Veterinary Microbiology 280 (2023) 109701

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Cluster analysis of bovine respiratory disease (BRD)-associated pathogens shows the existence of two epidemiological patterns in BRD outbreaks

Johan Manuel Calderón Bernal [a], Ana Fernández [b], José Luis Arnal [b], Cristina Baselga [b], Alfredo Benito Zuñiga [b], José Francisco Fernández-Garyzábal [a,c,*], Ana Isabel Vela Alonso [a,c], Dolores Cid [a]

[a] Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain
[b] Exopol. Veterinary Diagnostic and Autogenous Vaccine Laboratory, Polígono Río Gállego, D/8., 50840 San Mateo de Gállego, Zaragoza, Spain
[c] Centro de Vigilancia Sanitaria Veterinaria (VISAVET), Universidad Complutense, Madrid, Spain

ARTICLE INFO

Keywords:
Bovine respiratory disease
Hierarchical cluster analysis
Calves
Virus
Bacteria

ABSTRACT

A hierarchical cluster analysis was used to classify outbreaks of bovine respiratory disease (BRD; $n = 156$) in natural groups according to the detection of nine pathogens (parainfluenza 3 virus (PI-3), bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCV), bovine viral diarrhea virus (BVDV), and bovine herpesvirus 1 (BHV-1), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis*. Pathogens were detected by individual q-PCRs. Two clusters were identified. Cluster 1 was characterized by a relatively high frequency (40–72%) of four BRD-associated viruses, supporting their primary involvement in BRD. Cluster 2 was characterized by frequencies of PI-3, BRSV, or BVDV below 10% each. *P. multocida* and *M. haemolytica* were detected with high frequencies in both clusters ($P > 0.05$), while *M. bovis* and *H. somni* showed a significantly higher frequency in cluster 1and 2, respectively. Outbreaks in cluster 1 were associated with preweaning calves younger than 5 months (OR 2.2; 95% CI 1.1–4.5) and with cold months, whereas cluster 2 was associated with fattening calves older than 5 months after arrival to feedlots and without any seasonality. Thus, in addition to the classic epidemiological BRD pattern characterized by the primary involvement of viruses occurring preferably during winter and affecting young calves, there is a second pattern in which viruses would be less relevant, affecting mainly calves older than 5 months at any time of the year. This study allows a better understanding of the BRD epidemiology, which can be useful when implementing management and prophylaxis measures for a better control of this disease.

1. Introduction

Bovine respiratory disease (BRD) continues to be a major cause of economic loss, hampered animal welfare, and intensive antimicrobial use (AMU) in cattle worldwide (White and Larson, 2020; Lowie et al., 2021). BRD is a multifactorial infectious disease caused by the complex interaction between different viral and bacterial pathogens, the immune status of the host, and environmental and management factors, mainly stressing factors suppressing the defenses of the host (Pardon et al., 2020; Pratelli et al., 2020; Smith et al., 2020; Lowie et al., 2021). Pathogens involved in BRD etiology are viruses and bacteria. The main viral pathogens include parainfluenza 3 virus (PI-3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), and bovine herpesvirus 1 (BHV-1) (Fulton, 2020; Pardon and Buczinski, 2020). In addition, bovine coronavirus (BCV) is increasingly reported in association with BRD (ONeil et al., 2012; Pardon et al., 2020; Fanelli et al., 2021; Pratelli et al., 2021; Studer et al., 2021) although its role in the etiology of BRD is still under debate (Ellis, 2019; Fulton, 2020; Pardon et al., 2020). The most relevant bacteria associated with BRD infections of the respiratory tract are *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni,* and *Mycoplasma bovis* (Calcutt et al., 2018; Perez-Casal, 2020; Shirbroun, 2020; Snyder and Credille, 2020). *M. haemolytica* and *P. multocida* include different serotypes with important differences in their clinical and epidemiological significance (Snyder and Credille, 2020). Therefore, when studying BRD outbreaks it is essential to determine the serotypes involved in each of these pathogens. The bacteria associated with BRD are constituent of the upper respiratory microbiota of healthy cattle (McMullen et al., 2019) but they

* Corresponding author at: Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad Complutense, Madrid, Spain.
E-mail address: garayzab@ucm.es (J.F. Fernández-Garyzábal).

https://doi.org/10.1016/j.vetmic.2023.109701
Received 17 June 2022; Received in revised form 20 December 2022; Accepted 15 February 2023
Available online 17 February 2023
0378-1135/© 2023 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

may revert to pathogenicity when viral infections and stressing factors such as weaning, transportation, or commingling impair the systemic and the local host immune system (Zeineldin et al., 2017), increasing the risk that they colonize the epithelial surfaces of the upper respiratory tract, descent to the lungs, and finally result in bronchopneumonia (Timsit et al., 2016; McMullen et al., 2019).

Etiological BRD agents commonly act in synergy in coinfections with different viral and bacterial pathogens usually present in a single outbreak (O'Neill et al., 2014; Pardon et al., 2020; Fanelli et al., 2021), which contribute toward enhancing the severity of the disease. Due to the multi-etiological nature of BRD, the laboratorial diagnosis of BRD outbreaks is commonly based on the detection of the main pathogens involved, for which different quantitative polymerase chain reaction (q-PCR) assays have been used with success (O'Neill et al., 2014; Pardon et al., 2020; Zhang et al., 2022). Hierarchical cluster analysis is a standard method used in multivariate data analyses based on building a tree structure showing hierarchical relations between individuals or groups of individuals in order to detect associations and patterns in the observations under study (Michelacci et al., 2022). This methodology enables the construction of natural groups (clusters) of outbreaks based on the frequencies of detection of the BRD pathogens, thereby avoiding the need for multiple independent tests for comparison between variables that could increase the probability of yielding significant results by chance (Mills, 1993). In this study, we used a cluster analysis approach to study the patterns of BRD outbreaks according to the detection of the main pathogens associated with disease.

2. Material and methods

2.1. BRD outbreaks and clinical samples

A total of 156 outbreaks of BRD from 120 farms located in 30 provinces of Spain (Fig. 1), sent to the EXOPOL Laboratory (Zaragoza, Spain) for microbiological diagnosis between January 2020 and November 2021, were included in the study. In the present study an outbreak was defined as the presence of clinical signs consistent with BRD, such as nasal or ocular discharges, spontaneous cough, difficult breathing or rectal temperature of over 40 °C in > 5 animals or at least 5% of animals in the same farm simultaneously or within the next five days. Inclusion criteria were, in addition to the presence of respiratory signs, geographical localization, and complete results for the detection of viruses PI-3, BRSV, BCV, BVDV, and BHV-1 and of the bacteria *M. haemolytica*, *P. multocida*, *H. somni,* and *M. bovis*. Only diseased animals without antibiotic treatment in the past 15 days were sampled. Outbreaks were from farms in which affected animals were preweaned calves (< 5 months; $n = 51$), fattening beef calves (≥ 5 months; $n = 88$), replacement ($n = 8$) and adult ($n = 1$) animals, or of unknown age ($n = 8$). Clinical specimens were bronchoalveolar lavages in 96 outbreaks, lung tissues in 35 outbreaks, deep nasopharyngeal swabs in eight outbreaks, tracheal scrapes in two outbreaks, and a mixture of the above samples in 15 outbreaks Submissions from the same farm were recorded as different outbreaks when the timeframe between them was at least one month (range 1–10 months; mean 4.2 ± 2.7 months) or they were reported as outbreaks affecting different feedlots by veterinary clinicians. The number of samples per outbreak sent to the laboratory ranged between 1 and 30 (mean 6.2 ± 5.3), being in most outbreaks (88.2%) between 1 and 5 samples.

2.2. Detection of BRD-associated pathogens by individual q-PCR assays

From each outbreak, the detection of the nine BRD-associated pathogens indicated above was carried out in pools of up to five samples from different animals by q-PCR. For each sample, individual q-PCR reactions were performed targeting each of the nine respiratory

Fig. 1. Number of outbreaks analyzed by province and census of animals in feedlots operations.
(source of census: https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/estudiodelsectorespanoldecebodevacuno2019_tcm30–512343.pdf)
.

pathogens. The q-PCRs used were commercially available under the brand “EXOone” (Exopol, Spain) (https://www.exopol.com/es/exoone/list.php?especie=cattle) as individual kits for detection of PI-3 (ref PI3V), BRSV (ref BRSV), BCV (ref BCOV), BVDV (ref PEST), BHV-1 (ref BHV1), *M. haemolytica* (ref MANN), *P. multocida* (ref PAST), *H. somni* (ref HSOM), or *M. bovis* (ref MBOV).

Bronchoalveolar lavages and tracheal scrapes were pelleted through centrifugation of 1 mL samples at 4600 $\times g$ for 5 min and re-suspended with 200 μL of PBS. In the case of lungs specimens, pretreatment consisted of homogenization of 250 mg of tissue in a MagnaLyser device (Roche) with 400 μL of PBS and 0.1 mm zirconia beads. The swab content was collected in 700 μL of PBS by rotation movement. Nucleic acids (NA) from clinical specimens were extracted with the MagMAX™ CORE Nucleic Acid Purification Kit (Thermofisher) in an automated KingFisher Flex System (Thermofisher). Eluates were immediately used for all the aforementioned q-PCR assays and stored at − 80℃ for potential later purposes. The q-PCRs were performed using a 7500 Fast Real-Time PCR system (Applied Biosystems) with the following amplification protocol: reverse transcription (15 min at 45 °C), enzyme activation (5 min at 95 °C), 42 cycles of denaturation (15 s at 95 °C) and annealing (60 s at 60 °C). All the parameters, regardless of whether they were RNA or DNA pathogens, shared the same thermal profile. The thermocycler was set to acquire fluorescence on the FAM channel related to each pathogen and the HEX channel in order to verify the process though the detection of the respective endogenous control. The results were analyzed using the 7500 Software v2.3, and samples with a cycle threshold of 38 cycles or fewer were considered positive.

2.3. Detection of capsular types of M. haemolytica and P. multocida by multiplex q-PCR assay

Detection of capsular types of *M. haemolytica* and *P. multocida* was performed in 144 out of the 156 outbreaks investigated. Of these 144 outbreaks, 123 were positive for *P. multocida* and 93 for *M. haemolytica*. The same NA samples used for detection of BRD bacterial pathogens were used for capsular detection. Molecular detection of *M. haemolytica* A1, A2, and A6 capsular types was carried out using a commercially available multiplex q-PCR assay developed under the name “EXOone *M. haemolytica* types A1, A2, A6” (ref XMAN), (Exopol SL, Zaragoza, Spain; https://www.exopol.com/es/exoone/product.php?lang=es&tipo=cattle&_kp_Ppr=539) as described previously by Arnal et al. (2021).

The presence of *P. multocida* capsular types A, B, D, E, and F was determined using the commercially available individual q-PCRs developed under the name “PMCA-PMCF EXOone *P. multocida* capsular types A, B, D, E, and F,” respectively (Exopol SL, Zaragoza, Spain; https://www.exopol.com/es/exoone/product.php?lang=es&tipo=cattle&_kp_Ppr=539). These q-PCRs shared the same thermal profile that was described for the rest of the parameters. Specific probes for targeting each of the capsular types (A to F) were labeled with 6-FAM fluorophore. The results were analyzed using the 7500 Software v2.3, and samples with a cycle threshold of 38 cycles or fewer were considered positive.

2.4. Data analysis

Data were analyzed using the Epi-InfoTM 7 of the Centers for Disease Control and Prevention (CDC) (https://www.cdc.gov/epiinfo/index.html), SPAD V9.1, and SAS 9.1 programs. A cluster analysis of categorical variables was performed using the hierarchical agglomerative clustering method, using Ward's algorithm and squared Euclidean distance. Clustering was based on factorial coordinates obtained by the previous Multiple Correspondence Analysis (MCA). The Boolean variables (presence/absence) included as active variables to define clusters were detection of PI-3, BRSV, BCV, BVDV, BHV-1, *M. haemolytica*, *P. multocida*, *H. somni,* and *M. bovis.* For purpose analysis, the variable “season” was created from the data of outbreaks according to the categories: Spring (March 21 to June 21), Summer (June 22 to September 22), Autumn (September 23 to December 21) and Winter (December 22 to March 20). Associations between clusters and categorical variables or between categorical variables were analyzed using the chi-square test. The significance level was set at $P < 0.05$.

3. Results

3.1. Frequency of BRD-associated pathogen detection

The frequency of detection for the viruses and bacteria investigated in this study is indicated in Table 1. The most frequently detected virus was BCV (39.7%; 95% confidence interval (CI) 32.0–48.9), followed by PI-3 (26.3%; 95% CI 19.6–33.9), BRSV (19.9%; 95% CI 13.9–27.0), BVDV (17.3%; 95% CI 11.7–24.2) and BHV-1 (3.2%; 95% CI 1.1–7.3). *P. multocida* was the most frequently detected bacteria (85.9%; 95% CI 79.4–91.0), followed by *M. bovis* (77.6%; 95% CI 70.2–83.6), *M. haemolytica* (64.1%; 95% CI 56.0–71.6), and *H. somni* (42.3%; 95% CI 34.5–50.5). Capsular types A, B, D, and F of *P. multocida* were detected in 98.4%, 2.8%, 1.6%, and 4.1%, respectively, of the 123 outbreaks positive for *P. multocida* that were analyzed, whereas capsular types A1, A2, and A6 of *M. haemolytica* were detected in 55.9%, 50.5%, and 24.7%, respectively, of the 93 outbreaks positive for *M. haemolytica* that were analyzed.

3.2. BRD pathogen associations in outbreaks

Cluster analysis grouped outbreaks into two clusters (Table 2). Cluster 1 included 50 (32.1%) outbreaks in which the detection of the PI-3 (72.0%), BRSV (52.0%), BCV (72.0%), and BVDV (40.0%) viruses as well as of *M. bovis* (88.0%) was significantly higher while the detection of *H. somni* (22.0%) was significantly lower compared to the total number of outbreaks. Cluster 2 included 106 (67.9%) outbreaks in which the detection of the PI-3 (4.7%), BRSV (4.7%), BCV (24.5%), and BVDV (6.6%) viruses as well as of *M. bovis* (72.6%) was significantly lower while the detection of *H. somni* (51.9%) was significantly higher compared to the total number of outbreaks (Table 2). No statistically significant differences ($P > 0.05$) were observed between the frequencies of detection in cluster 1 and cluster 2 for *P. multocida* (90.0% and 84.0%, respectively) and *M. haemolytica* (76.0% and 61.3%, respectively) (Table 2). The frequencies of BHV-1 V were low with not statistically significant ($P > 0.05$) differences in both cluster 1 and 2 (4.0% and 2.9%, respectively; Table 2).

Statistically significant associations were found between clusters and the age of animals as well as the time of year when outbreaks were

Table 1
Frequencies of detection of the BRD associated pathogens investigated.

BRD agents detected by q-PCR	No. (%) of outbreaks		CI 95%[a]
Viruses			
BCV	62	39.7	32.0–48.9
PI-3	41	26.3	19.6–33.9
BRSV	31	19.9	13.9–27.0
BVDV	27	17.3	11.7–24.2
BHV-1	5	3.2	1.1–7.3
Bacteria			
P. multocida[b]	134	85.9	79.4–91.0
M. bovis	121	77.6	70.2–93–6
M. haemolytica[c]	100	64.1	56.0–71.6
H. somni	66	42.3	34.5–50.5
Total	156	100.0	

[a] Confidence interval of 95%.
[b] Capsular type A was detected in 98.4% of the 123 outbreaks positive for *P. multocida* analyzed. The frecuency of detection for other capsular types was less than 5% (B, 2.8%; D, 1.6% and F, 4.1%).
[c] Capsular types A1, A2 and A6 were detected in 55.9%, 50.5% and 24.7%, respectively in 93 of the outbreaks positive for *M. haemolytica* analyzed.

Table 2
Clustering of the 156 outbreaks according to the frequencies of detection of the BRD associated pathogens investigated.

Active Variables[1]	Cluster 1 (n = 50)		Cluster 2 (n = 106)		P
	n	%	n	%	
PI-3	36	72.0	5	4.7	< 0.05
BRSV	26	52.0	5	4.7	< 0.05
BCV	36	72.0	26	24.5	< 0.05
BVDV	20	40.0	7	6.6	< 0.05
H. somni	11	22.0	55	51.9	< 0.05
M. bovis	44	88.0	77	72.6	< 0.05
M. haemolytica	38	76.0	65	61.3	> 0.05
P. multocida	45	90.0	89	84.0	> 0.05
BHV-1	2	4.0	1	2.9	> 0.05

Table 3
Associations of outbreaks clusters with animal category and season of the year.

Variables	Total (n = 156)		Cluster 1 (n = 50)		Cluster 2 (n = 106)		OR (CI95%)[a]
	n	%	n	%	n	%	
Animal category							
Preweaned calves	51	32.7	23	46.0	28	26.4	**2.2 (1.1–4.5)**
Fattening beef calves	88	56.4	24	48.0	64	60.4	1
Others[b]	17	10.8	3	6.0	14	13.2	
Season[c]							
Autumn	34	21.8	8	16.0	26	24.5	1.5 (0.4–5.3)
Winter	46	29.5	22	44.0	24	22.6	**4.6 (1.5–14.1)**
Spring	46	29.5	15	30.0	31	29.2	2.4 (0.8–7.6)
Summer	30	19.2	5	10.0	25	23.6	1

[a] Odds ratio and confidence interval of 95%. In bold statistically significant differences
[b] Replacement (n = 8), adults (n = 1) and unknown age (n = 8).
[c] Spring: March 21 to June 21; Summer: June 22 to September 22; Autumn: September 23 to December 21; Winter: December 22 to March 20.

detected (Table 3). The odds of including BRD outbreaks of preweaned calves versus BRD outbreaks of fattening beef calves was significantly higher in cluster 1 than in cluster 2 (OR 2.2; 95% CI 1.1–4.5). The odds of including BRD outbreaks of winter was significantly higher in cluster 1 than cluster 2 (OR 4.6; 95% CI 1.5–14.1), but not significant differences were detected for including outbreaks of autumn (1.5; 95% CI 0.4–5.3) or spring (2.4; 95% CI 0.8–7.6), when comparing with including outbreaks of summer (Table 3). Outbreaks of cluster 1 were more frequently detected between December and March (Fig. 2).

4. Discussion

BRD represents one of the most important health problems in feedlots with a complex etiology that includes infection with different viruses and bacterial pathogens that lead to the development of lung lesions (Smith et al., 2020). In this study we investigated the presence of the most common BRD-associated pathogens by q-PCRs. This molecular technique has been adopted in many diagnostic laboratories to determine the presence of infectious agents contributing to BRD due to its high specificity and sensitivity and ability to detect simultaneously multiple infectious agents in a large number of samples. The study has several sources of potential bias, mainly related with the fact that it was based on diagnostic laboratory submissions. The use of a convenience sampling could cause selection bias. For instance, outbreaks with higher morbidity or mortality might be overrepresented in submissions. Nevertheless, submission of respiratory tract samples in outbreaks is nowadays more frequent in European countries given that laboratory diagnosis is necessary for a rational antimicrobial use (Pardon et al., 2020), increasing likely the diversity of outbreaks sent for diagnosis. Outbreak definition based on the observation of clinical signs in individual animals can be subjected to variation between farms because of differences in the individual observers, training of the personnel or routine monitoring of the animals. In addition, PCR diagnosis of viruses does not distinguish between field and vaccine viruses and therefore this could complicate the interpretation of the results in the absence of vaccination history, especially when viral modified live vaccines are used (Fulton et al., 2016; Walz et al., 2017). Despite these limitations, this study investigated the detection of the main pathogens associated with BRD in 156 outbreaks from 120 farms located in 30 provinces of Spain (Fig. 1) and therefore the results offer a representative overview of the circulating pathogens associated with BRD in this country. Less frequently reported viruses, such as influenza D, whose importance in BRD etiology remains to be confirmed (Fulton, 2020), were not analyzed in this study.

One of the most frequently identified viral pathogens was PI-3, with an overall frequency of detection of 26.3%, followed by BRSV and BVDV (19.9%, and 17.3%, respectively), frequencies of detection similar to those found in other European countries (O'Neill et al., 2014; Pardon et al., 2020; Fanelli et al., 2021). These viruses are commonly accepted as primary pathogens associated with BRD (Fulton, 2020; Pardon and Buczinski, 2020) and their detection at herd level likely indicate their involvement in the outbreaks (Pardon et al., 2020). Unlike the other viruses, the role of BCV as etiological agent in BRD is more controversial (Ellis, 2019; Fulton, 2020; Pardon et al., 2020) as it is also frequently detected in apparently healthy animals (Pratelli et al., 2021; Studer et al., 2021). In this study, BCV was detected in 39.7% of the outbreaks (Table 1), a rate that is in agreement with other studies in Europe in which BCV was also the virus most frequently detected in BRD outbreaks (O'Neill et al., 2014; Pardon et al., 2020; Fanelli et al., 2021). The high prevalence of BCV detected in different countries suggests the need for studies to determine whether BCV indeed has a primary role in BRD or whether it merely predisposes animals to secondary bacterial infections. The rate of BHV-1 frequency of detection was very low (3.2%), which is probably related to the existence from 2019 of a national program of control and eradication of infectious bovine rhinotracheitis (IBR).

Bacterial pathogens associated with BRD were present in all but two outbreaks (98.7%; Supplementary Table) with frequencies of detection that ranged between the 42.3% and 85.9% (Table 1). *M. bovis* was detected with a significantly higher frequency (77.6%; Table 1) than that found in other European countries (Fanelli et al., 2021; Pardon et al., 2020). This high frequency of detection of *M. bovis* is consistent with a herd-level seroprevalence of 100% and an individual animal seroprevalence of 65.5% found in a study that aimed to determine the seroprevalence of *M. bovis* in cattle in Spain (Martín-Espada et al., 2011), and it confirms the wider distribution of *M. bovis* in Spanish feedlots. *P. multocida* was the most frequently detected bacterial pathogen, with an overall frequency of detection of 85.9% (95% CI 79.4–91.0) of outbreaks, a result that is very similar to that found in Belgium by Pardon et al. (2020). Moreover, the incidence of *P. multocida* in BRD outbreaks has increased in the past few years (Timsit et al., 2017; Choudhary et al., 2019; Pardon et al., 2020), and it has also been considered the main agent involved in BRD outbreaks in cattle vaccinated against other pathogens associated with BRD (Crawshaw and Caldow, 2015). These data confirm the high frequencies of *P. multocida* isolation in clinical samples from BRD. The great majority of *P. multocida* isolates were of capsular type A (98.4%); the other capsular types were detected with frequencies lower that 5% (Table 1), which confirms the predominance of capsular type A among *P. multocida* isolates in BRD (Khamesipour et al., 2014; Snyder and Credille, 2020). Most of the current BRD commercial vaccines do not include *P. multocida*; however, due to the high frequency of detection of *P. multocida* detected in BRD outbreaks in this and other studies (Timsit et al., 2017; Pardon et al., 2020), it would be advisable to evaluate the convenience of its inclusion in the BRD vaccination programs. The overall frequency of detection of *M. haemolytica* was 64.1% (95% CI 56.0–71.6; Table 1), with more than half of the outbreaks being positive for serotype A1 (55.9% of

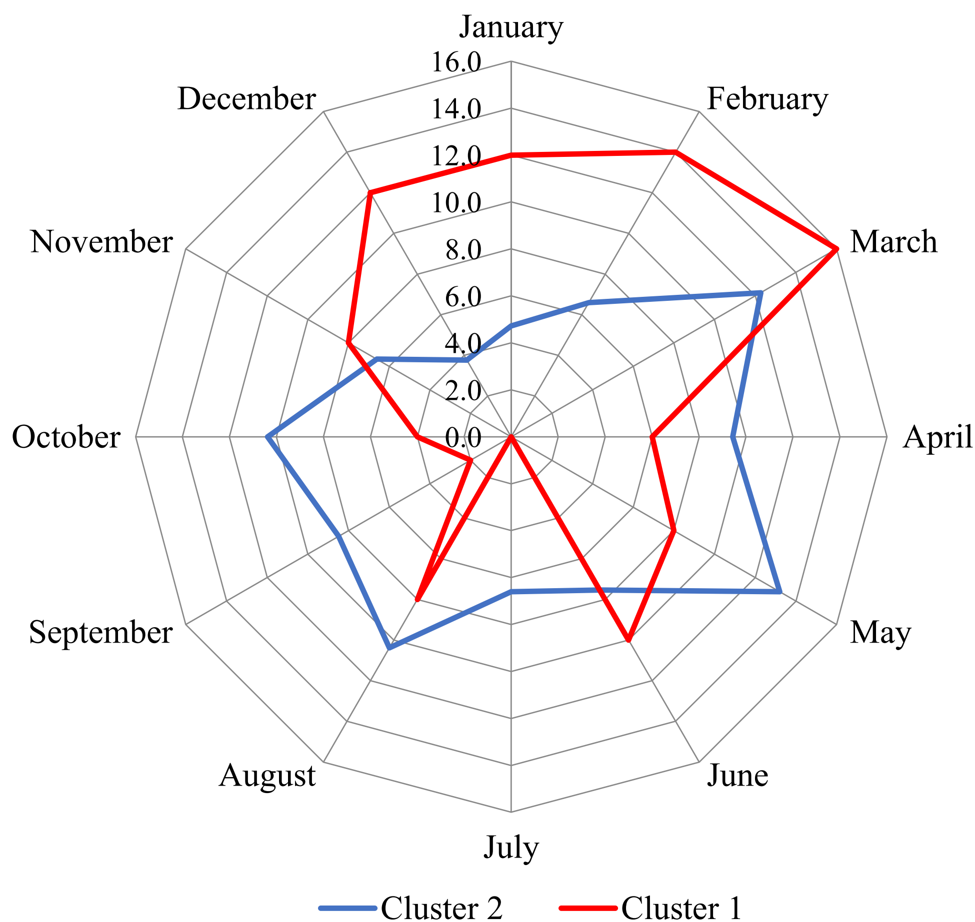

Fig. 2. Monthly distribution of BRD outbreak clusters.

93 *M. haemolytica*-positive outbreaks analyzed; Table 1), corroborating its frequent association with the disease (Smith et al., 2020; Snyder and Credille, 2020). *M. haemolytica* serotype A1 is able to invade and multiply rapidly intracellularly in bovine airway epithelial cells and to spread to adjacent cells causing extensive cellular damage (Cozens et al., 2019), which could explain the common involvement of A1 in BRD outbreaks. Nearly a quarter of *M. haemolytica* outbreaks were positive for serotype A6 (24.7% of 93 *M. haemolytica*-positive outbreaks analyzed; Table1), a result that highlights the increase in the frequency of detection of this serotype in clinical cases of BRD (Snyder and Credille, 2020). Serotype 2 is commonly prevalent in the nasopharynx of healthy cattle, and, unlike serotypes A1 and A6, is considered not to be associated with disease (Andrés-Lasheras et al., 2019; Arnal et al., 2021; Mason et al., 2022). A remarkable result in this study was the detection of serotype A2 in half of the 93 BRD outbreaks positive for *M. haemolytica* that were analyzed, with a frequency even higher than that of serotype A6 (Table 1). Moreover, it was detected in the absence of serotypes A1 or A6 in nearly one fifth of the lower respiratory tract samples (data not shown). These data do not support the generally accepted idea that *M. haemolytica* serotype 2 is merely a commensal microorganism of the upper respiratory tract. In this sense, experimental studies demonstrated that strains of serotype A2 are able to produce lung lesions comparable to those produced by the A1 strain of *M. haemolytica* (Gentry et al., 1983), and therefore its role as an opportunistic pathogen contributing to the induction of inflammatory response in the lung should be taken into account. Data on the detection of *H. somni* in BRD are limited (Timsit et al., 2017; Pardon et al., 2020), but the frequency of detection found in the present work (42.3% of the outbreaks; 95% CI 34.5–50.5; Table 1) indicates the relevance of this pathogen in BRD outbreaks. In opportunistic secondary pathogens, diagnosis based on their detection by PCR should be interpreted with caution, as their detection do not necessarily indicate a role in the aetiology of the outbreaks. However, the high frequencies of detection of these bacterial pathogens have a sanitary impact on the control of BRD, as they represent the main reason for the extensive use of antimicrobials in BRD treatment and for the further increase in antimicrobial resistances (Snyder and Credille, 2020).

An association between the presence of specific BRD-associated pathogens and risk factors has been commonly determined for individual pathogens (O'Neill et al., 2014; Pardon et al., 2020; Fanelli et al., 2021). In this study, we used a hierarchical cluster analysis to classify the BRD outbreaks in natural groups according to the detection of all nine pathogens investigated. This cluster analysis grouped BRD outbreaks into two clusters that differed mainly in the frequency with which the viruses were detected (Table 2 and Supplementary Table). Clusters were associated with the age of the affected animals and the time of the year in which the BRD outbreaks were detected (Table 3). Four of the viruses investigated were detected at relatively high frequencies (between 40% and 72%) in cluster 1 (Table 2). In addition, BRD-associated viruses were present in all BRD outbreaks of this cluster, usually as viral coinfections (Supplementary Table), supporting the generally accepted idea of the primary involvement of viruses in the development of BRD (Pardon and Buczinski, 2020; Pardon et al., 2020). The results of molecular tests as that used in this study can be affected by the use of modified live vaccines (MLV) to prevent BRD as they do not discriminate between field and vaccine isolates (Fulton et al., 2016; Walz et al., 2017). The use of MLV vaccines is a very common practice in fattening ($\geq$ 5 months) beef calves after arrival to feedlots in Spain, but it is not so common in preweaned (< 5 months) calves. The individual vaccination history of each farm was not available in this study, but it is unlikely that

the high frequencies of virus detection in outbreaks of cluster 1 associated with preweaned animals were biased by a high detection of vaccine virus rather than the detection of field virus. *M. bovis* is generally recognized as a potentially major pathogen in the etiology of the disease, involved not only in chronic and endemic respiratory cases but also in acute outbreaks with a clinical presentation undistinguishable from other bacterial BRD pathogens (Duket et al., 2020; Pardon and Buczinski, 2020; Pardon et al., 2020). *M. bovis* was significantly associated with BRD outbreaks of this cluster (88.0% cluster 1 vs. 72.6% cluster 2; Table 2). However, the fact that it was always detected in coinfections with at least one of the primary respiratory viruses investigated (Supplementary Table) precludes reaching any conclusion about its role in these BRD outbreaks. *M. haemolytica* and *P. multocida* are considered opportunistic pathogens highly prevalent in the upper respiratory tract of cattle (Smith et al., 2020); it is generally accepted that coinfections with respiratory viruses can compromise the respiratory tract defense mechanisms, facilitating the descent of these bacteria into the lungs, causing an inflammatory response and complicating the pneumonic lesions and clinical signs of the disease initiated by viruses (Smith et al., 2020). Consistent with this idea, *M. haemolytica* and *P. multocida* were detected, alone or together, in virtually all the BRD outbreaks in cluster 1 (Table 2) always in the presence of at least one of the viruses considered as primary BRD pathogens (Supplementary Table); this suggests a secondary role for both pathogens in the etiology of the BRD outbreaks investigated. These results indicate that in outbreaks of cluster 1, viruses most likely played a role as primary pathogens in the etiology of BRD outbreaks, while *M. haemolytica* and *P. multocida* would act as secondary opportunistic pathogens complicating the disease. The association found between BRD outbreaks in this cluster and preweaning calves younger than 5 months of age (OR 2.2; 95% CI 1.1–4.5; Table3) indicates a higher susceptibility of young animals to viral infections, which could be explained by the fact that the calf's immune system is not fully developed at those ages or by the absence of or a low maternal immunity. Respiratory viruses and *M. bovis* are more frequently detected in winter or in cold seasons (O'Neill et al., 2014; Dudek et al., 2020; Pardon et al., 2021), which could explain the seasonality detected in BRD outbreaks in cluster 1 (Table 3), being more frequently detected in cold months between December and March (Fig. 2).

Cluster 2 included most of the BRD outbreaks investigated ($n = 106$; 67.9%; Table 2). These outbreaks were distributed throughout the year without any seasonality (Fig. 2) affecting mainly calves older than 5 months after arrival to feedlots for fattening. This cluster was characterized by frequencies of PI-3, BRSV, or BVDV below 10% each (Table 2). Moreover, viruses were not detected in nearly 60% of BRD outbreaks in this cluster (Supplementary Table). On the other hand, *P. multocida* and *M. haemolytica* were detected in nearly all BRD outbreaks of cluster 2, with frequencies similar to those detected in cluster 1 (Table 2) and. *H. somni* showed a significantly higher frequency in cluster 2 (Table2), associated with fattening outbreaks (Table 3), which indicates a different epidemiology of *H. somni* in BRD. Overall, these data suggest that the presence of primary pathogen virus such as PI-3, BRSV, or BVDV should not be a *sine qua non* condition for BRD development as generally accepted (Pardon et al., 2020), and that in absence of these virus other infections or factors related with husbandry practices could predispose calves to secondary infections by opportunistic bacteria. BCV virus was detected in outbreaks of cluster 2 with frequencies five or four time higher than those found for PI-3, BRSV, or BVDV (Table 2). Despite the role of BCV as primary pathogen has not been clearly stablished (Ellis, 2019; Fulton, 2020; Pardon et al., 2020) it is possible that it could predisposes animals to secondary bacterial infections. In the same way, *M. bovis* was detected in almost three quarters of the outbreaks of this cluster (72.6%; Table 2. This result suggests that, in the absence of viruses, *M. bovis* could also be a primary cause of disease, impairing the host defenses of calves and promoting secondary bacterial infections (Pérez-Casal, 2020). In addition, various management and environmental conditions commonly present in feedlot operations such as high stock densities, commingling of calves of different sources and ages, transport to feedlots, or a deficient acclimatization to the feedlot are well known factors that have been associated with an increased risk of BRD (Pratelli et al. 2020; Smith et al., 2020; Padalino et al., 2021). These risk factors could not be recorded in this study but the contribution of one or several of these conditions to the occurrence of outbreaks in cluster 2 cannot be ruled out.

Overall, the results of this study show that in addition to the classic epidemiological BRD pattern characterized by the primary involvement of viruses occurring preferably during winter and affecting calves younger than 5 months, there is a second pattern, even more frequent than the first, in which the involvement of viruses is less relevant, affecting mainly calves older than 5 months at any time of the year.

Acknowledgments

The authors thank Ricardo Garcia Mata for his invaluable assistance in statistical analysis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109701.

References

Andrés-Lasheras, S., Zaheer, R., Klima, C., Sanderson, H., Polo, R.O., Milani, M.R.M., Vertenten, G., McAllister, T.A., 2019. Serotyping and antimicrobial resistance of *Mannheimia haemolytica* strains from European cattle with bovine respiratory disease. Res. Vet. Sci. 124, 10–12.

Arnal, J.L., Fernández, A., Vela, A.I., Sanz, C., Fernández-Garayzábal, J.F., Cid, D., 2021. Capsular type diversity of *Mannheimia haemolytica* determined by multiplex real-time PCR and indirect hemagglutination in clinical isolates from cattle, sheep, and goats in Spain. Vet. Microbiol. 258, 109121 https://doi.org/10.1016/j.vetmic.2021.

Calcutt, M.J., Lysnyansky, I., Sachse, K., Fox, L.K., Nicholas, R.A.J., Ayling, R.D., 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. Transbound. Emerg. Dis. 65 (Suppl. 1), 91–109.

Choudhary, M., Choudhary, B.K., Chandra Ghosh, R., Bhoyar, S., Chaudhari, S., Barbuddhe, S.B., 2019. Cultivable microbiota and pulmonary lesions in polymicrobial bovine pneumonia. Micro Pathog. 134, 103577.

Cozens, D., Sutherland, E., Lauder, M., Taylor, G., Berry, C.C., Davies, R.L., 2019. Pathogenic *Mannheimia haemolytica* invades differentiated bovine airway epithelial cells. Infect. Immun. 87, e00078–19.

Crawshaw, W.M., Caldow, G.L., 2015. Field study of pneumonia in vaccinated cattle associated with incorrect vaccination and *Pasteurella multocida* infection. Vet. Rec. 176, 17.

Dudek, K., Nicholas, R.A.J., Szacawa, E., Bednarek, D., 2020. *Mycoplasma bovis* infections-occurrence, diagnosis and control. Pathogens 9, 640. https://doi.org/10.3390/pathogens9080640.

Ellis, J., 2019. What is the evidence that bovine coronavirus is a biologically significant respiratory pathogen in cattle? Can. Vet. J. 60, 147–152.

Fanelli, A., Cirilli, M., Lucente, M.S., Zarea, A.A.K., Buonavoglia, D., Tempesta, M., Greco, G., 2021. Fatal calf pneumonia outbreaks in Italian dairy herds involving *Mycoplasma bovis* and other agents of BRD complex. Front. Vet., Sci. 8, 742785 https://doi.org/10.3389/fvets.2021.742785.

Fulton, R.W., 2020. Viruses in bovine respiratory disease in North America knowledge advances using genomic testing. Vet. Clin. Food Anim. 36, 321–332.

Fulton, R.W., d'Offay, J.M., Landis, C., Miles, D.G., Smith, R.A., Saliki, J.T., Ridpath, J.F., Confer, A.W., Neill, J.D., Eberle, R., Clement, T.J., Chase, C.C., Burge, L.J., Payton, M.E., 2016. Detection and characterization of viruses as field and vaccine strains in feedlot cattle with bovine respiratory disease. Vaccine 34, 3478–3492.

Gentry, M.J., Confer, A.W., Holland, S.G., 1983. Comparison of the toxic and antigenic properties of single bovine isolates of *Pasteurella haemolytica* representing five serotypes and an untypable strain. Vet. Microbiol. 16, 351–367.

Khamesipour, F., Momtaz, H., Azhdary Mamoreh, M., 2014. Occurrence of virulence factors and antimicrobial resistance in *Pasteurella multocida* strains isolated from slaughter cattle in Iran. Front. Microbiol. 5, 536.

Lowie, T., Callens, J., Maris, J., Ribbens, S., Pardon, B., 2021. Decision tree analysis for pathogen identification based on circumstantial factors in outbreaks of bovine respiratory disease in calves. Prev. Vet. Med. 196, 105469 doi: 10.1016.

Martín-Espada, C., Díez-Guerrier, A., Cid, D., 2011. Seroprevalencia de anticuerpos frente a *Mycoplasma bovis* en explotaciones bovinas de España. Anembe International Congress of Bovine Medicine. Ed. Asociación Nacional de Especialistas en Medicina Bovina de España (ANEMBE), Ávila, Spain, pp. 290–292.

Mason, C., Errington, J., Foster, G., Thacker, J., Grace, O., Baxter-Smith, K., 2022. *Mannheimia haemolytica* serovars associated with respiratory disease in cattle in Great Britain. BMC Vet. Res. 18, 5. https://doi.org/10.1186/s12917-021-03121-3.

McMullen, C., Orsel, K., Alexander, T.W., van der Meer, F., Plastow, G., Timsit, E., 2019. Comparison of the nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with Bovine Respiratory Disease. Vet. Microbiol. 231, 56–62.
Michelacci, V., Montalbano Di Filippo, M., Gigliucci, F., Arancia, S., Chiani, P., Minelli, F., Roosens, N.H.C., De Keersmaecker, S.C.J., Bogaerts, B., Vanneste, K., Morabito, S., 2022. Population analysis of O26 Shiga toxin-producing *Escherichia coli* causing hemolytic uremic syndrome in Italy, 1989-2020, through whole genome sequencing. Front. Cell Infect. Microbiol. 12, 842508.
Mills, J.L., 1993. Data Torturing. N. Engl. J. Med 329, 1196–1199.
O'Neill, R., Mooney, J., Connaghan, E., Furphy, C., Graham, D.A., 2014. Patterns of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective study. Vet. Rec. 175, 351. https://doi.org/10.1136/vr.102574.
Padalino, B., Cirone, F., Zappaterra, M., Tullio, D., Ficco, G., Giustino, A., Ndiana, L.A., Pratelli, A., 2021. Factors affecting the development of bovine respiratory disease: a cross-sectional study in beef steers shipped from France to Italy. Front. Vet. Sci. 8, 627894 https://doi.org/10.3389/fvets.2021.627894.
Pardon, B., Buczinski, S., 2020. Bovine respiratory disease diagnosis what progress has been made in infectious diagnosis? Vet. Clin. Food Anim. 36, 425–444.
Pardon, B., Callens, J., Maris, J., Allais, L., Van Praet, W., Deprez, P., Ribbens, S., 2020. Pathogen-specific risk factors in acute outbreaks of respiratory disease in calves. J. Dairy Sci. 103, 2556–2566.
Perez-Casal, J., 2020. Pathogenesis and virulence of *Mycoplasma bovis*. Vet. Clin. Food Anim. 36, 269–278.
Pratelli, A., Hodnik, A., Cirone, F., Capozza, P., Trotta, A., Corrente, M., Balestrieri, A., Buonavoglia, C., 2020. Bovine respiratory disease in beef calves supported long transport stress: An epidemiological study and strategies for control and prevention. Res. Vet. Sci. 135, 450–455. https://doi.org/10.1016/j.rvsc.2020.11.002.
Shirbroun, R.M., 2020. *Histophilus somni*, antigenic and genomic changes relevant to bovine respiratory disease. Vet. Clin. Food Anim. 36, 279–295.
Snyder, E., Credille, B., 2020. *Mannheimia haemolytica* and *Pasteurella multocida* in bovine respiratory disease. How are they changing in response to efforts to control them? Vet. Clin. Food Anim. 36, 253–268.
Smith, R.A., Step, D.L., Woolums, A.R., 2020. Bovine respiratory disease looking back and looking forward, what do we see? Vet. Clin. Food Anim. 36, 239–251.
Studer, E., Schönecker, L., Meylan, M., Stucki, D., Dijkman, R., Holwerda, M., Glaus, A., Becker, J., 2021. Prevalence of BRD-related viral pathogens in the upper respiratory tract of Swiss veal calves. Animals 11, 1940.
Timsit, E., Hallewell, J., Booker, C., Tison, N., Amat, S., Alexander, T.W., 2017. Prevalence and antimicrobial susceptibility of *Mannheimia haemolytica, Pasteurella multocida*, and *Histophilus somni* isolated from the lower respiratory tract of healthy feedlot cattle and those diagnosed with bovine respiratory disease. Vet. Microbiol. 208, 118–125.
Timsit, E., Workentine, M., Schryvers, A.B., Holman, D.B., van der Meer, F., Alexander, T.W., 2016. Evolution of the nasopharyngeal microbiota of beef cattle from weaning to 40 days after arrival at a feedlot. Vet. Microbiol. 187, 75–81.
Walz, P.H., Newcomer, B.W., Riddell, K.P., Scruggs, D.W., Cortese, V.S., 2017. Virus detection by PCR following vaccination of naive calves with intranasal or injectable multivalent modified-live viral vaccines. J. Vet. Diagn. Invest 29, 628–635.
White, B.J., Larson, B.L., 2020. Impact of bovine respiratory disease in U.S. beef cattle. Anim. Health Res. Rev. 21, 132–134.
Zeineldin, M., Lowe, J., de Godoy, M., Maradiaga, N., Ramirez, C., Ghanem, M., Abd El-Raof, Y., Aldridge, B., 2017. Disparity in the nasopharyngeal microbiota between healthy cattle on feed, at entry processing and with respiratory disease. Vet. Microbiol. 208, 30–37.
Zhang, J., Wang, W., Yang, M., Lin, J., Xue, F., Zhu, Y., Yin, X., 2022. Development of a one-step multiplex Real-Time PCR assay for the detection of viral pathogens associated with the bovine respiratory disease complex. Front. Vet. Sci. 9, 825257.