Clinical and pulmonary ultrasound evaluations after intranasal, parenteral, or both vaccine administration for bovine respiratory disease (BRD) in dairy calves

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SUMMARY

The bovine respiratory disease (BRD) can significantly reduce the health and welfare of dairy calves. Vaccination is a common practice to minimize the incidence of BRD both intranasal and parenteral. The aim of this study was to evaluate the clinical and lung ultrasound response of calves undergoing intranasal, parenteral, or both vaccination for BRD. Two-hundred one Holstein Friesian calves were enrolled and divided into four group: control group (Group A, n=41, without vaccination); intranasal-vaccination group (Group B, n=46, intranasal vaccination); parenteral-vaccination group (Group C, n=52, subcutaneous vaccination); double-vaccination group (Group D, n=62, intranasal and subcutaneous vaccinations). All animals received a clinical examination and lung ultrasonographic evaluation at 10-15 days of life (day of recruitment: T0), 17-22 (T1), 31-38 (T2), and 45-52 (T3) days of life. The Kruskall-Wallis and the Dunn tests were performed to assess differences between groups and over time, while the Chi-squared test was used to evaluate the differences between proportions. All vaccinated groups showed a lower ultrasonography score over time compared to Group A except for Group B at T3. Groups B and D presented a lower percentage of diseased animals compared to Group A at T1 and T2, while groups C and D were lower at T3. The odds ratio showed a lower risk of BRD in all vaccinated groups at T1 and T2, but only Group D continued to T3. Group D also showed a lower risk compared to Group C at T1, and groups B and C at T2. The respiratory score was greater in Group C except at T3. All vaccinated groups showed similar and lower mortality compared to the control group. Our results suggest that the lung ultrasound was more effective in identifying cases of BRD. Furthermore, the association of intranasal and parenteral vaccinations was more effective in reducing the risk of BRD.

KEY WORDS

Lung ultrasound; Vaccination protocols; BRD; Calves.

INTRODUCTION

The bovine respiratory disease (BRD) is a syndrome involving infectious agents, the host immune system, and environmental factors. The viral pathogens associated with BRD, such as bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI3V), bovine viral diarrhea virus (BVDV), and bovine respiratory syncytial virus (BRSV) can cause BRD and increase the susceptibility to secondary bacterial infections, mainly due to *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida*, and *Histophilus somni* [1,2].

Systemic signs and respiratory symptoms characterize the clinical presentation of BRD. Usually, observation of these clinical signs is used to diagnose BRD in-field [3]. However, clinical observation may fail to correctly identify cases of BRD. Several imaging devices are developing in veterinary medicine as diagnostic and preventive tools for in vivo, non-invasive, and rapid assessment of different structures [4,5]. Lung ultrasound is a tool that can be performed quickly and provides greater sensitivity and specificity in the ante-mortem assessment of BRD. These two parameters are important for early detection of diseased animals and for an accurate assessment of animal health status in order to avoid unnecessary antimicrobial treatment [2,6].

This disease can cause significant economic losses, especially for dairy calves under the age of 3 months [7]. Vaccination is a common practice to minimize the incidence of BRD by reducing its morbidity and mortality in pre-weaned dairy calves [6,7]. Routes of administration of BRD vaccines can be intranasal or parenteral, inducing a mucosal or systemic immunity against BRD pathogens, respectively. However, the colostrum-derived immunity may adversely affect parenteral vaccine-induced responses [7]. For this reason, mucosal immunization is usually used to provide immunity to young calves with ongoing passive immunity. Furthermore, mucosal immunity is more likely to prevent infection rather than reduce disease [8]. Nevertheless, a parenteral vaccination in the presence of maternal an-

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tibodies can provide an active immune response despite the lack of seroconversion. In addition, the neonatal immune system exhibits a weaker response to vaccination than older animals due to a deficiency of cellular components such as phagocytes and lymphocytes [9]. Moreover, the mother's metabolic condition during late gestation can limit the metabolites availability for fetal growth and colostrum production, adversely affecting calf health and increasing perinatal mortality [10,11].
The aim of this study was to evaluate the clinical and lung ultrasound response of calves undergoing intranasal, parenteral, or both vaccination for BRD.

MATERIALS AND METHODS

Ethical statement

Ethics committee approval was not required. No invasive medical procedures were executed to perform the study. The study was performed with the consent of the animals' owner during the routine clinical activity of the Veterinary Teaching Hospital, University of Padua. Animal care and procedures are in accordance with the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments (National law: D.L. 26/2014).

Animals and Experimental design

Two-hundred and one Holstein Friesian calves were recruited between October 2021 and March 2022 from four dairy farms located in Veneto (Italy). All animals came from eutocic calving, and immediately after calving they were dried and moved to individual outdoor elevated calf hutches of 0.9 x 1.8 x 1.8 m. Each animal received 10% of the body weight (BW) of colostrum within 4 h after the calving.
An interventional study was used as the experimental design. Animals were allocated to four groups: control group or Group A (n=41) without vaccination; intranasal-vaccination group or Group B (n=46) that received an intranasal vaccination (Bovalto® Respi Intranasal; Boehringer Ingelheim Animal Health Italia S.p.A., Noventa Padovana, Italy) against modified-live PI3V and BRSV; parenteral-vaccination group or Group C (n=52) that received a subcutaneous vaccination (Bovalto® Respi 3; Boehringer Ingelheim Animal Health Italia S.p.A., Noventa Padovana, Italy) against inactivated PI3V, BRSV, and *Mannhemia haemolytica*; and double-vaccination group or Group D (n=62) that received the intranasal and subcutaneous vaccinations. No animals included in the study received antibiotic treatment for respiratory diseases during the trial.
All animals received a clinical examination and lung ultrasound evaluation at 10±2 days for groups A, B, and D, and Group C was evaluated at day 15±2 (T0; day of recruitment). Immediately after the clinical and ultrasonographic evaluations, groups B and D received the intranasal vaccination, while Group C received the first parenteral vaccination. After 7 days (T1; 17±2 days for groups A, B, and D and 22±2 days for Group C), animals received a clinical examination and lung ultrasonographic evaluation after which the Group D received the first parenteral vaccination. The third clinical examination and lung ultrasonographic evaluation was performed 2 weeks after T1 for groups A, B, and C, and after 3 weeks for Group D (T2, 31±2 days for Group A and B, 36±2 days for Group C, and 38±2 days for Group D). At this time point, animals in groups C and D received parenteral vaccination booster. The fourth and last clinical examination and lung ultrasound evaluation was performed 2 weeks after T2 (T3; 45±2 days for Group A and B, 50±2 days for Group C, and 52±2 days for Group D; **Figure 1**).
At each time point, the animals identified as diseased according to the lung ultrasound score received a deep nasal swab, which was subsequently subjected to bacteriological exami-

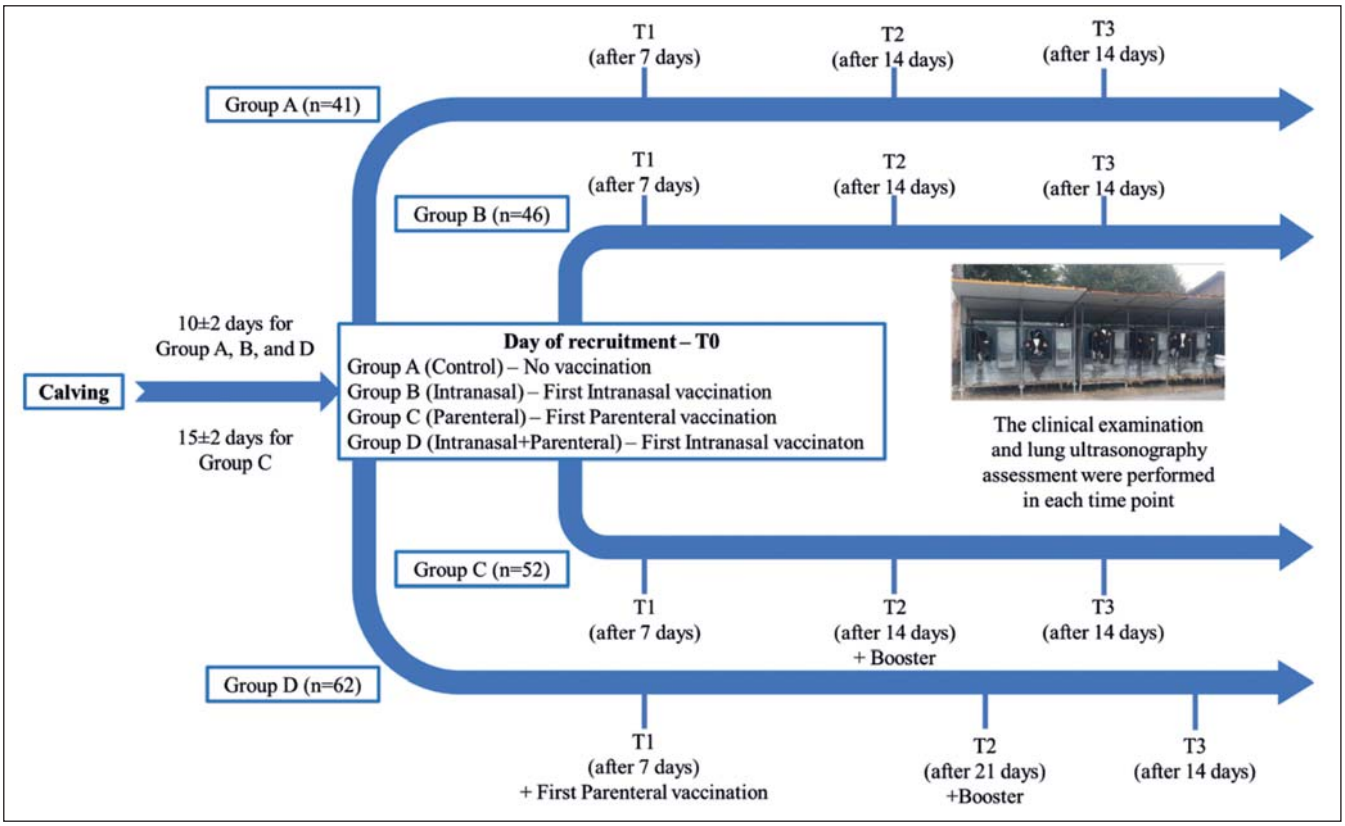

Figure 1 - Flowchart of the experimental design.

nation and assessment of the minimum inhibitory concentration (MIC) at the laboratory of Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe; Legnaro, Padua, Italy).

Clinical examination

The clinical examination was performed by veterinarians from the University of Padua Veterinary Teaching Hospital and included the general physical and special examinations of the respiratory system. Based on respiratory and systemic signs (cough, nasal discharge, ocular discharge and ear drop, and rectal temperature) the respiratory score was established as described by McGuirk & Peek (2014)[12]. The respiratory score considers animals as sick when the sum of the scores of the four parameters is greater or equal to 5, or at least two parameters showed a score of at least 2. Due to the different cut-offs for identifying sick animals, the percentage of sick animals was calculated based on respiratory score (SRS%).

Lung ultrasound evaluation

The lung ultrasound evaluation was performed with a portable ultrasound scanner (Draminski Blue; Draminski® S.A., Olsztyn, Polonia) equipped with a multi-frequency linear probe (Lineare L40/10 MHz, 6.0-15.0 MHz; Draminski® S.A., Olsztyn, Poland) after the use of ethyl alcohol (90%) as transducing agent. Six lung areas were investigated: between 10th and 7th intercostal space (ICS) for the caudal lung; between 6th and 5th ICS for the middle lung; and between 4th and 1st ICS for the cranial lung of both sides (**Figure 2**). All scans were performed with constant ultrasound settings frequency of 6.0 MHz, 15 cm depth acoustics window, 100% gray scale gain, and time-gain compensation was in a neutral position. Images were saved in a digital imaging and communications in medicine (DICOM) format.

The ultrasonography score on a 6-point scale [13] was established during lungs' ultrasonography. Based on this, a score of 0 corresponded to a normal aerated lung; 1 indicated diffuse comet-tail artifacts without consolidation; 2 indicated lobular consolidation; 3 indicated lobar consolidation; 4 lobar consolidation of two lobes; and 5 signified lobar consolidation of three or more lobes. The ultrasonography score greater or equal to 3 was consistent with bacterial bronchopneumonia. As well as for the respiratory score, the percentage of sick animals was also calculated based on ultrasonography score (SUS%).

In addition, lungs' lesions were converted in a numeric scale as follows: absence of lesions = 0; comet tail = 1; hepatization = 2; comet tail and hepatization = 3; fluid alveolograms = 4; comet tail and fluid alveolograms = 5; hepatization and fluid alveolograms = 6; comet tail, hepatization and fluid alveolograms = 7 (**Figure 3**). The score of the six investigated areas was summed to obtain a global lesion score (GL) as described by Fiore et al. (2022)[2].

Statistical analysis

Statistical analysis was performed using the R software ver. 4.2.0 implemented with "Rcmdr" package (R Core Team, Vienna, Austria). The data distribution was assessed by Shapiro-Wilk normality test before all statistical analysis. Considering the non-normal distribution, Kruskall-Wallis and the Dunn tests were performed to evaluate the differences between groups and over time, and to perform the multiple comparisons. A receiver operating characteristic (ROC) curve was performed to establish the GLS threshold value with the discriminant of ultrasonography score ≥ 3. The area under the curve (AUC) shows the diagnostic power of the test: AUC of 0.9 to 1.0 = excellent, 0.8 to 0.9 = good, 0.7 to 0.8 = fair, 0.6 to 0.7 = poor, and 0.5 to 0.6 = fail [14]. The Chi-square test in MedCalc software for Windows

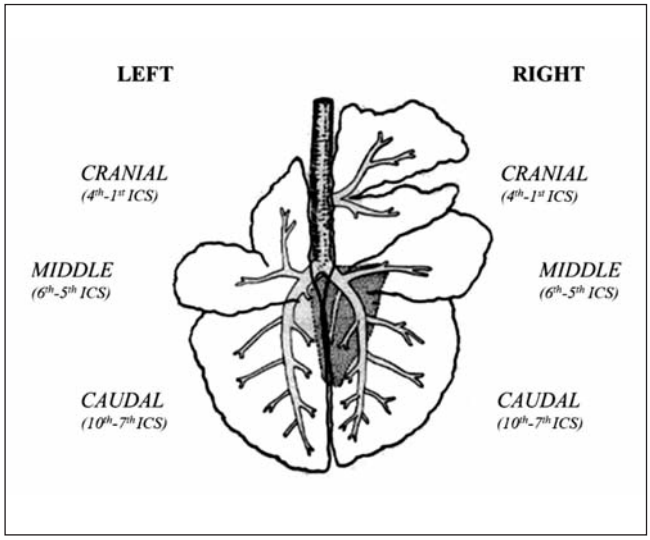

Figure 2 - Correspondence between the six areas investigated (4th-1st intercostal space (ICS): Cranial; 6th-5th ICS: Middle; 10th-7th ICS: Caudal) and the lung lobes. The lesions found in each of the six areas were summed to calculate the global lesion score (GL).

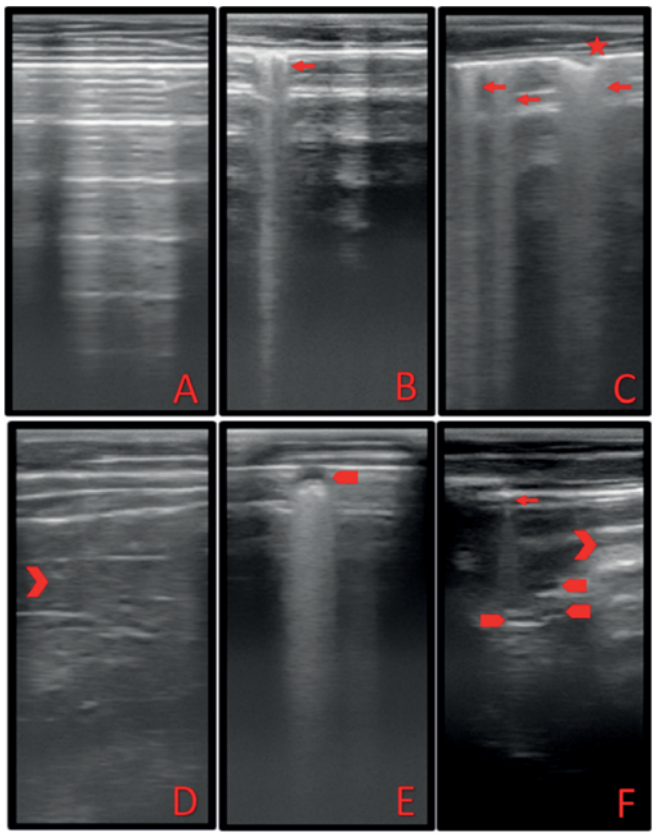

Figure 3 - Lung ultrasound of dairy calves: (A) healthy lung with reverberation artifacts; (B) comet tail (arrow); (C) comet tails (arrows) associated with pleural lesion (star); (D) lung hepatization (chevron arrow); (E) fluid alveologram (pentagon arrow); and (F) comet tail (arrow) associated with lung hepatization (chevron arrow) and fluid alveolograms (pentagon arrows). The lungs' lesions were converted in a numeric scale (lesion score). The score of the six investigated areas was summed to obtain a global lesion score (GL).

ver. 19.4 (MedCalc Software, Ostend, Belgium) was used to compare proportions. The same software was used to calculate the odds ratio to measure the association between vaccination and diseased animals according to ultrasonography score. In general, the accepted *p-value* was ≤ 0.05.

RESULTS

The diseased animals in Group A were tested positive for *Mannhemia haemolytica* throughout the study, while the sick animals in Group B were tested positive for *Mannhemia haemolytica* and *Pasteurella multocida* at the end of the study period. In Group C, animals were affected by *Pasteurella multocida* starting from about mid-study period, whereas in Group D two cases of Coronavirus were identified at the end of the study.

The ultrasonography and respiratory scores, and the GL showed differences between groups and over time, whereas no differences were found in rectal temperature (**Table 1**). Regarding the ultrasonography score, the groups did not present differences at the day of recruitment (T0). Groups B, C, and D showed lower values compared to Group A at T1 and T2, while only the values of groups C and D were lower compared to Group A at T3. The Group A worsened over time, while Group B improved at T1, and groups C and D at T2. Group C showed a higher level of respiratory score, except at T3 where the Group B showed the greater level. Additionally, only the Group D showed a difference over time with an increase of respiratory score at T3 compared to T0. Regarding the GL, the Group C showed a higher score at T0, followed by Group A, groups B and D respectively. Furthermore, groups B, C, and D showed a lower GL score than Group A at T1, Group B and D showed a low GL up to T2 and T3 respectively. Similar to the ultrasonography score, the GL worsened over time in Group A, while Group B improved at T1, Group C at T1 and T2, and Group D at T2.

The result of ROC analysis (**Figure 4**) indicated a GL threshold value of 10.50 to identify diseased animals with an AUC of 0.95 (95% Confidence Interval: 0.93-0.97; Sensitivity: 0.87; Specificity: 0.88).

The data about SRS%, SUS%, and the percentage of dead animals during the study were shown in **Table 2**. The SRS% showed a difference between the groups only at T0, with a higher number of diseased animals in groups A and C, followed by Group B, and after Group D. Furthermore, only Group D differed over time with an increase of diseased animals at T3. In contrast, the SUS% did not differ at T0. Groups B, and D showed the lower values at T1 and T2, while groups C and D showed a decrease at T3. The percentage of diseased animals in Group A increased over time, whereas in groups B and D there was only an increase at T3. Furthermore, more of the 50% of animals were sick in Group A between T1 and T3, and in Group B only at T3 whereas the percentage of diseased animals were always below 50% in groups C and D. Concerning the percentage animals that died during the study, it was higher in Group A compared to all other groups.

The odds ratio showed no differences at T0 (**Table 3**). On the contrary, all groups in which the animals were vaccinated reported a lower likelihood of sick animals according to the ultrasonography score at T1 and T2. Furthermore, Group D reduced this probability compared with Group C at T1 and with B and C at T2. However, only Group D was able to reduce the probability compared to groups A, B and C at T3.

DISCUSSION

BRD can significantly reduce the health and well-being of dairy calves and increase their mortality. Both intranasal and parenteral vaccinations are common practice to reduce the incidence of BRD. Mucosal immunity is usually employed to provide immunity to young calves with ongoing passive immunity. However, a parenteral vaccination can also provide an active immune response with variable clinical protection in presence of maternal antibodies [7,8]. The aim of this study was to evaluate the clinical and lung ultrasonography responses of calves receiving intranasal, parenteral, or both vaccination for BRD. As previously described, the respiratory score is based on the assessment of the presence and severity of nasal discharge, ocular discharge and ear position, cough, and rectal temperature [12]. However, the first three parameters are often considered subjective methods because they depend on the operator. Furthermore, animals may not exhibit clinical signs indicative of BRD due to discomfort caused by the presence of operators examining them or a subclinical BRD state. Rectal temperature, differently, is considered objective. Despite, body temperature rises due to other factors, such as stress, may occur in healthy animals, whereas sick animals may present normal temperature values. This results indicates a low sensitivity and specificity of clinical signs as the single method for diagnosing BRD, both being close to 60% [15,16]. The respiratory score showed no differences over time in the control, intranasal, and parenteral-vaccination groups in this study. A difference was shown at T3 compared to T0 in the double-vaccination group (Group D). This difference is also reflected in the SRS%, as no diseased animals were identified in Group D during the study until T3.

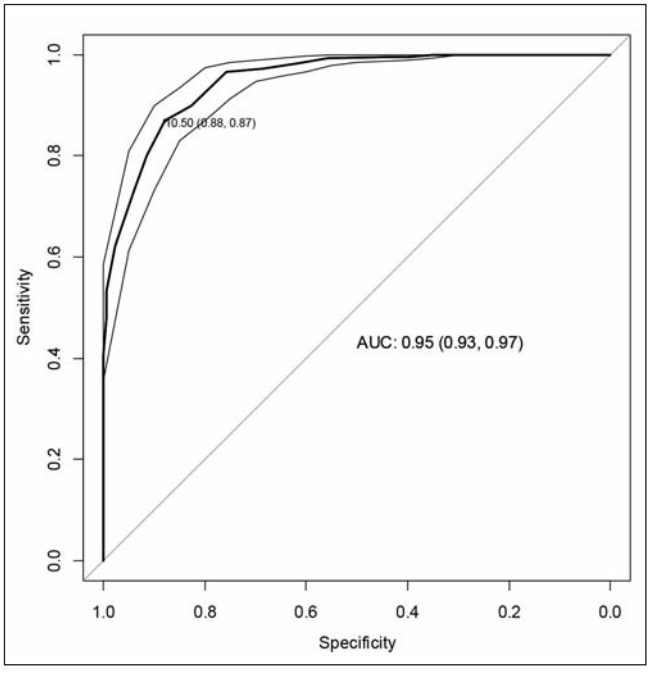

Figure 4 - Receiver operating characteristic (ROC) curve of the global lesion score (GL) using as discriminant an ultrasonography score ≥ 3. The area under the curve (AUC) with its 95% of confidence interval showed the diagnostic power of the test. The threshold value was associated with the specificity and sensitivity.

Table 1 - Ultrasonography and respiratory scores, rectal temperature, and global lesion score of groups A (without vaccination), B (intranasal vaccination), C (parenteral vaccination), and D (intranasal and parenteral vaccination) over time expressed as means ± SEM.

Ultrasonography score	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	SEM	*p-values*
T0 (10-15 days)	2.22 ^{y}	1.97 ^{y}	2.42 ^{x}	1.97 ^{x}	0.22	NS[2]
T1 (17-22 days)	3.97 ^{a,x}	1.17 ^{c,z}	2.40 ^{b,xy}	1.82 ^{b,xy}	0.23	< 0.01
T2 (31-38 days)	3.60 ^{a,xy}	1.41 ^{b,yz}	1.91 ^{b,y}	1.52 ^{b,y}	0.26	< 0.01
T3 (45-52 days)	3.64 ^{a,xy}	2.27 ^{ab,x}	2.11 ^{b,xy}	1.81 ^{b,xy}	0.27	< 0.05
SEM	0.26	0.22	0.26	0.24	/	/
p-values	< 0.05	< 0.01	< 0.05	< 0.05	/	/
Respiratory score	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	SEM	*p-values*
T0 (10-15 days)	0.96 ^{b}	1.77 ^{ab}	2.26 ^{a}	1.41 ^{b,y}	0.23	0.01
T1 (17-22 days)	1.16 ^{b}	1.29 ^{ab}	1.89 ^{a}	1.81^{a,xy}	0.25	< 0.01
T2 (31-38 days)	0.97 ^{b}	1.63 ^{ab}	1.92 ^{a}	1.79 ^{ab,xy}	0.27	< 0.01
T3 (45-52 days)	1.20 ^{b}	2.14 ^{a}	1.62 ^{b}	2.22 ^{a,x}	0.28	< 0.05
SEM	0.27	0.23	0.27	0.26	/	/
p-values	NS[2]	NS[2]	NS[2]	< 0.05	/	/
Rectal temperature	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	SEM	*p-values*
T0 (10-15 days)	38.54	38.72	38.84	38.65	0.09	NS[2]
T1 (17-22 days)	38.63	38.67	38.83	38.94	0.10	NS[2]
T2 (31-38 days)	38.50	38.79	38.79	38.74	0.11	NS[2]
T3 (45-52 days)	38.38	38.67	38.72	38.89	0.12	NS[2]
SEM	0.11	0.09	0.11	0.10	/	/
p-values	NS[2]	NS[2]	NS[2]	NS[2]	/	/
GL[1]	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	SEM	*p-values*
T0 (10-15 days)	8.60 ^{b,y}	6.13 ^{c,y}	12.30 ^{a,x}	6.50 ^{c,y}	1.22	< 0.01
T1 (17-22 days)	13.99 ^{a,x}	5.04 ^{c,z}	10.96 ^{b,y}	5.94 ^{c,yz}	1.39	< 0.05
T2 (31-38 days)	11.71 ^{a,x}	6.27 ^{b,y}	10.11 ^{a,y}	5.33 ^{b,z}	1.53	< 0.01
T3 (45-52 days)	11.96 ^{a,x}	10.10 ^{ab,x}	12.06 ^{a,x}	8.06 ^{c,x}	1.61	< 0.05
SEM	1.54	1.31	1.52	1.38	/	/
p-values	< 0.05	< 0.01	< 0.05	< 0.05	/	/

[1] Global lesion score; [2] Not significant; $^{a-c}$ Mean values in the same row which differ significantly; $^{x-z}$ Mean values in the same column which differ significantly.

The increase in the respiratory score and percentage of sick animals based on clinical signs may be related to the introduction of Coronavirus within the group and that those animals were not immunized. The differences among the groups at each time point showed higher values in the group that received parenteral vaccination, except at T3. However, Group C completed the parenteral vaccination with the booster at T2. Therefore, mucosal immunity against BRD pathogens, which is useful in

Table 2 - The SRS%, SUS%, and the percentage of diseased animals of groups A (without vaccination), B (intranasal vaccination), C (parenteral vaccination), and D (intranasal and parenteral vaccination) over time and during the study.

SRS% [1]	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	*p-values*
T0 (10-15 days)	10.00 ^{a}	6.52 ^{b}	11.54 ^{a}	0.00 ^{c,y}	< 0.05
T1 (17-22 days)	5.03	2.33	4.17	0.00 ^{y}	NS[3]
T2 (31-38 days)	4.50	0.00	4.16	0.00 ^{y}	NS[3]
T3 (45-52 days)	5.21	4.00	4.56	8.52 ^{x}	NS[3]
p-values	NS[3]	NS[3]	NS[3]	< 0.05	/
SUS% [2]	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	*p-values*
T0 (10-15 days)	47.06 ^{y}	34.78 ^{y}	46.15	30.14 ^{x}	NS[3]
T1 (17-22 days)	56.00 ^{a,xy}	30.23 ^{b,y}	45.83 ^{a}	15.86 ^{c,y}	< 0.01
T2 (31-38 days)	55.56 ^{a,xy}	35.48 ^{b,y}	41.67 ^{a}	18.52 ^{c,y}	< 0.01
T3 (45-52 days)	68.75 ^{a,x}	68.00 ^{a,x}	45.46 ^{b}	28.15 ^{c,x}	< 0.01
p-values	< 0.05	< 0.01	NS[3]	< 0.05	/
Dead animals %	Group A (n=41)	Group B (n=46)	Group C (n=52)	Group D (n=62)	*p-values*
T0 - T3	32.35 ^{a}	6.52 ^{b}	5.55 ^{b}	6.89 ^{b}	< 0.01

[1] Percentage of diseased animals based on respiratory score; [2] Percentage of diseased animals based on ultrasonography score; [3] Not significant; $^{a-c}$ Mean values in the same row which differ significantly; $^{x-z}$ Mean values in the same column which differ significantly.

Table 3 - Odds ratio between an exposed group *versus* an intercept group at each time point. An odds ratio =1 means exposure does not affect odds of outcome (diseased animals according to ultrasonography score); odds ratio >1 means exposure associated with higher odds of outcome; odds ratio <1 means exposure associated with lower odds of outcome.

Time	Exposed Group	Intercept Group	Odds Ratio	*p-values*	Time	Exposed Group	Intercept Group	Odds Ratio	*p-values*
T0	B	A	0.60	NS[1]	T1	B	A	0.34	< 0.01
	C	A	0.96	NS[1]		C	A	0.67	< 0.05
	D	A	0.36	NS[1]		D	A	0.17	< 0.01
	C	B	1.61	NS[1]		C	B	1.95	NS[1]
	D	B	0.60	NS[1]		D	B	0.50	NS[1]
	D	C	0.37	NS[1]		D	C	0.26	< 0.01
T2	B	A	0.44	< 0.05	T3	B	A	0.97	NS[1]
	C	A	0.57	< 0.05		C	A	0.38	NS[1]
	D	A	0.18	< 0.01		D	A	0.42	< 0.05
	C	B	1.30	NS[1]		C	B	0.39	NS[1]
	D	B	0.41	< 0.05		D	B	0.44	< 0.05
	D	C	0.32	< 0.01		D	C	0.41	< 0.05

[1] Not significant.

preventing infection, was not stimulated by vaccination and parenteral administration cannot prevent BRD after a single dose [8,9]. However, these results could also simply be due to a lack of effectiveness of the respiratory score to identify diseased animals [6].

Lung ultrasound shows a greater sensitivity and specificity, both close to 90%, for the diagnosis of BRD compared to clinical observations, providing a more accurate ante-mortem assessment of lung health. Furthermore, lung consolidation correlates with long-term effects on survival and reproduction of dairy calves [2,3,6]. The ultrasonography score did not differ among groups at the beginning of the study (T0: 10-15 days of life). However, animals in all groups already showed lung lesions as consolidation spots. These lesions are generally associated with infection by bacteria or viruses, or with chronic lesions [2] although chronic lesions in 10-15 day-old animals are uncommon. In fact, between 30 and 47% of the animals were sick according to the ultrasonography score (SUS%) in this time point. After one week (T1: 17-22 days of life), the ultrasonography score of all vaccinated groups was lower than the control group, which instead showed an increase indicating a worsening lung condition [17]. However, the percentage of sick animals was only lower in the intranasal and double-vaccination groups compared to control group. Furthermore, the odds ratio showed a lower risk of BRD in all vaccinated groups compared to control group, and a further lower risk in the double-vaccination group compared with the parenteral-vaccination group. These results suggest that both intranasal and parenteral vaccination began to provide immunity against BRD at T1. However, the greatest protection seemed to be related to mucosal immunity developed in the intranasal and double-vaccination groups[7,18]. The results of ultrasonography score, SUS%, and odds ratio two or three weeks after T1 (T2: 31-38 days of life) were similar to the previous time point. The only difference was that the odds ratio indicated a lower risk of BRD in the double-vaccination group than in all other groups. This result could be due to the development of both mucosal and parenteral-vaccination immunity, which allows a broader protection of the animals. This finding agrees with the hypothesis of Windeyer et al. (2012)[9] that multiple vaccinations before 30 days may reduce the incidence of BRD. The ultrasonography score at T3 (45-52 days of life) was lower only in the parenteral and double-vaccination groups as was the SUS%. The mucosal immunity developed in intranasal and double-vaccination groups should be protective up to 4 months after vaccination [18]. However, the worsening of the pulmonary condition in the intranasal-vaccination group could be due to the entry of *Mannhemia haemolytica* and *Pasteurella multocida*, major pathogens of BRD at 6-8 weeks of age [19], into the group and for which the animals were not immunized. According to the odds ratio, the double-vaccination group appeared to be the only group with a lower risk of BRD than the other groups in this time point. The better status of the double-vaccination group compared to the parenteral-vaccination group could be related to the wider immunity (both mucosal and parenteral-vaccination) or to entry of *Pasteurella multocida* into the Group C. Despite the entry of new pathogens into the vaccinated groups, all vaccinated animals showed similar and lower mortality than the control group in this study suggesting the importance of vaccination in limiting mortality due to BRD. The GL is a recently proposed score to discriminate between different types of lung consolidation and to better understand lung health status [2]. Based on this score, the groups showed differences starting from T0, with a worse lung health status in the parenteral-vaccination group, followed by the control group, and finally the intranasal and double-vaccination groups. According to the ROC analysis performed using the ultrasonography score accepted as a reference for animal classification, the GL threshold value for discriminating sick animals from healthy ones is 10.50. This result indicated that the parenteral-vaccination group was already sick at the beginning of the study. Regarding the differences in GL over time, the control group showed a significant worsening at T1 that continued until T3, identifying it as diseased. Both intranasal and double-vaccination groups presented a similar trend at the ultrasonography score. However, these groups remained healthy according to GL even at T3. The parenteral-vaccination group showed an improvement in GL from T1, in contrast to the ultrasonography score. In contrast, evaluating the differences among groups at each time point, the double-vaccination group showed one of lowest values at T1 and T3, differing from Group C in contrast to the ultrasonography score. These results indicating that the GL should be further investigated in order to improve the identification of lung health status.

CONCLUSION

In conclusion, the results of the present study showed that the use of vaccination protocol improved the pulmonary condition and thus the health status of the animals reducing the mortality due to BRD. In addition, the combination of intranasal vaccination followed by parenteral vaccination resulted in greater protective immunity with a reduction in the incidence of BRD. However, the epidemiological surveillance and the level of biosecurity were other key factors in choosing the most optimal vaccination protocol and preventing new pathogens from entering the herd.

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Author Contributors

Conceptualization, A.L., E.F., and E.M.; Methodology, A.L., E.F., and E.M.; Software, A.L.; Formal analysis, A.L., and E.S.; Investigation, A.L., E.F., G.Ca., and G.Ce.; Resources, E.F., E.S., N.M., and E.M.; Data curation, A.L.; Writing-original draft preparation, A.L., E.F., and E.M.; Writing-review and editing, G.Ca, E.S., G.Ce., C.T., and E.M.; Visualization, A.L.; Supervision, E.F.; Project administration, E.F.; Funding acquisition, E.F. All authors have read and agreed to the published version of the manuscript.

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Ethical approval

No approval from Ethics Committee was required. No invasive medical procedures were executed to perform the study. The study was performed with the consent of the animals' owner during the routine clinical activity of the Veterinary Teaching Hospital, University of Padua. Animal care and procedures are in accordance with the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments (National law: D.L. 26/2014).

Data Availability Statement

The data will be available by sending an email to the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Grissett GP, White BJ, Larson RL. Structured Literature Review of Responses of Cattle to Viral and Bacterial Pathogens Causing Bovine Respiratory Disease Complex. *J Vet Intern Med*. 2015;29(3):770-780. doi:10.1111/jvim.12597
2. Fiore E, Lisuzzo A, Beltrame A, et al. Lung Ultrasonography and Clinical Follow-Up Evaluations in Fattening Bulls Affected by Bovine Respiratory Disease (BRD) during the Restocking Period and after Tulathromycin and Ketoprofen Treatment. *Animals*. 2022;1:1-14.
3. Fiore E, Lisuzzo A, Beltrame A, Contiero B, Schiavon E, Mazzotta E. Clinical follow-up in fattening bulls affected by Bovine Respiratory Disease (BRD) after pharmacological treatment with Tulathromycin and Ketoprofen : clinical score and ultrasonographic evaluations. *Large Anim Rev*. 2021;27(5):243-249.
4. Gianesella M, Arfuso F, Fiore E, et al. Infrared thermography as a rapid and non-invasive diagnostic tool to detect inflammatory foot diseases in dairy cows. *Pol J Vet Sci*. 2018;21(2):299-305. doi:10.24425/122597
5. Mazzotta E, Lisuzzo A, Tognato E, et al. Evaluation of body surface temperature variations in dogs affected by spinal cord injuries during physiotherapy exercise in a water treadmill. *J Therm Biol*. 2022;106(July 2021):103247. doi:10.1016/j.jtherbio.2022.103247
6. Ollivett TL, Leslie KE, Duffield TF, et al. Field trial to evaluate the effect of an intranasal respiratory vaccine protocol on calf health, ultrasonographic lung consolidation, and growth in Holstein dairy calves. *J Dairy Sci*. 2018;101(9):8159-8168. doi:10.3168/jds.2017-14271
7. Chamorro MF, Palomares RA. Bovine Respiratory Disease Vaccination Against Viral Pathogens: Modified-Live Versus Inactivated Antigen Vaccines, Intranasal Versus Parenteral, What Is the Evidence? *Vet Clin North Am - Food Anim Pract*. 2020;36(2):461-472. doi:10.1016/j.cvfa.2020.03.006
8. Ellis J, Gow S, West K, et al. Response of calves to challenge exposure with virulent bovine respiratory syncytial virus following intranasal administration of vaccines formulated for parenteral administratio. *J Am Vet Med Assoc*. 2007;230(2):233-243.
9. Windeyer MC, Leslie KE, Godden SM, Hodgins DC, Lissemore KD, LeBlanc SJ. The effects of viral vaccination of dairy heifer calves on the incidence of respiratory disease, mortality, and growth. *J Dairy Sci*. 2012;95(11):6731-6739. doi:10.3168/jds.2012-5828
10. Fiore E, Piccione G, Gianesella M, et al. Serum thyroid hormone evaluation during transition periods in dairy cows. *Arch Anim Breed*. 2015;58(2):403-406. doi:10.5194/aab-58-403-2015
11. Fiore E, Lisuzzo A, Tessari R, et al. Milk Fatty Acids Composition Changes According to -Hydroxybutyrate Concentrations in Ewes during Early Lactation. *Animals*. 2021;11(5):1371. doi:https://doi.org/10.3390/ani11051371
12. McGuirk SM, Peek SF. Timely diagnosis of dairy calf respiratory disease using a standardized scoring system. *Anim Heal Res Rev*. 2014;15(2):145-147. doi:10.1017/S1466252314000267
13. Ollivett TL, Buczinski S. On-Farm Use of Ultrasonography for Bovine Respiratory Disease. *Vet Clin North Am - Food Anim Pract*. 2016;32(1):19-35. doi:10.1016/j.cvfa.2015.09.001
14. Xia J, Broadhurst DI, Wilson M, Wishart DS. Translational biomarker discovery in clinical metabolomics: An introductory tutorial. *Metabolomics*. 2013;9(2):280-299. doi:10.1007/s11306-012-0482-9
15. Abutarbush SM, Pollock CM, Wildman BK, et al. Evaluation of the diagnostic and prognostic utility of ultrasonography at first diagnosis of presumptive bovine respiratory disease. *Can J Vet Res*. 2012;76(1):23-32.
16. Timsit E, Dendukuri N, Schiller I, Buczinski S. Diagnostic accuracy of clinical illness for bovine respiratory disease (BRD) diagnosis in beef cattle placed in feedlots: A systematic literature review and hierarchical Bayesian latent-class meta-analysis. *Prev Vet Med*. 2016;135:67-73. doi:10.1016/j.prevetmed.2016.11.006
17. Hussein HA, Binici C, Staufenbiel R. Comparative evaluation of ultrasonography with clinical respiratory score in diagnosis and prognosis of respiratory diseases in weaned dairy buffalo and cattle calves. *J Anim Sci Technol*. 2018;60(29):1-11.
18. Ellis JA, Gow SP, Mahan S, Leyh R. With Bovine Respiratory Syncytial Virus of Young Passively Immune Calves. *J Am Vet Med Assoc*. 2013;243(11):1602-1608.
19. Shewen PE, Carrasco-Medina L, McBey BA, Hodgins DC. Challenges in mucosal vaccination of cattle. *Vet Immunol Immunopathol*. 2009;128(1-3):192-198. doi:10.1016/j.vetimm.2008.10.297.