wisconsin at Madison (McGuirk, 2008, figure 1). Wisconsin calf respiratory scoring chart (WCRSC) consider 5 clinical signs: rectal temperature, cough, nasal discharge, ocular discharge and hear/head position. To every category a clinical score, where 0 (normal), 1 (variation of or slightly abnormal), 2 (abnormal), and 3 (severely abnormal), is assigned. The maximum score is equal to 12, the scores of the temperature, coughing and nasal discharge categories, plus the highest of the scores from the ocular discharge and ear carriage categories, are summed (Glover, 2017). Calves with a score lower than 4 are considered healthy; a score equal to 4 means that the calf is at risk to develop BRDC and prompts heightened observation of the calf during the following days (Glover, 2017); a total score of five or more, or that have two or more clinical parameters with score 2 or 3 is indicative of BRDC (McGuirk, 2008; McGuirk & Peek, 2014), and need to be treated. The WCRSC score is a very suitable scoring system, with published score weights and a decision rule (Love et al., 2014).

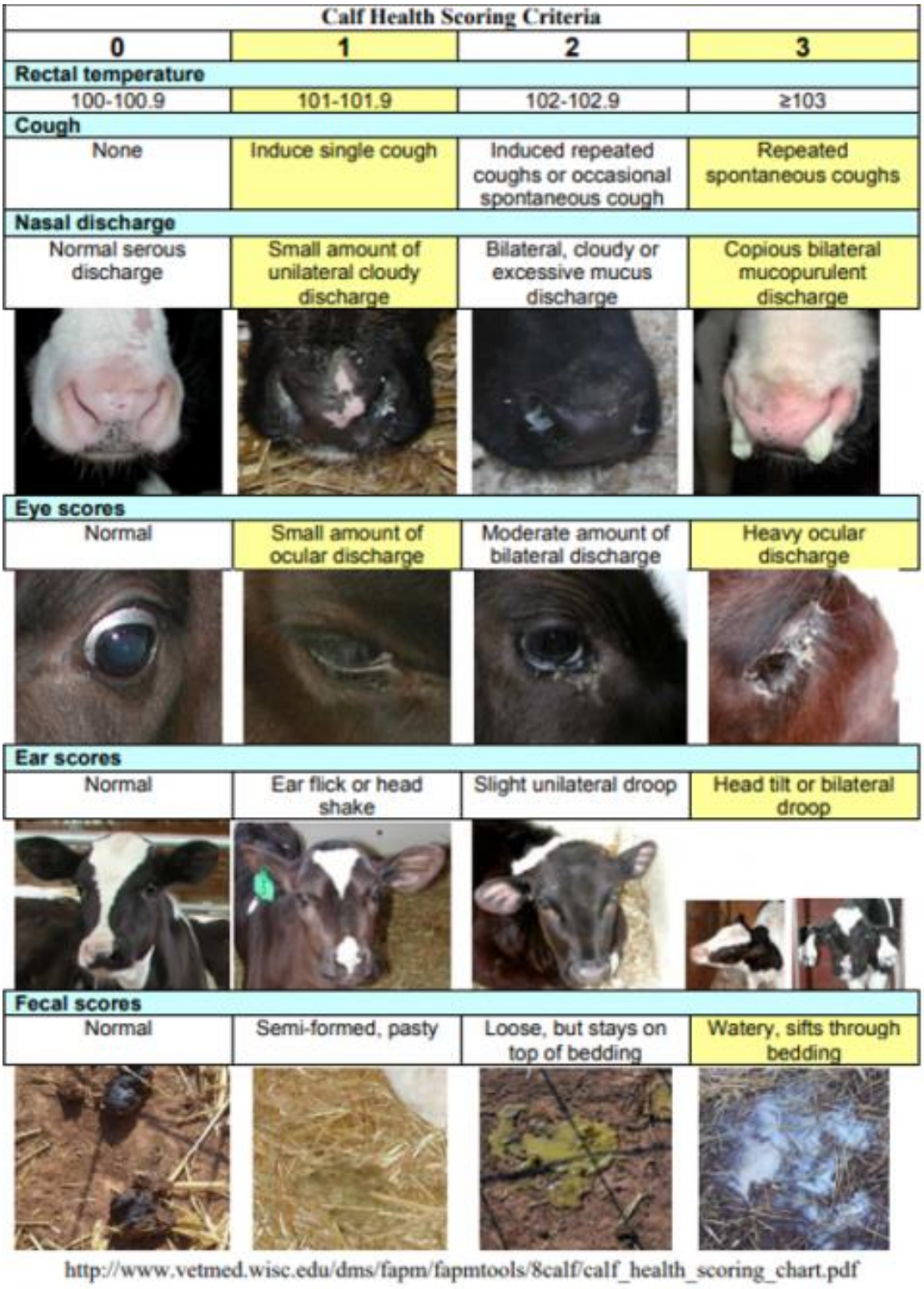


Figure 1 Wisconsin calf respiratory scoring chart which consider 5 clinical signs: rectal temperature, cough, nasal discharge, ocular discharge and hear/head position. To every category a clinical score, where 0 (normal), 1 (variation of or slightly abnormal), 2 (abnormal), and 3 (severely abnormal), is assigned.

Recently UC Davis developed a new scoring system, based on the WCRSC, with the goal to assess the weight of each to each severity level of clinical sign. The objective of this study was to develop a simple scoring system with objectively assigned score weights for on-farm diagnosis of BRDC in pre-weaned dairy calves. The final model was fit to create a system that included only dichotomous clinical signs and minimized calf handling in terms of laryngeal manipulation, which can be time consuming and a biosecurity concern because it often required entry into the hutch (Love et al., 2014). One of the advantages of this new clinical scoring system is that each clinical sign is assessed using a dichotomous way (normal vs. abnormal) Figure 2 (Buczinski et al., 2018).

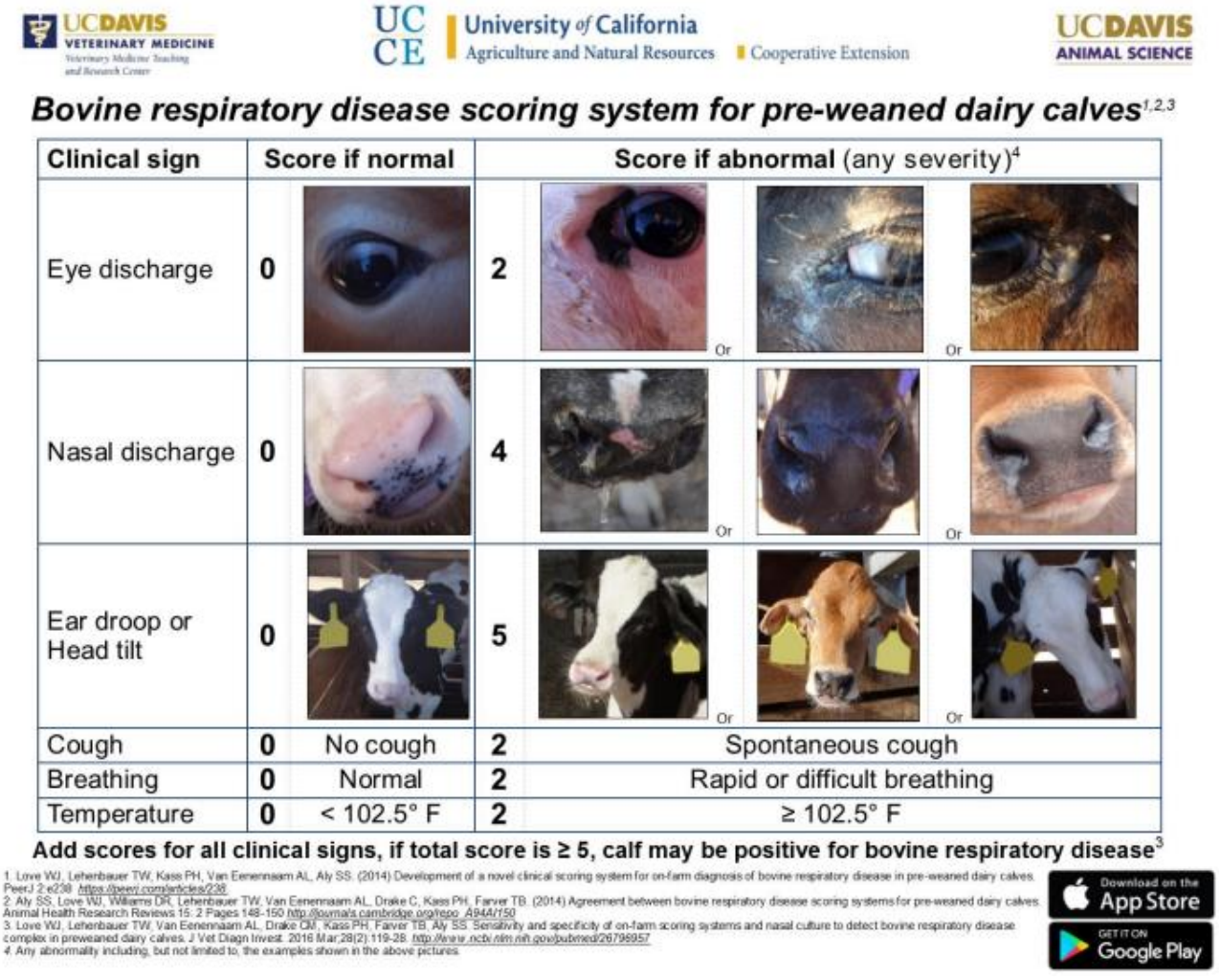


Figure 2 Bovine respiratory scoring system for pre-weaned dairy calves developed by UC DAVIS. It considers each clinical sign in a dichotomous way: normal (0) or abnormal (score depending on the clinical sign) (Aly et al.; 2014;Love et al., 2014; Love et al., 2016)

A study has estimated the sensitivity and specificity of the Wisconsin scoring system as 55.4% and 58.0%, respectively (Buczinski et al., 2013). However, a published study by Buczinski et al. has estimated the sensitivity (Se) and specificity (Sp) of the WCRSC scoring system as 55.4% and 58.0%, respectively compared with lung ultrasonographic exam. The accuracy of this clinical scoring system was recently assessed using a Bayesian latent class framework (Buczinski et al., 2015). The score Se for detection of active BRDC was 62.4% and Sp was 74.1% (Buczinski et al., 2018).

Clinical illness scores are considered a subjective tool, as they rely on the visual appraisal of animals, and are nonspecific relative to BRDC (Baruch et al., 2019). These systems are useful but fail to differentiate between upper and lower airway disease and do not identify calves with subclinical pneumonia (Ollivett and Buckzinski 2016).

Thoracic auscultation

For veterinarians, diagnosis of BRDC is based on clinical signs in association with abnormal lung sounds, such as increased bronchial sounds, crackles, wheezes, or the absence of respiratory sounds on thoracic auscultation (AUSC) (Berman et al., 2019). Auscultation is the practice of listening to and interpreting sounds from the lungs and airways. Proper auscultation requires a systematic routine with careful attention to what one is hearing rather than a careless, ritualistic approach (Curtis et al., 1986). In fact, auscultatory exam has to be performed on each side of the thorax, starting from the upper -middle line, right behind the cranial edge of lung projection area, proceeding by horizontal or vertical lines until all area has been auscultate. To perform the auscultation correctly, it's important to stay focused on each point for 2-3 inspiratory acts. To auscultate both the cranial fields is possible (Belloli & Tesei, 2014), arranging the stethoscope under the forelimb or by moving forward the forelimb. Normal breath sounds are louder on inspiration than on expiration because inspiration is active with more rapid airflow, whereas expiration is passive in normal animals and associated with lower rates of airflow in healthy animals (Radostis, 2006). Normal breathing sounds are loudest over the base of the trachea and quietest over the diaphragmatic lobes of the lung (Jackson & Cockcroft, 2007). It is important to remember that auscultation is subjective and opinions as to what constitute 'normal' lung sounds may differ, besides background noise is common on farms and may further reduce sensitivity (Glover, 2017). Very few studies have been performed concerning the validity of thoracic auscultation for the diagnosis of pneumonia in veterinary medicine (Buczinski et al., 2014). Buczinski et al. (2014) performed a study that compared thoracic auscultation and the ultrasonographic evidence of lung consolidation. The sensitivity results have been found to be poor (between 0 and 16%) whereas specificity was high. Another study executed on sheep, compared thoracic auscultation with both ultrasonography and necropsy, demonstrating that severe lung lesions can be missed easily using thoracic auscultation (Scott et al., 2010). In conclusion if abnormal lung sounds are detected, the animal is very likely to have BRDC, but not detecting such sounds is no guarantee of disease absence. Moreover, obvious abnormal lung sounds likely represent severe pathology or chronic cases. A computer aided auscultation device, the Whisper Stethoscope (Merck Pharmaceuticals), has been developed which automatically detects abnormal lung sounds. This new technology is promising as a rapid and accurate method of BRDC diagnosis. Sensitivity and specificity have been estimated at 93 and 90% respectively (Mang et al., 2015), and results from the device are useful for predicting mortality of affected calves (Noffsinger et al., 2014).

Thoracic ultrasonography

Ultrasonography (US) is a non-invasive method of diagnosing pneumonia *in vivo* (Ollivett & Buczinski, 2016), independently of clinical signs (Ollivett et al., 2015). Thoracic Ultrasonography (TUS) can be performed on the field for clinical or research purposes (Buczinski et al., 2013).

TUS was found to be applicable to the diagnosis of lung disease in calves, in fact this method is able to identify the presence of lung lesions and their extension as well (Rabeling et al., 1998). These features allow TUS to be useful for the detection of BRDC in both individuals and herds (Ollivett et al., 2015), allowing to establish an appropriate treatment plan and to avoid unjustified and inappropriate use of antimicrobials (Berman et al., 2019). When a systematic clinical score, such as WCRSC is used, calves can be categorized by BRDC subtypes: upper respiratory infection, clinical pneumonia, or subclinical pneumonia.

One limitation of TUS is that lesions located deep in aerated parenchyma cannot be visualize (Rabeling et al., 1998; Reinhold et al., 2002), moreover atelectatic parenchyma has the same aspect of consolidated parenchyma (Babkine & Blond, 2009). Finally, the bones of the ribcage can limit the visualization of lung parenchyma, but this impediment can be overcome by scanning through adjacent intercostal spaces and angling of the ultrasound beam (Constable et al., 2017).

To perform the examination is possible to use a linear or sectorial probe with a frequency of 7.5 to 3.5MHz, depending on the depth of the lesion observed (Babkine & Blond, 2009). Ollivett et al. (2015) showed that the shape of a linear probe for transrectal examination, is conducive to a better examination of the cranial thorax, specifically the right 1st and 2nd inter costal space (ICS).

During examination, restraint should be minimal in dairy calves, depending on their age. On the field, the hair of the thorax is unclipped, for this reason as transducing agent 70% isopropyl alcohol, coupling gel or vegetable oil, can be used allowing to eliminate the air between the hair (Ollivett & Buczinski, 2016).

When TUS is performed on a single animal, the goal is to scan the entire lung field bilaterally. When screening a group, a different approach should be applied: only the most common lung lobes affected in early pneumonia will be scanned (cranial aspect of the right cranial lobe, right middle lung lobe and the caudal aspect of the left cranial lung lobe) (Ollivett & Buczinski, 2016).

In the last decades many techniques to perform TUS have been described in the literature (Buczinski et al., 2013; Flöck, 2004; Jung & Bostedt, 2004, Pravettoni et al., 2020). Ultrasonographic examination of the thorax should be performed in a consistent manner that ensures thorough examination of the thorax (Constable et al., 2017). In general, the recommended TUS examination extends caudocranially and dorsoventrally. An intercostal approach is used moving from the 10° ICS

to the 1st ICS on the right hemithorax and from the 10° to the 2nd ICS on the right hemithorax. The probe should move parallel to the ribs. To ensure that the high-risk locations for pneumonia are examines, the ventral imagine landmarks play the role of unique identifiers. The most difficult area to access, especially for novice ultrasonographers, are the 1st and 2nd ICS, which corresponds to the most common location for bronchopneumonia in dairy calves (Ollivett & Buczinski, 2016).

Ultrasonographic findings

Normal appearance

Normal scans reveal a thin, echogenic, pleural line and reverberation artifact (evenly spaced parallel echogenic lines caused by the presence of air in the lungs, that block the sound waves progression) deep to the pleural surface. The pleural line represents visceral pleura and lung surface. As a result, normal air-filled pulmonary tissue cannot be imaged (Babkine & Blond, 2009; Streeter & Step, 2007).

Pleural Lesions

As proved by Braun et. al (1997), pleural effusion (defined as the accumulation of fluid between the parietal and the visceral pleura, caused by a pathology of the pleura itself or a condition affecting the surrounding tissues) can be visualize by ultrasonography. In this case a liquid-like content would separate the parietal and the visceral pleura from each other. Usually, effusion are located in the ventral part of thorax, and TUS can be employed to perform an aspiration of the fluid that will allow further analysis. (Babkine & Blond, 2009; Streeter & Step, 2007). If the liquid-like material is a transudate it will appear anechoic, otherwise if it is a transudate it will be more echoic, as result of cells or fibrin presence (Babkine & Blond, 2009). Other pleural anomaly is an irregular or fragmented appearance and thickening (Babkine & Blond, 2009).

Comet-tail artifacts

Comet-tail artifacts in the form of bright, closely situated echo bands starting at the lung surface and running perpendicular to the pleura in the lung tissue (Flöck, 2004), are a particular form of reverberation indicating the focal accumulation of a small amount of highly reflective material, often gas bubbles (Babkine & Blond, 2009).

Pulmonary Lesions

Lobular lesions are small discreet areas of consolidation within an otherwise aerated lung lobe. The hyperechoic pleural interface with reverberation artifact of normal lung can be seen both dorsal and

ventral to the lobular lesion when the probe is placed vertically within the rib space (Ollivett & Buczinski, 2016). Using ultrasonography only pulmonary lesions next to the visceral pleura are visible, due to the block of the progression of the ultrasound waves when there is air contained in the lung tissue blocks. In those cases, reverberation artefacts are visible on the scan. Therefore, only if the air is replaced with tissue or fluids, the parenchyma would become visible on ultrasound (Babkine & Blond, 2009).

Pulmonary consolidation

Consolidation of pulmonary tissue extending to the pleura is seen as absence of reverberation artefacts and instead a homogenous moderately echogenic area the depth of which (representing the depth of lung consolidation) may be assessed. Despite the lack of aeration of consolidated lung, small areas of gas may still be visible as hyperechogenic patches. Comet tail artefacts echogenic 'streaks' radiating from the pleura into the lung, represent pleural lesions or emphysema at the lung surface, but may be present in animals with and without BRDC (Buczinski et al., 2014; Buczinski et al., 2013).
Pulmonary consolidation can be classified as lobular or lobar. In the context of the TUS, lobular and lobar lesions reflect the extent of which the lung lobe is consolidated on the US image. Lobular lesions are small discreet areas of consolidation within an otherwise aerated lung lobe. The hyperechoic pleural interface with reverberation artifact of normal lung can be seen both dorsal and ventral to the lobular lesion when the probe is placed vertically within the rib space. Lobar lesions indicate full-thickness consolidation of the lung lobe that extends proximally from the tip of the lobe. In the US image, the hypoechoic parenchyma of the entire distal lung lobe is visible, and aerated lung cannot be seen ventral to the lesion (Ollivett & Buczinski, 2016).

Pulmonary abscesses

Abscesses may occasionally be observed if they are near the body wall or within consolidation, as hypoechogenic areas circumscribed by a more echogenic capsule (Glover, 2017). In cattle, the masses or cavitary lesions that are observed in pulmonary tissue are most commonly abscesses. An abscess is characterized by a well-defined circular region with a content of variable echogenicity. It is possible to observe these abscesses only if they are located against the visceral pleura. An ultrasound-guided aspiration allows analysis of the contents for diagnostic purpose.

Atelectasis

Atelectasis and consolidation are the extreme situation, where most of the air is removed by compression or infiltration and exudation, respectively. Atelectatic lung is classically hypoechoic,

homogenous, devoid of air bronchograms in the periphery, and it typically floats within pleural effusion (Streeter & Step, 2007). Atelectasis of a pulmonary lobe occurs after increased pressure in the pleural cavity caused by pulmonary effusion, a pneumothorax, or the inability to adequately inflate the lung (eg. bronchial obstruction or severe aspiration of amniotic fluid in newborns). In the presence of a pneumothorax, pulmonary atelectasia cannot be diagnosed by ultrasonography because of the presence of air between the two pleura. In the presence of pleural effusion, however, the pulmonary lobe affected by atelectasis is imaged and appears smaller, triangular and well defined, and mildly hyperechoic.

Ultrasonography scoring

Fewer ultrasonography scoring systems have been developed (Buczinski et al., 2013; Ollivett & Buczinski, 2016; Reinhold et al., 2002). Since measuring the depth of consolidation wasn't a fast method, Ollivett and Buczinski (2016) developed a categorical scoring system, easier to apply on the field. In order to properly score, a prerequisite is to recognize the difference between aerated lung, aerated lung with diffuse pleural roughening (comet tail artifacts), lobular lung lesions (lobular consolidations or lobular pneumonia) and lobar lung lesions.

The ultrasonographic scoring system is based on a score from 0 to 5; where 0 indicates normal aerated lung with no consolidation and none comet-tail artifacts; 1 indicates diffuse comet-tail artifacts without consolidation; 2 indicates lobular or patchy pneumonia; 3 indicates lobar pneumonia affecting only 1 lobe; 4 indicates lobar pneumonia affecting two lobes (where the cranial and caudal aspects of the cranial lobe are scored individually; 5 indicates lobar pneumonia affecting three or more lobes (Ollivett & Buczinski, 2016). In general, scores 0 to 1 are considered normal and scores higher or equal to 3 are consistent with bacterial bronchopneumonia. Other abnormalities such as pneumothorax, pleural fluid, abscesses, and necrosis are not included in the scoring system but as a comment (Ollivett & Buczinski, 2016).

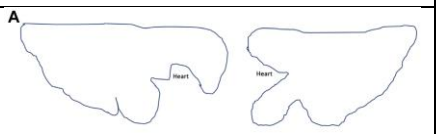
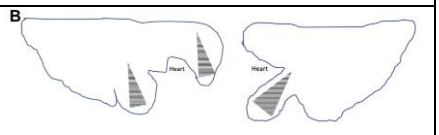
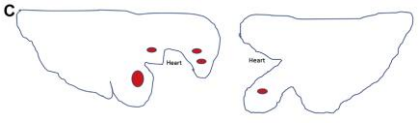
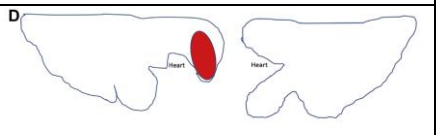
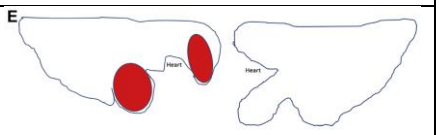
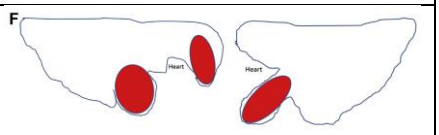
Depth of consolidation can be assessed, and it may be that depth of consolidation, which is correlated with the number of areas of consolidation, is a useful prognostic indicator (Buczinski et al., 2014). Small lobular lesions are most likely to be viral and may not warrant an antimicrobial treatment (Ollivett & Buczinski, 2016). It has been suggested that thoracic ultrasound may be used to assess the diagnostic ability of farm staff (Ollivett & Buczinski, 2016).

Recently, TUS was validated as an accurate (Ollivett & Buczinski, 2016), rapid, on-farm diagnostic tool to identify lung consolidation associated with BRD in preweaned dairy calves (Buczinski et al., 2013, 2014; Ollivett et. al, 2015). Buczinski et al. (2015) reported Bayesian estimates for TUS sensitivity (79.4%, 95% CI: 66.4–90.9) and specificity (93.9%; 95%CI: 88.0–97.6). Sensitivity and

specificity refer to the proportion of calves with and without pneumonia, respectively that are correctly identified by TUS. Within-herd prevalence of subclinical pneumonia can range from 23 to 67% (Ollivett and Buczinski, 2016), which represents a large source of unidentified disease. Thoracic ultrasonography, which can detect pneumonia regardless of clinical status (Buczinski et al., 2014; Ollivett et al., 2015), might improve our ability to accurately assess the impact of BRDC on calf growth and assist with early management decisions.

Thoracic ultrasound provides accurate BRDC diagnosis. In two studies (Ollivett et al., 2015; Rabeling et al., 1998), sensitivity for detection of lung consolidation has been found to be 85 and 94 %, while specificity was almost perfect at 99 and 100%. Agreement between operators is also very good (Buczinski et al., 2013).

Table 1 TUS scoring description from score 0, healthy lungs, to score 5, lung consolidation lesions in three or more lung lobes.

Score	Ultrasound finding	Description	Diagnosis	Appearance
0	Reverberation artifacts	Echogenic pleural line with parallel reverberation artifacts receding from the probe	Healthy well-ventilated lung	
1	Comet-tailed artifact	Hyperechogenic artifact originating at the level of the pleurae and receding vertically from the probe	Focal irregularity or thickening of the pleurae	
2	lobular lung lesions	Hyperechoic subpleural lesion at least 1 cm in diameter within properly aerated lung tissue	Lobular pneumonia	
3	Lung consolidation	Portion of consolidated, hepatized, nonaerated lung parenchyma at the level of one lung lobe	Lobar pneumonia affecting one lung lobe	
4	Lung consolidation	Portion of lung parenchyma that is consolidated, hepatized, and not aerated at the level of two lung lobes	Lobar pneumonia affecting two lung lobes	
5	Lung consolidation	Portion of consolidated, hepatized and non-areated lung parenchyma at the level of three lung lobes	Lobar pneumonia affecting three or more lung lobes	

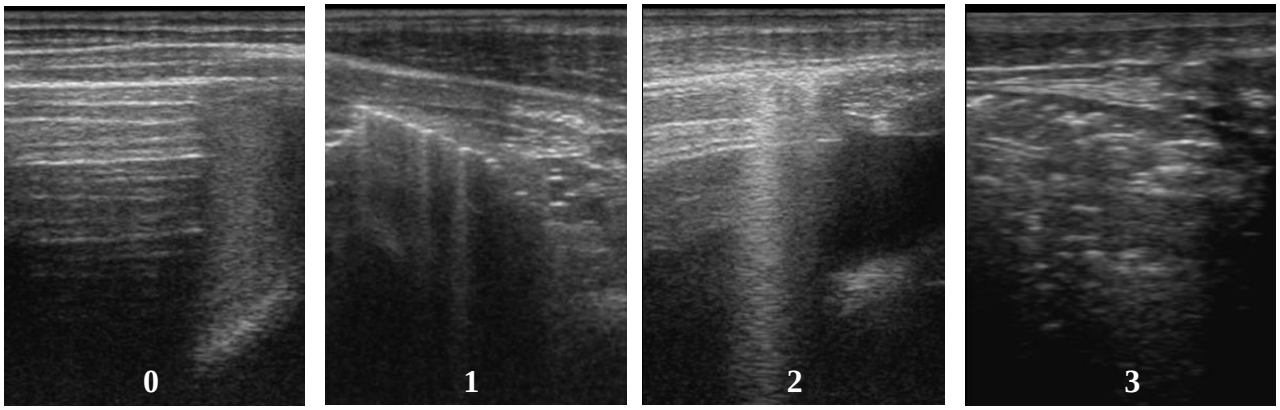


Figure 3: 0 ultrasonographic image of healthy lung with reverberation artefacts. 1 ultrasonographic image of lungs with comet tails. 2. ultrasonographic image of lungs with lobular lung lesion. 3. ultrasonographic image of lungs with lung consolidation lesion.

Diagnostics tools for pathogens identification

Sampling techniques

Sampling techniques for the field need to be economically feasible both in terms of equipment/disposables cost and also invested time. The deep nasal swab (DNS), transtracheal wash (TTW), and non-endoscopic bronco alveolar lavage (nBAL) best suit this profile and are currently most frequently used in the field (Pardon & Buczinski, 2020). As mentioned by Pardon and Buczinski (2020), although laboratory costs are fixed, the return on investment of an analysis depends on selection of appropriate animals to sample and on the technical sampling skills of the veterinarian. The first choice should be an animal in the acute phase of the disease, not previously treated with antimicrobials and without severe respiratory signs. By selecting this kind of animal there are higher odds to detect the viral component, the probability that the antibiogram derived is useful for empiric therapy increases and last the odds of aggravation of disease or even mortality can be decreased.

DNS or nasopharyngeal swab is an easy, fast and cheap technique to apply on a large group of animals (Godinho et al., 2007). From one side, this technique has significance to identify upper respiratory tract viruses, on the other hand, the significance of bacterial isolates or negative test results should be questioned (Cooper & Brodersen, 2010). In fact, a major issue associated to this technique is related to the presence of polymicrobial samples, influenced by both technique and hygiene (Van Driessche et al., 2017). The contamination may be reduced by rinsing the nostrils or by using a guarded DNS, but there is no study in cattle confirming the reduction of nasal contamination using a guarded swab (Pardon & Buczinski, 2020). The largest disadvantage of deep nasal swab is that it do not sample the lower respiratory tract (Pardon & Buczinski, 2020) even if it has been suggested that results for *M. haemolytica*, *P. multocida* or *M. bovis* are similar to the ones obtained with BAL and TTW (Doyle et al., 2017). Complication during sampling, such as hemorrhage and damages are rare.

TTW is a technique that allows to overcome nasal contamination. To perform this procedure restraint and local anesthesia is required, as the surgical preparation of the skin to avoid infection of cutaneous tissue around the tracheal puncture site (Cooper & Brodersen, 2010; Pardon & Buczinski, 2020). To perform TTW, sterile saline instilment (better if warmed to body temperature) and immediate aspiration is required (Cooper & Brodersen, 2010; Pardon & Buczinski, 2020). Several studies in the literature, in different states of the world, refer to this method of tracheo-bronchial fluid sampling using different types of catheters all with similar characteristics (Slathia et al., 2020; Woolums et al., 2019; Zhang et al., 2019; Basqueira et al., 2017). An angiocatheter has also been used in Italy in order to perform a TTW in a sterile way (Pravettoni et al., 2020). Some counterarguments for the representativity of the TTW are that first the mucociliary system can be heavily impaired by

bronchopneumonia, therefore the efflux of the mucociliary system could not come together in the bronchial bifurcation. Second, microbial aspiration from the nasopharynx into the upper trachea is likely frequent. Third, normal pathogenesis involves gradual descent of bacteria down the respiratory tree toward the lung. In conclusion a positive TTW culture may represent a bacterial tracheitis, a colonization or a contamination (Pardon & Buczinski, 2020).

Non-endoscopic bronco alveolar lavage and endoscopic bronchoalveolar lavage are performed introducing a BAL catheter or flexible endoscope through the nose and trachea into the lower airways until it wedges into a bronchus. Sterile saline is injected in the catheter and immediately aspirated. With Endoscopic bronchoalveolar technique the major advantage is that a specific lung lobe, previously shown to be affected during TUS, can be sampled. The major disadvantage are the high operating costs and the risk for equipment damage in the farm setting. Also, the sampling of multiple animals takes time because the sterilization time of the equipment between animals (Pardon & Buczinski, 2020). Alternatively, with adequate training, BAL can be performed without endoscope using a BAL catheter. Advantages are that BAL catheters are cheap and can be sterilized and reused. Moreover, sampling a representative number of animals in a limited time frame is possible (Van Driessche et al., 2016). Van Driessche et al. (2016) demonstrated that, if a BAL catheter introduced without endoscopic guidance, samples a random lung lobe, when sedation is applied, the dorsocaudal lung lobes, which are less frequently affected, are systematically sampled.

Van Driessche et al. (2017) reported that non-endoscopic BAL results less contaminated (and therefore more easily to interpret) compared to DNS. Moreover, with nBAL it returns an interpretable result in 79.2% of the cases, compared to 31.2% in DNS. nBAL has also a better isolation rate for *H. somni*. Moreover, agreement between DNS and BAL samples was moderate for *M. haemolytica*, *P. multocida*, and *M. bovis*. Agreement was much lower for *H. somni*, which can be explained by the fact that *H. somni* is easily overgrown by other bacteria. Given their polymicrobial nature, DNS samples are likely to be falsely negative for *H. somni*, when no selective media are used (Quinn et al., 1994).

Laboratory test

Serology is used to detect antibody in blood and is mainly focused on viruses (eg. virus neutralization test (VNT) or enzyme-linked immunosorbent system (ELISA)). Often is performed using acute and convalescent serum samples (Fulton & Confer, 2012), that means that the turn around time (TAT) is of 3 weeks (Buczinski & Pardon, 2020). Moreover, seroconversions can follow either vaccinal or natural exposure, in fact seroconversion is not diagnostic for disease but suggests exposure to the pathogen (Fulton & Confer, 2012). On the other hand, based on their experience, Fulton and Confer (2012) highlighted that cattle with high antibodies against viral or bacterial agents are occasionally susceptible to experimental BRDC challenge, and individual cattle low antibody responder can be highly resistant to challenge (Fulton & Confer, 2012). Finally, serologic tests are useful to target vaccination programs, to determine protective status and to evaluate infection dynamics in larger scale.

Microbial culture is most frequently used for identification of bacteria; their quantification, the possibility of antimicrobial susceptibility testing and low costs are important advantages. Disadvantages are that for the identification live organisms are needed (animals have to be sampled in early stages and before any antimicrobial treatment), in general the turnaround time require days up to weeks (*Mycoplasma* spp). Moreover, secondary pathogens may overgrow causing false negatives results. At last, for most bacteria involved in BRDC, Se and Sp have not been determined (Buczinski & Pardon, 2020; Fulton & Confer, 2012)

Polimerase Chain Reaction is commonly used for identification of bacterial and viral genotyping. In conventional PCR detection, a genetic region is amplified in a thermocycler using polymerase to produce an amplified segment of nucleic acid. Those products are then compared to known positive controls using gel electrophoresis or sequenced and compared to published sequence for the specific agent. These gel-based PCR assays are qualitative, indicating only presence or absence of the product of PCR (Fulton & Confer, 2012). PCR multiplex is an advancement that allows multiple viruses and/or bacteria to be detected with one test decreasing costs. Moreover, quantitative real-time PCR allows to distinguish the specimens containing a higher infectious agent nucleic acid load, compared to a specimen with a lower infectious agent load, through the rapidity with which PCR amplification begin (Fulton & Confer, 2012). This result is more informative, in fact the pathogen load in BRDC is important (Pardon & Buczinski, 2020) considering that respiratory tract is normally colonized by bacteria. One limit of PCR is that requires specific primers, for this reason pathogens of interest needs to be determined before, causing a potential bias and the possibly lead to false-negative results. Another problem is that viral genomes evolve rapidly, and primers might become outdated, limiting the efficient detection of the pathogens of interest. The largest disadvantage of PCR is interpretative

difficulty because PCR can identify dead pathogens, opportunists currently not involved in the infection, and contaminants, none of which signify a clinically meaningful test result (Pardon & Buczinski, 2020) considering that respiratory tract is normally colonized by bacteria. At last, it is important to remember the rate of development and use of molecular diagnostic tests have outpaced validation, standardization, and standards for interpretation relative to their use in BRDC diagnostics (Fulton & Confer, 2012).

Recently, matrix-assisted laser desorption/ionization time-offlight mass spectrometry (MALDI-TOF MS) has entered the routine diagnostic laboratory for bacterial infections (Seng et al., 2009; Sauer and Kliem, 2010). This technique has shown promising results in obtaining rapid antimicrobial susceptibility results for human pathogens (Lange et al., 2014).

It is mainly used for the identification of bacteria after culture, including *Mycoplasma* species. However, MALDI-TOF MS can also be applied directly to the sample after a very short period of enhanced growth in a liquid medium.

MALDI-TOF MS represents a universal, fast and cost-effective alternative to phenotypic as well as genetic assays. Moreover, it is an open system that can be complemented with own reference data. (Kuhnert et al., 2010). The method is based on analysis of bacterial proteins, mainly ribosomal, which are particularly abundant in the bacterial cells and are present in similar numbers of copies per cell. Each bacterial species contains a characteristic set of proteins called a molecular fingerprint (Sun et al., 2006).

Compared with classical microbial culture, there are direct MALDI-TOF MS techniques in growth broths that allow, in a short time (within 6 hours) to obtain correct bacterial identification in 73% of samples (sensitivity = 59.1%; 95% confidence interval [CI], 47.2-71.0; specificity =100% [100-100]) (Van Driessche *et al.*, 2019).

The technique provided less accurate results in polymicrobial samples and in mixed infection affected animals. A culture-enriched direct MALDI-TOF MS technique was also developed for *M. bovis*, which in a Bayesian latent class model including PCR and microbial culture on solid agar was 86.6% sensitive (95% CI, 54-99) and 86.4% specific (95% CI, 80-96). The time required for availability of the test results was reduced from more than 5 days to less than 3 days. (Bokma et al., 2020)

In a study of (Van Driessche *et al.*, 2018), they estimated sensitivity and specificity of MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) for detection tetracycline resistance in *P. multocida* and them turned out to be of 95.7% and 100%, respectively, classifying 98% of the isolates correctly after only three hours of incubation.

Blood biomarkers

The acute phase response (APR) is a reaction of an organism to homeostatic disturbances, that could be due to an infection, tissue injury, neoplastic growth or immunological disorders (Jawor et al., 2013). Acute inflammatory conditions that are severe enough to raise blood plasma concentrations of cytokines, such as IL-1 and TNF, modify the blood concentrations of acute phase proteins (APPs). APP are plasma proteins, synthesized in the liver, whose concentrations increase or decrease by 25% or more during inflammation. On one side, the concentration of positive acute phase proteins such as C-reactive protein, acid glycoprotein, haptoglobin, mannose-binding protein, fibrinogen usually increases during inflammation, on the other side the concentration of prealbumin and albumin decreases (Ackermann, 2007). Therefore measuring concentrations of different APPs in plasma can give us valuable information on the APR and the course of disease (Nikunen et al., 2007). As result of the need to early identification of calves affected by BRDC, in the last decades many studies have been performed to investigate the presence of blood markers that could help in this task and to prognosis evaluation. Among domestic animals, it is of major clinical interest to define markers of subclinical inflammation in ruminants, because their leukocyte acute phase response is often not evident (Kovačević Filipović et al., 2012).

Therefore in calves with respiratory tract diseases, the following APPs were examined: Haptoglobin (Hp), Fibrinogen, Serum Amyloid A (SAA), transferrin (Tf), lipopolysaccharide binding protein (LBP), α1-acid glycoprotein (AGP), α1-antitripsin (α1-AT), seromucoid (Sm), ceruloplasmin (Cp), albumin, α1-antichymotrypsin and α2-macroglobulin (Jawor et al., 2013). Procalcitonin, neopterin (El-Deeb et al., 2020).

Acute phase protein (APP)

Haptoglobin (Hp) has a major role in bovine acute phase response (Eckersall & Bell, 2010), therefore is one of the most investigated APPs in calves affected by BRDC. The primary function of Hp is to bind free hemoglobin in the blood, in order to avoid oxidative damage in tissues. This bond also reduces the availability of the iron, contained in the heme molecule, from bacterial growth, acting indirectly as an antibacterial (Tóthová et al., 2013). It has been demonstrated that Hp reacts to bacterial and viral infections (Gånheim et al., 2003; Heegaard et al., 2000), showing a bigger increase compared to serum amyloid A (Heegaard et al., 2000). Moreover Carter et al. (2002) compared the reliability of the diagnostic and predictions derived from the Hp:SAA values to the one of Hp. Results showed that predictions derived from Hp:SAA were less reliable than the ones of Hp, suggesting to use the Hp serum concentration to the development of a diagnostic tool. Moisà et al. (2019) assessed Hp sensitivity (46.4%) and specificity (86.1%). In contrast Joshi et al. (2018) suggests that Hp may

be use as biomarker because of its high sensitivity, easily quantifiable and capable of diagnosis BRDC at an early stage even in mild cases and monitoring of treatment efficacy (Joshi et al., 2018). Humblet et al. (2004) reported that due to its high specificity, Hp could detect truly healthy calves among recovered calves.

Serum amyloid A is a positive APP, which presents multiple isoforms. Depending on the disease, isoforms occur in diverse plasma concentration ratios (Alsemgeest et al., 1995). SAA plays several roles such as opsonization, inhibition of phagocyte oxidative burst, platelet activation and reverse transport of cholesterol from tissue to hepatocytes (Tóthová et al., 2013). Moreover, SAA directly influences the cells involved in immunity by acting as chemoattractant and mediates migration of neutrophils and monocytes to the site of infection (Abdallah et al., 2016). Heegaard et al. (2000) demonstrated that SAA can react to viruses infections reacting more rapidly and sensitively than Hp.

Fibrinogen is the circulating precursor of fibrin in the blood clotting cascade, and it was the first APP routinely used in diagnostics to evaluate inflammatory and traumatic disease in cattle (Nikunen et al., 2007; Tóthová et al., 2013). Humblet et al. (2004) pointed out that the detection of Fibrinogen alone has a high specificity, but only combined with other APP is able to increase its sensitivity. Carter et al. (2002) reported that their results of the Fib assay did not appear to be a useful prognostic or diagnostic tool for monitoring respiratory tract disease and treatment in their study.

Albumin is a negative APP, in fact during acute phase response there is a reduction of 10-40% of serum albumin concentration as effect of reprioritization of the hepatic protein synthesis (Joshi et al., 2018; Tóthová et al., 2013). The alteration of Alb serum concentration has been demonstrated to be associated to clinical severity of BRDC diseased calves (Joshi et al., 2018).

Others blood biomarkers

Cytokines are a group of proteins, produced by many cell types, that plays many roles during inflammatory response. Their primary purpose is to modulate the functional expression of other cell types during the inflammatory response, moreover they promote chemotaxis (Ackermann, 2007). Ozkanlar et al. (2012) reported that cytokines production has been detected only in the calves at the initiation stage of BRDC, and mostly related to microbiological identification of BVDV and *M. haemolytica*.

Peroxonase 1 (PON1) is known to be a negative APP in humans. For this reason Giordano et al. (2013) investigated its role in bovines, and it was found that PON1 serum concentration is low in new born calves but is significantly higher in adults. Moreover, the serum concentration of PON1 increases from 2 to 21 days of age, but in calves affected by BRDC its concentration was significantly lower in calves > 28 – to 120-days-old. These results suggest that the detection of this negative APP may be used to diagnose inflammation in neonatal calves when age specific reference intervals are used (Giordano et al., 2013).

Lipopolysaccharide binding protein (LBP) role is to bind bacterial lipopolysaccharide and Lipoteichoic acid (present on the cell wall of Gram-positive bacteria) and to present it to immunity cells. LBP serum concentration has been examined by Idoate et al. (2015), the results showed that LPB, as well as Hp, concentrations were considerably higher for BRDC clinical cases (enrolled based on CHSC) than for controls.

Procalcitonin (PCT) is a precursor of calcitonin hormone involved in homeostasis of calcium. PCT belongs also to the group of APP and is considered as a quantifiable marker in bacterial and parasitic infections, due to its ability to increase quickly after the production of specialized cytokines (TNF-α, IL-6 and IL-8) (Reinhart et al., 2000).

Neopterin (NPT) is a biomarker associated with cell-mediated immunity and produced by dynamic monocyte/macrophage (El-Deeb et al., 2020).

El-Deeb et al. (2020) measured APPs, proinflammatory cytokines, PCT and NPT blood concentration in BRDC infected calves and those recovered ones and evaluate their accuracy in diagnosing BRDC and as treatment efficacy biomarkers. The results showed that BRDC was associated with a significant alteration in serum PCT, NPT, APPs and proinflammatory cytokines levels. Moreover, these biomarkers were higher in calves affected by BRDC then in recovered ones. For these reasons, authors suggested to employ the measurement of APPs, cytokines, PCT and NPT together with the clinical examination as diagnostic and prognostic tool for assessment of calves affected by BRDC.

A low sensitivity was registered for the APPs when used alone. It was necessary to consider Hp and Fib to get to the best combination of sensitivity (71%) and specificity (83%) for pointing out sick

animals. The most significant result was the relationship between presence of *P. multocida* and both clinical signs and increased concentrations of APPs. No associations between tested viruses and clinical signs or APPs were found, this suggests a weak role of these viruses in BRDC. This study confirms the strong pathogenic role of *P. multocida* and the value of APPs in exploring respiratory disease in calves under field conditions. During coinfection of viruses and bacteria, a similar response of Hp to SAA and fibrinogen has been reported (Gånheim et al., 2003). Moreover, a supra-normal APP-value was reported for clinical asymptomatic calves and could be useful to identify animals that are, or have recently been, clinically or sub-clinically diseased. However, the short duration of the APP elevation limits the possibilities to discover diseased animals. Moreover, a low level does not exclude early viral infection as the APP concentrations were not elevated during the incubation period (Gånheim et al., 2003).

Respiratory exchanges and hemogasanalysis

Arterial blood gas analysis allows us to acquire information regarding the patient's oxygenation and ventilatory capacity and allows us to assess the patient's acid-base balance (Rieser, 2013). The recommended sites for arterial sampling are: the common carotid artery and the middle branch of the caudal auricular artery (Muylle, 1996). Although the gold standard for arterial sampling involves the use of a glass syringe, plastic syringes are cheaper and analytical errors due to gas diffusion through the plastic can be limited if samples are analyzed immediately (Knowles et al., 2006; Picandet et al., 2007). The technology and portability of portable blood gas analyzers has improved dramatically in recent years making them available to improve therapeutic decisions in the field (Kreuder, 2020).
In the field of buiatrics, arterial blood gas analysis finds application in the clinical evaluation of the newborn and particularly in the diagnosis of early and late hypoxia (McGuirk, 2015). The list of parameters that can be measured with these instruments is shown in Table 1.

Table 1-Blood gas analysis values with respective ranges of normality (Constable et al., 2017)

Parameters	Reference range	Unit of measure
pH	7.35 - 7.45	Unità
pCO_2	35 - 50	mmHg
pO_2	85 - 100	mmHg
$cHCO_3^-$	20 - 35	mmol/L
BE (ecf)	-4 / +4	mmol/L
cSO2	>95	%
Na^+	135 - 145	mmol/L
K^+	3.5 – 5	mmol/L
Ca^{2+}	1.1 – 1.3	mmol/L
Cl^-	90 - 105	mmol/L
$cTCO_2$	30 - 40	mmol/L
AGapK	8 - 16	mmol/L
Hct	18 - 24	%
cHgb	8 - 12	mmol/L
Glucose	2.5 – 6.6	mmol/L
Lactates	0.1 – 2.2	mmol/L
Creatinine	0.5 – 1.2	mg/dL
BUN	10 - 18	mg/dL
Urea	3.3 – 6.5	mmol/L
BUN/Crea	10 - 20	mg/mg
Urea/Crea	20 - 40	mmol/mmol
A	≈100	mmHg
A-a ($AaDO_2$)	5 - 10	mmHg
a/A	≈40	%

pCO_2: arterial CO_2 pressure; pO_2: arterial O_2 pressure; Na^+ : blood sodium; K^+: blood potassium; Ca^{2+}: blood calcium; Cl- : blood chlorine; Hct: hematocrit; BUN: blood urea nitrogen; cHgb: hemoglobin; $cHCO_3^-$: bicarbonate; $cTCO_2$: blood CO_2 content; BE (ecf): Base-excess; cSO_2: hemoglobin saturation AgapK: aniongap; A: alveolar O_2 pressure; $AaDO_2$: difference between alveolar O_2 pressure arterial O_2 pressure; a/A: ratio of arterial O_2 pressure to alveolar O_2 pressure.

Ventilation

Ventilation is defined as the ability to move air to and from the lungs to facilitate gas exchange (Reiser, 2013). CO_2 is a very soluble gas that readily crosses the blood-brain barrier, and its increase (hypercapnia) in the CNS alters its interstitial pH, which leads to activation of the chemoreceptors of the respiratory center at the level of the medulla, this leads, as a response, to a rapid increase in ventilation (Reiser, 2013).

Alveolar partial pressure of O_2 (pAO_2) can provide information regarding alveolar ventilation: alveolar hypoventilation leads to an increase in $pACO_2$ (arterial pressure of CO_2) and a decrease in pAO_2; this can occur in cases of CNS depression, damage to the phrenic nerve and diaphragmatic innervation, trauma to the chest or respiratory muscles, severe airway obstruction, or as a result of lung disease that decreases alveolar function (Robinson, 2017). Conversely, alveolar hyperventilation leads to a decrease in $pACO_2$ and an increase in pAO_2; this occurs following stimuli such as hypoxia, acidosis, or as a result of increased body temperature (Robinson, 2017).

Oxygenation

Blood oxygenation occurs by passive diffusion according to a pressure-type gradient (Robinson, 2017). The pAO_2 has a normal value of about 100 mmHg while venous blood reaching the lungs has pO_2 of about 40 mmHg. This difference creates a pressure gradient that promotes gaseous exchange between the alveolus and blood. The gaseous exchange continues until the hemoglobin is completely saturated or until the pressure gradient between the alveolus and blood has cleared (Robinson, 2017). The partial pressure of CO_2 (pCO_2) in venous blood returning to the lungs is about 46 mmHg and $pACO_2$ has an average value of 40 mmHg, the pressure gradient for CO_2 diffusion, from venous blood to the alveolar lumen, is therefore only 6 mmHg. Despite such a low value, the diffusion of CO_2 is comparable to that of O_2: in fact, the solubility of CO_2 is higher than that of O_2 thus compensating for the low diffusion gradient. For this reason, it is rare during lung disease for CO_2 diffusion between blood and alveolus to be altered (Robinson, 2017).

For proper blood oxygenation, each alveolus must receive gas and blood in adequate amounts so that alveolar ventilation (Va) and perfusion (Q) are equivalent (Robinson, 2017). During disease, the Va/Q ratio can alter leading to hypoxemia (drop in paO_2). Any alveolus with a decreased Va/Q ratio is relatively hyperperfused and hypoventilated; this frequently occurs during lung disease due to partial airway obstruction or due to loss of elasticity lung due to the ongoing inflammatory process (Robinson, 2017). In contrast, an increase in the Va/Q ratio may indicate the presence of vascular obstruction or pulmonary hypotension; in this case, arterial blood will show elevated pO_2 values and a decrease in pCO_2 (Robinson, 2017). As the difference between Va and Q increases, and as a result

of decreased gas exchange efficiency, the difference between pAO_2 and paO_2 ($AaDO_2$) increases. Normally, $AaDO_2$ is between 5 and 10 mmHg and may increase during anesthesia or during lung disease (Robinson, 2017).

Several researchers have found decreased paO_2 values in arterial blood samples in calves with BRDC (Ellis et al., 2012; Šoltésova et al., 2015; Ider et al., 2021).

In venous blood samples, in addition to the decrease in paO_2, an increase in SO_2 and PCO_2 values was also shown (Ider et al., 2021). These authors believe that paO_2 may be a valid indicator of the extent of lung injury during BRDC by providing important prognostic information (Ellis et al., 2013; Šoltésova et al., 2015A).

In contrast, in a 2010 study, no correlation was shown between the paO_2 values and lung pathology caused by *Mannheimia haemolytica* (Hanzlicek et al., 2010).

In a 2014 study, calves were classified by a score that considered the characteristics of nasal discharge and cough: calves that possessed a cough score of 3 (cough evoked at access) showed a decrease in paO_2 and the value of $AaDO_2$ (difference between the partial tension of oxygen at the alveolar level and at the arterial) (Neary et al., 2014).

Acid-base balance

The traditional approach to acid-base balance assessment focuses on how PCO_2, bicarbonate concentration [HCO_3^-], equilibrium constant (pK_1') and solubility
of CO_2 in plasma (S) interact in determining plasma pH (Constable, 1999).

These interactions are expressed in the Henderson-Hasselbalch equation (Henderson, 1908; Hasselbalch, 1916):

$$pH = pK_1' + log\frac{HCO_3^-}{SPCO_2}$$

where pK1' is the equilibrium constant of the reaction:

$$CO_{2(aq)} + H_2O \leftrightarrow H^+ + HCO_3^-$$

Assessment of acid-base status through the Henderson-Hasselbalch approach uses pH as a general assessment, CO_2 as an independent measure of the respiratory component, and BE as an independent measure of the metabolic component (Constable, 1999). Using this approach, four disorders of acid-base balance can be established:

- Respiratory acidosis (increase in PCO_2)
- Respiratory alkalosis (decrease in PCO_2)
- Metabolic acidosis (decrease in extracellular HCO_3^-)
- Metabolic alkalosis (increase in extracellular HCO_3^-)

Although of great help in understanding acid-base disorders in ruminants, this approach does not explain the dependence of plasma pH on temperature, nor why the value of pK1' depends on pH, sodium concentration, and total plasma protein, nor does it account for the linear relationship between pH and log pCO2 (Constable 1997).

The lack of accuracy of this approach in defining acid-base imbalances prompted Singer and Hastings to propose a new model in 1948 that suggested that plasma pH was determined by two independent factors: pCO_2 and the net charge of strong ions (strong ion difference (SID+)). It was later suggested to add an additional variable namely the total plasma concentration of non-volatile weak acids (A_{tot}) comprising albumins, globulins and phosphates (Stewart 1978; 1981; 1983).

The Strong Ion model, and its simplified version, reduces plasma reactions to those of ions in solution; this is made possible because the cations (Na^+, K^+, Ca^{2+}, Mg_2^+) and glianions (Cl^-, HCO_3^-, $proteins^-$, $lactates^-$, $sulfates^-$, $ketoacids^-$) most represented in plasma bind together to form salts (Constable, 1997; Leeuwen, 1964). Other ions, however, are not considered because their plasma concentrations are irrelevant to pH (Constable, 1997).

Simple plasma ions are divided into: strong ions, which are completely dissociated at physiological pH, and weak ions with a buffering effect. Strong ions exert an electrical effect because the sum of the fully dissociated cations does not correspond to the sum of the fully dissociated anions: this difference equals the SID, which can be measured in mEq/L (Constable, 1999).

Weak ions arise from weak bases and weak acids that are not completely dissociated in the plasma; the dissociation reaction of a weak acid (HA) from a strong base (A^-) is:

$$HA \leftrightarrow H^+ + A^-$$

At equilibrium, the dissociation constant can be calculated:

$$Ka = \frac{[\mathrm{H+}][\mathrm{A-}]}{[\mathrm{HA}]}$$

Where $[H^+]$ is the concentration of hydrogenions and [HA] and $[A^-]$ represent the concentrations plasma of weak bases and acids and their conjugates.

Buffer ions can be divided into volatile ions (bicarbonates) and nonvolatile ions (Constable, 1999).
In the Strong Ion model, moreover, HA and A^- do not participate in plasma reactions that result with the actual destruction or creation of HA and A^- and, for this reason, the sum of HA and A^-, termed A_{tot}, remains constant (Stewart 1978; 1981; 1983).
By combining the law of mass conservation, the law of charge conservation, and four dissociation equations (carbonic acid, bicarbonate, water, and plasma weak acids) Stewart developed an equation that associated plasma $[H^+]$ with three independent variables (pCO_2, [SID], $[A_{tot}]$) and five constants (Stewart, 1981).
With the Strong Ion approach, it is possible to understand what happens to hydrogen ions in a living organism (Stewart, 1981) and allows us to explain how alterations in blood protein concentration change pH (Constable, 1997). The main limitation for this approach is the difficulty in obtaining accurate values of the [SID+], since identification and measurement of all strong plasma ions is required (Constable, 1999). In ruminants, an estimate of it can be calculated by considering the plasma ions most represented and the concentration of lactates.
Later, Constable showed that two of the eight factors considered by Stewart had no quantitative effect on pH and formulated the simplified equation of the Strong Ion with three independent factors (pCO_2, SID, A_{tot}) and three constants (S, constant of apparent dissociation for plasma carbonic acid, [K1'] and Ka) (Constable, 1997):

$$pH = pK_1' + log\left\{SID - \left[\frac{A_{tot}}{1+(10^{pKa-pH})}\right]\right\} / SP_{CO_2}$$

With the Strong Ion approach, six acid-base imbalances can be defined: acidosis and alkalosis respiratory, metabolic acidosis and alkalosis, and A_{tot} acidosis and alkalosis. This should be the preferred clinical approach, especially when protein and phosphate concentrations serum are markedly abnormal (Constable, 2010).

Evaluation of arterial blood gas analysis parameters in calves with lung lesions attributable to BRDC

Given the importance of BRDC in dairy cattle breeding, the lack of a gold diagnostic standard presents a challenge to the veterinarian. To date, the diagnosis of BRDC is issued by combining the results of one or more imperfect diagnostic tests. In a study of 2021, for example, an in vivo reference method was used as a diagnostic panel consisting of: history, clinical examination, chest auscultation, haemogasanalysis on arterial blood, complete blood count, and profile biochemistry (Berman et al., 2021). Arterial blood gas analysis can represent an important source of information during BRDC outbreaks as it can provide many data, including pH, electrolytes, and oxygenation parameters, based on a simple blood draw and analyzing it in a few minutes. There are few studies in the literature that have correlated the presence of lung injury and altered blood gas parameters.

Šoltésova et al. (2015) found a marked correlation between pO_2, SO_2 values and severity of pulmonary pathology diagnosed by necropsy; they also attributed negative prognostic significance to pCO_2 and plasma lactate values (Šoltésova et al., 2015A).

The authors justify the decrease in pO_2 and the simultaneous increase in pCO_2 with the presence of partial airway obstruction that occurs in cases of catarrhal bronchopneumonia (Šoltésova et al., 2015A). The increase of pCO_2 has been mainly found in calves affected by severe forms, in which the mechanisms of compensation of hypoxia, namely hyperventilation and increased cardiac output, fail (Šoltésova et al., 2015A).

Similarly, Ellis et al. (2013) identified a significant association between values of paO_2 and the severity and extent of lung injury, as assessed on necropsy, during course of experimental infection with BRSV.

Similar results were obtained by Nagy et al. (2006) who showed alterations of pO_2 even during mild respiratory infections and an increase in pCO_2 in more severe cases.

In 2014, Neary et al. classified calves with a scoring system that considered the characteristics of nasal discharge and cough: subjects with cough evoked at accesses showed decreased values of PaO_2 and $AaDO_2$ (difference between the tension partial oxygen at alveolar and arterial levels) (Neary et al., 2014).

Increased blood lactate concentration may indicate tissue hypoperfusion, and different authors agree that blood lactate values may provide prognostic information during BRDC (Coghe et al., 2000; Šoltésova et al., 2015A ; Ellis et al., 2013; Buczinski et al., 2014). Šoltésova et al. (2015) and Ellis et al. (2013) reported an increase in blood lactate concentration in severe disease cases in which pO_2 fell below 50 mmHg.

Cut-offs for lactate values during BRDC have been proposed: Coghe et al. (2000) suggest that lactate concentrations $\geq$ 4 mmol/L are indicative of death in the following 24h during BRDC; Šoltésova et

al. (2015) agree with this cut-off (Coghe et al., 2000; Šoltésova et al., 2015A). Buczinski et al. (2014) proposed a cut-off for lactate of 5 mmol/L above which drug treatment is not recommended.
During BRDC, compensated respiratory acidosis is a consistent finding (Šoltésova et al., 2015A; Ellis et al., 2013; Nagy et al., 2006; Hanzlicek et al., 2010).
The Strong Ion Difference (SID) approach is gaining momentum in the assessment of acid-base balance during neonatal calf diarrhea and may represent an additional source of information for the diagnosis of BRDC. There are no studies in the literature evaluating changes in IBS during BRDC. However, Ostermann et al. (2014) and Reinhold et al. (2010) used a Strong Ion approach in assessing acid-base imbalance in cows and pigs infected with *Chlamydia* spp.
In the first study, a venous blood sample was collected from the jugular vein and used to measure the values of pH, pCO_2, Na^+ , K^+ , Ca^{2+}, Cl^- , glucose, L^-lactate, HCO_3^-, BE, AG, PT, SID, SIG, A_{tot}. The results showed that in the acute phase of the disease, a decrease in albumin concentration and consequently in A_{tot} was recorded due to the inflammatory state induced by *Chlamydia psittaci* inoculation.
During the early stage of the disease, there is evidence of a decrease in SID caused by hyponatremia, which is much more severe than hypokalemia. The decrease in SID balances the alkalizing effect due to the decrease in A_{tot}.
(Ostermann et al., 2014). The pCO_2 was found to be decreased in the acute phase of the disease, due to hyperventilation induced by the state of hypoxemia caused by the pathology. The decrease in pCO_2 led to a rise in pH, again in the acute phase, which probably triggered a lowering of HCO_3^- and BE which occurred at a later time. The SID itself also led to a probable elevation of pH at a later time due to continued hypochloremia and the partial resolution of hyponatremia. The A_{tot} in the chronic phase was elevated, and this variation was explained by the physiological immune response of hypergammaglobulinemia.
In the second article, venous sampling was performed from the jugular vein to assess pH, pCO_2, hemoglobin, Na^+, K^+, Ca^{2+}, Cl^-, glucose, L^-lactate, sPT, phosphates, albumin, globulin, Hct, BE, HCO_3^-, SIG, SID, and A_{tot}. A strong ion acidosis was recorded in infected subjects due to anaerobic metabolism resulting from respiratory distress caused by *Chlamydia suis* inoculation, which led to an increased L^-lactate production. The increase in pCO_2, due to the alveolar hypoventilation caused by airway obstruction, the drop in HCO_3^- and BE were explained by the author as phenomena due to the endotoxemia that was triggered. Indeed, in the pathogenesis of *Chlamidya suis* pneumonia, LPS plays a predominant role; while endotoxemia is a common cause of pulmonary hypertension, changes in the systemic circulation in endotoxemic pigs include arterial hypotension, decreased cardiac output, decreased blood flow in femoral and mesenteric arteries, decreased plasma volume and increased

oxygen consumption. The hemodynamic alterations due to the endotoxemic state could also have contributed to the anaerobic metabolism manifested by hyper L^-lactatemia (Reinhold et al., 2009).

Therapy and prevention

Therapy

Antimicrobials are used to treat pneumonia, in order to reduce bacterial charge, which may complicate the development of the disease (Stöber, 2004). The choice of antimicrobials depends on tentative diagnosis, previous experience and result of antibiotic sensitivity test. The selected treatment has to be active against the causative agent, to achieve the therapeutic concentration in diseased lung, finally should be convenient to administer and not least it has to be affordable (Constable et al., 2017). Most commonly used antimicrobials are macrolide, triamilide (eg. tulatromicin), fluoroquinolones (eg. danofloxacin, enrofloxacin), fluorfenicol, betalactam (eg. penicillin, ceftiofur) (Constable et al., 2017), tilmicosin, gamitromycin, oxytetracycline (Peek et al., 2018). A clinical improvement in response to appropriate antibiotic therapy will appear as better attitude, better appetite and a decreasing of fever within 24 hours (Peek et al., 2018). Moreover, animals treated in early stages with adequate dose of antibiotics usually recover quickly (24-72h), quite the opposite, severe cases of pneumonia require daily treatment for several days (Constable et al., 2017). Depending on which active substance is used, a minimum of 3 days of antibiotic coverage is often required and more often 5 to 7days of continuous therapy is necessary and less likely results in recurrence (Peek et al., 2018). If two days after the beginning of the therapy there is not a clinical improvement, the active substance should be changed, better if using the antimicrobial sensitivity test (Stöber, 2004). In conjunction to antibiotic therapy, anti-inflammatory drugs, such as prednisone or NSAID (eg. Flunixin meglumine, ketoprofen, carprofen, meloxicam), can be used. NSAID has to be used warily, in fact prolonged use concur to GI side effects (Peek et al. 2018; Stöber 2004). In early stages, it is indicated the administration of broncho spasmolytic and secretory products, later, mucolytic products can be administered. In calves, combinations with vitamin E and selenium are also recommended (Stöber, 2004). Dehydration may appear as a complication of toxemia and fever, or as result of severe dyspnea, inducing loss of appetite and reduces water consumption. In these cases, IV fluid volume has to be appropriate to avoid the onset of pulmonary edema (Peek et al., 2018). At last fresh air is utmost important and is better cold fresh air than a poorly ventilated or drafty but warm enclosure (Peek et al., 2018).

Prevention

Temperature, ammonia concentrations above 4 ppm in 24-h measurements and presence of an air velocity > 0.8 m/s are associated with lung consolidation in group-housed calves (Van Leenen et al., 2020).

In order to reduce the risk factors previously described, there are management changes to be evaluated to improve the quality of housing and animal care.

Both biosecurity and welfare should be improved. With a good biosecurity plan, pathogen circulation can be reduced by acting on internal biosecurity (improving ventilation, reducing overcrowding, and implementing feeding) and external biosecurity (avoiding mixing animals, especially of different species). In addition, increased animal welfare reduces stress. (Callan & Garry, 2002). In fact, analyzing and resolving calves housing risk factors, can reduce the exposure to aerosolized bacteria and the prevalence of respiratory disease (McGuirk, 2008). Calf housing facilities should ensure high air volumes to reduce the concentration of irritant gases, such as ammonia, without the formation of air currents that direct impact the animals (Van Leenen et al., 2020). One of the main cornerstones of external biosecurity is the quarantine of new animals. The inclusion of quarantine facilities is often overlooked in the dairy farm design plan. However, off-site heifer rearing and the purchase of replacement herd animals pose a significant risk for disease, so quarantine procedures must be provided for new arrivals and sick animals. (Gorden & Plummer, 2010). With this mind, a regular implementation of screening examinations can help to find sick calves in an early stage, either among newly purchased animals or in the replacement herd, because during the early stage of BRDC the treatment is extremely effective. Moreover, scoring calves after a treatment is useful to identify animals that require additional treatment. Furthermore, calves scored before moving into a group pen can result in fewer uncured pneumonia cases causing a respiratory disease outbreak in the weaning pen (McGuirk, 2008). An important aspect of BRDC management among calves and heifer is the vaccination protocol for the herd (McGuirk, 2008). Vaccination should be considered part of a biosecurity management system, in fact it allows to reduce pathogens shedding, decreasing transmission within a susceptible population. It should be administrated also to adult cattle as well, to provide optimal colostral immunity to the calves and to decrease shedding (Callan & Garry, 2002). Vaccines administrated intranasal (IN), lead to development of immune globulins, primarily IgA, on the mucosal surface where potential pathogens will be invading (Chase et al., 2008; Gorden & Plummer, 2010). IN is more likely to prevent infection rather than just reduce disease, moreover an advantage of IN vaccines is to induce interferon that on one side has an antiviral effect and on the other may aid in the development of a mature immune system (Chase et al., 2008). Moreover, in case of high incidence of FPT in the herd, calves result to be at high risk to contract diseases. For this reason vaccination in the first month of life may be warranted (Gorden & Plummer, 2010). To minimize induced immunosuppression and interference by maternal antibody result, the use of mucosal vaccination routes can be helpful (Chase et al., 2008).

Economic losses of BRDC

Bovine respiratory disease complex is associated with considerable economic costs (Schaffer et al., 2016). The cost related to respiratory disease is difficult to quantify and includes prevention, treatment and loss of productivity (Gorden & Plummer, 2010), such as death, lower ADG and reduced carcass value (Fulton et al., 2002). In dairy cows, this means increased age at first calving, and decreased milk production at first lactation (Quick et al., 2020). In recent USDA surveys (2012) percentage of heifers that died as the result of respiratory disease was 2.3 % of preweaned heifers. BRDC represents the principal cause of death (56.1%) in weaned heifers calves. In 2001, an economic model to calculate farm-specific losses due to bovine respiratory disease in dairy heifers has been created by Fels-Klerx et al.(2002), and 31.2 € was the average loss per heifer present on the farm (range 18.4 - 57.1 €).

In calves affected by BRDC, it is reasonable to hypothesize that weaning weights are reduced on average by 15.87 kg (Wittum & Perino, 1995). If the gain is approximately 1.10 $/kg, it means that the loss would be worth 17.50 $. If treatment costs including labor are 20 $/calf, and assuming no death loss, then the cost of one calf needing treatment would be $37.50. If the percentage of calves becoming ill during the suckling phase is 10%, then the cost of illness for each calf in the herd would be 3.75 $. When death occurs and is included in this calculation, the cost of illness increases considerably. If the value of each lost calf is equal to the cost of keeping a cow on an annual basis (eg, 500 $), then each 1% death loss increases the cost of respiratory disease for each surviving calf by 5.00 $/calf. Thus, if the percentage of calves needing treatment is 10% and death loss is 1%, then the total cost of respiratory disease would be 8.75 $ for each surviving calf. Moreover, it would be necessary to add time and labor costs associated with treatment or death losses (Stokka, 2010). As suggested by Dubrovsky (2019), there is still room for improvement on decreasing overall calf mortality due to BRD, therefore a proper herd health management, attention to common risk factors and the use of specific vaccines should be applied to prevent BRDC outbreaks (Stokka, 2010).

Performing a systematic review and meta-analysis of the relevant scientific literature, Buczinky et al. in 2021 reported that heifers diagnosed with BRD during the first few months of life were 2.85 times more likely to die (95 percent confidence interval: 1.22 to 6.69) and 2.30 times more likely to be removed from the herd (i.e., dead, euthanized or sold) before the first calving (95 percent confidence interval: 1.75 to 3.03) than heifers not diagnosed with the respiratory disease. Heifers with BRD at calving had also a reduced average daily gain of 0.067 kg/d (95% confidence interval: -0.099 to -0.034) and produced 121.2 kg (95% confidence interval: -184.9 to -57.5) less milk during first lactation.

Aim of the study

To date, the Bovine Respiratory Disease Complex is a disease that has been much studied, but because of the complexity of its etiopathogenesis, further studies are still needed for its deeper comprehension. The consequences that BRDC has are numerous. It has direct effects on both animal welfare and an impact on the dairy farm economy.

This work aims to increase knowledge regarding BRDC by analyzing it from multiple perspectives. The first purpose was to fill a gap that so far exists in Italy regarding the prevalence of viral and bacterial etiological agents related to BRDC in dairy calves. The second aim was to evaluate diagnostic methods that would be able to increase the accuracy of diagnosis, since to date we do not have a diagnostic gold standard available, and these methods would be accessible and applicable on a large scale under field conditions. To achieve this goal, we evaluated the diagnostic accuracy of fast ultrasound: Focused Lung Ultrasonography (FLUS) compared with systematic thoracic ultrasound (TUS). We also evaluated the diagnostic accuracy of lung auscultation through a Bayesian method by standardizing audible sounds and perceptible noises. Finally, information regarding the changes that occur in blood gas level in calves with BRDC is scarce, and also there are not enough data regarding the evaluation of these changes with a modern approach (Strong Ion approach), for these reasons, this thesis aims to investigate what are the blood-gas changes on arterial blood and the variations in gas exchanges, electrolyte equilibrium and base acid balance evaluated in groups of calves suffering from BRDC classified through clinical scores and by ultrasound scores.

Prevalence of lower airway respiratory pathogens in dairy calves of northern Italy

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Introduction

Bovine respiratory disease complex (BRDC) is a disease, caused by the interaction of multiple factors that afflicts cattle worldwide. All types of cattle can be affected by the disease regardless of sex, age, breed, and affects both dairy and beef production lines. Diagnosis of BRDC remains a challenge to date because of the not specific symptoms and because of the presence of asymptomatic or paucisymptomatic animals. Respiratory symptomatology may be due to an inflammatory or infectious process affecting the upper respiratory tract, however, BRDC is defined as when the disease process reaches and affects the lower airways with a pathogen causing active infection (Smith et al., 2020). The most commonly reported bacteria are, *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, *Mycoplasma bovis*, and *Trueperella pyogenes*. The viruses mainly involved in the pathogenesis of BRDC are bovine respiratory syncytial virus (BRSV), parainfluenza virus type 3 (PI-3), bovine herpesvirus type 1 (BHV-1) which is the etiologic agent of infectious bovine rhinotracheitis (IBR), bovine viral diarrhea virus (BVDV) and bovine coronavirus (BCoV) (Woolums et al., 2009).

In calf husbandry, BRDC is the main reason for the use of antimicrobials during the feeding period (Nickell and White, 2010; Brault et al., 2019). In dairy calves, BRDC and diarrhea are the most important diseases observed during the pre- and post-weaning period (Windeyer et al., 2014).

To preserve the efficacy of antibiotics and reduce the phenomenon of antimicrobial resistance, it is important to avoid their reckless and careless use by basing treatment choices on the results of etiologic investigations and antibiotic susceptibility testing, reducing mass treatments, and favouring individual case therapy (Ungemach et al., 2006; Baptiste and Kyvsgaard, 2017). Recent guidelines for antibiotic therapy encourage the use of diagnostic procedures and clinical trials before prescribing drugs and to perform susceptibility testing as often as possible (EU Reg., 2019/6). To date, the main reason for the use of antibiotic drugs in cattle breeding is respiratory diseases (Pardon et al., 2012; Windeyer et al., 2014; Dubrovsky et al., 2019). A proper setting of a treatment plan should always include targeted etiologic therapy; nasal swabs provide useful but not always indicative information for lower airway disease. Therefore, in an attempt to follow published guidelines, non-endoscopic bronchoalveolar lavage (nBAL) is increasingly being performed in many countries to obtain lower

respiratory tract specimens for bacteriological analysis (Van Driessche et al., 2017) but even this technique does not exclude contamination by bacteria living in the upper airways. For this reason, the present study aimed to evaluate the prevalence in Lombardy (Northern Italy) of bacterial and viral pathogens causing respiratory pathology, in preweaning dairy calves, sampling them by trans tracheal wash (TTW).

Materials and methods

Animals and housing

The research protocol was reviewed and approved by the Animal Welfare Organization (OPBA) of the University of Milan (Approval number 104/2022).

The study was conducted between February 2021 and May 2022 in 10 dairy farms in Lombardy (northern Italy).

To be eligible for this study, calves had to be male or female Italian Holstein breed, aged between 1 to 3 months, and bred in farms that had BRDC outbreaks during the last week. Moreover, the animals must not have had concomitant diseases (enteric diseases and diarrhea, umbilical infections, etc.) and must not have received antibiotic treatment in the three weeks prior to the study. At the time of clinical examination, the unique identification number on the ear tag of each calf was recorded on a working chart, and the age and sex were noted.

For the first month of life, animals were housed in individual boxes at ground level or raised on slatted floors. After this period, animals were housed in groups of 20 to 25. Within 4 hours after calving, calves received 4 litres of maternal colostrum if it was of good quality (>24° Brix evaluated whit hand refractometer) , or otherwise they were given colostrum from the bank. The diet consisted of pelleted feed, forage, and reconstituted milk powder, fed automatically by self-feeders or given to them by attendants two or three times a day. None of the sampled animals had undergone vaccine prophylaxis against respiratory pathogens except against BVD. The absence of an immunization plan for at least 3 months was a condition for the study. In some farms trivalent inactivated vaccine against parainfluenza type 3, respiratory syncytial virus and *Mannheimia haemolytica* was being used but vaccination protocol had been discontinued in the recent period. 240 calves, which came from 10 different farms, were included in the study; among these animals, a total of 60 calves (6 animals per farm) were sampled for etiological investigations.

Study design

A prevalence study was conducted using a convenience sample based on the following inclusion criteria:

1) dairy farms that were regularly inspected by a veterinarian operating in northern Italy,
2) availability for calf capture,
3) number of milking cows per farm (between 200 and 500),
4) farms located in the area easily accessible from the Department of Veterinary Medicine and Animal Sciences of the University of Milan, so that samples could be consigned to the Laboratory on the same day of on-farm sampling (Figure 1).

Notifications of dairy farms with recent BRDC problems were collected, and 10 farms met the inclusion criteria

All pens were visually evaluated looking for animals with respiratory signs (cough, nasal or ocular discharge). Pens in which at least one calf showed respiratory signs were included and the whole group of calves, composed by 20 to 25 animals, was evaluated by GS using the Wisconsin Calf Respiratory Scoring Chart (McGuirk, 2008). All animals also received lung auscultation performed by AB (Curtis et al. 1986) and thoracic ultrasound performed by one of the principal authors (VF or AB (Ollivett and Buczinski, 2016). A total of 240 calves were clinically evaluated to determine their health status and prevalence of respiratory disease. A total of 60 Trans Tracheal Wash (TTW) was performed. In brief, 6 calves were recruited from each herd. The number of 6 calves per farm was decided by consulting the available scientific literature and to limit the risk of not finding an animal positive for a specific pathogen (Gorden and Plummer,2010; Pardon and Buczinski, 2020). Animals were included in the study and underwent TTW if TUS was positive with a score ≥ 2 and/or WCRSC was ≥ 5 and/or auscultation was positive with score ≥ AUSC 3.

Microbiological isolation

Trans tracheal wash was performed by one of the principal authors (VF or DP) as previously described (Pravettoni et al. 2020). The procedure involved, shaving the hair of a 5-cm square area at the level of the ventral region of the neck in the middle third of the trachea, and surgical skin preparation was performed with alternating serial passes of alcohol and iodopovidone. Before proceeding with a 1-cm skin incision with a sterile blade, local anesthesia of the skin at the trachea was performed by subcutaneous injection of 2 ml of procaine hydrochloride subcutaneously (Procamidor; Izo s.r.l., Brescia, Italy). A 14G needle cannula, contained in the Cavafix® Certo Kit, was inserted, passing perpendicularly between two tracheal rings and then tilting and sliding the cannula within the tracheal lumen. Placement of the 32-cm Cavafix® Certo catheter was then performed. The kit consists of three

components: the catheter, a yellow graft to connect it to the cannula, and a red part associated with a clear plastic coating that covers the catheter and preserves its sterility during the placement procedures. After the yellow component is grafted to the cannula, the catheter is slid inside the tracheal lumen, the red part with the sheath is removed, and once the yellow part is removed, the base of the catheter can be screwed to the cannula cone. In this way, the tip of the catheter is at the level of the bifurcation of the trachea.

Trans-tracheal lavage was performed by injecting 20 ml of sterile saline, and immediately the material was aspirated with the same syringe to be analyzed. The procedure was considered successful if at least 4 ml of fluid was collected. After completion, the cannula and catheter were removed and a single surgical stitch was applied at the level of the skin incision using a non-resorbable polyamide, which was removed after 10 days.

The resulting sample was transferred into a sterile 9-mL tube and refrigerated at 4°C during transport to the laboratory.

The samples, within 12 hours after collection, were divided into two aliquots, one part was destined for microbiological investigations conducted in the microbiology laboratory of the Department of Veterinary Medicine and Animal Sciences of the University of Milan, while the remaining aliquot was taken to the laboratory of the Zooprophylactic Institute of Lombardy and Emilia Romagna to investigate, by PCR method, positivity to *Mycoplasma bovis* and the following viral agents: bovine respiratory syncytial virus (BRSV), bovine parainfluenza-3 virus (BPI3V), bovine coronavirus (BCoV) bovine viral diarrhea virus (BVDV) and bovine herpesvirus-1 (BHV-1). Real-time PCR analyses were first performed on a pool of the 6 samples constituted by taking 200 µL from each sample; in case of positivity, tests were performed on all 6 different samples for individual identification.

Trans-tracheal wash samples were processed by one of the principal authors (GG or LFP) for isolation of bacteria, the liquid was shaken and resuspended to equalize the sample, and 100 µL was taken with an automatic pipettor and placed in the respective plate, with the help of a plastic stick the sample was evenly distributed on the blood agar plate and were incubated for 24 h at 37 °C and then evaluated. Bacterial colonies were isolated and provisionally identified based on morphology and hemolysis patterns. The number of colony-forming units (CFU) per mL was reported, and according to the degree of bacterial growth, the samples were divided into: sterile samples; samples characterized by mixed microbial flora (polymicrobial) < to 100 CFUs; samples characterized by a mixed microbial flora with a predominance of one microorganism (>100 $<10^3$ CFU); samples characterized by a pure culture with abundant growth of only one bacterial species ($>10^4$ CFU) or a mixed culture i.e., equal growth of 2 bacterial species (primary or secondary pathogens). Matrix-

assisted laser desorption ionization-time of flight mass spectrometry (MALDI - TOF MS) was used for their species-level identification.

Antibiotic sensitivities of major respiratory pathogens were tested for each herd. The Kirby Bauer disk diffusion technique was used, as described in the literature. Antimicrobials tested included amoxicillin-clavulanic acid, ampicillin, ceftiofur, flumequine, enrofloxacin, marbofloxacin, florfenicol, lincospectin, oxytetracycline, penicillin, and sulfonamide-trimethoprim.

Data analysis

The collected data were organized in an excel spreadsheet. The following variables were annotated for each animal included in the study: identification number, sex, age, labored breathing, rectal temperature, Wisconsin clinical score, Auscultation score, TUS score, bacteriological test positivity, virological PCR test positivity, and *Mycoplasma bovis* PCR test positivity.

Both global animal and farm-level prevalences were calculated by the following formula: (number of positive cases/total population number) X 100.

Figure 1. Distribution of selected farms.

Distribution over the territory of the Lombardy region of the 10 farms investigated: 5 in the Lodi district, 4 in the Cremona district, and 1 in the Varese district. In each farm, 6 animals with respiratory symptoms were sampled by trans tracheal wash (TTW) and subjected to etiological investigations.

Results

Considering all 240 calves that were tested, 70% had at least lobular lesions of pneumonia, or more severe lesions, detectable by TUS, the prevalence of ultrasound-detected BRDC was variable among farms and was on average 68% (values ranged from 9% to 100% prevalence at farm level). On all farms, tracheobronchial fluid samples yielded a positive etiologic diagnosis in 75% of the animals tested; the remaining 25% of samples yielded negative results on RT-PCR investigations and bacterial culture were sterile (8%) or with various and sparse microbial flora (17%).

The calf population included in the study was characterized by animals with an average age of 59.6 days (min 28, max 92, SD 19.5). The values obtained from the compilation of the Wisconsin clinical score gave a mean value of 4.5 (min 0, max 10, SD 1.9) while the mean score of the auscultation score gave a value of 1.6 considering a range from 0 to 3 (SD 1.2) All calves received lung ultrasound resulting in an average TUS score of 3 with a range of 1 to 5 (SD 1.5). Considering the calves that were subjected to thoracic ultrasound, 12 had TUS 1 (20%); a TUS score of 2 was obtained in 13 calves (22%); 13 animals had TUS 3 (22%); and the remaining 22 calves (36%) had a TUS score greater than or equal to 4.

Bacterial pathogens

Pasteurellaceae was the family with the highest prevalence in the lower airways of pre-weaning calves, particularly *Pasteurella multocida* (22/60, 37%), followed by *Mannheimia haemolytica* (7/60, 12%). These two pathogens were identified in 7 out of 10 farms and 4/10 farms, respectively. Regarding those farms found to be positive for *Pasteurella multocida*, the farm-level prevalence ranged from a maximum of 100% to a minimum of 17%, while for farms positive for *Mannheimia haemolytica*, the pathogen prevalences were a maximum of 50% and a minimum of 17%.

The presence of *Mycoplasma bovis* was detected in two animals from one farm by RT-PCR method, and in all the 6 animals of another farm, its prevalence was 13% (8/60).

The fourth pathogen found most frequently was *Bacillus pumilus* in 4 out of 60 animals but these were all from one farm, while *Streptococcus pluranimalium* was detected in 2 animals from 2 different farms. Through bacterial culture on agar plates, other bacterial colonies were also identified: on one farm three animals had *Escherichia coli*, *Streptococcus gallolyticus* and *Enterococcus gallynarum*, respectively, and the presence of *Streptococcus suis*, *Bacillus altitudinis*, *Staphylococcus aureus* and *Actinobacillus succinogenes* was also detected finally in two individuals showed traces of *Aerococcus viridans* and *Bibersteinia teahalosi*.

Viral pathogens

Bovine coronavirus and bovine respiratory syncytial virus were demonstrated to be present in 9/60 animals (15%) and 4/60 (7%), respectively. The farm-level prevalence for BCoV is relatively high despite the few cases and amounted to 60 % of the farms visited, while BRSV was found in only 2 farms. No presence of PI3V, BVDV and BHV-1 was found. The prevalence of the etiological agents is shown in Table 1.

Co-infection Analysis

Only co-infections determined by 2 pathogens were found, and no multi-infections with high presence of multiple etiological agents were detected. The relationships between the pathogens detected are shown in Table 2.

Pasteurella multocida was found simultaneously with another pathogen 8 times/22 (36%), 3 times with *Mannheimia haemolytica*, 2 times with *Mycoplasma bovis*, 2 times with BRSV, and once with *Streptococcus pluranimalium*.

Mannheimia haemolytica in addition to the 3 animals in which it was found with *Pasteurella multocida* was also detected twice with BCoV and once with *Mycoplasma bovis*.

Mycoplasma bovis was detected in 8 animals in which two in co-infection with *P. multocida*, one in co-presence with *M. haemolytica* and one with *Stahpylococcus aureus.*

Bacillus pumilus was isolated in pure culture 3 times out of 4 while in one calf it was in association with *Bacillus altitudinis.*

Regarding viral agents Bovine coronavirus gave co-infections 56% of the times it was detected, 2 times with *Mannheimia* and once with BRSV, *Streptococcus suis*, and with *Escherichia coli*, while bovine respiratory syncytial virus was always found in association with other etiological agents 2 times with *Pasteurella* and once with BCoV and with *Streptococcus pluranimalium*.

BRSV, *Streptococcus pluranimaliu,. Escherichia coli and Bacillus altitudinis* were found only in association with other viral or bacterial pathogens.

Table 1. Prevalence of pathogens.

	CFU/ml (average)	Number of Positive Samples	Overall Prevalence	Number of Positive Farms (%)
Sterile		5	8%	
Various Microbial Flora	$< 10^2$	10	17%	
Pasteurella multocida	10^5	22	37%	7 (70%)
Mannheimia haemolytica	10^5	7	12%	4 (40%)
Bacillus pumilus	10^4	4	7%	1 (10%)
Streptococcus pluranimalium	10^3	2	3%	2 (20%)
Streptococcus suis	10^5	1	1.7%	1 (10%)
Bacillus altitudinis	10^4	1	1.7%	1 (10%)
Stahpylococcus aureus	10^4	1	1.7%	1 (10%)
Escherichia coli	10^3	1	1.7%	1 (10%)
Streptococcus Gallolyticus	10^3	1	1.7%	1 (10%)
Actinobacillus succinogenes	10^3	1	1.7%	1 (10%)
Enterococcus gallynarum	10^2	1	1.7%	1 (10%)
Aerococcus viridans	$<10^2$	1	1.7%	1 (10%)
Bibersteinia teahalosi	$<10^2$	1	1.7%	1 (10%)
Mycoplasma bovis		8	13%	2 (20%)
BCoV		9	15%	6 (60%)
BRSV		4	7%	2 (20%)
PI3V		0	0%	0
BVDV		0	0%	0
BHV-1		0	0%	0

Pathogens found in the lower airways of pre-weaning dairy calves sampled through Trans Tracheal Wash (TTW) with the number of positive samples, and their overall and farm-level prevalences.
CFU, Colony Forming Units; BCoV, bovine coronavirus; BRSV, bovine respiratory syncytial virus; PI3V, parainfluenza-3 virus; BVDV, bovine viral diarrhea virus; BHV-1, bovine herpesvirus-1.

Table 2. Pathogens identified in multiple infections.

	Tot	single infection	co-infection	*Pasteurella multocida*	BCoV	*Mycoplasma bovis*	*Manneimia haemolytica*	*Bacillus pumilus*	BRSV	*Streptococcus pluranimalium*	*Escherichia coli*	*Stahpylococcus aureus*	*Bacillus altitudinis*	*Streptococcus suis*	% singl-inf./tot	% co-inf./tot
Pasteurella multocida	22	16	8	/		2	3		2	1					73%	36%
BCoV	9	4	5		/		2		1		1			1	44%	56%
Mycoplasma bovis	8	4	4	2		/	1					1			50%	50%
Manneimia haemolytica	7	1	6	3	2	1	/								14%	86%
Bacillus pumilus	4	3	1					/					1		75%	25%
BRSV	4	0	4	2	1				/	1					0%	100%
Streptococcus pluranimalium	2	0	2	1					1	/					0%	100%
Escherichia coli	1	0	1		1						/				0%	100%
Stahpylococcus aureus	1	0	1			1						/			0%	100%
Bacillus altitudinis	1	0	1					1					/		0%	100%
Streptococcus suis	1	0	1		1									/	0%	100%

Pathogens that showed presence during multiple infections. The percentage of pure isolates and the percentage of isolates under co-infection conditions are reported.

Discussion

Most of the pathogens found in our study at the lower airway level have also been reported to be present in analyzed animals by other studies in Europe and worldwide. In some studies, aimed at investigating the viral or bacterial etiological agents causing BRDC, sampling has been done by BAL post-mortem at slaughter (Klima et al, 2019), or by using post-mortem histological techniques on tissue samples (Rahe et al., 2022), while others have used BAL for *in vivo* diagnosis in groups of beef calves (Zeineldin et al., 2017) or in dairy herds (Doyle et al., 2017) still others used trans tracheal wash (TTW) as a sampling method in both groups of beef animals (Timsit et al., 2017; Nicola et al., 2017; Timsit et al., 2018) and dairy cattle (Härtel et al., 2004; Tortorelli et al., 2017; Bottinelli et al., 2017; Gaeta et al., 2018).

Many studies conducted to investigate the prevalence of respiratory pathogens in dairy cattle have been performed from nasal or deep nasopharyngeal swabs (Lee et al., 2022; Aly et al., 2021; Studer et al., 2021; Francoz et al., 2015). Due to the increasing complexity of execution, fewer studies have used BAL and even less have performed sampling with TTW of which some were conducted in Italy for example, the study by Bottinelli et al., (2017) in which they were focused on investigating only the genus *Mycoplasma*.

In our population, the pathogens with the highest prevalence were *P. multocida* (37%), BCoV (15%), *Mycoplasma bovis* (13%) and *M. haemolytica* (12%) followed then by BRSV and *Bacillus pumilus*,

both with a prevalence of 7% and *Streptococcus pluranimalium* with prevalence of 3% other bacteria were found in 1 case out of the 60 animals sampled, and no other viruses were detected. Other studies have also predominantly shown an increased presence of *Pasteurella multocida*, but this is a recent trend as a few decades ago the prevalent pathogens were different.

For example, regarding beef cattle, Welsh et al. (2004) reported that the ratio of *M. haemolytica* and *P. multocida* isolates from the lungs of beef cattle with pneumonia decreased from 3.1 in 1994 to 0.8 in 2000. Furthermore, in a recent study of beef cattle with BRD, 30 percent were positive for *P. multocida*, 18 % for *M. haemolytica*, and 11 % for *H. somni* on bronchoalveolar lavage fluid samples (Capik et al., 2017). In another study conducted by Timsit et al., (2017) the most prevalent bacterium was *P. multocida*, isolated from 54.8% of these cattle, followed by *M. haemolytica* (30.5%) and *H. somni* (22.9%).

The cause of this change in bacterial prevalence is not well known, but could be related to changes in the virulence of these bacterial pathogens, changes in bacterial resistance to several antimicrobials (Welsh et al., 2004), and/or the availability of more effective vaccines against *M. haemolytica* than *P. multocida* (Larson and Step, 2012). Regardless of the cause, the high prevalence of *P. multocida* indicates the need for more effective control of this bacterium in cattle breeding.

In a study conducted on trans tracheal washes performed on 37 calves referred to the Veterinary Teaching Hospital of the University of Milan in 2015, the most frequently isolated pathogens were *Pasteurella multocida* (13 cases, 35.14%) and *Mycoplasma bovis* (11 cases, 29.76%). In 8 patients (21.62%), other species of *Mycoplasma* were identified. Four samples (10.81%) were positive for bovine respiratory syncytial virus (BRSV), and 4 (10.81%) for parainfluenza type 3 virus (PI-3). In 7 (19%) cases no pathogens were isolated. Five out of 37 of the sampled calves tested positive for *Trueperella pyogenes*, this finding is probably due to the severity and particularly the chronicity of pneumonia with the formation of foci of suppuration, abscesses, and necrosis that characterized the severe respiratory problem in these animals and prompted owners to hospitalize their calves (Pravettoni et al., 2020). The same bacterium, previously called *Arcanobacterium pyogenes,* was reportedly found in Argentina in beef calves with severe chronic pneumonia that resulted in a fatal outcome (Margineda et al., 2017). The absence of *Trueperella pyogenes* in the samples analyzed in this study could be explained by the fact that the investigations were performed a few days after receiving reports of BRDC symptomatology in the herds and therefore most of the animals were in the acute stage of the disease.

To the authors' best knowledge, *Bacillus pumilus* findings in cattle breeding or in correlation with respiratory diseases have not been described. In the literature of human medicine, this type of infection has been described during neonatal septicemia or causing dermatitis in humans (Branquinho

et al., 2014; Kimouli et al., 2012; Tena et al., 2007). In our sample of animals, this bacterium was found in 4 calves belonging to a single farm, its presence in the lower respiratory tract with such a high farm prevalence (4/6 or 67%) may have been influenced by farm management conditions. In fact, the environment in which the calves were housed was very dusty, and since *Bacillus pumilus* is a sporigenous bacterium typical of the soil, it may have been inhaled by the calves and at the level of the lower airways may have behaved as an opportunistic microorganism, also because the number of CFUs that were found in the sample was high (10*3/ml to 10*6/ml) and therefore cannot be attributed to fortuitous contamination.

In a study conducted by Bottinelli et al. (2017), 49 calves, from a dairy farm, were sampled with nasal swab and TTW Of these 49 animals, 42 were positive for *Mycoplasma* spp. but none showed the presence of *Mycoplasma bovis*. On the contrary in Canada the prevalence for *Mycoplasma bovis* in 11 farms was 20 % (Francoz et al., 2015). In our sample, considering the overall population, 13 % of animals were positive and the percentages of affected farms were low (2/10), but the problem becomes relevant if we consider the farm situation where 33% and 100% of calves were positive for *Mycoplasma bovis*.

The finding of *Streptococcus suis* in a calf is an event that has already occurred in other cases. It has been described in Canada (Higgins et al.,1990), Switzerland (Vogel et al., 2001) and in the United States (Okwumabua et al., 2017). Pneumonia caused by this pathogen in calves has the same caracteristics that in swine, without the complication of polyserositis. Positivity for *Streptococcus plurianimalim* has already been described in cattle; it can be found in milk or vulvar drains but can cause different types of diseases in many animals, including respiratory diseases that have been described in sheep and humans (Yan et al., 2022; Mahmood & Kahya, 2022).

The other microorganisms that we detected, were singularly evidenced in only one animal in the group, and the number of CFU was on average lower than the pathogenic ones more classically associated with BRDC. This finding may be associated with inhalation of these microorganisms due to dusty or excessively contaminated environments, as presumably was the case on the farm that showed the presence of *E. coli*, *Streptococcus gallolyticus* and *Enterococcus gallynarum*, probably because the calves were housed in an environment overpopulated by pigeons and other birds.

The absence of *Histophilus somni* can be attributed to the fact that cultures were not done in anaerobiosis but mainly aerobic bacteria were investigated.

Among viral agents, bovine coronavirus was the most represented (9/60) and was found both in consociation with other viral or bacterial pathogens (in 56% of cases) and as the sole etiologic agent (44% of times). Many studies support the active role of BCoV in the pathogenesis of bovine respiratory disease. In some studies, the prevalence of BCoV during BRDC was very high, greater

than 80%, but the prevalence was obtained by analyzing nasal swabs (Storz et al., 2000; Decaro et al., 2008). The overall lower airway prevalence value in our whole sample was 15%, it is very interesting to note that 60 % of the farms were found to have BCoV. It is therefore much more widespread in the area than other pathogens and in more than half of the cases acted allowing co-infections. The role of this pathogen towards respiratory disease deserves further investigation and clarification.

In Lombardy, there are voluntary farm-level control plans for the surveillance of IBR and BVD, those are considered important BRDC-associated pathogens (Constable et al., 2016). These control plans are based on herd surveys, vaccination and control of purchased animals. The farms we investigated had vaccination protocols in place for the control of BVDV, BHV-1, PI3V, BRSV and against *Mannheimia haemolytica*. The lack of positivity for BVDV and BHV-1 indicate that the efforts being made regionally to limit the spread of these diseases are achieving the desired results. Regarding the cases of TTW that were found to be sterile (5/60) this may be because, although TUS remains the best method for in-vivo diagnosis of BRDC, it is an imperfect Gold Standard. In fact, these animals may have presented lung lesions due to other causes (atelectasis, trauma, etc.) or may have received antibiotic treatments more than three weeks prior to the clinical investigation and still presented lung lesions, sequela of previous pneumonia, but no more active and without an ongoing infection.

Conclusions

This is the first study that considers the prevalence of bacterial and viral agents causative of respiratory disease in the lower airways of dairy calves in northern Italy. It has been found that the prevalence of *Mycoplasma bovis* although low in the population reaches very high values at the farm level. In our population the etiological agents with a higher presence were *Pasteurella multocida* and bovine coronavirus. As required by the European Community it is necessary to strengthen of the prudent use of antimicrobials, avoiding their routine metaphylactic and prophylactic use, and to achieve this objective, diagnoses must also be thoroughly investigated from the etiological aspect, so that therapeutic choices can be set up in the most effective way.

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Short communication: Diagnostic accuracy of focused lung ultrasonography as a rapid method for the diagnosis of respiratory disease in dairy calves

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ABSTRACT

This study estimates the accuracy of the focused lung ultrasound (FLUS) compared with systematic thoracic ultrasonography (TUS) as the reference test for diagnosing pneumonia in pre- and postweaned dairy calves. One hundred thirty-five Holstein Friesian calves, aged between 1 to 6 mo were enrolled and were kept in the same pen with one or more animals showing signs of bovine respiratory disease complex (BRDC). One operator performed FLUS on each calf, and then a second, blinded operator performed TUS on the same calf. For the FLUS, we only scanned the lung lobes that are most frequently affected during BRDC and are thus easier to detect, such as the caudal aspect of the cranial lobe of the left lung (fifth and fourth left intercostal spaces; ICS), the middle lobe of the right lung (fifth right ICS), and the caudal aspect of the cranial lobe of the right lung (fourth right ICS). Pneumonia was diagnosed when a calf had a minimum of one small lobular lung lesion that was at least 1 cm deep within a normally aerated lobe (TUS score of ≥2). Diagnostic accuracy indexes of the FLUS were calculated using TUS as the gold standard. The McNemar test was performed to evaluate the differences between the 2 techniques. In addition, an intertest agreement was assessed using the weighted kappa test. A total of 76 out of 135 calves had a TUS score of ≥2 and were therefore considered to be affected by BRDC. The FLUS had a sensitivity of 81.6% (95% CI = 71.0–89.5%), specificity = 100% (95% CI = 93.9–100%), positive predictive value was 100%, negative predictive value was 96.6% (95% CI = 94.7–97.9%), and accuracy was 97% (95% CI = 92.6–99.2%). The McNemar test highlighted a difference of 10.3% between the FLUS and TUS. The agreement between the TUS and FLUS was substantial (weighted kappa test 0.78). Although FLUS shows some limitations in diagnosing lung lesions associated with BRDC compared with the systematic approach, this study shows that the focused method could be used as an additional tool for evaluating consolidation, especially when examining a large number of postweaned dairy calves.

Key words

bovine respiratory disease, calves, focused ultrasonography, thoracic ultrasonography

Short Communication

Thoracic ultrasonography (TUS) is a useful calf-side diagnostic tool for detecting lung lesions associated with bovine respiratory disease complex (BRDC; Ollivett et al., 2015). There are several techniques for performing TUS in dairy calves, which vary in terms of the number of intercostal spaces (ICS) evaluated and the interpretation of the positivity of the test based on the depth and extension of the observed lesions (Teixeira et al., 2017; Ollivett and Buczinski, 2016; Buczinski et al., 2018a; Ollivett et al., 2015; Cramer and Ollivett, 2019). Ollivett and Buczinski (2016) described an ultrasonographic 6-point scoring system based on the portion of lung tissue involved in 3 types of ultrasonographic lung lesions (i.e., comet tails, lobular, and lobar pneumonia), identified by scanning the right lung from the tenth to the first ICS and the left lung from the tenth to the second ICS, with each ICS characterized by a specific intrathoracic anatomical landmark (Ollivett et al., 2015). Assessing the entire lung field on both sides of the thorax has proven useful (Buczinski et al., 2014) and enables the extent of lung lesions to be evaluated, thus facilitating the prognostic evaluation of the affected subjects (Ollivett and Buczinski, 2016). Unfortunately, the forelimb musculature precludes access to the cranial thorax in older, heavily muscled calves, thus preventing a complete ultrasonographic examination (Ollivett and Buczinski, 2016). The hypothetical added value of assessing the cranial part of the lung compared with the extra time needed was recently investigated (Berman et al., 2019). Although the study was conducted in a low prevalence context of the disease that may have altered the power to estimate sensitivity (Se) accurately, these authors found that the presence of a lung consolidation caudal to the heart, with a depth of 3 cm or more, yielded an excellent Se and specificity (Sp) (89 and 95%, respectively) in diagnosing pneumonia in preweaned calves using a latent class model (Berman et al., 2019).

Currently, there are no data on a simplified ultrasonographic technique that could be used in both preweaned and postweaned dairy calves with a mid to high prevalence of lung lesions. Our study therefore aimed to compare the results of focused lung ultrasonography (FLUS) for the diagnosis of lung lesions associated with BRDC in dairy calves 1 to 6 mo old with traditional systematic TUS used in preweaned dairy calves. The FLUS was performed by rapidly evaluating the lung lobes that are frequently involved in pneumonia and which are also the easiest to reach ultrasonographically in older calves, such as the caudal aspect of the cranial left lung lobe (fifth and fourth left ICS), the middle right lung lobe, and the caudal aspect of the right cranial lung lobe, (fifth and fourth right ICS). We hypothesized that FLUS could be a clinically appropriate method for diagnosing lung lesions associated with BRDC and that there would be a good level of agreement with systematic TUS.

A prospective diagnostic accuracy study was performed according to the relevant standards (Bossuyt et al., 2015), using a convenience sample selected from dairy farms that were regularly checked by our ambulatory clinic from September 2018 to April 2019. Criteria for the selection of the farms were a recent history of BRDC in at least 1 calf pen detected by the herd practitioner, location of the farms (no further than a 1-h drive from the Veterinary Teaching Hospital), willingness to capture calves, and milking cows per farm ranging from 180 to 230. We selected calves in pens on each farm only if at least 1 calf showed signs of

BRDC. In brief, an experienced veterinarian (AB) examined the calves to see if any of them presented a spontaneous cough. Coughing calves were scored using the Wisconsin calf respiratory scoring chart (McGuirk, 2008). If one of the coughing calves reached a total respiratory score of 5 or more, it was considered as having BRDC. All calves within the pen were then considered eligible for the study, irrespective of their external clinical status. This selection was performed to ensure a minimum prevalence of lung lesions in the affected pen to optimize the sample size for both groups of calves (calves with and without lesions). The selected animals were both male and female Holstein Friesian calves aged between 1 and 6 mo. A maximum of 10 calves were examined per pen. A randomization application (Randomizer, Darshan Institute of Engineering and Technology, Rajkot, India), run on an Android smartphone, was used when more than 10 calves were present. Calves were excluded if they belonged to breeds other than Holstein Friesian, were older than 6 mo, or had other concurrent diseases.

The publication of data was approved by the Ethical Committee of the University of Milan (approval number 47/2017, November 28, 2017).

Enrolled calves were submitted to thoracic ultrasonography using a portable unit (Ibex Pro, EI Medical) with a 7.5 MHz linear transducer designed for a transrectal purpose, set to a depth of 8 cm and gain of 16 dB. Vegetable oil was sprayed over the thoracic skin (Brethour, 1992). The thorax was not shaved to reflect the rapid use in a field setting (Ollivett and Buczinski, 2016). The FLUS was performed by an experienced veterinarian (AB). The technique used was an abbreviation of the examination described in Ollivett and Buczinski (2016), based on the ventral landmarks described by Ollivett et al. (2015). Focused ultrasonography of the left lung was performed first. The probe was placed between the middle and dorsal third of the fifth ICS, immediately under the elbow. Ultrasonography scanning was performed by moving the probe ventrally to the costochondral junction (CCJ). The probe was then slipped cranially into the fourth ICS, starting between the middle and dorsal third and moving again ventrally to the CCJ and pleural deviation. Similarly, on the right site, the probe was placed over the fifth right ICS and then slipped cranially toward the fourth ICS to visualize the lung tissue until the heart was ventrally visualized.

After performing FLUS on all calves in the same selected pen, systematic TUS (ICS 10–1 on the right, and ICS 10–2 on the left) was performed by the first author (DP) following the procedures described by Ollivett and Buczinski (2016) by randomly re-catching all previously scanned calves. This procedure was used to ensure blinding between FLUS and TUS examinations. Lung lesions were scored directly on the farms. For both FLUS and TUS, we adopted the 6-point ultrasonographic scoring system described by Ollivett and Buczinski, (2016). A positive ultrasonographic examination test was defined when a calf had a TUS score of 2 or more (lobular pneumonia: consolidation $\geq$1 cm; Ollivett and Buczinski, 2016). Because FLUS enables 3 lung lobes to be visualized [the caudal aspect of the cranial left lung lobe (fifth and fourth left ICS), the middle right lung lobe and the caudal aspect of the right cranial lung lobe, (fifth and fourth right ICS)], the whole 6-point scoring scale and the same cut-off point as the full technique were used.

Data storage and analysis was done with IBM SPSS Statistics version 25.0 for Macintosh (IBM Corp., Armonk, NY). Sex was reported as frequency and percentage of males and females. Age was not normally distributed and reported with median, interquartile range (IQR) from the 25th to the 75th percentile, minimum (min) and maximum (max) values. The TUS and FLUS scores were reported as frequencies and percentages. Because the initial aim was to quantify the significance of information loss due to FLUS versus complete examination, we based our sample size calculation first on a minimal agreement beyond the chance of reaching agreement between the 2 tests. Specifically, we based our sample size calculation on an acceptable kappa level of at least 0.80, with a minimal lower bound of 0.61. The 0.61 level was determined a priori as the lower bound of strong agreement between 2 different measures (Dohoo et al., 2003). Different scenarios were tested. Sample size of at least 120 calves would fit most of the scenarios using type 1 error 5%, type 2 error 20%. The accuracies of FLUS, Se, Sp, positive predictive value (PPV), and negative predictive value (NPV) were calculated using TUS as the gold standard.

To investigate whether there was a systematic, significant difference in the number of cases resulting as positive between TUS and FLUS, the McNemar test was performed. In addition, the intertest agreement between FLUS and TUS scores was assessed using the weighted kappa (κw; Dohoo et al., 2003). The clinical application of the differences between TUS and FLUS findings was further investigated, modeling various previous scenarios from 0 to 1 with 0.05 increment steps. The apparent disease prevalence based on FLUS findings was calculated based on the true prevalence of TUS positive cases and FLUS accuracy (Se and Sp). These were derived from the formula by Dohoo et al. (2003): Apparent prevalence = Prev × Se + (1 ¬– Sp) × (1-Prev).

The uncertainty around the estimates was obtained using low and high 95% confidence intervals limits of Se and Sp, respectively. A logistic regression model was also performed to underline the possible effect of age and the interaction between age and FLUS on TUS results. Specifically, in the logistic regression model, the outcome of interest was TUS (positive when a calf had a TUS score ≥2, negative with a score of <2), and the independent variables checked were the FLUS score, age, and the interaction between FLUS and age, using backward stepwise regression. Age was used as a categorical variable and classified according to the median (i.e., lower than the median, and greater than or equal to the median).

Eleven male (8.1%) and 124 female calves (91.9%) belonging to 10 dairy farms were enrolled in the study with a total number of 135 calves. The median age of the calves was 84 d (IQR 25% = 54 d; IQR 75% = 115 d; min 30 d; max 183 d). Sixty-two calves (45.9%) had a FLUS score of 2 or more. Seventy-six calves (56.2%) had a TUS score of 2 or more and were therefore considered to have pneumonia. For each FLUS and TUS scoring class, Table 1 summarizes the number of animals and percentages, also split by the calf age. Using a TUS threshold score of ≥2, FLUS had a Se of 81.6% (95% CI = 71–89.5%), a Sp of 100% (95% CI = 93.9–100%), a PPV of 100%, an NPV of 96.6% (95% CI = 94.7–97.9%), and an accuracy of 97% (95% CI = 92.6–99.2%).

Table 1. Ultrasonography scores used to classify the severity of lung lesions associated with bovine respiratory disease complex for 2 different methods of lung ultrasonography [focused lung ultrasonography (FLUS), and thoracic ultrasonography (TUS)] in 135 Holstein Friesian calves from 10 dairy herds in Italy[1]

Group	Method	Score						Tested negative	Tested positive
		0	1	2	3	4	5		
Total cases	FLUS	51 (37.9)	22 (16.3)	19 (14.1)	14 (10.4)	13 (9.6)	16 (11.9)	73 (54.1)	62 (45.9)
	TUS	38 (28.1)	21 (15.6)	25 (18.5)	11 (8.1)	12 (8.9)	28 (20.7)	59 (43.7)	76 (56.3)
Calves 1 mo old (n = 13)	FLUS	2 (15.4)	4 (30.8)	4 (30.8)	—	2 (15.4)	1 (7.7)	6 (46.2)	7 (53.8)
	TUS	2 (15.4)	3 (23.1)	4 (30.8)	1 (7.7)	2 (15.4)	1 (7.7)	5 (38.5)	8 (61.5)
Calves 2 mo old (n = 48)	FLUS	17 (35.4)	7 (14.6)	6 (12.5)	10 (20.8)	4 (8.3)	4 (8.3)	24 (50.0)	24 (50.0)
	TUS	13 (27.1)	5 (10.4)	8 (16.7)	6 (12.5)	5 (10.4)	11 (22.9)	18 (37.5)	30 (62.5)
Calves 3 mo old (n = 28)	FLUS	11 (39.3)	5 (17.9)	4 (14.3)	3 (10.7)	1 (3.6)	4 (14.3)	16 (57.1)	12 (42.9)
	TUS	7 (25.0)	7 (25.0)	5 (17.9)	2 (7.1)	3 (10.7)	4 (14.3)	14 (50.0)	14 (50.0)
Calves 4 mo old (n = 26)	FLUS	11 (42.3)	3 (11.5)	3 (11.5)	—	4 (15.4)	5 (19.2)	14 (53.8)	12 (46.2)
	TUS	7 (26.9)	4 (15.4)	5 (19.2)	—	2 (7.7)	8 (30.8)	11 (42.3)	15 (57.7)
Calves 5 mo old (n = 17)	FLUS	8 (47.1)	2 (11.8)	2 (11.8)	1 (5.9)	2 (11.8)	2 (11.8)	10 (58.8)	7 (41.2)
	TUS	7 (41.2)	1 (5.9)	3 (17.6)	2 (11.8)	—	4 (23.5)	8 (47.1)	9 (52.9)
Calves 6 mo old (n = 3)	FLUS	2 (66.7)	1 (33.3)	—	—	—	—	3 (100)	—
	TUS	2 (66.7)	1 (33.3)	—	—	—	—	3 (100)	—

Columns report animals grouped according to the TUS score and the binary interpretation of the same score (i.e., animals testing positive vs. animal testing negative). Rows report total numbers of animals as well as grouped according to age. Values are presented as n (%). For both FLUS and TUS, the 6-point ultrasonographic scoring system described by Ollivett and Buczinski (2016) was applied: calves with a TUS score of 0 or 1 were considered healthy, whereas calves with a TUS score of 2 (lobular consolidation), 3 (consolidation of 1 lung lobe), 4 (consolidation of 2 lung lobes), or 5 (consolidation of 3 or more lung lobes) were considered to be affected by bovine respiratory disease.

Figure 1: shows the effect of the simulated true prevalence of calves with TUS lesions on the apparent prevalence using FLUS. The effect of using FLUS for prevalence assessment versus TUS as a gold standard revealed that the difference between the 2 methods was minimal in prevalence settings lower than 30 to 50%. A comparison between FLUS and TUS in the current setting revealed 10 false negatives with lesions detected by TUS. Of these false negatives, 8 calves were affected by lobular consolidation in the cranial aspect of the right or left cranial lung lobes, whereas 2 calves showed a consolidation of the entire cranial aspect of the right cranial lung lobe. None of the 10 false-negative cases showed lesions in other lung lobes. There were no false positives when using FLUS. The McNemar test underlined a difference of 10.37% between FLUS and TUS (P = 0.001; 95% CI = 5.23–15.51%). The agreement between FLUS and TUS, calculated using the κ_w test, was substantial (κ_w 0.778; 95% CI = 0.718–0.837). The comparison between FLUS and TUS scores is shown in Table 2. According to the applied logistic regression model, age did not influence the relationship between FLUS and TUS (P = 0.453). Furthermore, the interaction between age and FLUS did not influence the TUS results (P = 1.00).

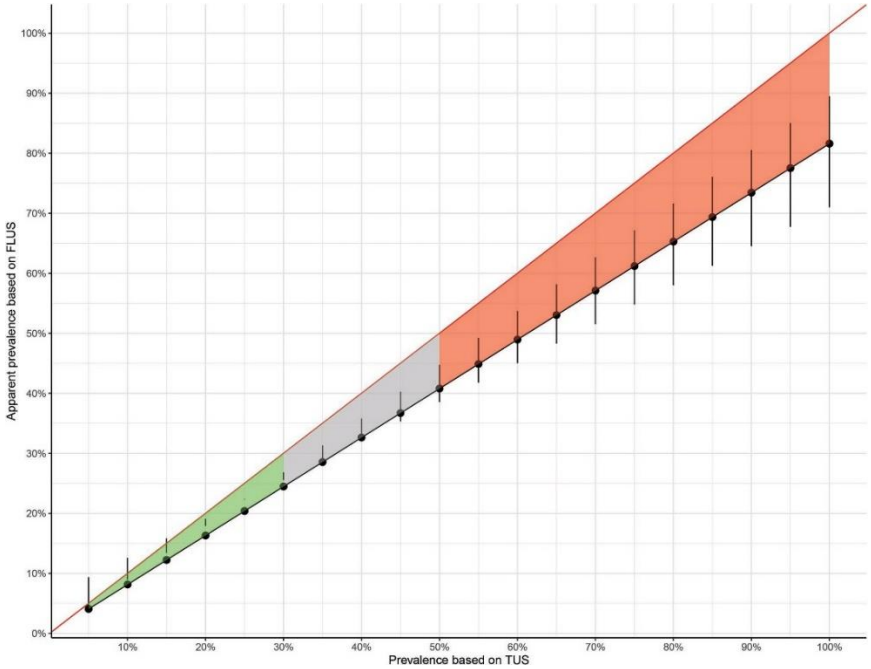

Figure 1. Apparent prevalence of lung lesions using focused lung ultrasonography (FLUS; consolidation threshold of ≥1 cm within a normally aerated lung lobe; score 2) according to different disease prevalence scenarios of lung lesions using thoracic ultrasonography (TUS) as a gold standard. This figure depicts the relation between apparent prevalence based on FLUS versus the true prevalence of lung lesions when assessed by TUS using the observed FLUS sensitivity of 81.6% (95% CI = 71.0–89.5%) and specificity of 100% (95% CI = 93.9–100%) based on various simulated ranges of lung lesion prevalence (from 0 to 1 by 0.05 increment steps). The black line and associated dots represent the mean estimate. The upper and lower bounds around each specific dot are based on the apparent prevalence calculation using low and high Se and Sp bounds, respectively. The identity line (y = x) is indicated in red. The green shaded area depicts the prevalence range where TUS and FLUS prevalence estimations are less than 5%. The red area depicts an underestimation by FLUS of 10% or less of the real prevalence ranges. The gray shaded area depicts an underestimation by FLUS of between 5 and 10% of the true prevalence.

Table 2. Contingency table and weighted kappa (κw) value for the comparison between focused lung ultrasonography (FLUS) scores and thoracic ultrasonography (TUS) scores

TUS score[1]	FLUS score						No. of calves	κ_w[2]
	0	**1**	**2**	**3**	**4**	**5**		
0	38	0	0	0	0	0	38	0.78
1	8	13	0	0	0	0	21	
2	4	6	15	0	0	0	25	
3	0	3	2	5	1	0	11	
4	1	0	2	6	2	1	12	
5	0	0	0	3	10	15	28	
No. of calves	51	22	19	14	13	16	135	

1 For both FLUS and TUS, the 6-point ultrasonographic scoring system described by Ollivett and Buczinski (2016) was applied: calves with a TUS score of 0 or 1 were considered healthy, whereas calves with a TUS score of 2 (lobular consolidation), 3 (consolidation of 1 lung lobe), 4 (consolidation of 2 lung lobes) or 5 (consolidation of 3 or more lung lobes) were considered to be affected by bovine respiratory disease.
2 The weighted kappa coefficient was 0.78 (95% CI = 0.72–0.84), indicating a substantial agreement (Dohoo et al., 2003).

The results of this study were expected because the protocol chosen for FLUS was based on the literature that evaluated the topographical distribution of lung abnormalities in suppurative bronchopneumonia, which is the most common form of pneumonia in young dairy calves (Panciera and Confer, 2010). These results reflect those of Berman et al. (2019), who found that the diagnostic accuracy indices obtained with the scan of the caudal part of the lung (eleventh to third ICS right and left) alone were satisfactory compared with those obtained by ultrasonographically scanning the full entire lung fields. Moreover, as Ollivett and Buczinski (2016) reported and confirmed by our results, the lung lobes caudal to the fifth ICS are only rarely involved in pneumonia, and when they are, they are associated with more substantial cranial lesions. Although the results mentioned above were satisfactory, a note of caution is warranted for at least 2 reasons. Eight out of 10 false-negative calves using FLUS had lobular lesions cranially to the fourth ICS on the left or the right side of the thorax. Lobular pneumonia can be associated with viral infections and bacteria such as *Pasteurella multocida* or *Mycoplasma bovis*, which are responsible for lobular necrotic pneumonia or bronchiole-centered lesions with a progressive spread along with the surrounding tissue (Panciera and Confer, 2010).

In addition, 2 out of 10 false negatives had deep consolidation of the cranial lung lobes. Although consolidation in these areas may also be due to fibrosis and atelectasis not directly correlated with

BRDC pathogens (Berman et al., 2019), pneumonia cannot be ruled out in these false-negative cases because typically, lung lesions generally start in the cranial aspect of the right cranial lung lobe (accessible by scanning first and second ICS) with a subsequent caudal progression (Dagleish et al., 2010). Furthermore, Ollivett et al. (2015) demonstrated that lobar lesions of the right cranial lobe are consistent with bacterial pneumonia based on histological examination of the lung tissue. In our opinion, these results illustrate the intrinsic limitation of focused ultrasound techniques, which are not 100% accurate and cannot fully replace systematic ultrasonography.

The McNemar test substantiated this observation and showed a significant discrepancy between cases testing positive/negative at FLUS and TUS, respectively. Another source of uncertainty is related to the variation in the FLUS accuracy based on the prevalence of the disease. As expected, in the presence of a high Sp and imperfect Se, the FLUS examination could be used as a good alternative to TUS when the prevalence of lung lesion is not too high (e.g., <30–50%). However, this was based on simulations of different prevalence contexts, assuming a constant accuracy of FLUS across these ranges. In scenarios with a high prevalence of lung lesions, an underestimation associated with FLUS is likely to be clinically relevant, highlighting the need to add the right cranial part of the right cranial lung in the scanning, especially when it can be easily done (in calves less than 2 mo old).

Although an in-depth evaluation of the effect of age on the diagnostic accuracy of FLUS was beyond the scope of the current study, we found that age did not influence the FLUS results in this case study group. This supports the hypothesis that the loss of information due to the FLUS technique did not depend on age but probably on the technique. The use of FLUS could thus be recommended in diagnosing pneumonia in larger calves, when reaching the cranial fields of the thorax is difficult due to the muscles of the thoracic limbs (i.e., when the calves are 4–6 mo old). On the other hand, TUS should be preferred to FLUS in smaller calves to prevent the loss of valuable information (e.g., the early stage of the disease which commonly affects most cranial lobes). In addition, TUS should be preferred in high-risk herds, considering that the full technique does not present any limitation in these animals and highlights the extent of the consolidation, thus representing more useful information in terms of prognostic factors.

The main limitation of this study was that FLUS was compared with TUS, which itself is an imperfect gold standard. Although it is important to consider the possible bias of this technique, the detection of lobar or lobular lesions by systematic TUS is the most accurate in vivo diagnostic tool. Furthermore, because FLUS and TUS are conditionally dependent, the covariance between the 2 tests could lead to the same classification error. Nonetheless, FLUS can help the clinician and can be used for the early diagnosis of lung lesions associated with BRDC in older animals because it is reasonably

accurate, easy and rapid to perform. Two different clinicians performed TUS and FLUS, respectively. This could be perceived as a limitation of the study. However, it was difficult to guarantee that a specific clinician performing the 2 tests would remain blind to the first examination results (therefore inducing a risk of recall bias falsely inflating the test accuracy). We therefore preferred to use 2 experienced clinicians to perform the examination. Including a gap between the 2 examinations performed by the same clinician could also have been a solution. However, there would have been a risk of lesion progression between the 2 examinations as previously reported (Ollivett and Buczinski, 2016), which would also have introduced a risk of bias. Due to the high level of experience of both ultrasonographers, we are confident that using 2 clinicians was the best way to limit the current risk of bias.

A further potential limitation affecting the differences observed between the FLUS and TUS could have been a disagreement in interpreting the lung lesions between the 2 clinicians performing the examinations. Although the discrepancy could have been assessed using an agreement study between the 2 operators, interrater agreement for the lung ultrasonography is generally good for evaluating lung consolidation, comet tails, and pleural effusion (Buczinski et al., 2018b). Another limitation of the current study is the lack of investigations on the etiological agents involved. This information would have been very useful to correlate the lobular lesions to a specific etiological agent, especially in the FLUS false-negative calves. Further studies are therefore warranted to investigate this topic.

The results of this study have various clinical implications. An important finding is that FLUS represents a practical method to diagnose lung lesions in field conditions and can be performed rapidly using a linear probe. Focused lung ultrasonography may help practitioners to recognize lung lesions in postweaned calves. The easy recognition of sick animals would facilitate specific management versus healthy subjects such as early separation, thereby preventing unnecessary antimicrobial treatments of healthy cohabitants and spreading pathogens among the groups. In conclusion, we believe that FLUS is a promising tool to improve the diagnosis of pneumonia in older calves where there is a limited access to the cranial part of the thorax.

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Bayesian evaluation of the accuracy of a thoracic auscultation scoring system in dairy calves with bronchopneumonia using a standard lung sounds nomenclature

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Abstract:

Background: Although thoracic auscultation (AUSC) in calves is quick and easy to perform, the definition of lung sounds is highly variable and leads to poor/moderate accuracy for diagnosing bronchopneumonia (BP).

Hypothesis/Objectives: We hypothesized that using a standard nomenclature of lung sounds could increase the accuracy of BP diagnosis. Therefore, we evaluate the diagnostic accuracy of a standardized auscultatory scoring accounting for the absence of a gold standard test for BP diagnosis.

Animals: Three hundred and thirty dairy calves.

Methods: Thoracic auscultation was categorized as follows: AUSC1 (all pathological sounds), AUSC2 in which wheezes, crackles, increased bronchial sounds, and pleural friction rubs were considered, and AUSC3 when only increased bronchial sounds or pleural friction rubs were evaluated. The accuracy of the AUSC categorizations was determined using three imperfect diagnostic tests by a Bayesian latent class model and sensitivity analysis (informative vs. weakly informative vs. non-informative priors and with vs. without covariance between ultrasound and clinical scoring).

Results: Based on the priors used, the sensitivity (95%BCI) of AUSC1 ranged between 0.89 (0.80-0.97) to 0.95 (0.86-0.99), with a specificity (95%BCI) of 0.54 (0.45-0.71) to 0.60 (0.47-0.94). Removing the increased breath sound from AUSC categorizations results in a pronounced increase in specificity (for AUSC3, specificity ranged between 0.97 [0.93-0.99] and 0.98 [0.94-0.99]) but at

the cost of a decreased sensitivity (0.66 [0.54-0.78] to 0.81 [0.65-0.97]). The impact of priors on posterior densities of AUSC was limited.
Conclusions and clinical importance: A standardized definition of lung sounds improved AUSC accuracy for BP diagnosis in calves.

Introduction

There is great interest in studying bronchopneumonia (BP) diagnostic strategies in calves because a more accurate indication of antimicrobial treatment for sick patients is needed, and no practical and affordable gold standard test currently exists.[1] One of the main obstacles to diagnosing the individual BP case is that clinical signs expressed by affected animals are often non-specific or lack sensitivity.[2-4] Among these, thoracic auscultation (AUSC) is considered a cornerstone of the ruminant respiratory tract examination, often the first diagnostic approach used by practitioners.[4,5] In dairy calves, it has recently been observed that the interpretation of respiratory sounds results in low inter-rater reliability of the test when performed by different practitioners[4] and poor/limited accuracy[2,5] when correlated with ultrasonographically defined lung lesions. One of the critical limitations is that the method and definition of lung sounds are highly variable and complex.[6] Confusion about the terminology to be used and numerous problems in interpreting auscultated sounds have been mentioned.[7,8] Another issue encountered is the subjectivity of the auscultated sounds[5] and the little agreement on the definition of sick calves in published studies.[2,5,9-11] These same difficulties have made it a relatively underused clinical method in respiratory research, even in human medicine. Different authors argue that standardizing and simplifying the description of pathological sounds in human respiratory AUSC could improve international communication and accuracy.[12,13] In this regard, the task force on lung sounds of the European Respiratory Society has aimed to standardize the nomenclature of human thorax AUSC findings.[6] From this consensus, it emerged that "crepitations or crackles" should be used to describe crackling sounds and that "rhonchi" should describe low-pitched wheezes. Similarly, Curtis and colleagues[7] recommend the use of terms such as "increased breath sounds," "crackles," "wheezes," and "increased bronchial sounds" to describe pathological sounds commonly heard in large animals.

A standard definition and classification of lung sounds following Curtis et al. 7 (1986) guidelines might increase the diagnostic accuracy of affected calves. The objective of this study was to evaluate the diagnostic accuracy of a scoring system (index test) characterized by a precise lung sound classification and definition for the diagnosis of BP compared to thoracic ultrasounds (TUS; imperfect reference standard test) using a Bayesian latent class approach in a population of dairy calves.

Materials and Methods

Study design and animal selection

A cross-sectional diagnostic accuracy study was performed according to standards for reporting diagnostic accuracy studies using Bayesian latent class models (STARD-BLCM).[14] A convenience sample was selected from dairy farms that requested the respiratory disease diagnostic service of the Clinic for Ruminants and Swine, Department of Veterinary Medicine and Animal Sciences, University of Milan, from January 2019 to December 2020. All calves included in the study were managed according to standard protocols for diagnosing BP in compliance with the professional ethics of veterinarians and the standards for protecting calves.[15] Criteria for farm inclusion were a history of BP-related clinical signs in at least one calf pen detected by the herd practitioner, no history of treatment for BP in calves in the previous 15 d before the day of the study, the farm's location (no more than 1-hour drive from our clinic), and willingness to use data for scientific purposes. The publication of data from the ordinary activity of our clinic was approved by the Ethics Committee of the University of Milan" (approval number 47/2017, Nov. 28, 2017). On these farms, calves were usually separated from the dam immediately after birth and received 4 L of good-quality colostrum (Brix ≥22%) within 6-8 h after birth. Calves were housed individually for up to 15-20 days and fed with 4-5 L of milk replacer twice a day with a bucket before entering multiple pens with an automated calf feeder, where they remained for up to 75-80 days. The automated calf feeder was programmed to ensure adequate feeding based on age. The calves moved from approximately 9 L/d to zero to get out of this pen completely weaned in the last period. After weaning, calves were transferred to multiple pens for up to 6 months of life. We selected both pre-and-post weaned dairy calves in these two pens.

On the days of the study, calves were observed by one experienced veterinarian for the detection of spontaneous cough. This selection was performed to ensure a minimum prevalence of lung lesions in the affected pen to optimize the sample size for both groups of calves (calves with and without lesions) because the cough is associated with an increased probability of lung consolidation.[16] Coughing calves were caught and scored using the Wisconsin calf respiratory scoring (WCRS) chart by a postdoc-trained veterinarian. The scoring system resulted in a minimum score of 0 and a maximum of 12. Each calf was examined and assigned a clinical score of 0 (normal), 1 (variation of or slightly abnormal), 2 (abnormal), or 3 (severely abnormal) for temperature, nasal discharge, cough, eye discharge, and ear position, considering the highest score of the last 2 signs.[17] If one coughing calf reached a total respiratory score of 5 or more, it was considered a positive case.[17] All calves within the pen were considered eligible for the study unless they showed lameness, dehydration, or umbilical pathologies. The selected animals were male and female Holstein Friesian calves aged

between 1 and 6 months. A maximum of 20 calves was examined per farm. If more than 20 eligible calves were present, the recruited calves were randomly selected with an application that runs on an Android smartphone (Randomizer, Darshan Institute of Engineering and Technology, Rajkot, India) using the list of identification numbers (written on the ear tag of each calf) provided by the farmer. For each enrolled animal, identification, date of birth, age at the clinical examination, and sex were recorded.

Thoracic auscultation

Enrolled calves were submitted to AUSC by the first author using the stethoscope Littmann® Master Classic II Veterinary Stethoscope. The clinician who performed AUSC was blind to the calves' clinical evaluation and ultrasound scoring. This procedure was used to ensure as much as possible that the investigator responsible for AUSC did not observe in detail the enrolled animals. For evaluating the lung sounds, the area of AUSC was divided topographically into the ventral, middle, and dorsal thirds (Figure 1). Each field was auscultated for at least three respiratory cycles (inspiration and expiration phases). During the procedure, the clinician observed the right costo-abdominal region to differentiate the sounds from inspiration and expiration. Auscultation was then scored according to the nomenclature proposed by Curtis et al.[7] and summarized in Table 1. According to Curtis et al.,[7] the term "normal breath sounds" was used to describe the sounds produced during inspiration by the normal aired lung parenchyma. "Increased breath sounds" were defined as a moderate increase in loudness of breath sounds audible during inspiration and expiration in which the difference between inspiration and expiration is always identifiable. Signs of bronchial diseases, including "wheezes" and "crackles," were also noted. Signs of lung consolidation as "increased bronchial sounds" were defined as an actual increase in expiratory sounds reaching the same inspiration tone, simulating the sounds generally audible during trachea auscultation and causing an evident difficulty in distinguishing between inspiration and expiration sounds.[7,19,20] Finally, signs of pleural anomalies, including "pleural friction rubs," were recorded. In the case of multiple pathological sounds in the same calf, the highest score was recorded based on auscultation at the six different sites.

Categorization of AUSC data

To further explore the impact of different lung sounds classification, data from the clinical examination were analyzed using three categorizations (see Table 1). The first one (AUSC1) assessed the accuracy of the AUSC when all pathological lung sounds (increased breath sounds, wheezes, crackles, increased bronchial sounds and/or pleural friction ribs) were considered to qualify a positive case (calves with a score ≥ 1). Calves with a score of 0 (normal breath sounds) were considered

negative. A second categorization (AUSC2) assessed the accuracy of the AUSC when wheezes, crackles, increased bronchial sounds, and pleural friction rubs were considered to qualify as a positive case (AUSC score ≥ 2) while calves with a score < 2 (normal breath sounds or increased breath sounds) were considered negative. A third categorization (AUSC3) assessed AUSC when only severe pathological sounds (increased bronchial sounds and/or pleural friction ribs) were considered to qualify as a positive case (AUSC score ≥ 3) while calves with a score < 3 (patients with normal breath sounds, increased breath sounds, wheezes or crackles) were considered negative.

Thoracic ultrasonography

Systematic TUS (intercostal spaces [ICS] 10–1 on the right and ICS 10–2 on the left) was then performed in all auscultated calves based on the landmarks described by Ollivett et al.[21] by one of the main authors (DP) blinded to AUSC results. Ultrasonographic examination was performed using a portable unit (Ibex Pro, EI Medical) with a 7.5 MHz linear transducer designed for a transrectal purpose, set to a depth of 8 cm and gain of 16 dB. The thorax was not shaved, and 70% isopropyl alcohol was applied to the hair as a transducing agent. Lung lobes were examined and scored based on the mass of lung tissue involved according to Berman et al.[22], that showed ≥3 cm of depth yielded excellent accuracy for diagnosing active pneumonia in calves, and Dunn et al.[23] that considered consolidation positive if ≥3 cm of the consolidated lung was present. Ultrasonography score ranged between 0 to 3 (0 = no lesions or <1 cm consolidation; 1 = diffuse comet tails; 2 = patchy lesions; consolidation of ≥1 cm but < 3 cm, between normal aired lung parenchyma; 3 lungs consolidation = consolidation area ≥ 3 cm). Consolidation was defined when the normal reverberation artifact was replaced by a hypoechoic structure similar to the liver. For each calf, the maximal depth of consolidation on TUS was recorded. The maximal depth of lung consolidation (cm) was calculated by manual count using the lateral grid of the ultrasound image.

Categorization of TUS data

Data from the ultrasonography were then analyzed using the following categorization: calves with a score of 3 (consolidation depth ≥ 3cm) were considered positive, and calves with a score < 3 were considered negative.

Descriptive statistics

Data storage and analyses were performed with IBM SPSS Statistics version 27.0 for Macintosh (IBM Corp., Armonk, NY). Descriptive statistics were performed, and age was reported with median, interquartile range (IQR), minimum and maximum because non normally distributed (Shapiro-Wilk

test). At the same time, categorical variables (sex, AUSC score, and TUS score) were expressed as frequencies and percentages.

Contingencies tables

A first contingency table was obtained considering the 5-degree score of AUSC combined with the 4-degree score of TUS (Table 2). A second cross-classification table was built using data from WCRS (positive calves when WCRS scores ≥ 5 and negative calves when WCRS scores < 5), TUS dichotomized as previously reported (positive calves having a score = 3 and negative calves having a score < 3) and the three categorizations for AUSC (AUSC1, AUSC2 and AUSC3) (Table 3).

Bayesian latent class model

The accuracy of the different AUSC definitions was determined using one population and three imperfect diagnostic tests under a Bayesian latent class analysis framework. The latent variable was the true BP status of examined animals, which was evaluated using AUSC, WRSC, and TUS. We a priori used a model with no covariance between tests. However, because it was difficult to rule out a conditional dependence between WCRS and TUS as previously reported,[22] the possibility of conditional dependence between these tests (in truly affected (covDp) and truly non-diseased (covDn) calves was positively modeled using Dendukuri and Joseph[24] parametrization as follows:

- covDp~Uniform (0, min (sensitivity-WRSC, sensitivity-TUS) – sensitivity-WRSC× sensitivity-TUS)
- covDn~Uniform (0, min (specificity-WRSC, specificity-TUS) – specificity -WRSC× specificity-TUS).

Informative priors could be used to feed the model using previously obtained accuracy information from the WRSC[25] and TUS at a specific threshold of positivity of ≥ 3cm depth.[22] For the WRSC, the median sensitivity (95% credible intervals) and specificity were 62% (48-76%) and 74% (65-83%), respectively. Elicited beta distribution was obtained using the PriorGen package.[26] The equivalent beta distributions were beta (21.47, 13.29) and beta (53.79, 19.11) for WRSC sensitivity and specificity, respectively. For TUS accuracy, the median sensitivity and specificity were 89% (55-100) and 95% (92-98%). The equivalent beta distributions were beta (4.62-0.86) and beta (77.55,4.4) for TUS sensitivity and specificity, respectively. Non-informative models using uniform probabilities from 0 to 1 (beta [1,1]) were used for all tests' accuracy and the true prevalence of the disease. Because using purely non-informative prior has also been criticized since flat distribution can strongly influence posterior findings and could therefore be considered informative,[27] a third approach using a weakly informative approach was used using horseshoe prior-like type with the most probable value

of 60% and 95th percentile of the distribution at 95% leading to a distribution beta (1.58, 1.16). Therefore, a total of 6 different models were run for each auscultation definition (informative vs. weakly informative vs. non-informative, with or without conditional dependence). Models were run in OpenBUGS[28] using the interface of the package R2OpenBUGS in RStudio.[29,30] Three different chains were run starting from different units. Thirty thousand iterations were performed with a 5'000 burn-in resulting in a total of 25'000. A specific thinning was added if needed based on autocorrelation plots.

Evaluation of the models

Convergences of the different models were first assessed by visual inspection of the history and density plots.[29] This was further formalized by using the Brooks-Gelman-Rubin (BGR) statistics measuring the ratio of the total variability combining multiple chains (between-chain plus within-chain) to the within-chain variability, which is close to 1 when convergence is achieved. Posterior distributions of each parameter were reported as medians and corresponding 95% Bayesian credible interval (BCI). The models were evaluated using deviance information criteria (DIC). A difference in DIC of five or more was considered an indicator of a better fit and used to assess model differences.[31]

Results

Fifteen males (4.5%) and 315 female calves (95.5%) belonging to 18 dairy farms were enrolled in the study, totaling 330 calves. Within each farm, all tests were performed on the same day. In 14 farms, there were more than 20 eligible calves; therefore, the randomization system was used. The calves' median age was 65 days (IQR [47-85 days]; range [12-171 days]). Cross-tabulated results of AUSC and TUS are shown in Table 2 and Figure 2. One hundred and fifteen calves (34.8%) had physiological lung sounds (AUSC score = 0). Of these, 69 (60%) calves showed normal aerated parenchyma at TUS (TUS scores of 0 and 1). On the other hand, 46 (40%) calves showed lung lesions with a TUS score of ≥ 2 (21 calves with TUS score = 2, and 25 with TUS score = 3). Two hundred fifteen calves showed an AUSC score ≥ of 1 (pathological lung sounds). Of these, 56 (26%) calves had normally ventilated parenchyma (TUS scores of 0 and 1), and 159 (74%) had a TUS score of ≥ 2. Ninety-seven (45%) calves showed severe pathological lung sounds related to lung consolidation or pleuritis. Of these, 14 had a TUS score of 2, and 83 had a TUS score of 3. At TUS examination, none of these calves showed normal aired parenchyma (TUS scores of 0 or 1). Of the 233 (70%) calves that had lung sounds not related to consolidation or pleuritis (AUSC scores of 0, 1, 2), 63

showed ≥ 3 cm of lung consolidation at TUS (TUS score of 3), and 45 showed a patchy lesions pattern (TUS score = 2). The remaining 125 calves showed normally aerated lungs (TUS scores of 0 and 1). The posterior densities of the different auscultation definitions' sensitivity and specificity (AUSC1, AUSC2, and AUSC3) are presented in Table 4 and Figure 3. The DIC was relatively similar when comparing TUS and WRSC models with vs. without covariance parameters. The informative models had a higher DIC than others (non-informative or weakly informative models). The main differences between informative priors and other models were the limited coverage of AUSC2 and AUSC3 specificities (i.e., the median estimate of the informative model included in the 95% BCI but close to the 95% BCI bounds) and no coverage of TUS specificity BCI (no coverage between informative model posterior median in the 95% BCI of non-informative or weakly informative priors). The auscultation sensitivity and specificity (95% BCI) of informative models with no covariance were 90.3% (81.2-98.4%) and 57.4% (46.6-71.5%) for AUSC1, 81.7% (69.1-94.5%) and 90.8% (81.1-99.0%) for AUSC2, and 67.9% (55.7-82.8%) and 98.5% (93.8-99.9%) for AUSC3 respectively. Positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) of informative models with no covariance are shown in Table 5.

Discussion

In this study, we reported the accuracy of 3 different AUSC criteria for BP case definition. Using all pathological sounds in AUSC1 allowed having a high sensitivity but a low specificity. Instead, avoiding the "increased breath sounds" in AUSC2 or using only the severe pathological sounds in AUSC3 shows a high specificity at the cost of lower sensitivity.

Results obtained from the assessment of the accuracy of the AUSC1 regarding all pathological lung sounds were in line with a previous study.[5] Unfortunately, the interpretation of all pathological sounds of AUSC1 suffers from a lower specificity. These results could be related to "increased breath sounds" included in the score. This lung sound occurs in the early stages of BP but also in conditions unrelated to BP, such as exercise or stress.[19]

On the other hand, when "increased breath sounds" were not considered, the specificity of both AUSC2 and AUSC3 was high (90.8% and 98.5%, respectively). The specificity for these models is higher than previous studies that reported a specificity of 53.3%.[5] In addition, regarding AUSC3, the high specificity was characterized by a tightly 95% BCI in all Bayesian latent class models, further strengthening the observed data's reliability. Beyond the absence of the "increased breath sounds" within the model, another possible explanation for these results could also be related to an unambiguous sound classification. For Curtis et al.,[7] the "increased bronchial sound" is the essential pathological sound in any lung disease in which one or more large bronchi remain open around a lung

tissue that has been replaced by consolidated tissues. In these conditions, forced respiratory sounds are better transmitted than in normally ventilated parenchyma leading to a harsh sound similar to what is usually heard during trachea auscultation. Following Curtis et al.[7] and Bohadana et al.[18] recommendations, we defined "increased bronchial sounds" when the sounds from inspiration were identical to that from expiration, differentiating it from "increased breath sounds" in which the difference between inspiration and expiration is identifiable. Although, according to Curtis et al.,[7] "there is no sharp line of demarcation between increased breath sounds and increased bronchial sounds", we believe that, in clinical practice, it is not challenging to recognize tracheal-like sounds auscultating the thorax when severe lung consolidation is concurrent. From the results of our study, the high specificity of AUSC3 showed that these sounds infrequently occur in healthy calves. Moreover, in our study sample, observing the contingency table 2, all calves with "increased bronchial sounds" had at least 1 cm of lung consolidation at TUS, whereas the increase in bronchial sounds did not occur in TUS-negative calves. The low sensitivity of AUSC3 could be related to the fact that these sounds are often not produced even in the face of obvious lung damage. To produce the "increased bronchial sounds," at least one large bronchus must still be patent at the site of sound generation. In dairy calves, severe or chronic suppurative BP is characterized by evident intrabronchial purulent exudate, bronchiectasis, and/or abscessation, which increases the risk of total obstruction of the airways.[32] Obstruction leads to a lack of ventilation of the distal airways with complete gas reabsorption, resulting in poor-ventilated atelectasis areas.[33] Furthermore, with partial obstruction of the airways, which may also occur during a BP episode, it is possible to observe a concomitant decrease in the strength of the sounds due to a loss of intensity of the turbulent flow. This mechanism plays a pivotal role in the genesis of both physiological and pathological lung sounds.[18] Airflow limitation during lung inflammation has also been reported in calves inoculated experimentally with *Pasteurella multocida*.[34]

Results from AUSC2 were expected. Although wheezes and crackles generally indicate bronchial affections, both can be found in cases of BP. For these sounds, there were different described mechanisms of origin, i.e., high-velocity air passage through intrathoracic airways obstruction (wheezes) and the sudden opening of airways or rupture of fluid menisci in case of moderate density exudate (crackles).[6,19] Whatever the pathogenesis, most calves' bronchial anomalies come from an increased amount of fluid from inflammation associated with BP. However, intrabronchial mucopurulent exudate is not always necessarily abundant. Its quantity is also related to the stage of the inflammatory process (i.e., it usually increases in chronic stages of the disease).[32] In addition, it is possible to have a pathological process at the bronchial level without injury to the alveolar tissue, especially in the case of infection by moderately virulent pathogens.[35] These factors may explain the

relatively lower robustness of AUSC2 compared with AUSC3, particularly concerning the greater width of the BCI that characterize the (albeit high) specificity of the AUSC2 model.

The results of this study raise intriguing questions about the use of AUSC for diagnosing BP in dairy calves. Severe pathological sounds (increased bronchial sounds and pleural friction rubs) were found to be highly specific pathological sounds for diagnosing BP. The low Se of these sounds results in missed BP cases due to a high false negative rate, emphasizing using a composite reference to better identify positive cases. At the same time, these observations support the hypothesis that standardizing and simplifying the description of lung sounds may be helpful in the diagnosis of lung diseases in farm animals. Because of the constant use of imperfect diagnostic tests, AUSC should be considered a still helpful tool, especially in field conditions. For example, to correctly identify affected animals, TUS could be beneficial in addition to AUSC, especially in calves in which increased bronchial sounds are not auscultated, or to better identify calves with pathological sounds less specific to lung disease. Thus, higher accuracy in diagnosing BP could be achieved and generate a more judicious use of antibiotics.

We performed various Bayesian latent class models allowing for covariance between TUS and WRSC and with various types of priors for TUS and WRSC. It is essential to point out that this sensitivity analysis is inherent to latent class modeling.[14] Using the different priors' scenarios helped to look for the stability of AUSC accuracy posterior density findings. The impact of priors was limited except for TUS specificity. One must remember that the informative priors from TUS and WRSC were taken from a previous dairy study performed in North America.[22,25] The relative variability between the different models could be partially associated with the fact that these priors do not account for the BP pathogens, which may vary from one geographical area to another. The accuracy of the diagnostic tests, primarily TUS may also depend on specific pathogens involved in BP, which can cause different ultrasonographic signs, leading to a more variable accuracy of lung consolidation for BP diagnosis. Unfortunately, we could not test this hypothesis.

There are some limitations to our study. First, this study was performed using a convenience sample of farms enrolling a limited number of animals with a high BP prevalence. Secondly, although two independent operators performed AUSC and TUS to ensure blinding between exams, only one operator performed AUSC; therefore, future studies on the inter-observer agreement of AUSC to detect BP are recommended. In the human pneumology field, these studies have led to improved standardization of lung sound definitions.[12] It was impossible to perform microbiological tests to determine the relative prevalence of the different respiratory pathogens in BP observed on this farm. Therefore, we cannot determine the auscultation accuracy findings depending on the specific pathogens encountered.

In conclusion, this study showed that AUSC3 described here has, on average, high specificity for detecting BP in dairy calves. These results showed that a clear definition of lung sounds could help practitioners to detect BP based on inexpensive tools and easy-to-obtain clinical parameters. Further studies are needed to assess the accuracy of AUSC in populations having a different prevalence of BP in dairy calves and the inter-rater agreement of different operators using a precise definition of lung' sounds.

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Figure legends

Figure 1. Illustration of thoracic auscultation sites. The total auscultation area ranges from the 10th intercostal space in the dorso-caudal regions of the lung projection area to approximately the third intercostal space located below the axillary region of the cranial thorax. The total area was divided into dorsal (d), median (m) to ventral (v) thirds and auscultated for at least three respiratory cycles. The auscultation area was identical for both the right and left hemithorax.

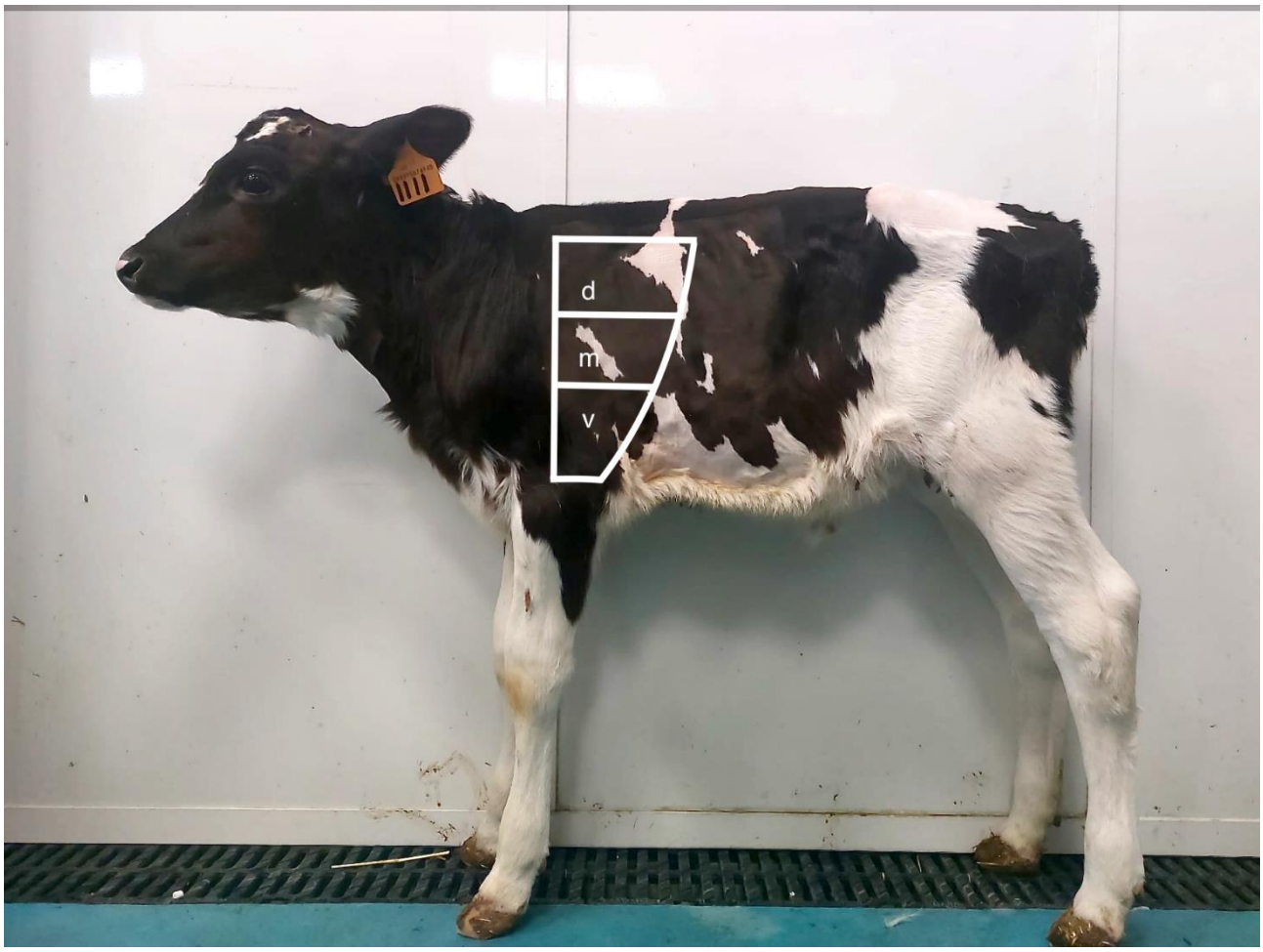

Figure 2. Distribution of auscultation score in 330 dairy calves (a) and correspondence between auscultation and thoracic ultrasonography (TUS) findings (b). Systematic TUS (intercostal spaces [ICS] 10–1 on the right and ICS 10–2 on the left) score ranged between 0 to 3 (0 = no lesions or <1 cm consolidation; 1 = diffuse comet tails; 2 = patchy lesions; consolidation of ≥1 cm but < 3 cm, between normal aired lung parenchyma; 3 lungs consolidation = consolidation area ≥ 3 cm). Thoracic auscultation scoring system ranged from 0 to 4 (0= normal breath sounds; 1= increased breath sounds; 2= wheezes or crackles in at least one auscultation site; 3= increased bronchial sounds; 4= pleural friction rubs or absence of breath sounds).

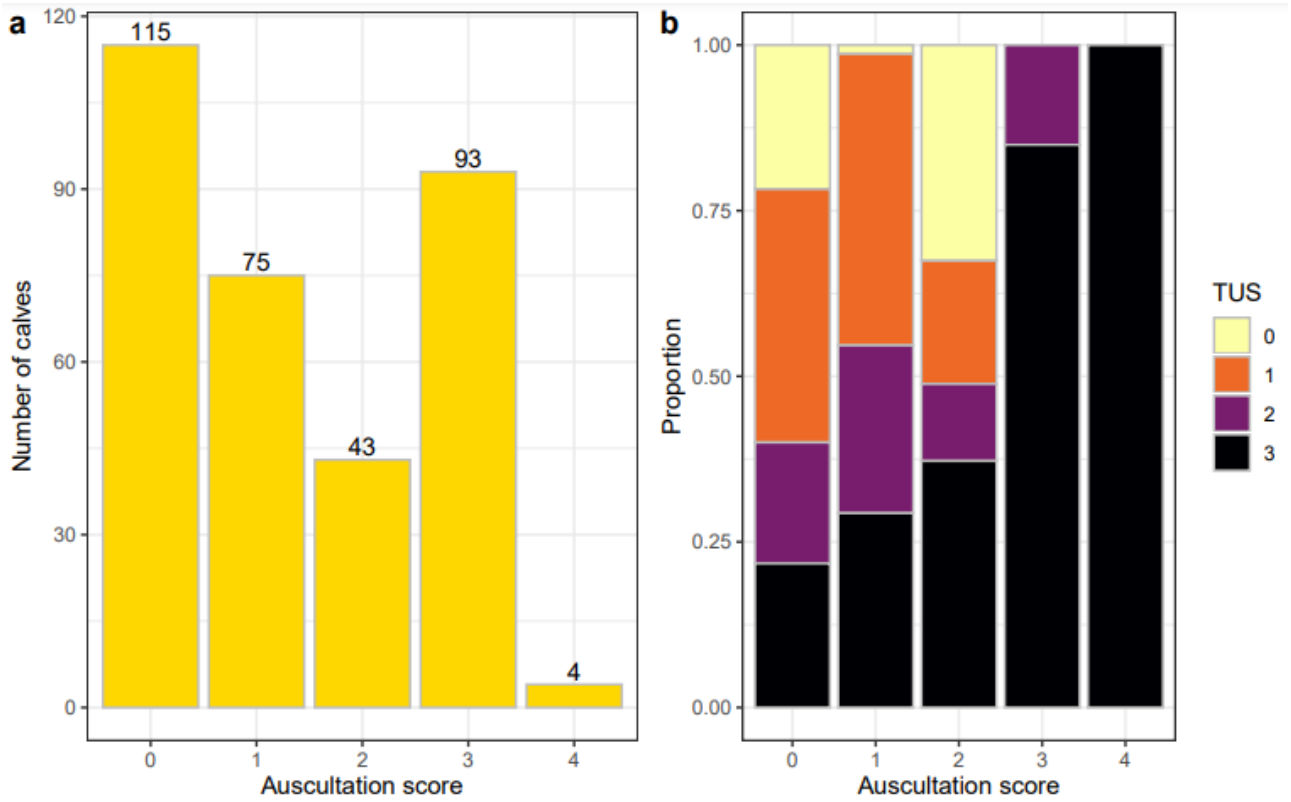

Figure 3. The distribution of posterior densities (median estimate and 95% Bayesian credible intervals of the accuracy [i.e., sensitivity (Se) and specificity (Sp]) of thoracic auscultation (AUSC), thoracic ultrasonography (TUS), and Wisconsin clinical respiratory score (CRS) presented based on different modeling strategies (informative vs. weakly informative vs. non-informative priors and with vs. without covariance between TUS and CRS)) in three different auscultation categorizations (Auscultation 1, 2 and 3).

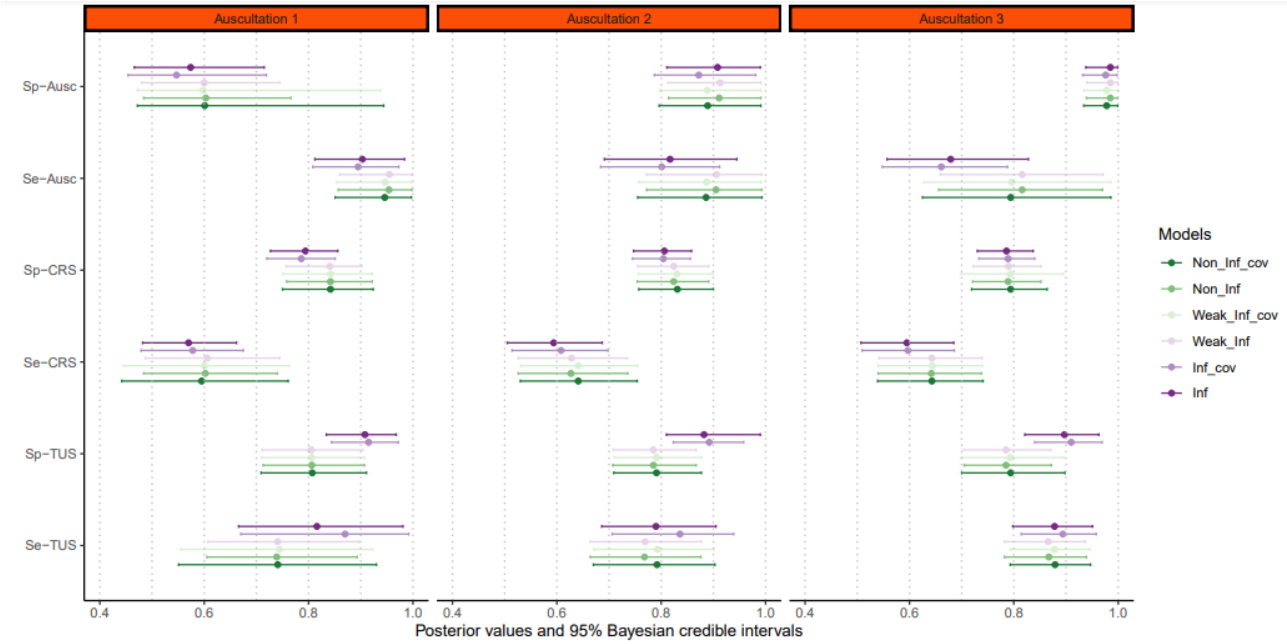

Table 1. Thoracic auscultation (AUSC) scoring system and categorizations used to explore the impact of different lung sounds classification for distinguishing calves with and without bronchopneumonia.

Score	AUSC findings	Description	AUSC1	AUSC2	AUSC3
0	Normal breath sounds	Soft blowing sounds, longer and louder on inspiration than on expiration. In heavier subjects, the sound from expiration may be non-audible (lung sounds file 1)	–	–	–
1	Increased breath sounds	Increase in loudness of breath sounds mainly on inspiration but also on expiration. The difference between inspiration and expiration is always identifiable (lung sounds files 2 and 3)	+	–	–
2	Signs of bronchial disease: a) Wheezes or b) Crackles in at least one auscultation site	a) Variable-toned, intermittent, or continuous musical whistling sounds ("huin")[18] that are usually heard on expiration but can also be heard on inspiration (lung sounds file 4) b) Crepitating non-musical sounds ("knack")[18] (lung sounds file 5)	+	+	–
3	Signs of lung consolidation: Increased bronchial sounds in at least one auscultation site	High and harsh audible tone like what is usually possible to hear during trachea auscultation. Difficulty in assessing the difference in tone between expiration and inspiration (lung sounds file 6)	+	+	+
4	Signs of pleuritis: Pleural friction rubs	Grating sounds during inspiration and first phase of expiration (lung sounds file 7)	+	+	+

For this study, we evaluated the accuracy performance of respiratory sounds by categorizing them into three different models: AUSC1 (score of 0 [negative] vs. ≥ 1 [positive]), AUSC2 (score of 0 or 1 [negative] vs. ≥ 2 [positive]), and AUSC3 (score ≤ 2 [negative] vs. ≥ 3 [positive]).
+: Sound considered in categorization of positive cases
–: Sound not considered in categorization of negative cases

Table 2. Cross-tabulated results of thoracic auscultation (AUSC) and thoracic ultrasounds exam (TUS) on 330 dairy calves for diagnosing bronchopneumonia.

TUS (0-3)	AUSC (0-4)					
	0	1	2	3	4	Total
0	25	1	14	0	0	40
1	44	33	8	0	0	85
2	21	19	5	14	0	59
3	25	22	16	79	4	146
Total	115	75	43	93	4	330

Systematic TUS (intercostal spaces [ICS] 10–1 on the right and ICS 10–2 on the left) score ranged between 0 to 3 (0 = no lesions; 1 = diffuse comet tails; 2 = patchy lesions; consolidation of ≥1 cm but < 3 cm, between a normal aired lung parenchyma; 3 = lung consolidated area ≥ 3 cm). Thoracic auscultation scoring system ranged from 0 to 4 (0 = normal breath sounds; 1 = increased breath sounds; 2 = wheezes or crackles in at least one auscultation site; 3 = increased bronchial sounds; 4 = pleural friction rubs).

Table 3. Cross-tabulated results of Wisconsin calf respiratory score (WCRS), three different thoracic auscultation (AUSC) examinations definitions and thoracic ultrasonography (TUS) examinations with score ≥ 3 conducted on 330 dairy calves for the diagnosis of bronchopneumonia.

AUSC1					**AUSC2**					**AUSC3**				
		Ausc +	Ausc –	Tot.			Ausc+	Ausc–	Tot.			Ausc +	Ausc –	Tot.
WCRS +	TUS +	68	5	73	WCRS +	TUS +	21	35	56	WCRS +	TUS +	52	21	73
	TUS –	31	15	46		TUS –	38	117	155		TUS –	10	36	46
WCRS –	TUS +	53	20	73	WCRS –	TUS +	61	12	73	WCRS –	TUS +	31	42	73
	TUS –	63	75	138		TUS –	20	26	46		TUS –	4	134	138
		215	115	330			140	190	330			97	233	330

AUSC +; calves with a AUSC score ≥ of 1, ≥ 2 or ≥ 3 for AUSC1, AUSC2 and AUSC3 respectively (pathological lung sounds); AUSC –; calves with a AUSC score < of 1, < 2 or < 3 for AUSC1, AUSC2 and AUSC3 respectively (physiological lung sounds)
WCRS +; calves with a Wisconsin calf respiratory score ≥ 5 (considered positive)
WCRS –; calves with a Wisconsin calf respiratory score < 5 (considered negative)
TUS +; calves with a TUS score of 3 (calves with lung consolidation ≥ 3 cm considered as positive)
TUS –; calves with a TUS score < 3 (calves considered negative).

Table 4. Posterior densities of the different Bayesian latent class models using thoracic auscultation (AUSC), thoracic ultrasound (TUS) and Wisconsin calf respiratory score (WCRS) for the diagnosis of bronchopneumonia in 330 dairy calves using 3 different auscultation definitions.

			AUSC1 (0 vs. 1+)		**AUSC2 (0,1 vs. 2+)**		**AUSC3 (0,1,2 vs. 3+)**	
		Informative priors for TUS and WCRS						
Test	**Parameters**	**Priors**	**No covariance Median (95%BCI)**	**Covariance Median (95%BCI)**	**No covariance Median (95%BCI)**	**Covariance Median (95%BCI)**	**No covariance Median (95%BCI)**	**Covariance Median (95%BCI)**
AUSC	Se	beta (1,1)	0.903 (0.812-0.984)	0.895 (0.808-0.973)	0.817 (0.691-0.945)	0.801 (0.684-0.912)	0.679 (0.557-0.828)	0.661 (0.548-0.788)
	Sp	beta (1,1)	0.574 (0.466-0.715)	0.547 (0.454-0.719)	0.908 (0.811-0.990)	0.872 (0.787-0.981)	0.985 (0.938-0.999)	0.976 (0.932-0.998)
TUS	Se	beta (4.62,0.86)	0.816 (0.666-0.981)	0.870 (0.670-0.992)	0.790 (0.686-0.905)	0.836 (0.706-0.939)	0.878 (0.798-0.951)	0.894 (0.814-0.958)
	Sp	beta (77.55,4.4)	0.908 (0.834-0.0.968)	0.915 (0.844-0.972)	0.882 (0.810-0.990)	0.892 (0.823-0.958)	0.897 (0.821-0.963)	0.910 (0.840-0.969)
WCRS	Se	beta (21.47, 13.29)	0.570 (0.482-0.662)	0.578 (0.479-0.675)	0.594 (0.505-0.687)	0.608 (0.514-0.698)	0.595 (0.507-0.685)	0.597 (0.509-0.686)
	Sp	beta (53.79, 19.11)	0.794 (0.727-0.856)	0.786 (0.720-0.851)	0.806 (0.747-0.858)	0.804 (0.745-0.856)	0.786 (0.730-0.837)	0.789 (0.733-0.840)
BP prevalence	P(BP)	beta (1,1)	0.469 (0.349-0.607)	0.447 (0.342-0.608)	0.459 (0.352-0.570)	0.443 (0.348-0.552)	0.420 (0.329-0.508)	0.425 (0.342-0.508)
Covariance	covDn	a	-	-0.006 (-0.024-0.022)	-	-0.009 (-0.025-0.017)	-	-0.007 (-0.024-0.018)
	covDp	b	-	-0.054 (-0.008-0.048)	-	-0.017 (-0.054-0.033)	-	-0.021 (-0.049-0.010)
DIC	DIC		46.7 (39.1-56.8)	47.6 (39.7-58.1)	47.0 (38.9-59.1)	47.5 (39.3-60.3)	46.0 (38.3-56.9)	44.8 (37.3-56.1)
		Weakly Informative priors for TUS and WCRS						
AUSC	Se	beta (1,1)	0.955 (0.860-0.998)	0.947 (0.853-0.997)	0.906 (0.772-0.993)	0.887 (0.757-0.992)	0.816 (0.659-0.971)	0.796 (0.628-0.986)
	Sp	beta (1,1)	0.600 (0.480-0.745)	0.598 (0.472-0.938)	0.913 (0.813-0.991)	0.888 (0.797-0.991)	0.985 (0.940-0.999)	0.978 (0.934-0.999)
TUS	Se	beta (1.58,1.16)	0.741 (0.607-0.896)	0.744 (0.555-0.923)	0.769 (0.664-0.877)	0.793 (0.671-0.901)	0.866 (0.782-0.937)	0.878 (0.793-0.946)
	Sp	beta (1.58,1.16)	0.805 (0.711-0.904)	0.806 (0.710-0.907)	0.785 (0.708-0.867)	0.791 (0.710-0.877)	0.785 (0.704-0.871)	0.793 (0.699-0.895)
WCRS	Se	beta (1.58,1.16)	0.606 (0.487-0.745)	0.600 (0.445-0.763)	0.628 (0.526-0.735)	0.641 (0.531-0.755)	0.643 (0.542-0.739)	0.644 (0.540-0.740)
	Sp	beta (1.58,1.16)	0.841 (0.757-0.902)	0.842 (0.751-0.922)	0.824 (0.755-0.891)	0.830 (0.756-0.899)	0.790 (0.722-0.852)	0.793 (0.699-0.895)
BP prevalence	P(BP)	beta (1,1)	0.454 (0.318-0.611)	0.458 (0.313-0.683)	0.411 (0.315-0.518)	0.405 (0.307-0.517)	0.349 (0.270-0.438)	0.351 (0.260-0.452)
Covariance	covDn	a	-	-0.005 (-0.030-0.032)	-	-0.004 (-0.028-0.031)	-	0.000 (-0.029-0.039)
	covDp	b	-	0.006 (-0.056-0.071)	-	-0.010 (-0.052-0.044)	-	-0.018 (-0.048-0.016)
DIC	DIC		41.0 (36.2- 50.5)	41.6 (36.4-52.2)	40.8 (36.6-50.2)	41.6 (36.7-52.0)	40.7 (35.8-50.3)	40.4 (35.4-50.9
		Non-Informative priors						
AUSC	Se	beta (1,1)	0.954 (0.857-0.998)	0.946 (0.851-0.997)	0.905 (0.772-0.993)	0.886 (0.755-0.993)	0.816 (0.656-0.970)	0.794 (0.625-0.986)
	Sp	beta (1,1)	0.603 (0.484-0.766)	0.601 (0.472-0.944)	0.911 (0.814-0.991)	0.889 (0.796-0.991)	0.985 (0.939-0.999)	0.978 (0.934-0.999)
TUS	Se	beta (1,1)	0.739 (0.605-0.893)	0.741 (0.551-0.930)	0.768 (0.664-0.876)	0.792 (0.670-0.903)	0.867 (0.782-0.939)	0.879 (0.793-0.947)
	Sp	beta (1,1)	0.806 (0.713-0.907)	0.807 (0.709-0.911)	0.785 (0.707-0.867)	0.791 (0.709-0.877)	0.785 (0.705-0.872)	0.794 (0.700-0.898)
WCRS	Se	beta (1,1)	0.602 (0.484-0.740)	0.595 (0.442-0.761)	0.627 (0.526-0.736)	0.641 (0.530-0.754)	0.642 (0.540-0.738)	0.643 (0.539-0.741)
	Sp	beta (1,1)	0.842 (0.758-0.922)	0.842 (0.750-0.924)	0.824 (0.754-0.891)	0.831 (0.757-0.900)	0.789 (0.721-0.852)	0.794 (0.719-0.864)
BP prevalence	P(BP)	beta (1,1)	0.457 (0.320-0.613)	0.461 (0.312-0.688)	0.412 (0.315-0.519)	0.406 (0.307-0.518)	0.348 (0.269-0.440)	0.353 (0.260-0.454)
Covariance	covDn	a	-	-0.005 (-0.030-0.032)	-	-0.004 (-0.028-0.031)	-	-0.000 (-0.029-0.038)
	covDp	b	-	0.007 (-0.055-0.072)	-	-0.009 (-0.053-0.044)	-	-0.018 (-0.047-0.015)
DIC	DIC		40.9 (36.2-50.6)	41.7 (36.4-52.4)	40.9 (36.5-50.2)	41.6 (36.7-51.8)	40.7 (35.9-50.1)	40.4 (35.3-50.9)

Se: sensitivity, Sp: specificity, covDn: covariance between TUS and WCRS in truly negative animals, covDp: covariance between TUS and WCRS in truly positive animals, BP: bronchopneumonia, DIC: deviance information criterion.
AUSC1 (score of 0 [negative] vs. ≥ 1 [positive]), AUSC2 (score of 0 or 1 [negative] vs. ≥ 2 [positive]), and AUSC3 (score ≤ 2 [negative] vs. ≥ 3 [positive]) were associated with different definitions based on auscultation findings (see Table 1).
Calves with a WCRS ≥ 5 were considered positive.
Calves with a maximal consolidation depth ≥ 3 cm with TUS were considered positive.
a = min (Se-WCRS, Se-TUS) – Se-WCRS× Se-TUS
b = min (Sp-WCRS, Sp-TUS) – Sp-WCRS × Sp-TUS

Table 5. Positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR) and negative likelihood ratio (NLR) of models with informative priors without covariance between thoracic ultrasonography (TUS) and Wisconsin respiratory calf scoring (WRCS) in three clinical categorizations of thoracic auscultation (AUSC) used for the diagnosis of bronchopneumonia in 330 dairy calves.

Informative priors for TUS and WCRS; no covariance			
Parameter	**AUSC1**	**AUSC2**	**AUSC3**
PPV	0.653 (0.499-0.814)	0.884 (0.734-0.989)	0.971 (0.874-0.999)
NPV	0.869 (0.721-0.982)	0.854 (0.715-0.963)	0.808 (0.699-0.914)
PLR	2.124 (1.620-3.213)	8.949 (4.194-82.42)	45.075 (10.690-1112.075)
NLR	0.168 (0.027-0.350)	0.201 (0.060-0.346)	0.327 (0.175-0.453)

AUSC1 (score of 0 [negative] vs. ≥ 1 [positive]), AUSC2 (score of 0 or 1 [negative] vs. ≥ 2 [positive]), and AUSC3 (score ≤ 2 [negative] vs. ≥ 3 [positive]) were associated with different definitions based on auscultation findings (see Table 1).

Arterial blood gases, acid-base status, and metabolic parameters in pre-weaned dairy calves diagnosed with bronchopneumonia based on the combination of clinical scores and thoracic ultrasonography: a cross-sectional study.

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INTRODUCTION

One of the most significant challenges in bronchopneumonia (BP) management in calves is the lack of a 100% precise diagnostic test (Buczinski & Pardon, 2020). A possible strategy to improve diagnostic accuracy without a perfect gold standard could be using a composite reference test (Buczinski et al., 2021). Although this method cannot exclude risks of diagnostic error (Schiller et al., 2016), the use of thoracic ultrasound (TUS) in addition to BP-related clinical signs can help clinicians better define a positive or negative case (Ollivett & Buczinski, 2016; van Leenen et al., 2020; Reynolds & Brennan, 2021).

Several methods have been described to evaluate BP-related clinical signs and TUS. Existing research has reported that TUS's cutoffs of ≥1 to ≥3 cm has high diagnostic accuracy and are correlated with reduced performance of affected calves (Cramer & Ollivett, 2019; Decaris et al., 2022; Berman et al., 2019). Wisconsin (WISC) and California (CALIF) scores (McGuirk & Peek, 2014; Love et al., 2014) or their alternative versions (Cramer & Ollivett, 2019; Decaris et al., 2022) are commonly used to identify calves with BP. Although the diagnostic accuracy of these scoring systems is still debated (Buczinski et al., 2016; Lowie et al., 2022), also clinical signs were correlated to low average daily gain (Cramer & Ollivett, 2019) and commonly used in recent literature combined to TUS (Jourquin et al., 2022; Cuevas-Gómez et al., 2020; Denis-Robichaud et al., 2021). Nevertheless, defining the different pathological categories remains challenging. Recent evidence suggests that the classification of calves using clinical signs and TUS did not coincide with the bacterial isolation rate and neutrophil percentage of bronchoalveolar fluid (van Leenen et al., 2020).

Respiratory-related functional markers could be a possible method to assess the severity of BP episodes. Blood oxygenation data and respiratory-related acid-base disturbances are objective measures of lung lesions (Ellis et al., 2013). In addition, L-lactate levels or alteration of acid-base balance using the simplified strong ion difference (sSID) approach have recently been described as related to the severity of respiratory diseases (Reinhold et al., 2010; Ostermann et al., 2014; Buczinski

et al., 2015; Niedzwiedz et al., 2019).

Therefore, the study's objectives were to explore the effect of BP detected with different combinations of clinical scores and TUS on 1) blood gas status, 2) acid-base abnormalities using the traditional Henderson-Hasselback (H-H) approach and sSID theory, and 3) biochemical findings.

We hypothesized that these metabolic alterations could define which categorization methods best described BP severity. In addition, we aimed to explore which abnormalities are most significantly involved in affected dairy calves. Increased knowledge and understanding of how the combination of TUS and clinical signs affect lung function and metabolic status may improve BP case definition.

MATERIALS AND METHODS

Study design and animal selection

Using the strengthening of the reporting of observational studies in epidemiology (STROBE) guidelines, we conducted a cross-sectional study in Lombardy using a convenience sample selected from dairy farms that our ambulatory clinic regularly checked from 2018 to 2021. All calves included in the study were managed according to standard protocols for diagnosing BP in compliance with the professional ethics of veterinarians and the standards for protecting calves (European Union Council, 2008). The publication of data from the routine extramural clinical activity of the Ruminant and Swine Clinic of the University of Milan was approved by the Ethics Committee of the University of Milan (approval number 47/2017, November 28, 2017). The criterium for the selection of the farms was a history of cough detected by the herd practitioner in pre-weaned Holstein heifers raised within multiple pens with an automatic calf feeder with no history of treatment for BP in the previous 15 d before the day of the study. This selection was performed to ensure a minimum prevalence of lung lesions and BP-related clinical signs to optimize the sample size for each group of calves (calves with and without lung lesions and clinical respiratory signs). The sample size was calculated with G*Power (Ver. 3.1, Heinrich-Heine-Universität, Düsseldorf, Germany). To determine the minimum number of required calves a logistic regression analysis was used with Poisson X distribution, the odds ratio of 1.5, α error of 5% (type I), a confidence interval of 95%, and a test power of 80%. The result was a minimum of 210 calves.

Calves on each farm were maintained according to the farm's calf management. Enrolled farms reflect the typical mid-size (cows ranging from 150 to 300) milk production systems of the Po Valley. Briefly, calves were separated from the dam immediately after birth and received 4 L of good-quality colostrum (Brix ≥22%) within 6-8 h after birth. Calves were housed individually for up to 15-20 days and fed with a milk replacer before entering multiple pens with an automated calf feeder, where they

remained for up to 75-90 days. A minimum of 15 to 25 calves per farm were examined. Selected animals were randomly chosen with an application that runs on an Android smartphone (Randomizer, Darshan Institute of Engineering and Technology, Rajkot, India) using the list of identification numbers (written on the ear tag of each calf) provided by the farmer. For each enrolled animal, identification, date of birth, age at the clinical examination, and sex were recorded. Calves were eligible for the study unless they showed lameness, dehydration, or umbilical disorders.

Calves were assessed with the same one-gate design protocol. Two helpers gently captured each calf; without moving it from the capture site, one of the principal authors (VF or DP) performed arterial blood sampling. After the procedure, GS performed a clinical evaluation of BP-related clinical signs. At this point, the calf was subjected to jugular vein sampling and moved to a designated corner outside the pen for TUS examination. Calves from the same herd were subjected to the clinical protocol on a single day. The clinical score results and ultrasound results of each intercostal space were added to the predetermined table that contained the all data regarding the identification of the enrolled animals

Clinical score assessment, thoracic ultrasound, and categorizations

Each calf was examined and assigned a clinical score from Wisconsin (WISC) and California (CALIF) respiratory scoring charts. Wisconsin's scoring ranged from 0 to 3 based on the severity of 5 criteria: temperature, nasal discharge, cough, eye discharge, and ear position. Calves with a score of 5 or higher (WISC5) were considered positive cases (McGuirk & Peek, 2014). In addition, WISC was interpreted using an alternative approach based on the observations of Cramer and Ollivett (2019), and Decaris et al., (2022), in which calves with respiratory scores ≥2 in 2 or more categories were considered positive (WISC2points). California scoring is based on a dichotomous scoring system of 6 criteria: nasal discharge, ocular discharge, rectal temperature, ear position, spontaneous cough, and abnormal breathing, and is characterized by the assignment of different weights for each of the included clinical signs. California score was considered positive if the total score was ≥5 (CALIF5) (Love et al., 2014).

Bilateral TUS (intercostal spaces [ICS] 10–1 on the right and ICS 10–2 on the left) was performed in all scored calves based on the ventral landmarks described by Ollivett et al. (2015) by an experienced veterinarian (AB) blinded to clinical scoring results. Ultrasonography was performed using a portable unit with a 7.5 MHz linear transducer set to a depth of 8 cm and a gain of 16 dB. Ultrasonography score ranged between 0 to 3 (0 = no lesions or <1 cm consolidation; 1 = diffuse comet tails; 2 = patchy lesions; consolidation of ≥1 cm but < 3 cm, between a normal aired lung parenchyma; 3 lungs consolidation = consolidation area ≥ 3 cm). Consolidation was defined when the normal reverberation artifact was replaced by a hypoechoic structure similar to the liver. For each calf, the maximal depth

of consolidation on TUS was recorded. Depth (cm) was calculated by manual count using the lateral grid of the ultrasound image. Thoracic ultrasonography was interpreted in two different ways, and calves with a consolidation of ≥1 cm (TUS1cm) (Buczinski et al., 2015) or with a consolidation of ≥3 cm (TUS3cm) (Berman et al., 2019) were considered positive.

Based on the combination of TUS and clinical scoring, enrolled calves were categorized as follows:

- Group WISC5/TUS1cm; healthy (WISC<5 and no consolidation on ultrasound), upper respiratory tract infection (WISC≥5 but no lesions on ultrasound), subclinical BP (WISC<5 but TUS>1cm), and clinical BP (WISC≥5 and TUS>1cm).
- Group WISC5/TUS3cm; healthy (WISC<5 and no consolidation on ultrasound), upper respiratory tract infection (WISC≥5 but no lesions on ultrasound), subclinical BP (WISC<5 but TUS>3cm), and clinical BP (WISC≥5 and TUS>3cm).
- Group WISC2points/TUS1cm; healthy (WISC<2points and no consolidation on ultrasound), upper respiratory tract infection (WISC≥2points but no lesions on ultrasound), subclinical BP (WISC<2points but TUS>1cm:), and clinical BP (WISC≥2points and TUS>1cm).
- Group WISC2points/TUS3cm; healthy (WISC<2points and no consolidation on ultrasound), upper respiratory tract infection (WISC≥2points but no lesions on ultrasound), subclinical BP (WISC<2points but TUS>3cm:), and clinical BP (WISC≥2points and TUS>3cm).
- Group CALIF5/TUS1cm; healthy (CALIF<5 and no consolidation on ultrasound), upper respiratory tract infection (CALIF≥5 but no lesions on ultrasound), subclinical BP (CALIF<5 but TUS>1cm), and clinical BP (CALIF≥5 and TUS>1cm).
- Group CALIF5/TUS3cm; healthy (CALIF<5 and no consolidation on ultrasound), upper respiratory tract infection (CALIF≥5 but no lesions on ultrasound), subclinical BP (CALIF<5 but TUS>3cm), and clinical BP (CALIF≥5 and TUS>3cm).

Blood sample collection and calculations

In all cases, arterial blood had been anaerobically collected from the medial intermediate auricular branch of the caudal auricular artery (Bleul et al., 2007). Hairs on the dorsal surface (in the middle position and near basal attachment) of the pinna were removed with a disposable razor. The tip of a butterfly needle (23G; Terumo) was placed into the lumen of the artery until the plastic conduit was filled up to the end of the final connector. A 2.5 mL disposable heparinized syringe was connected until 0.5 mL of blood was aspirated. After the procedure, the needle was removed from the artery and closed with a rubber stopper. The syringe was not disconnected from the butterfly needle until the blood gas analysis was carried out immediately using an automated POC blood analyzer (Marca e modello) commonly used in cattle practice (Coenen et al., 2022: Ro et al., 2022). The processing of

samples was performed according to the manufacturer's guidelines. Briefly, the automatic blood gas analyzer was automatically calibrated through the insertion of a commercially available test card. During the calibration (approximately 60-70 seconds), each enrolled calf's rectal temperature, serial number, and the 21% inspired oxygen fraction (FiO_2) were entered.

After the arterial blood sample, each enrolled calf was subjected to a venous blood sampling via jugular venipuncture using a 20-gauge hypodermic needle and stored in a sterile Vacutainer tube without an anticoagulant. Blood samples were transported to the clinic in a portable cooler and then centrifuged at 20 C for 10 min at 900g within 6 h of collection. The serum obtained was used to determine serum total protein (STP) using a temperature-compensating optical refractometer (Rifrattometro Milwaukee mod. MR514ATC, Milwaukee, Gallarate, Italy). Before use, the refractometer was calibrated with distilled water.

At the end of the study day, ear tag number, age, calf serial number, clinical scores, TUS score, STP concentration (g/L), and all data stored in the blood gas analyzer were manually transferred into a spreadsheet (Excel; Microsoft).

The sSID variables were calculated according to the formulas used by Gomez et al. (2017). The concentration of nonvolatile weak acids (A_{tot}, mmol/L) was calculated from the STP concentration (g/L) according to the experimentally determined values for calf plasma (Constable et al., 2005):

$Atot$ = 0.343 × STP concentration

Measured strong ion difference was calculated from blood concentrations of Na, Cl, K, Ca, and L-lactate (SID5, mmol/L):

$SID5 = (Na^+ + K^+ + Ca^{2+})$ - (Cl^- + L-Lactate)

Unmeasured strong anions (USI) concentration was obtained by deducting from the SID blood HCO_3^- concentration and the total negative charge of the plasma proteins (A-):

$USI = SID5 – HCO_3^– – ([A_{tot}^–]/ (1 + 10(pKa- pH))$

where pKa (7.08) is the effective dissociation constant of bovine plasma weak acids (Constable et al., 2005).

The device used for this study measures and calculated more than 24 parameters. However, the focus was on the following:

- H-H acid-base model: temperature-adjusted blood pH, partial pressure of carbon dioxide (pCO_2), blood bicarbonate (HCO_3^-), base excess (BE), and anion gap (AG).
- sSID acid-base model: (SID5, A_{tot}, and USI).
- blood oxygenation data: partial pressure of arterial oxygen (PaO_2), oxygen saturation (SO_2), the alveolar-arterial difference for oxygen (A-aDO_2), and Horowitz index for lung function (P/F Ratio)
- electrolytes values: sodium (Na^+), potassium (K^+), chloride ($Cl^–$), and ionized calcium (Ca^{2+}).

- metabolic parameters: glucose, L-lactate, creatinine, blood urea nitrogen (BUN), and urea.

Data analysis

Data storage and analysis were performed with IBM SPSS Statistics 27.0 (IBM Corp. Armonk. NY). Continuous variables were reported with median and interquartile range (IQR) from the 25^{th} to the 75^{th} percentile because the data were not normally distributed (tested with the Shapiro-Wilk test). In contrast, categorical variables were expressed as frequencies and percentages. A multivariable logistic regression model was made for each group to investigate how considered parameters change in correlation with the clinical scores and TUS findings. Univariable analysis was performed with the Kruskal-Wallis test to identify which parameters could change based on BP classification. The parameters with $P < 0.1$ were inserted in the multivariable logistic model using backward stepwise. Spearman's correlation index was evaluated, and variables with an $R > 0.6$ were considered collinear. Only parameters that presented a $P < 0.05$ on the final regression model were considered correlated with BP classification. The final models obtained for each group were evaluated against each other with McFadden's pseudo-R-squared (pseudo-R^2)

RESULTS

The research team visited 13 dairy herds. The final sample consisted of 231 Holstein-Friesian female calves aged between 12 to 94 days old (median = 57 days; IQR 25% = 42 days; IQR 75% = 72 days). The median values of the studied parameters subdivided by categories and groups are available in Supplementary File 1. The distribution of calves based on the combination of TUS and clinical were:

- Group WISC5/TUS1cm; 60 healthy calves (26%), 11 upper respiratory tract infection calves (4.8%), 106 subclinical BP calves (45.8%), and 54 clinical BP calves (23.4%).
- Group WISC2points/TUS1cm; 59 healthy calves (25.5%), 12 upper respiratory tract infection calves (5.2%), 105 subclinical BP calves (45.5%), and 55 clinical BP calves (23.8%).
- Group WISC5/TUS3cm; 91 healthy calves (39.4%), 27 upper respiratory tract infection calves (11.7%), 75 subclinical BP calves (32.5%), and 38 clinical BP calves (16.5%).
- Group WISC2points/TUS3cm; 91 healthy calves (39.4%), 27 upper respiratory tract infection calves (11.7%).73 subclinical BP calves (31.6%), and 40 clinical BP calves (17.3%).
- Group CALIF/TUS1cm; 44 healthy calves (19%), 27 upper respiratory tract infection calves (11.7%), 74 subclinical BP calves (32%), and 86 clinical BP calves (37.2%).

- Group CALIF/TUS3cm; 67 healthy calves (29%), 51 upper respiratory tract infection calves (22.1%), 51 subclinical BP calves (22.1%), and 62 clinical BP calves (26.8%).

For each group, the median values of arterial blood gas status, acid-base, and biochemical abnormalities are reported in Table 1. In each group, the multivariable analysis showed that BP was not correlated with oxygenation parameters (pCO_2, pO_2, A-a, P/F, cSO). The results of multivariate analyses are reported below, divided by group:

- Group WISC5/TUS1cm; the independent variables included in the multivariate model were Na^+, K^+, glucose, BUN, urea, and USI. The variables that were significantly correlated with the clinical score and TUS findings were K^+ (p-value 0.004), glucose (p-value <0.001), and USI (p-value 0.027). The pseudo R^2 value for this model was 0.079.
- Group WISC2points/TUS1cm; the independent variables included in the multivariate model were Na^+, K^+, glucose, BUN, urea, A_{tot}, and USI. The variables that were significantly correlated with the clinical score and TUS findings were K^+ (p-value 0.001), glucose (p-value <0.001), A_{tot} (p-value 0.044), and USI (p-value 0.015). The pseudo-R^2 value for this model was 0.099.
- Group WISC5/TUS3cm; the independent variables included in the multivariate model were sodium, glucose, BUN, urea, creatinine, and USI. The variable significantly correlated with the clinical score and TUS findings was glucose concentration (p-value <0.001).

However, although not significant, Na^+ (p-value 0.074) and creatine (p-value 0.084) are also considered in the final model. The pseudo-R^2 value for this model was 0.066.

- Group WISC2points/TUS3cm; the independent variables included in the multivariate model were Na^+, glucose, BUN, urea, A_{tot}, and USI. The variable significantly correlated with the clinical score and TUS findings was glucose concentration (p-value <0.001). Again, although not significant, Na^+ (p-value 0.060) was also considered in the final model. The pseudo-R^2 value for this model was 0.051.
- Group CALIF/TUS1cm; the independent variables included in the multivariate model were Na^+, K^+, glucose, BUN, urea, A_{tot}, and USI. The variables that were significantly correlated with the clinical score and TUS findings were glucose (p-value 0.003), BUN (p-value 0.047), Atot (p-value 0.020), and USI (p-value 0.022). The pseudo-R^2 value for this model was 0.076.
- Group CALIF/TUS3cm; the independent variables included in the multivariate model were sodium, glucose, BUN, urea, creatinine, A_{tot}, and USI. The variables significantly correlated with the clinical score and thoracic ultrasonography findings were glucose (p-value 0.002) and creatinine (p-value 0.029). However, although it is not significant, sodium (p-value 0.088) is also considered in the final model. The pseudo-R^2 value for this model was 0.053. The results of the multivariate analysis for each group are reported in Table 1.

DISCUSSION

This is the first study that evaluates arterial blood gas status and metabolic abnormalities between different diagnostic groups of dairy calves diagnosed with BP. It was hypothesized that classifying calves into healthy, upper respiratory tract infection, subclinical BP, and clinical BP could correspond to increasing severity of alterations in blood oxygenation, acid-base balance, and metabolic abnormalities. Contrary to expectations, most parameters studied were found to have no or limited value in describing metabolic changes using clinical and TUS categorization. In contrast, relative hypoglycemia successfully emerged as a good parameter to describe disease severity in all groups of calves.

An important finding was that none of the classifications used corresponded to alterations in oxygenation status values and H-H acid-base respiratory parameters. This finding was unexpected and contrasted with previous studies, which have suggested that blood oxygenation values and respiratory acidosis represent good predictors of BP's severity (Nagy et al., 2006; Ellis et al., 2013; Šoltésová et al., 2015). It is not easy to compare our results with those of Šoltésová et al. (2015) and Nagy and colleagues (2006) because these studies are performed in different conditions using calves with chronic diseases often affected by life-threatening conditions. On the other hand, Ellis et al. (2013) showed that PaO_2 was significantly associated with the extent of lung lesions and was considered predictive of disease severity in calves experimentally infected with the respiratory syncytial virus. The differences from the findings presented here could be related to the disparity in the nature and distribution of lung lesions induced by experimental inoculation of the syncytial virus compared with our sample population's naturally occurring disease process. In the study by Ellis et al. (2013), the respiratory syncytial virus caused lesions on approximately 24% of the lung mass that severely affected gas exchanges during the acute phase of the post-infection disease. Conversely, our findings reflect that Hanzlicek et al. (2010) did not detect hypoxemia and hypercapnia after endoscopic inoculation of Mannheimia haemolytica. These authors argued that lung injury had not been extensive enough to alter ventilation rates or reduce pulmonary gas exchange. Similarly, Ostermann et al. (2013) showed that pCO_2 did not increase in calves treated with a low to high intrabronchial dose of Chlamydia psittaci, evidencing only an increase in $A\text{-}aDO_2$ 2-3 days after infection in calves treated with a high dose of pathogens. In our study sample, calves were considered to have a positive TUS when consolidation of ≥1 or ≥3 cm of depth was identified. Especially regarding ultrasonographic classification with a cutoff of ≥3 cm, many calves had more extensive lobar lesions. However, they were not sufficiently severe to produce significant alveolar hypoventilation. To emphasize this outcome, it is also possible to use the results from the L-lactate concentration. This marker is commonly used as an indicator of hypoperfusion and hypoxemia. In

beef calves, L-lactate concentration higher than 4 to 5 mmol/L was a good predictor of death and could use to assess BP severity (Coghe et al., 2000; Buczinski et al., 2015). There was no correlation between L-lactate, pCO_2 and clinical and TUS categorization in calves of our study population, even between the farthest groups, such as those healthy and clinically affected with ultrasound lesions. This observation may support the hypothesis that lung lesions were not particularly severe in inducing hypoxemia or respiratory acidosis. Therefore, it can be hypothesized that in our study population, blood gas exchanges and the respiratory acid-base imbalances associated with the effect of pCO_2 were found to be unrelated to the classification of calves based on the combination of clinical scores and TUS due to co-infection with other pathogens presumptively less severe than the respiratory syncytial virus and a smaller lung mass involved in naturally occurring BP. Further research should be undertaken to investigate the percentage of lung mass that results in reduced respiratory exchange and respiratory acidosis using TUS techniques that permit a better grading of lung injury.

Between the clinicopathological variables, glucose concentration showed the most interesting trends within and between the different diagnostic groups. Blood glucose was the only parameter systematically included in the multivariable logistic regression and, almost in all cases, significantly lower as clinical and ultrasonographic categorization severity increased in all groups. In critically ill calves, severe hypoglycemia increases the risk of mortality (Trefz et al., 2016; Trefz et al., 2017). Moreover, plasma glucose concentration could indicate a poor prognosis for abdominal emergencies and endotoxemia (Gomez et al., 2019; Lausch et al., 2021). Due to limited energy reserves, young patients can show hypoglycemia for any disease that limits energy intake (Stämpfli & Oliver-Espinosa, 2020). Recent evidence on metabolic monitoring used as biomarkers to indicate the presence of diseases suggests that feedlot cattle suffering from clinical BP had lower relative plasma glucose concentrations than non-affected ones (Blakebrough-Hall et al., 2020).

Interestingly, relative hypoglycemia in affected calves seems to be associated only with the secondary bacterial infection due to the increased energy expenditure required for immune cell activity to counteract the bacterial infection (Blakebrough-Hall et al., 2020; Santos-Rivera et al., 2021). Based on these data, we can infer that the different combinations of clinical scores and ultrasonographic lesions used in this study had a good ability to characterize disease severity based on impaired glucose metabolism. It is fascinating how the decrease in mean glucose concentration was markedly more significant in calves with ultrasonographic lesions and persistently lower in the BP-clinical groups. This result suggests that the severity of systemic involvement of the glucose metabolism during the BP episode might be related to an ultrasonographic lesion; however, the simultaneous clinical symptoms provoke a more severe condition.

The concentration of some individual electrolytes, such as Na^+ and K^+, showed typical alterations in some groups (see Table 1). However, their significance in terms of mean absolute changes and magnitude of the impact was relatively low. Similarly, SID and Atot also showed no or marginal importance. These findings contrast with previous studies suggesting that respiratory disease may affect the metabolic compounds of the sSID theory (Reinhold et al., 2010; Ostermann et al., 2014; Niedzwiedz et al., 2019). The sSID approach is based on three independent variables (pCO_2, SID, Atot), which can constantly modify the water dissociation process, directly determining the blood pH and HCO_3^- (Constable, 2000). Unlike the metabolic component differs considerably between the two types of approach (HCO_3^- vs. SID and Atot), both for the traditional H-H approach and the sSID approach, the respiratory acid-base imbalances are associated with the effect of pCO_2; however, the respiratory component is not disconnected from the metabolic aspect of the sSID approach (Niedzwiedz et al., 2019). For example, HCO3 concentration increases with hypercapnia or decreases during hyponatremia (Niedzwiedz et al., 2019; Ostermann et al., 2014). In previously published papers, it has been observed that a series of pathological events related to the severity of lung disease (hypercapnia accompanied increase in HCO_3^- concentration, hyperalbuminemia, hypernatremia, and hypochloremia; [Niedzwiedz et al., 2019]) or hyperacute reaction after pathogens inoculation (hypoalbuminemia resulted in a decrease of Atot, hypochloremia, and hyponatremia; [Ostermann et al. 2014]) altered numerous biological constituents that were, therefore, able to change sSID metabolism substrates. As already noted for the oxygenation status values, one possible explanation for our results could be the absence of severe lung disturbances that resulted in a limited alteration of the acid-base balance. On the other hand, although it was identified as significant in the final regression model only in group WISC5/TUS 1cm between healthy and clinical-BP calves, the performance of USI appears attractive for at least two reasons. The mean value in calves with lung lesions of ≥1 cm was considerably lower than in those without; this tendency disappeared when ≥3 cm lesions were considered. Evaluation of unmeasured strong anions contributes to the acid-base balance assessment through the alternative paradigm of the sSID. This parameter represents the logical extension of the AG concept of the traditional H-H approach. However, unlike AG, USI concentration is obtained by deducting HCO_3^- and the total negative charge of plasma protein from the SID. It follows that a decrease in USI accounts for the contribution of increased anions concentrations whose origin or source is not yet entirely clear but intimately linked to adverse disease outcomes (Moviat et al., 2008; Gomez et al., 2017; Gomez et al., 2020). The reasons for the increased concentration of unmeasured anions in calves with BP remain unclear. Although the decrease in USI concentration was not clearly significant between categorizations, we find it interesting that the ≥1 cm lesion has the propensity to lower this value, which is often correlated with major metabolic issues

such as septicemia, change in Atot due to the production of inflammatory proteins, alteration of the Krebs's cycle, or increased ketogenic metabolism (Kellum et al., 1995; Moviat et al., 2008; Gomez et al., 2020; Porta et al., 2006).

The results of this study can help us understand the metabolic alterations during naturally-occurring BP in preweaned dairy calves. An important finding was that arterial blood gases and acid-base status values were limited in describing the metabolic change in calves with BP. Our results suggest that categorizing calves according to clinical signs and ultrasonographic lesions effectively describes disease severity only based on alterations in glucose metabolism. This result could be interesting because it can be related to all that pre-existing literature correlating BP with reduced weight gain due to less energy available for growth in the presence of lung diseases (Cramer & Ollivett, 2019; Cuevas-Gómez et al., 2021). Through the goodness of fit in terms of pseudo-R^2, we found that the WISC2points/TUS1cm group achieves the best performance in explaining changes in metabolic abnormalities. This result again confirms the knowledge about ≥1 cm lung consolidation and positive WISC2points score in reducing weight gain and increasing the specificity of BP diagnosis (Cramer & Ollivett, 2019; Decaris et al., 2022). Because we only highlighted glucose as a factor that always correlated with the severity of ultrasound lesions and clinical signs, we do not have data that clearly show the difference between subclinical and clinical BP calves. Further work is needed to determine whether the presence of clinical signs accompanying ultrasound lesions can be a differentiation between an active BP or not.

In cross-sectional studies, the results represent a snapshot of the health status of the population studied over a specific period; this type of study is potentially subject to bias. The most important is the absence of a temporal relationship between putative etiologic exposures and the outcomes under investigation. The addition of incident cases, evaluated, for example, over a longer study period, could have diversified the results obtained. In addition, selection and information bias could also distort the results, particularly when convenience samples are used. We tried to mitigate these problems by adopting validated methods to measure metabolic parameters from calves classified by standardized methods of clinical scoring and TUS examination performed by experienced veterinarians. In addition, using a one-gate design (which usually includes doubtful cases returning results generally closer to the expected in a practical context; Buczinski and Pardon [2020]) could have mitigated the selection bias. Unfortunately, the other side of the coin was that the one-gate design did not allow us to have a uniform number of calves within the categorizations used. This was especially true for the calves of the upper respiratory tract infection group, which were effectively numerically smaller than other groups. Therefore, the results from these calves need to be interpreted with caution.

CONCLUSIONS

The present study was designed to determine the effect of BP diagnosed with a combination of clinical scores and TUS cutoffs on blood oxygenation data, acid-base, and metabolic parameters to evaluate which diagnostic set might be most relevant to define the severity of metabolic alterations during BP events. Except for glucose concentration, which was significantly lower in all calves with ultrasonographic lesions and clinical signs, other parameters were of limited value. Using a cutoff≥1 cm at TUS and a WISC considered positive when at least 2 characters scored ≥2 also seems a promising method to describe the severity of metabolic changes during a BP episode. However, we had hypothesized a more significant presence of abnormalities that could have better-defined calves with positive TUS. Further studies with more effective respiratory-related markers are needed to better define an active or inactive lesion.

Table 1. Results from a multivariable logistic regression models identifying factors associated with the clinical score and thoracic ultrasonography findings.

Group	N	Variable	Median (IQR range)	p value	OR	CI 95%	
Group WISC5/TUS1cm							
Healthy	60	K+ (mmol/L)	4.00 (0.40)	0.006*	0.22	0.07	0.65
		Glu (mmol/L)	5.92 (1.22)	0.000*	2.38	1.59	3.56
		USI	2.73 (3.80)	0.046*	1.11	1.00	1.22
Upper respiratory tract infection	11	K+ (mmol/L)	4.10 (0.40)	0.366	0.42	0.06	2.76
		Glu (mmol/L)	5.70 (0.80)	0.016*	2.23	1.16	4.28
		USI	3.50 (5.00)	0.454	1.07	0.90	1.26
Subclinical BP	106	K+ (mmol/L)	4.20 (0.43)	0.534	1.20	0.68	2.12
		Glu (mmol/L)	5.30 (1.72)	0.028*	1.44	1.04	1.99
		USI	0.95 (3.46)	0.556	0.97	0.89	1.06
Clinical BP	54	K+ (mmol/L)	4.15 (0.40)				
		Glu (mmol/L)	5.20 (1.79)	Reference category for each term.			
		USI	0.71 (5.97)				
Group WISC2points/TUS1cm							
Healthy	59	K+ (mmol/L)	4.00 (0.40)	0.020*	0.25	0.08	0.81
		Glu (mmol/L)	5.89 (1.20)	0.000*	2.25	1.48	3.40
		A tot	18.52 (2.06)	0.933	0.99	0.79	1.24
		USI	2.59 (3.73)	0.062	1.11	0.99	1.24
Upper respiratory tract infection	12	K+ (mmol/L)	4.15 (0.85)	0.873	0.86	0.14	5.21
		Glu (mmol/L)	5.82 (1.10)	0.031*	2.05	1.07	3.94
		A tot	18.87 (3.09)	0.327	0.83	0.57	1.21
		USI	4.09 (4.45)	0.236	1.10	0.94	1.28
Subclinical BP	105	K+ (mmol/L)	4.20 (0.45)	0.111	1.88	0.86	4.09
		Glu (mmol/L)	5.30 (1.76)	0.222	1.24	0.88	1.73
		A tot	18.52 (2.74)	0.018*	0.80	0.66	0.96
		USI	1.00 (3.61)	0.381	0.96	0.86	1.06
Clinical BP	55	K+ (mmol/L)	4.10 (0.40)				
		Glu (mmol/L)	5.20 (1.70)	Reference category for each term.			
		A tot	19.21 (2.74)				
		USI	0.43 (5.79)				
Group WISC5/TUS3cm							
Healthy	91	Na+ (mmol/L)	138 (2.00)	0.349	1.06	0.94	1.20
		Glu (mmol/L)	5.70 (1.33)	0.000*	2.60	1.69	3.98
		Creatinine (mg/dL)	0.93 (0.30)	0.533	1.75	0.30	10.09
Upper respiratory tract infection	27	Na+ (mmol/L)	137 (2.00)	0.642	0.95	0.78	1.16
		Glu (mmol/L)	5.80 (0.70)	0.000*	2.56	1.52	4.31
		Creatinine (mg/dL)	0.86 (0.25)	0.849	1.24	0.14	11.31
Subclinical BP	75	Na+ (mmol/L)	137 (3.00)	0.180	0.90	0.78	1.05
		Glu (mmol/L)	5.26 (1.83)	0.001*	2.02	1.33	3.08
		Creatinine (mg/dL)	0.83 (0.32)	0.409	0.46	0.08	2.86
Clinical BP	38	Na+ (mmol/L)	136 (3.50)				
		Glu (mmol/L)	5.05 (1.79)	Reference category for each term.			
		Creatinine (mg/dL)	0.90 (0.26)				
Group WISC2points/TUS3cm							
Healthy	91	Na+ (mmol/L)	138 (3.00)	0.403	1.05	0.93	1.18
		Glu (mmol/L)	5.70 (1.33)	0.000*	2.33	1.57	3.47
Upper respiratory tract infection	27	Na+ (mmol/L)	137 (2.00)	0.866	0.98	0.81	1.19
		Glu (mmol/L)	5.90 (0.70)	0.001*	2.42	1.47	4.00
Subclinical BP	73	Na+ (mmol/L)	137 (3.00)	0.108	0.89	0.76	1.03
		Glu (mmol/L)	5.26 81.90)	0.003*	1.81	1.22	2.69
Clinical BP	40	Na+ (mmol/L)	136 (4.00)				
		Glu (mmol/L)	5.03 (1.56)	Reference category for each term.			
Group CALIF/TUS1cm							
Healthy	44	Glu (mmol/L)	5.79 (1.26)	0.004*	1.79	1.21	2.64
		BUN (mg/dL)	7.00 (4.00)	0.287	0.95	0.86	1.05
		USI	2.61 (3.77)	0.120	1.08	0.98	1.18
		A tot	18.52 (2.06)	0.823	0.97	0.78	1.22
Upper respiratory tract infection	27	Glu (mmol/L)	5.94 (1.16)	0.006*	1.92	1.21	3.05
		BUN (mg/dL)	6.00 (4.00)	0.016*	0.81	0.68	0.96
		USI	3.09 (3.91)	0.098	1.10	0.98	1.24
		A tot	19.21 (2.06)	0.533	1.09	0.83	1.43
Subclinical BP	74	Glu (mmol/L)	5.32(1.61)	0.429	1.14	0.83	1.57
		BUN (mg/dL)	6.00 (4.00)	0.263	0.96	0.89	1.03
		USI	0.50 (3.79)	0.160*	0.93	0.85	1.03
		A tot	17.84 (2.74)	0.010*	0.78	0.64	0.94
Clinical BP	86	Glu (mmol/L)	5.20 (1.61)				
		BUN (mg/dL)	7.00 (4.75)	Reference category for each term.			

		USI	0.99 (4.22)				
		A tot	18.87 (2.74)				
Group CALIF/TUS3cm							
Healthy	67	Na+ (mmol/L)	138.00 (2.00)	0.246	1.07	0.95	1.21
		Glu (mmol/L)	5.70 (1.45)	0.001*	1.91	1.33	2.74
		Creatinine (mg/dL)	0.90 (0.31)	0.025*	5.75	1.24	26.61
Upper respiratory tract infection	51	Na+ (mmol/L)	137.00 (3.00)	0.679	1.03	0.90	1.18
		Glu (mmol/L)	5.80 (0.76)	0.003*	1.77	1.21	2.58
		Creatinine (mg/dL)	0.94 (0.28)	0.030*	5.47	1.18	25.36
Subclinical BP	51	Na+ (mmol/L)	137.00 (3.00)	0.137	0.89	0.77	1.04
		Glu (mmol/L)	5.50 (1.62)	0.073*	1.40	0.97	2.03
		Creatinine (mg/dL)	0.87 (0.32)	0.107	4.18	0.73	23.82
Clinical BP	62	Na+ (mmol/L)	137.00 (3.00)				
		Glu (mmol/L)	5.00 (1.70)	Reference category for each term.			
		Creatinine (mg/dL)	0.83 (0.28)				

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Discussion and Conclusion

With this study, it was possible to increase our knowledge to date regarding Bovine Respiratory Disease Complex (BRDC) by analyzing various aspects of it.

- First, a focus on etiologic diagnosis was performed to explore one of the three fundamental factors (pathogens, host, and environment) involved in the pathogenesis of BRDC.
- Then, the clinical approach to the diagnosis of BRDC was considered by examining the clinical tools currently available, to increase the accuracy and earliness of diagnosis; in detail, the potential of ultrasonography and thoracic auscultation was reviewed.
- Finally, through the blood gas analysis, we focused our attention on the changes that BRDC causes at the level of the organism of the animals that it affects and the impact that pulmonary lesions have.

Correct management and environmental housing conditions of calves are fundamental to the control of BRDC, but sometimes they are not enough. The other tools available to veterinarians to contain this disease are vaccinations and antibiotic therapy. In the management of BRDC, antibiotic treatment plays a crucial role. Nonetheless, when dealing with a multifactorial disease, the risk of incurring inappropriate treatment choices is possible by increasing the risk of therapy failure and antibiotic resistance development. This can have repercussions in both veterinary and human medicine. The rise of antimicrobial resistance to human and veterinary medicines is a rising health problem in the European Union and around the world (Holschbach et al., 2020; De Jong et al., 2014). The impact of the issue, due to its complexity, affects society as a whole and requires urgent and coordinated action according to the "One Health" approach. Such action includes strengthening the prudent use of antimicrobials, avoiding their routine metaphylactic and prophylactic use, limiting the use in animals of antimicrobials that are critically important for the treatment of life-threatening infections in humans, as well as encouraging and incentivizing the development of new antimicrobials (EU Reg., 2019/6).

The first aim of this study was achieved by investigating both bacterial and viral etiological agents of BRDC at the level of the lower airway in preweaning dairy calves. To date, this information was still lacking in Italy. What emerged was that the most frequent lower airway microorganism causing respiratory disease in calves is *Pasteurella multocida,* which was isolated in 37 percent of the samples. Although expected, this result highlights how the on-farm vaccine control applied in practice toward other bacterial etiological agents, e.g., *Mannheimia haemolytica,* has significantly reduced the on-farm prevalence. It also became clear that the second most important etiological agent was bovine Coronavirus, which can induce a disease condition individually or associated with other pathogens. The presence of BCoV in the territory is widespread and characterized in 6 out of 10 farms examined.

Increasing control measures against this virus may reduce the incidence of BRDC in dairy cattle farms. The presence of other pathogens known to be related to BRDC, such as *M. haemolytica*, *Mycoplasma bovis*, or Bovine Respiratory Syncytial Virus, was detected. They represent a relevant problem at the farm level, but considering their overall prevalence in the territory and on the sample of farms we examined, they appear to be of secondary importance. Also, our work demonstrated how a method of lower respiratory tract sampling (Trans Tracheal Wash), although more indaginous than simpler methods (nasal swab), turns out to be feasible in the field and is justified by the accuracy of the results obtained that allow setting in therapeutic plan and targeted vaccine prophylaxis.

Another point this work aimed to address was to give an extra diagnostic tool to the buiatrics practitioner dealing with BRDC. The ability to issue an accurate and early diagnosis can often result in better disease control, both at the individual animal level and from a farm management perspective. With the development of the FLUS "focused lung ultrasound" method, we wanted to create a technique that can help the veterinarian in certain situations, such as when many animals need to be screened or in the case of older animals with more pronounced shoulder muscle masses. The FLUS method basis on scanning the lung lobes that are most frequently involved in pneumonia and are also the easiest to reach ultrasonographically in older calves, such as the caudal aspect of the left cranial lung lobe (left fifth and fourth ICS), the right middle lung lobe, and the caudal portion of the right cranial lung lobe, (right fifth and fourth ICS). This test was compared with the TUS "systematic thoracic ultrasonography" method, which is considered the imperfect gold standard for diagnosing the respiratory disease in vivo (Ollivett et al., 2015). Using a TUS threshold score of ≥2, FLUS had a Se of 81.6% (95% CI = 71–89.5%), a Sp of 100% (95% CI = 93.9–100%), a PPV of 100%, an NPV of 96.6% (95% CI = 94.7–97.9%), and an accuracy of 97% (95% CI = 92.6–99.2%). The concordance between the two methods resulted to be significant. An important finding is that FLUS represents a practical method to diagnose lung lesions in field conditions and can be performed rapidly using a linear probe. The FLUS method may help practitioners to recognize lung lesions in postweaned calves. The easy recognition of sick animals would facilitate specific management versus healthy subjects, such as early separation, thereby preventing unnecessary antimicrobial treatments of healthy cohabitants and the pathogens spread among the groups. This opportunity would optimize the use of on-farm facilities, i.e., judicious use of the quarantine box or infirmary, and also allows better monitoring of animals to check the effectiveness of treatments.

For the same reasons and benefits just listed and to obtain an evaluation of diagnostic techniques, Thoracic auscultation (AUSC) was also reviewed.

The AUSC is often the first diagnostic approach used by operators. In dairy calves, it has recently been observed that the interpretation of respiratory sounds involves some difficulty due to

interpretation, subjectivity, and terminology, which results in poor accuracy and reproducibility of the test. Therefore, another objective of this study was to evaluate the diagnostic accuracy of a scoring system characterized by a precise classification and definition of lung sounds for diagnosing bronchopneumonia compared to thoracic ultrasound using a Bayesian latent class approach in a population of dairy calves.

First, auscultatory pathological sounds were defined and listed as follows: increased breath sounds, wheezes, crackles, increased bronchial sounds and/or pleural friction ribs. We defined "increased breath sounds" in which the difference between inspiration and expiration is identifiable, differentiating it from "increased bronchial sounds" when the sounds from inspiration were identical to that from expiration. After that, the auscultatory findings collected from 340 dairy calves were compared with the presence of ultrasound-detectable lung lesions ascribable to bronchopneumonia. TUS was used to distinguish animals into healthy/sick as it is considered the imperfect reference standard test.

In this study, we reported the accuracy of 3 different AUSC criteria for bronchopneumonia case definition. Using all pathological sounds in AUSC1 allowed having a high sensitivity but a low specificity. Instead, avoiding the "increased breath sounds" in AUSC2 or using only the severe pathological sounds in AUSC3 shows a high specificity at the cost of lower sensitivity.

The auscultation sensitivity and specificity (95% BCI) of informative models with no covariance were 90.3% (81.2-98.4%) and 57.4% (46.6-71.5%) for AUSC1, 81.7% (69.1-94.5%) and 90.8% (81.1-99.0%) for AUSC2, and 67.9% (55.7-82.8%) and 98.5% (93.8-99.9%) for AUSC3 respectively. From the results of our study, the high specificity of AUSC3 showed that these sounds infrequently occur in healthy calves. As a practical example, to correctly identify affected animals, TUS could be beneficial in addition to AUSC, especially in calves in which increased bronchial sounds are not auscultated, or to better identify calves with pathological sounds less specific to lung disease. Thus, higher accuracy in diagnosing BP could be achieved and generate a more judicious use of antibiotics. Also, due to the growing interest in and the ever-increasing possibility for the buiatrics veterinarian to have and use portable blood gas analyzer instruments, the last aim of this thesis was to evaluate whether Respiratory-related functional markers could be a possible method to assess the severity of BP episodes. Blood oxygenation data and respiratory-related acid-base disturbances are objective measures of lung lesions (Ellis et al., 2013). In addition, L-lactate levels or alteration of acid-base balance using the simplified strong ion difference (sSID) approach have recently been described as related to the severity of respiratory diseases (Buczinski et al., 2015; Niedzwiedz et al., 2019). Therefore, the effect of BP diagnosed with different combinations of clinical scores and TUS was

explored in relation to 1) blood gas status, 2) acid-base abnormalities using the traditional Henderson-Hasselback (H-H) approach and sSID theory, and 3) biochemical findings.

We hypothesized that these metabolic alterations could define which categorization methods best described BP severity. In addition, we aimed to explore which abnormalities are most significantly involved in affected dairy calves. Increased knowledge and understanding of how the combination of TUS and clinical signs affect lung function and metabolic status may improve BP case definition. For this study 231 Holstein-Friesian female calves were enrolled, each of them was examined and clinical information was collected and subjected to TUS. A combination of 3 different clinical scores WISC5 (McGuirk & Peek, 2014), WISC2points (Decaris et al., 2022), CALIF5 (Love et al., 2014), and 2 TUS scores: TUS≥1cm (Buczinski et al., 2015) and TUS≥3cm (Berman et al., 2019) were evaluated, resulting in 6 combinations of the two methods.

Based on these combinations, the enrolled calves were assigned to one of the following 4 categories: healthy; upper respiratory tract infection; subclinical BP; and clinical BP.

To these classifications with gradually increasing severity, we expected that alterations in parameters detectable by hemogasanalysis would also be correlated with progressively more significant changes. It is reported in the literature that PaO_2 (Ellis et al., 2013) and A-aDO_2 (Ostermann et al., 2013) were associated with the extent of lung lesions and other markers take on prognostic value as in the case of L-lactate concentration >5 mmol/L (Buczinski et al., 2015). Furthermore, the concentration of some electrolytes, such as Na and K, but also the measured values of SID and A_{tot} showed no or marginal importance.

In contrast, relative hypoglycemia successfully emerged as a good parameter to describe disease severity in all groups of calves. Based on these data, we can assume that the combinations of clinical scores and ultrasonographic lesions used in this study were able to characterize disease severity based on altered glucose metabolism. In fact, the decrease in mean glucose concentration was more significant in calves with ultrasonographic lesions and significantly lower in the BP-clinical groups.

Therefore we found that the WISC2points/TUS1cm group achieves the best performance in explaining changes in metabolic abnormalities. This result again confirms the knowledge about ≥1 cm lung consolidation and positive WISC2points score in reducing weight gain and increasing the specificity of BP diagnosis (Cramer & Ollivett, 2019; Decaris et al., 2022).

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