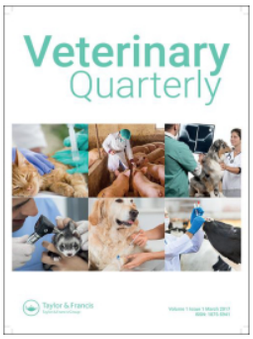

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Cross-sectional study: can endogenous procalcitonin differentiate between healthy and bovine respiratory disease-affected preweaned dairy calves?

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ABSTRACT

Bovine respiratory disease (BRD) represents a significant challenge in cattle management due to its multifactorial nature and lack of a gold standard diagnostic method. Procalcitonin (PCT) has emerged as a potential biomarker for bacterial infections in various species, including cattle. This study aimed to investigate plasma PCT concentration variations in pre-weaned dairy calves categorized as BRD-positive using clinical scores (WRSC; BRD-positive ≥5), thoracic ultrasonography with two cut-off (TUS; BRD-positive ≥1 or ≥3), or a combination of both methods (WRSC/TUS1cm or WRSC/TUS3cm). Additionally, the accuracy of PCT in diagnosing BRD was evaluated. A cross-sectional study was conducted on a convenience sample of 226 pre-weaned Italian-Friesian female calves. Clinical scoring, TUS, and plasma PCT analysis were performed. Calves were categorized based on TUS findings, clinical scores, or a combination of both methods. Statistical analyses were conducted to assess the differences in PCT concentrations among different groups and to determine the diagnostic accuracy of PCT. Results showed a significant increase in PCT levels in calves with lung consolidation detected by TUS using a 1 cm cutoff. However, the diagnostic accuracy of PCT in discriminating BRD-positive cases was poor (area under the curve 0.62). The optimal cutoff value for PCT was determined to be 86.63 pg/mL, with sensitivity of 49.7%, specificity of 71.8%, positive predictive value of 79.4% and negative predictive value of 39.5%. In conclusion, while PCT showed potential as a biomarker for BRD, its diagnostic accuracy was limited in this study. Future research should focus on integrating PCT measurements with other diagnostic methods and conducting longitudinal cohort studies to better understand its role in BRD diagnosis and management.

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Procalcitonin; dairy calves; bovine respiratory disease; thoracic ultrasonography; clinical scores

Introduction

Bovine respiratory disease (BRD) includes a wide spectrum of respiratory diseases that can affect upper and lower respiratory tract in cattle (Buczinski and Pardon 2020), representing an important cause of morbidity, mortality, economic losses, and use of antimicrobials across cattle sectors (Lava et al. 2016; Blakebrough-Hall et al. 2020; Diana et al. 2020; Buczinski et al. 2021; Crosby et al. 2023). Bovine respiratory disease is considered a multifactorial disease in which viral and bacterial pathogens, environmental conditions, and host immunity interplay making its management challenging (Ackermann et al. 2010). No practical and affordable gold standard test for on-field diagnosis of BRD is available, thus the definition of BRD-positive cases remains non-uniform. Despite the use of different classification methods have been assessed by literature, their diagnostic accuracies remain variable (Buczinski and Pardon 2020). Common diagnostic methods for a BRD-positive diagnosis in calves involve thoracic ultrasonography (TUS) (Berman et al. 2019; Pravettoni et al. 2021; Baxter-Smith et al. 2022), clinical scoring systems (McGuirk and Peek 2014; Love et al. 2014; Cramer and Ollivett 2019) and lung auscultation (Pardon et al. 2019; Boccardo et al. 2023). Between these techniques, TUS offers the highest achievable diagnostic accuracy in calves under field conditions. Recent studies have shown that TUS performs as well as radiography, with a sensitivity of 81% and specificity of 90% compared to thoracic computed tomography (Berman et al. 2020). Clinical scoring systems showed different levels of diagnostic accuracy when compared to TUS, ranging from low to moderate across different studies with a sensitivity ranging between 18.8% and 62.4%, and a specificity

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between 80.8% and 74.1% (Buczinski et al. 2015; Lowie et al. 2022). Similarly, lung auscultation also shows variable diagnostic accuracy compared to TUS (sensitivity range: 5.9%–89%, specificity range: 53.3%–97%) (Buczinski et al. 2014; Buczinski et al. 2016; Boccardo et al. 2023). Recent literature about BRD cases classification categorized calves into 4 groups based on a combination of TUS findings and clinical signs: healthy (absence of clinical signs and absence of consolidation at TUS), those affected by upper respiratory tract infections (presence of clinical signs, but absence of lesions at TUS), calves with subclinical bronchopneumonia (absence of clinical signs but presence of consolidation at TUS), or those with clinical bronchopneumonia (both clinical signs and consolidation at TUS were present) (Denis-Robichaud et al. 2021; Jourquin et al. 2022). Difficulties in BRD diagnosis often leads to metaphylaxis which is implied in the increasing of antimicrobial resistance (Lubbers and Hanzlicek 2013; Ives and Richeson 2015; Noyes et al. 2015; Buczinski and Pardon 2020; Lowie et al. 2022; Crosby et al. 2023). Thus, the interest for a BRD diagnostic strategy able to easily and quickly discriminate between animals requiring antimicrobial treatment *vs* those who did not is raising.

Biomarkers (BIOs) have been studied as diagnostic tools for diseases (Abdallah et al. 2016; Li et al. 2022). In bovine, PCT has been evaluated with good results in case of sepsis (Ercan et al. 2016; Bonelli et al. 2018), cryptosporidiosis (El-Deeb et al. 2022), BRD in beef calves (El-Deeb et al. 2020; Koshiishi et al. 2023), aspiration pneumonia (Akyüz et al. 2022), adult animals with conditions that lead to hospitalization (Bonelli et al. 2023), and cows with subclinical and clinical mastitis (El-Deeb et al. 2021; Neumann et al. 2023; Sala et al. 2023). PCT is the prohormone of calcitonin (Maruna et al. 2000), regulated by the calcitonin gene-I (CALC-I). In healthy humans, the CALC-I gene is predominantly active within the thyroid, resulting in minimal release of PCT into the bloodstream (Christ-Crain and Müller 2007). During bacterial infections, lipopolysaccharides (LPS) from the bacterial cell wall and inflammatory cytokines, such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), induce the up-regulation of the CALC-I gene in various tissues, resulting in the substantial release of PCT into the bloodstream from multiple cell types (Maruna et al. 2000; Riedel 2012). PCT levels rise rapidly, typically within 2–6 h following the onset of bacterial infection and decrease by half within 24 h once the bacterial infection and/or host immune response are controlled (Meisner et al. 1997). In human medicine, PCT is utilized in antibiotic decision algorithms for various diseases, including respiratory diseases (Schuetz et al. 2013; Schuetz et al. 2018; Smith et al. 2020).

In BRD, PCT was investigated only in beef-calves (El-Deeb et al. 2020; Koshiishi et al. 2023). In these studies, PCT were evaluated in BRD-positive cases; animals were considered sick based on clinical signs and the isolation from nasopharyngeal swabs of at least one BRD pathogen (El-Deeb et al. 2020; Koshiishi et al. 2023). In both studies, PCT showed a Despite limitations of clinical studies, especially related to BRD diagnostic tools, which may have influenced results, researchers found that PCT significant increase in clinically BRD-positive cases compared with clinically healthy controls (El-Deeb et al. 2020; Koshiishi et al. 2023). Currently, there are no data on PCT concentration in pre-weaned dairy calves with BRD diagnosed by TUS and examination of clinical signs. Thus, the present study aimed to investigate the variations of plasma PCT concentration in pre-weaned dairy calves categorized as BRD-positive using clinical scores, TUS, and the combination of the two diagnostic methods. An additional aim was to determine the accuracy of PCT in naturally BRD-affected calves. We hypothesized that lung consolidation determined by TUS is associated to a higher plasmatic PCT concentration compared to calves without TUS lesion, regardless the severity of clinical signs in pre-weaned dairy calves.

Material and methods

Study design and population

In accordance with STROBE guidelines, we conducted a cross-sectional study employing a convenience sample of dairy farms selected between those served from the mobile clinic of the University of Milan between January 2020 and February 2022.

The data set used in this study consisted in 2 groups of calves enrolled in previous studies. The first group was composed by a population of 330 calves originating from 18 dairy farms (Boccardo et al. 2023 – study A). The second group was represented by 231 calves originating from 13 dairy farms (Boccardo et al. 2024 – study B). The aim of study A was to evaluate the diagnostic accuracy of lung auscultation using Bayesian evaluation, while study B aimed to evaluate the blood gases, acid-base balance, and metabolic alterations in calves with BRD. Among these groups, we selected animals still having residual stored plasma (minimum quantity of plasma stored: 1 ml) for a total of 226 calves originating from 13 farms (36 calves from study A and 190 calves from study B). A Wilcoxon-Mann-Whitney analysis was employed to determine the minimum sample size of calves using G-power v. 3.1 (Heinrich-Heine-Universität, Düsseldorf, Germany) required to detect BRD-related PCT differences between healthy and affected calves. An effect size of 0.5 (medium), an α error of 5% (type I), a confidence interval of 95%, and a test power of 95% were applied, resulting in a minimum sample size of 220 calves for detecting differences in the median of PCT concentration.

The study protocols were approved by the Institutional Animal Care and Use Committee of the University of Milan (approval number 104/2020, January 15, 2020). Residual blood samples collected for clinical screening were employed following the

guidelines of the ethical committee of the University of Milan (approval number 2/16, February 15, 2016).

The criterion for farm selection during the just mentioned studies was a history of cough in pre-weaned Italian Friesian calves housed in multiple pens with automatic calf feeders, with no record of treatment for BRD in the 15 d before the study. A maximum of 25 pre-weaned calves *per* farm were examined, with selected animals chosen randomly using an Android application (Randomizer, Darshan Institute of Engineering and Technology, Rajkot, India) based on individual ear tags provided by the farmer. Calves with signs of lameness, cachexia, dehydration, diarrhea, or umbilical pathologies were excluded. Calves from the same farm underwent to the clinical protocol on the same day.

Clinical scoring and thoracic ultrasonography

Selected calves underwent a standardized one-gate design protocol, including a clinical evaluation made by the Wisconsin Calf Respiratory Scoring Chart (WCRSC). Clinical data were used to assess the WCRSC as described in the literature (McGuirk and Peek 2014). Briefly, the WCRSC uses five different clinical signs (rectal temperature, presence of cough, nasal discharge, eye score, and ear score) on a 4-level scale (from 0 to 3). Calves were considered BRD-positive when the total score exceeded 5.

Bilateral TUS was also performed on selected calves as described in prior studies (Ollivett and Buczinski 2016; Cramer and Ollivett 2019; Pardon et al. 2019) by the same operator, who remained blinded to the clinical scoring results. Ultrasonography was carried out with a portable unit (Esaote MyLab Five Vet, Esaote, Italy) equipped with a 7.5 MHz linear transducer set to a depth of 8 cm and a gain of 16 dB. The thoracic scan was performed without shaving the calves and using vegetable oil as an interface. TUS was conducted first on the left side (from the 10th intercostal space to the 2nd intercostal space) and then on the right side (from the 10th intercostal space to the 1st intercostal space). Each intercostal space was scanned from dorsal to ventral until reaching the landmarks described by Ollivett and Buczinski (2016). The consolidation was defined as the replacement of a normal reverberation artifact with an echoic structure. The maximum depth of consolidation on TUS was recorded for each calf. Depth (cm) was manually counted using the lateral grid of the ultrasound image. In cases of uncertainty or when lesions approached a cutoff measurement, the integrated measurement tool in the ultrasound scanner was employed. Comet tail artifacts, pleural irregularities, and lesions <1 cm were excluded from analysis due to the ongoing lack of clarity in the literature regarding the diagnostic value of these specific lesions.

Blood venous sampling

After the clinical examination, blood samples were collected from the jugular vein in lithium heparin tubes and transported to the clinic in a portable cooler (5 °C). Samples were centrifuged at 20 °C for 10 min at 900 g within 6 h of collection and the resulting plasma was carefully transferred to sterile tubes and stored at −80 °C until analysis. All samples were analyzed within 8 months from collection. To preserve the bioactivity of the samples, they were thawed on ice for approximately 2 h before the analysis (Schuetz et al. 2010).

PCT analysis

The concentration of PCT in plasma samples was measured using a commercial kit designed for cattle (Bovine Procalcitonin ELISA kit, Cusabio, Houston, TX, USA). Briefly, the kit employs a sandwich enzyme immunoassay technique. Reagents preparation, incubations, and washes were performed according to the manufacturer's instructions. The kit included a standard solution, which was diluted to obtain concentrations of 1250, 625, 312.5, 156.25, 78.12, and 39.6 pg/mL, with a final blank well containing only the standard diluent (0 pg/mL PCT).

The optical density (OD) of the samples was measured using a microplate reader set to 450 nm. The OD of the blank well was subtracted from each sample's OD. A standard curve was created by plotting the mean absorbance of the standards against their known concentrations, enabling the calculation of PCT concentrations in the samples. The calibration curve and kit characteristics (LOD, calibration range, intra-assay, and inter-assay variation) were provided by the manufacturer for plasma, serum, and tissue homogenates. The calibration range was 39.0–2500.0 pg/mL, intra-assay and inter-assay CVs were <8.0% and <10.0%, respectively, and the LOD was 9.77 pg/mL.

Categorizations of BRD-positive and negative cases for PCT evaluation

Due to the absence of a perfect gold standard, calves were classified as BRD-positive based on three different approaches: (1) using TUS only (Ollivett and Buczinski 2016); (2) using WCRSC only (McGuirk and Peek 2014); and (3) using a combination of TUS and WCRSC (Denis-Robichaud et al. 2021; Jourquin et al. 2022).

TUS approach defined BRD-positive calves by using two different cut off:

- Lung consolidation ≥ 1 cm (Buczinski et al. 2015) (TUS1cm);
- Lung consolidation ≥ 3 cm (Berman et al. 2019) (TUS3cm).

WCRSC approach defined calves with a WCRSC score of 5 or higher as BRD-positive cases (McGuirk and Peek 2014).

The WCRSC/TUS approach was used followed what already described in the literature (Denis-Robichaud et al. 2021; Jourquin et al. 2022):

- WCRSC/TUS1cm: cases were defined as (1) healthy (WCRSC < 5 without consolidation on TUS), (2) upper respiratory tract infection (WCRSC ≥ 5 without consolidation on TUS), (3) subclinical pneumonia (WCRSC < 5 with consolidation on TUS ≥ 1 cm), and (4) clinical pneumonia (WCRSC ≥ 5 with consolidation on TUS ≥ 1 cm).
- WCRSC/TUS3cm: cases were defined as (1) healthy (WCRSC < 5 without consolidation on TUS), (2) upper respiratory tract infection (WCRSC ≥ 5 without consolidation on TUS), (3) subclinical pneumonia (WCRSC < 5 with consolidation on TUS ≥ 3 cm), and (4) clinical bovine pneumonia (WCRSC ≥ 5 with consolidation on TUS ≥ 3 cm).

Data analysis

Collected data were stored and analyzed using IBM SPSS Statistics v. 27.0 (IBM Corp. Armonk, NY). Descriptive statistics were conducted, with categorical variables expressed as frequencies and percentages, while continuous variables were presented as median, 25° and 75° percentile due to non-normal data distribution (tested with the Shapiro-Wilk test). Non-parametric tests were employed for statistical analysis, given the non-normal distribution of the data.

Differences between parameters were assessed using the Mann-Whitney U test for TUS1cm, TUS3cm and WCRSC, while the Kruskal–Wallis test and Bonferroni's post hoc test were applied for WCRSC/TUS1cm and WCRSC/TUS3cm. Statistical significance was established at p value < .05.

If differences between the healthy and pathological groups were identified using various categorizations, a diagnostic accuracy analysis was conducted using MedCalc® Statistical Software version 22.003 (MedCalc Software Ltd, Ostend, Belgium). Cut-offs were determined using the Receiver Operating Characteristic (ROC) curve, and the cut-off point was chosen through the Youden index (J) (Youden 1950). The Youden index (Y) ensures that sensitivity and specificity are maximized, assigning equal weight to false-positive and false-negative results (J = Sensitivity + Specificity − 1).

Moreover, the areas under the curve (AUC) and their 95% confidence intervals (CI) were calculated as indicators of test accuracy. The interpretation of AUC was based on a 1.00 perfect test, 0.99 – 0.9 excellent test, 0.89–0.80 good test, 0.79–0.70 fair test, 0.69 – 0.51 poor test, 0.50 failed test (Hanley and McNeil 1982). The selected cut-off values were then used to estimate sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV).

Results

The age of the 226 Italian-Friesian female calves included in this study ranged from 15 to 94 d old (median 58 d; IQR 29.25 d). The percentage of BRD-positive calves following the different categorizations were reported in Table 1, along with PCT concentrations.

Mann–Whitney U test results showed that PCT was significantly different only using the TUS1cm (p value .004) to distinguish BRD-negative (median 67.63 pg/ml; 25°P 49.38 pg/ml; 75°P 96.88 pg/ml) and BRD-positive (median 86.25 pg/ml; 25°P 61.00 pg/ml; 75°P 125.00 pg/ml) calves. PCT resulted not significantly different using TUS3cm (p value 0.098) to distinguish BRD-negative (median 75.00 pg/ml; 25°P 57.63 pg/ml; 75°P 110.09 pg/ml) and BRD-positive (median 87.12 pg/ml; 25°P 61.50 pg/ml; 75°P 123.03 pg/ml) calves.

With the Kruskal–Wallis test, PCT was different for the categorization WCRSC/TUS1cm (p value 0.015). The multiple comparison underlined a statistical difference only between healthy (median 66.87 pg/ml; 25°P 50.63 pg/ml; 75°P 90.43 pg/ml) and clinical pneumonia (median 88.00 pg/ml; 25°P 65.23 pg/ml; 75°P 123.03 pg/ml) categories (p value .010).

The ROC curves (Figures 1–3) were calculated for all methods of categorizations. The resulted cut-off values for different method of classification were: TUS1cm 86.63 pg/ml (Y 0.215); TUS3cm 87.77 pg/ml (Y 0.155); WRSC 69.00 pg/ml (Y 0.138); WRSC/TUS1cm 81.69 pg/ml (Y 0.190) for distinguish healthy from upper respiratory tract infection animals, 84.00 pg/ml (Y 0.227) for distinguish healthy from subclinical pneumonia and clinical pneumonia; WRSC/TUS3 cm 69.00 pg/ml (Y 0.164) for distinguish healthy from upper respiratory tract infection animals, 115 pg/ml (Y 0.157) for distinguish healthy from subclinical pneumonia, 87.77 pg/ml (Y 0.228) for distinguish healthy from clinical pneumonia.

The AUC and accuracy indexes indicated poor test accuracy for all methods of categorizations:

- TUS1cm: AUC 0.621 (95% CI: 0.554–0.684); Se 49.7% (95% CI: 41.6%–57.8%), Sp 71.8% (95% CI: 59.9%–81.9%), PPV 79.4% (95% CI: 72.0%–85.2%), NPV 39.5% (95% CI: 34.5%–44.7%).
- TUS3cm: AUC 0.565 (95% CI: 0.498–0.631); Se 50.0% (95% CI: 40.3%–59.7%), Sp 65.5% (95% CI: 56.1%–74.1%), PPV 58,6% (95% CI: 50.8%–66.0%), NPV 58.4% (95% CI: 52.9%–63.8%).
- WRSC: AUC 0.576 (95% CI: 0.508–0.641); Se 68.8% (95% CI: 57.3%–78.9%), Sp 45.0% (95% CI: 36.8%–53.3%), PPV 38.6% (95% CI: 33.6%–43.7%), NPV 72.4% (95% CI: 64.6%–79.1%).
- WRSC/TUS1cm:
  - Healthy vs. upper respiratory tract infection animals: AUC 0.560 (95% CI: 0.437–0.678); Se 50.0% (95% CI: 24.7%–75.3%), Sp 69.1% (95% CI: 55.2%–80.9%), PPV 32.0% (95% CI: 20.0%–47.0%), NPV 82.6% (95% CI: 73.8–88.9%).
  - Healthy vs. subclinical pneumonia: AUC 0.619 (95% CI: 0.535–0.697); Se 50.0% (95% CI: 42.4%–68.5%), Sp 72.7% (95% CI: 59.0%–83.9%), PPV 75.8% (95% CI: 66.1%–83.4%), NPV 46.0% (95% CI: 39.6%–52.4%).

Table 1. Descriptive statistics for procalcitonin (PCT) levels and bovine respiratory disease (BRD) categorization using various thresholds and scoring criteria.

BRD categorization	Frequency	Percentage	PCT pg/ml median (25°P–75°P)
Thoracic ultrasonography (TUS)			
TUS1cm negative	71	31.4%	67.63 (49.38–96.88)[a]
TUS1cm positive	155	68.6%	86.25 (61.00–125.00)[b]
TUS3cm negative	116	51.3%	75.00 (57.63–110.09)
TUS3cm positive	110	48.7%	87.12 (60.5–129.72)
Wisconsin Respiratory Scoring Chart (WRSC)			
WRSC negative	149	65.9%	75.94 (57.75–113.50)
WRSC positive	77	34.1%	86.25 (61.50–123.03)
WCRSC/TUS1cm			
Healthy	55	24.3%	66.87 (50.63–90.43)[a]
Upper respiratory tract infection	16	7.1%	77.09 (12.34–158.12)[a,b]
Subclinical pneumonia	94	41.6%	83.78 (60.25–126.88)[a,b]
Clinical pneumonia	61	27.0%	88.00 (65.23–123.03)[b]
WCRSC/TUS3cm			
Healthy	82	36.3%	71.05 (55.75–105.77)
Upper respiratory tract infection	34	15.0%	80.5 (57.88–121.12)
Subclinical pneumonia	67	29.6%	82.83 (58.00–132.5)
Clinical pneumonia	43	19.0%	91.5 (62.00–128.80)

For the thoracic ultrasonography (TUS) assessment, calves with lung consolidations smaller than 1 cm are categorized as TUS1cm negative, while those with consolidations of 1 cm or more are classified as TUS1cm positive. Similarly, calves with consolidations smaller than 3 cm are categorized as TUS3cm negative, and those with consolidations of 3 cm or larger as TUS3cm positive. The Wisconsin Respiratory Scoring Chart (WRSC) categorizes calves scoring below 5 as WRSC negative, while those with a score of 5 or above are classified as WRSC positive. When combining WRSC and TUS assessments, calves are classified as follows: in the WRSC/TUS1cm categories, calves are considered healthy if WRSC <5 and have no TUS consolidation, classified as having an upper respiratory tract infection if WRSC ≥5 without TUS consolidation, subclinical pneumonia if WRSC <5 with TUS ≥1 cm, and clinical pneumonia if WRSC ≥5 with TUS ≥ 1 cm. In the WRSC/TUS3cm categories, calves are healthy if WRSC <5 without TUS consolidation, are classified as having an upper respiratory tract infection if WRSC ≥5 without TUS consolidation, subclinical pneumonia if WRSC <5 with TUS ≥3 cm, and clinical pneumonia if WRSC ≥5 with TUS ≥3 cm.

The only statistically significant differences observed were between TUS1cm negative and positive categories, and between the WRSC/TUS1cm healthy and clinical pneumonia categories.

Categories (TUS and WRSC/TUS1cm) with different superscript letters (a, b) were statistically different from each other ($p < .05$).

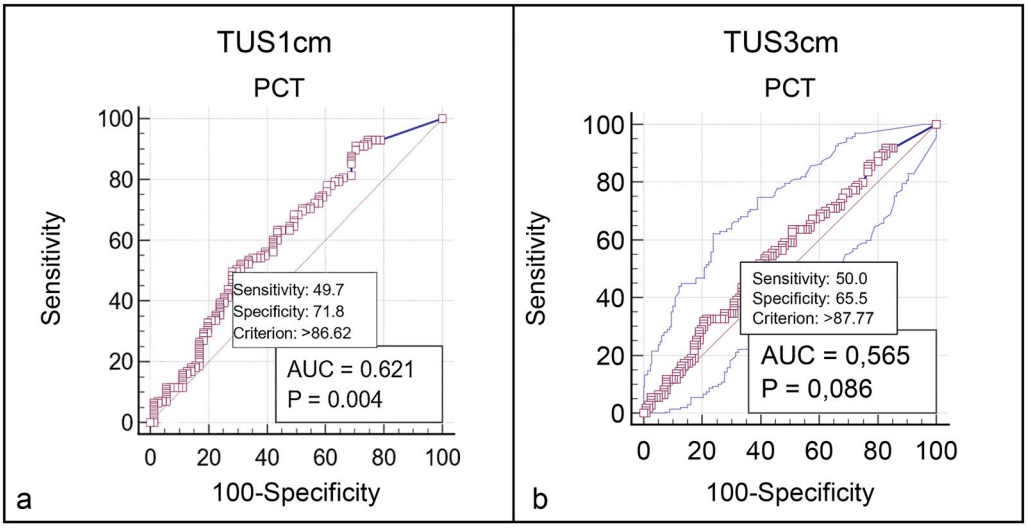

Figure 1. Receiver Operating Characteristic (ROC) curves and Area under the curve (AUC) showing the optimal procalcitonin (PCT) cut-off for detecting bovine respiratory disease (BRD) in 226 calves, as diagnosed *via* thoracic ultrasonography (TUS). BRD is categorized using two common thresholds for lung consolidation: 1 cm and 3 cm. (a) TUS1cm: calves with consolidations <1 cm (negative) versus those with consolidations ≥1 cm (positive); (b) TUS3cm: calves with consolidations <3 cm (negative) versus those with consolidations ≥3 cm (positive).

  - Healthy vs. clinical pneumonia: AUC 0.671 (95% CI: 0.578–0.756); Se 55.7% (95% CI: 42.4%–68.5%), Sp 72.7% (95% CI: 59.0%–83.9%), PPV 69.7% (95% CI: 58.5%–78.9%), NPV 59.4% (95% CI: 51.4%–66.9%).
- WRSC/TUS3cm:
  - Healthy vs. upper respiratory tract infection animals: AUC 0.569 (95% CI: 0.474–0.661); Se 67.7% (95% CI: 49.5%–82.6%), Sp 48.8% (95% CI: 37.6%–60.1%), PPV 35.4% (95% CI: 28.6%–42.8%), NPV 78.4% (95% CI: 68.1%–86.1%).
  - Healthy vs. subclinical pneumonia: AUC 0.558 (95% CI: 0.475–0.639); Se 32.8% (95% CI: 21.8%–45.4%), Sp 82.9% (95% CI: 73.0%–90.3%), PPV 61.1% (95% CI: 46.6%–73.9%), NPV 60.2% (95% CI: 55.4%–64.7%).
  - Healthy vs. clinical pneumonia: AUC 0.628 (95% CI: 0.537–0.724); Se 55.8% (95% CI: 39.9%–70.9%), Sp 67.1% (95% CI: 55.8%–77.1%), PPV 47.1% (95% CI: 37.2%–57.2%), NPV 74.3% (95% CI: 66.7%–80.7%).

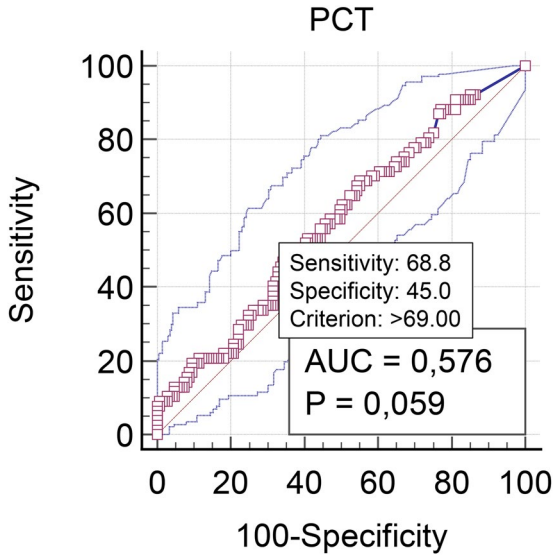

Figure 2. Receiver Operating Characteristic (ROC) curves and Area under the curve (AUC) for the optimal procalcitonin (PCT) cut-off in 226 calves to identify bovine Respiratory disease (BRD) diagnosed *via* the Wisconsin Respiratory Scoring Chart (WRSC). WRSC negative: calves with a WRSC score <5; WRSC positive: calves with a WRSC score ≥5.

Discussion

When calves were classified as BRD-positive/negative based on a single diagnostic tool approach (TUS1cm, TUS3cm or WCRSC), PCT concentrations increased in BRD-positive case defined by TUS1cm, and not by TUS3cm or WCRSC. While the ability of PCT to distinguish between the presence or the absence of an infection disease is already well-documented across various species (Chadorneshin et al. 2023; Sala et al. 2023; Nocera et al. 2024), the debate between the best TUS cut-off (≥1 *vs* ≥3 cm) for definition of BRD-positive case is still open. Recent studies showed that TUS is the most accurate diagnostic method for identifying animals affected by BRD, even with subclinical forms (Cuevas-Gómez et al. 2020; Cuevas-Gómez et al. 2021; Jourquin et al. 2022). Berman et al. (2021) demonstrated that ultrasound lesions ≥1cm was correlated with active BRD with a Se and Sp of 84% and 74%, respectively. Therefore, variations in PCT concentrations in calves with TUS1cm can be explained by the higher accuracy of TUS in detecting lesions probably associated with infections and related immunological changes (Moisá et al. 2019; Berman et al. 2021; Porter et al. 2021). The absence of significatively differences between groups in WCRSC can be related to the poor diagnostic accuracy of this clinical scoring system when used alone (Se 62.4% and Sp 74.1%) (Buczinski et al. 2015). Interestingly, with the categorization using A1-TUS3cm, the variation in PCT is not statistically significant. This could be attributed to the misclassification of calves with ultrasonography lesions ranging between 1 cm and 3 cm. Specifically, these animals were identified as BRD-positive with TUS1cm, but BRD-negative with TUS3cm. Such variance in classification may have impacted the observed differences in statistically significant PCT concentrations between the two ultrasonography cutoffs.

The second interesting result of this study was that PCT concentration varies significantly between healthy calves and those with clinical pneumonia when two diagnostic tools were used together (WCRSC/TUS1cm). The definition of clinical pneumonia using both WCRSC and TUS has been proposed in recent studies for increasing the accuracy of the two diagnostic methods (Cuevas-Gómez et al. 2020; Denis-Robichaud et al. 2021; Jourquin et al. 2022). Furthermore, it has been highlighted that animals with clinical pneumonia have an active form of BRD, which usually leads to evident hematological alterations (Cuevas-Gómez et al. 2020; Berman et al. 2021). These results support PCT variations found in BRD-positive calves as soon as the mechanism by which active bacterial infection leads to an increase in PCT has been widely demonstrated in human medicine (Schuetz et al. 2018; Schuetz et al. 2019). It has been noted that PCT levels rise due to stimulation by TNF-α and IL-1β, typically involved in bacterial infection (Maruna et al. 2000). While a direct link between PCT and these cytokines was not established in cattle, El-Deeb et al. (2020) evaluated the specific increase of PCT, TNF-α and IL-1β during respiratory disease confirming their implication in BRD.

In our study, the optimal cut-off values identified for PCT was higher than the values reported in feedlot calves with BRD (48.62 pg/ml El-Deeb et al. 2020 and 40 pg/ml Koshiishi et al. 2023). We determinate PCT on plasma, while El-Deeb et al. (2020) and Koshiishi et al. (2023) used serum; additionally, the age of calves differed among these studies (our study: 15–94 d old; El-Deeb et al. 2020: 4–12 months old; Koshiishi et al. 2023: 39–399 d old), as did the breed and sex, where our study included only female dairy calves, while the other two studies included both male and female beef calves (El-Deeb et al. 2020; Koshiishi et al. 2023). Unfortunately, there is a lack of knowledge regarding potential variations of PCT concentration due to the matrix used, sex, age, and breed of the assessed animals, but the influence of these factors on other biomarkers and blood parameters is well-documented (Mohri et al. 2007; Hughes et al. 2014).

Although a statistically significant difference was found, the diagnostic accuracy of PCT in discriminating the presence/absence of lung consolidation is not satisfactory for all diagnostic methods. Since now, only one study evaluated the diagnostic accuracy of PCT during BRD with a Se of 100% and a Sp of 90% (El-Deeb et al. 2020) which are not in line with our findings. The differences in diagnostic accuracy can be explained by the different designs of the two studies. Our study is a cross-sectional study in which all forms and stages of BRD were included, while the study of El-Deeb et al. (2020) was designed as a case-control study. Case-control studies may lead to the selection of much more severe cases with an overestimation of the diagnostic accuracy of the diagnostic test, leading to spectrum bias (Buczinski and Pardon 2020). Additionally, the selection of BRD-positive calves in the

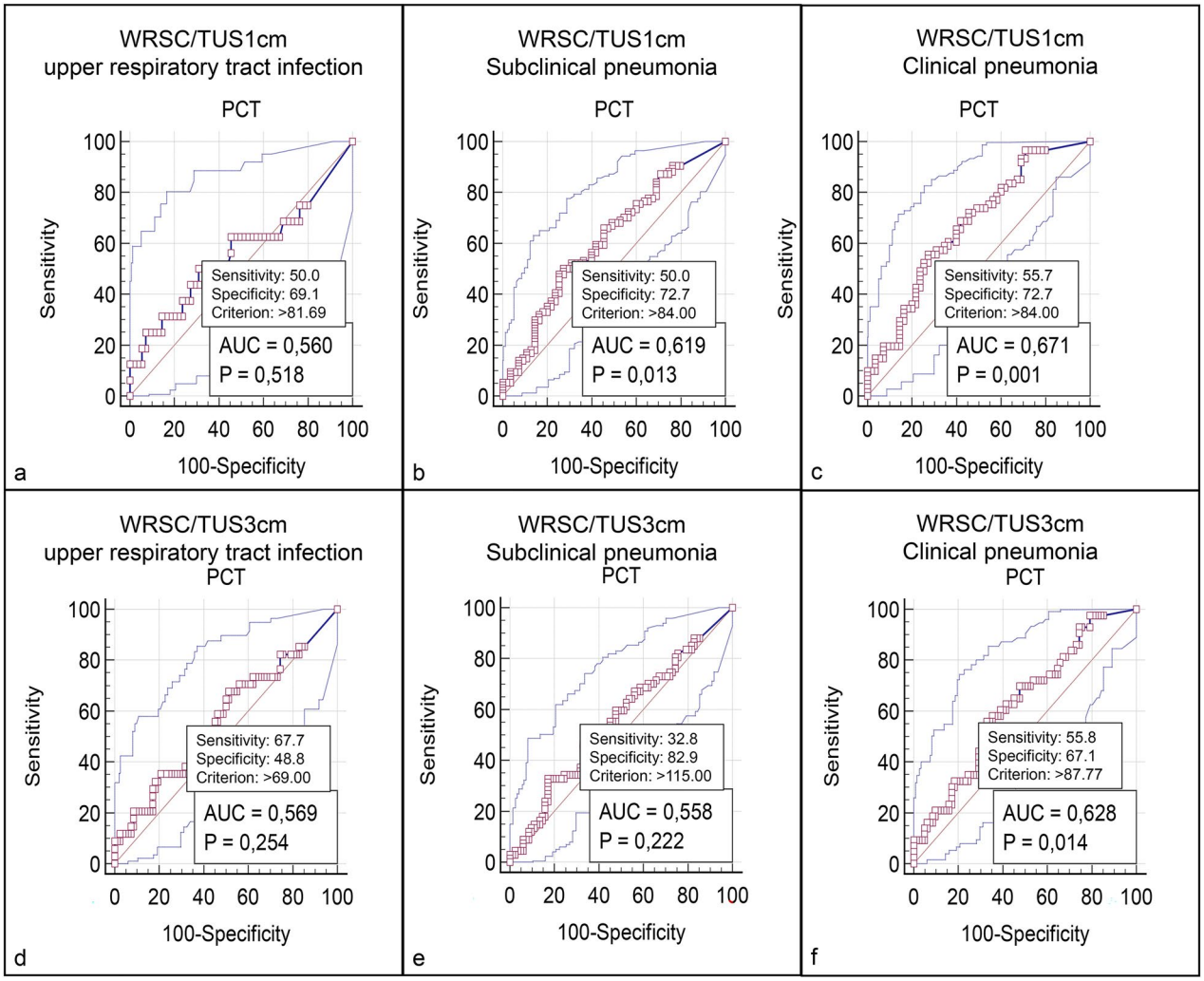

Figure 3. Receiver Operating Characteristic (ROC) curves and Area under the curve (AUC) for the optimal procalcitonin (PCT) cut-off in 226 calves for the detection of bovine respiratory disease (BRD), diagnosed with a combined approach using the Wisconsin respiratory scoring Chart (WRSC) and thoracic ultrasonography (TUS) with lung consolidation thresholds of 1 cm and 3 cm. (a) WRSC/TUS1cm negative: WRSC < 5 without TUS consolidation; WRSC/TUS1cm positive (upper respiratory tract infection): WRSC ≥ 5 without TUS consolidation (b) WRSC/TUS1cm negative: WRSC <5 without TUS consolidation; WRSC/TUS1cm positive (subclinical pneumonia): WRSC < 5 with TUS ≥ 1 cm; (c) WRSC/TUS1cm negative: WRSC < 5 without TUS consolidation; WRSC/TUS1cm positive (clinical pneumonia): WRSC ≥ 5 with TUS ≥ 1 cm; (d) WRSC/TUS3cm negative: WRSC < 5 without TUS consolidation; WRSC/TUS3cm positive (upper respiratory tract infection): WRSC ≥ 5 without TUS consolidation (e)WRSC/TUS3cm negative: WRSC < 5 without TUS consolidation; WRSC/TUS3cm positive (subclinical pneumonia): WRSC < 5 with TUS ≥ 3 cm; (f) WRSC/TUS3cm negative: WRSC < 5 without TUS consolidation; WRSC/TUS3cm positive (clinical pneumonia): WRSC ≥ 5 with TUS ≥ 3 cm.

study of El-Deeb et al. (2020) was based on clinical signs *plus* the simultaneous isolation of at least one bacterial pathogen from nasal swabs. Selection of cases with at least the participation of a bacterial pathogen may have further increased the accuracy of the marker. As previously observed, PCT can be considered a biomarker specific of bacterial infection (Schuetz et al. 2019), however, in the etiopathogenesis of BRD bacteria are not the only pathogen involved. Interestingly, studies in human medicine showed that viral infection suppresses PCT production by inducing inhibitory interferon-γ (Linscheid et al. 2003; Linscheid et al. 2005; Gilbert 2017). This mechanism could partly explain the poor diagnostic accuracy of PCT in our study. Since data on the etiology of BRD were unavailable in this study, some animals may have had viral infection without bacterial involvement, typical of the early stages of the disease (Smith 2014). Moreover, PCT decreases rapidly (3–6 h) after the resolution of bacterial infection (Riedel 2012; Afsar and Sener 2015), while ultrasound-identifiable lung lesions may persist for longer (Fiore et al. 2022). The design of our study does not allow for indications regarding the previous stage of disease of included calves. Finally, the absence of a gold standard diagnostic tool feasible in field conditions is still missing for BRD (Buczinski and Pardon 2020). Thoracic ultrasound has some limitations in the diagnosis of BRD: the inability to identify lesions in the deep lung parenchyma may increases false negatives, while the failure to determine the etiology of consolidation may lead to consider animals that have had lesions for causes other than BRD (for example atelectasis or aspiration pneumonia) as positive, increasing false positives (Buczinski and Pardon 2020).

In diseases due to bacteria, such as sepsis or mastitis, PCT has provided good results in diagnostic accuracy and as a prognostic factor in cattle (Bonelli et al. 2018; El-Deeb et al. 2021; Bonelli et al. 2023). Future perspectives include investigating PCT during BRD cases diagnosed by the simultaneous use of clinical examination, TUS, and etiological isolation in a cohort study. This would allows studying all stages of BRD to understand PCT intriguing behavior better and setting PCT pathogen-specific cut-off.

This study showed some limitations: the already mentioned lack of etiological isolation does not allow for an in-depth study of PCT variations based on the involved pathogen. Furthermore, the study's observational design does not allow for identifying PCT variations in the different stages of the pathology. In general, there is a gap of knowledge about the exact mechanism for which PCT rise and fall during infections in bovine and no gold standard test for its determination in serum or plasma.

Furthermore, in this retrospective study, leftover plasma from previous studies was used and this might have affected the determination of PCT concentrations, as the plasma could have deteriorated over time. To minimize this issue, the plasma was stored at −80 °C, a temperature commonly used for long-term storage of biological samples (Schuetz et al. 2010).

There is still a long way to go for making PCT feasible for field monitoring in veterinary species. One of the main limitations is the availability of only ELISA kits with a minimum requirement of 40 samples for running a single butch as a tool for PCT dosage in bovine. This makes individual testing laborious and costly, along with showing the limitation typically related to the ELISA technique itself; consequently, routine laboratory facilities do not offer this method. To integrate PCT into bovine medicine practice, there is a need to develop routine methodologies that are cost-effective, like those used in human medicine where laboratory responses are rapid (30 min), providing an objective basis for initiating antibiotic therapy or otherwise (Samsudin and Vasikaran 2017). However, understanding the pattern of PCT concentrations in healthy and sick animals is preparatory.

In conclusion, PCT results correlated with ultrasound lesions ≥ 1 cm, confirming the importance of TUS in on-field BRD diagnosis. PCT may represent a helpful biomarker for BRD, but further studies are necessary to better understand its utility in this pathology.

Authors contributions

Conceptualization, F.B., G.S and D.P.; methodology, F.B., A.B. and G.S.; formal analysis, V.M. and L.D.M.; investigation, A.B., V.F. and G.S.; data curation, G.S.; writing—original draft preparation, G.S.; writing—review and editing, F.B., G.S., A. B. and D.P.; visualization, F.B. and M.S.; supervision, F.B. All authors have read and agreed to the published version of the manuscript.

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Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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