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Evaluation of *Mollicutes* Microorganisms in Respiratory Disease of Cattle and Their Relationship to Clinical Signs

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Background: Bovine respiratory disease (BRD) is an important problem in cattle production that is responsible for economic losses in dairy herds. *Mycoplasma* spp. are described as an important etiological agent of BRD.

Hypothesis: To evaluate the occurrence of the most important mycoplasmas in the lower respiratory tract of healthy and BRD cattle in relationship to clinical signs of BRD.

Animals: Sixty young dairy cattle were classified as healthy (n = 32) or cattle showing clinical signs of BRD (n = 28).

Methods: Tracheal lavage samples were collected and added to tubes containing Hayflick media. *Mycoplasma* spp. were identified by the presence of "fried egg" like colonies, biochemical tests and polymerase chain reaction (PCR). Occurrence of *Mollicutes, M. bovis, M. mycoides* subsp. *mycoides SC* and *M. dispar* was evaluated. The association between clinical signs of BRD and the presence of *Mycoplasma* spp. also was evaluated.

Results: Colonies were obtained from a 1-year-old BRD calf only. However, species identification was not possible. *Mollicutes* ($P = .035$) and *M. dispar* ($P = .036$) were more common in BRD cattle. The relationship between *Mollicutes* and crackle ($P = .057$) was not significant. *M. dispar* was associated to tachypnea ($P = .045$) and mixed dyspnea ($P = .003$). Relationships to heart rate ($P = .062$) and crackle ($P = .062$) were not significant.

Conclusions and clinical importance: The results confirmed the importance of mycoplasma as an etiologic agent of BRD and suggested *M. dispar* as part of the respiratory microbiota and its possible role in the development of BRD.

Key words: Bovine; Buiatrics; Diseases of cattle; Mycoplasma.

Bovine respiratory disease (BRD) is an important problem in cattle production that remains responsible for economic losses in dairy and feedlot herds because of high morbidity and mortality rates.[1–3] It is observed in young calves,[4] particularly between 2 and 6 weeks of age.[5,6] In addition, BRD negatively impacts growth, reproductive performance, and longevity.[5,7]

Mycoplasma spp. belong to the *Mollicutes* class, and they are described as important etiological agents of BRD.[3,8,9] *M. mycoides* subsp. *mycoides* small colony (MmmSC), *M. bovis*, and *M. dispar* are the most important species related to BRD. *M. mycoides* subsp. *mycoides* small colony is the etiological agent of contagious bovine pleuropneumonia,[10] and it is considered the most pathogenic mycoplasma. *Mycoplasma mycoides* subsp. *mycoides* SC has never been detected in Brazilian cattle, although its detection in the external auditory meatus of clinically healthy goats was described elsewhere.[11] *M. bovis* is an opportunistic bacterium considered part of the bovine respiratory tract microbiota.[12] After stressful situations, *M. bovis* becomes pathogenic and clinical signs of BRD are observed, especially in young calves.[13] *M. dispar* was first isolated from pneumonic lungs of cattle,[14] and it has been described as a potential pathogen associated with BRD.[15,16]

Considering the importance of *Mycoplasma* spp. in the development of BRD, the aim of our study was to evaluate the occurrence of the most important mycoplasma species in the lower respiratory tract of healthy and sick Brazilian cattle in relationship to clinical signs of BRD.

Abbreviations:

BRD	bovine respiratory disease
MmmSC	*Mycoplasma mycoides* subsp. *mycoides* small colony
PCR	polymerase chain reaction

Materials and Methods

The study was conducted at the Internal Medicine Department, School of Veterinary Medicine and Animal Science, University of São Paulo and at the Laboratory of Mycoplasmas, Institute of Biological Sciences, University of São Paulo, Brazil. All procedures were carried out in agreement with the guidelines of Ethical Principles in Animal Research adopted by the Ethic Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo.

Sixty young dairy cattle were randomly selected and enrolled in the study. Fifty-eight cattle were from 10 farms located in the state

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of São Paulo, Brazil. Calves were immediately separated from their mothers after birth. They received colostrum and milk by farm employees, and after weaning, they received a pasture and barley-based diet and mineral salt. Two cattle were presented at the Veterinary Hospital of the School of Veterinary Medicine and Animal Science, University of São Paulo.

Case Definition

Bovine respiratory disease was diagnosed when the animal showed ≥2 of the following clinical signs: mucopurulent or purulent nasal discharge, cough, rectal temperature >39.5°C, respiratory rate >40 breaths/min, and increased cranioventral lung sounds or crackle.[6,17,18] The limits of the lung field were 12° intercostal space at iliac line and 11° intercostal space at sciatic line. Two experienced veterinarians on our research team performed the physical examinations in all cattle. Animals were allocated in 2 groups: healthy (n = 28) and BRD cattle (n = 32).

Sample Collection

The distal part of the neck was shaved and decontaminated with 70% alcohol and iodopovidone. Twenty milliliters of sterile saline 0.9% was instilled with a 16 × 40 mm needle and up to 5 mL was recovered. Samples were added to tubes containing Hayflick media and transported on ice to the laboratory.

Cultivation of Mycoplasma spp.

Clinical samples were diluted (10^0, 10^{-1}, 10^{-2}, 10^{-3}) in phosphate-buffer saline (PBS). *Mycoplasma* spp. isolation was performed by plating 100 μL of each dilution in Hyflick media growth plates and adding 200 μL of each dilution in 1800 μL of liquid media containing Hyflick media.[19] Plates and liquid media were incubated at 37°C for 21 days and evaluated on a daily basis. Plates containing "fried-egg" colonies and glucose fermentation with or without arginine hydrolysis were considered positive. Liquid media containing glucose fermentation with or without arginine hydrolysis and absence of turbidity were considered positive.

Molecular Detection

Molecular investigation of *Mycoplasma* spp was performed using DNA extraction according to a previously described procedure.[20] Polymerase chain reaction was performed to investigate the presence of *Mollicutes* class bacteria.[21] Positive samples were used to detect *M. bovis*,[22] *M. dispar*,[23] and *Mmm*SC.[24]

Statistical Methods

Descriptive analysis was performed to determine absolute and relative frequencies. The occurrence of *Mycoplasma* spp. was considered the dependent variable. Health status and clinical signs were considered the independent variables. The association between the presence of *Mycoplasma* spp. and health status and clinical signs of BRD was compared by applying the Pearson's chi-square test or Fisher's exact test using a 95% confidence interval. Clinical data were analyzed by the Statistical Package for Social Sciences 19.0. Variables with $P < .05$ were considered significant.

Results

Clinical signs detected during physical examination of cattle are described in Table 1. Most healthy cattle showed only normal findings. However, some cattle showed lethargy (6%), expiratory or inspiratory dyspnea (3%), mixed dyspnea (both expiratory and inspiratory dyspnea) (3%), crackle (3%), or snoring (3%).

Colonies were obtained from 1 BRD calf only. However, species identification was not possible because of the low quality of the sample.

Polymerase chain reaction was performed to detect *Mollicutes, M. dispar, M. bovis,* and *Mmm*SC. *Mollicutes* were increased in BRD cattle (68%) compared to healthy cattle (41%; $P = .035$; Table 2). Specific PCR tests to detect *M. dispar, M. bovis,* and *Mmm*SC were performed in 25% (8 of 32) and 64% (18 of 28) of samples from healthy and BRD groups, respectively, because of the low quality of the other samples. *M. dispar* was increased in BRD group (61%) compared to the healthy group (12.5%; $P = .036$), *M. bovis* was detected in BRD animals only (5%), and no difference between groups was noted ($P = .497$;

Table 1. Clinical signs detected after clinical examination of healthy and BRD cattle.

Clinical Signs	Healthy % (N/T)	BRD % (N/T)	Total % (N/T)
Behavior			
Alert	94 (30/32)	39 (11/28)	72 (41/60)
Lethargic	06 (02/32)	68 (17/28)	28 (19/60)
Ocular mucous membrane			
Normal	100 (32/32)	75 (21/28)	88 (53/60)
Pale	–	25 (07/28)	12 (07/60)
Heart rate			
<100 bpm	100 (32/32)	21 (06/28)	63 (38/60)
>100 bpm	–	79 (22/28)	37 (22/60)
Respiratory rate			
<40 breaths/min	100 (32/32)	36 (10/28)	70 (42/60)
>40 breaths/min	–	64 (18/28)	30 (18/60)
Body temperature			
<39.5°C	100 (32/32)	50 (14/28)	77 (46/60)
>39.5°C	–	50 (14/28)	23 (14/60)
Nasal discharge			
Absent	97 (31/32)	18 (05/28)	60 (36/60)
Serous	–	11 (03/28)	05 (03/60)
Mucous	03 (01/32)	50 (14/28)	25 (15/60)
Mucopurulent/purulent	–	21 (06/28)	10 (06/60)
Cough			
Absent	100 (32/32)	18 (05/28)	62 (37/60)
Productive	–	100 (15/28)	25 (15/60)
Nonproductive	–	100 (08/28)	13 (08/60)
Dyspnea			
Absent	94 (30/32)	21 (06/28)	60 (36/60)
Inspiratory	03 (01/32)	11(03/28)	07 (04/60)
Expiratory	–	25 (07/28)	12 (07/32)
Mixed[a]	03 (01/32)	43 (12/28)	22 (13/60)
Crackles			
Absent	97 (31/32)	11 (03/28)	57 (34/60)
Present	03 (01/32)	71 (20/28)	35 (21/60)
Snoring			
Absent	97 (31/32)	43 (12/28)	72 (43/60)
Present	03 (01/32)	57 (16/28)	28 (17/60)
Whistling			
Absent	100 (32/32)	73 (20/28)	87 (52/60)
Present	–	27 (08/28)	13 (08/60)

[a]Inspiratory and expiratory dyspnea.

Table 2. *Mollicutes*, *M. dispar,* and *M. bovis* associated to bovine respiratory disease in the state of São Paulo, Brazil.

Microorganism	Healthy % (N/T)	BRD % (N/T)	OR (CI 95%)	*P*-value
Mollicutes	41 (13/32)	68 (19/28)	3.085 (1.067–8.919)	.035
M. dispar	12.5 (01/08)	61 (11/18)	11.00 (1.103–109.674)	.036
M. bovis	00 (00/08)	06 (01/18)	–	.497

Table 2). *Mycoplasma mycoides* subsp. *mycoides* SC was not detected. Undetermined species were observed in both healthy (87.5%; 07/08) and BRD groups (33%; 06/18).

The association between the bacteria detected and clinical signs of BRD was evaluated. (Table 3). With regard to *M. dispar,* tachypnea was more common in positive animals (66.7%) as compared to negative animals (21.4%; $P = .045$). Mixed dyspnea (inspiratory and expiratory dyspnea) was more common in the positive group (66.7%) compared to the negative group (7%; $P = .003$). No significant association between clinical signs and *M. dispar* was observed (Table 4).

Discussion

To better understand the importance of *Mycoplasma* spp. in BRD, we evaluated the occurrence of *Mycoplasma bovis, Mycoplasma dispar,* and *Mycoplasma mycoides* subsp. *mycoides SC*. in tracheal wash samples of healthy and BRD cattle in association with clinical signs of BRD. Our results indicated that *M. dispar* was common in BRD animals, confirming its importance as a pathogen of BRD. Association between *Mollicutes* and some clinical signs of respiratory diseases was detected.

Colonies were obtained from 1 sample only, unlike the high isolation rates of *Mycoplasma* spp. described elsewhere.[5,15,25,26] *Mycoplasma* spp. are well-known as fastidious and slow-growing bacteria for which isolation takes an extended time.[27] Polymerase chain reaction is a quick and sensitive test that can detect nucleic acid from only 1 microorganism when it is used to detect *Mollicutes*.[21] Often, culture negative samples are positive for molecular detection, as observed in our study.

Mollicutes was increased in the BRD group ($P = .035$). Similarly, *Mollicutes* have been reported frequently in more BRD calves (90.96%; 53%) compared to healthy calves (52.05%; 23%).[27,28] *Mollicutes* are well characterized as part of the bovine respiratory tract microbiota[27,29] but several species have been described as etiologic agents of respiratory diseases.[30]

M. dispar was increased in BRD cattle ($P = .036$). Our data are in agreement with a previous study,[31] which also described the increased occurrence of *M. dispar* in both healthy and BRD groups, especially in the latter group. Two other studies also detected a high occurrence of *M. dispar* in BRD cattle compared to healthy cattle.[15,27] *Mycoplasma mycoides* subsp. *mycoides* SC is the etiologic agent of contagious bovine pleuropneumonia, and it is considered the most important mycoplasma species related to BRD.[10] In our study, this species was not detected, and this result is in accordance with other Brazilian studies.[27,31] *M. bovis* is another important mycoplasma related to BRD.[29,32] In

Table 3. *Mollicutes* associated with clinical signs of bovine respiratory disease in the state of São Paulo, Brazil.

	Mollicutes			
Clinical Sign	Absent (%)	Present (%)	OR	*P*-value
Behavior				
Alert	75	62.5	1.800 (0.590–5.491)	.299
Lethargic	25	37.5		
Mucosas				
Pink	96.4	81	6.231 (0.701–55.364)	.109
Pale	3.6	19		
Heart Rate				
<100 bpm	75	53	2.647 (0.880–7.966)	.079
>100 bpm	25	47		
Respiratory Rate				
<40 breaths/min	78.6	62.5	2.200 (0.695–6.962)	.175
>40 breaths/min	21.4	37.5		
Rectal Temperature				
<39.5°C	85.7	67	2.727 (0.746–9.966)	.140
>39.5°C	14.3	31		
Purulent Nasal discharge				
Absence	93	87.5	1.857 (0.313–11.005)	.675
Presence	7	12.5		
Serous nasal discharge				
Absence	96.4	94	1.800 (0.154–20.987)	1.000
Presence	3.6	6		
Mucous nasal discharge				
Absence	78.6	72	1.435 (0.438–4.702)	.550
Presence	21.4	28		
Productive cough				
Absence	75	75	1.000 (0.310–3.226)	1.000
Presence	25	25		
Nonproductive cough				
Absence	96.4	78	7.560 (0.868–65.866)	.057
Presence	3.6	22		
Mixed dyspnea				
Absence	85.7	72	2.348 (0.634–8.695)	.226
Presence	14.3	28		
Expiratory dyspnea				
Absence	82	90.6	0.476 (0.103–2.203)	.454
Presence	18	9.4		
Inspiratory dyspnea				
Absence	96.4	94	1.800 (0.154–20.987)	1.000
Presence	3.6	6		
Crackles				
Absence	64.3	53	1.588 (0.562–4.489)	.382
Presence	35.7	47		
Snoring				
Absence	78.6	65.6	1.921 (0.197–1.379)	.267
Presence	21.4	34.4		
Whistling				
Absence	93	81	3.000 (0.553–16.260)	.187
Presence	7	19		

Table 4. *M. dispar* associated with clinical signs of bovine respiratory disease in the state of São Paulo, Brazil.

	M. dispar			
Clinical Sign	Absent (%)	Present (%)	OR	*P*-value
Behavior				
Alert	64.3	41.7	2.520 (0.516–12.296)	.249
Lethargic	35.7	58.3		
Mucosas				
Pink	78.6	75	1.222 (0.197–7.594)	1.000
Pale	21.4	25		
Hear Rate				
<100 bpm	64.3	25	5.400 (0.983–29.668)	.062
>100 bpm	35.7	75		
Respiratory Rate				
<40 breaths/min	78.6	33.3	7.333 (1.272–42.294)	.045
>40 breaths/min	21.4	66.7		
Rectal Temperature				
<39.5°C	78.6	50	3.667 (0.666–20.191)	.218
>39.5°C	21.4	150		
Purulent Nasal discharge				
Absence	93	75	4.333 (0.386–48.610)	.306
Presence	07	25		
Serous nasal discharge				
Absence	85.7	100	–	.483
Presence	14.3	0		
Mucous nasal discharge				
Absence	71.4	58.3	1.786 (0.349–9.127)	.683
Presence	28.6	41.7		
Productive cough				
Absence	85.7	50	6.000 (0.919–39.185)	.090
Presence	14.3	50		
Nonproductive cough				
Absence	78.6	75	1.222 (0.197–7.594)	1.000
Presence	21.4	25		
Mixed dyspnea				
Absence	93	33.3	26.000 (2.451–275.826)	.003
Presence	07	66.7		
Expiratory dyspnea				
Absence	85.7	91.7	0.545 (0.043–6.889)	1.000
Presence	14.3	08.3		
Inspiratory dyspnea				
Absence	85.7	100	–	.483
Presence	14.3	00		
Crackles				
Absence	64.3	25	5.400 (0.983–29.668)	.062
Presence	35.7	75		
Snoring				
Absence	65.3	50	1.800 (0.373–8.681)	.462
Presence	35.7	50		
Whistling				
Absence	78.6	75	1.222 (0.197–7.594)	1.000
Presence	21.4	25		

our study, however, this bacterium was detected in 1 BRD calf only. Similar results were obtained in another study.[33]

Undetermined mycoplasma species were observed in both groups. *Ureaplasma diversum*, *Acholeplasma* spp. and other mycoplasma species such as *M. bovirhinis, M. alkalensis,* and *M. arginini* have been detected in the bovine respiratory tract.[15,16,25–27] The genus *Mycoplasma* has several species, and culture-independent techniques are indispensable to determine all species present in the respiratory tract.

Regarding clinical signs of BRD, our data identified *Mollicutes* and *M. dispar* associated with respiratory problems. Our results establish association but not necessarily causation. Another study found that the presence of a clinical sign of BRD (stony dull sound on percussion of the thorax) was related to the absence of *Mollicutes*.[31] Regarding *M. dispar,* our data indicated an association between this bacterium and tachypnea and mixed dyspnea. In an experimental infection with *M. dispar* in calves, most calves showed no clinical signs of BRD.[34] However, only 1 calf showed persistent

nonproductive cough and dyspnea, besides increased respiratory rate and fever. Recently, another study indicated that coarse crackles and whistling were associated with the absence of *M. dispar*.[31] *Mycoplasma dispar* is regularly isolated from bovine pneumonic lungs, but its presence has been associated with mild infection.[30,34] Discrepancies in results allow researchers to continue studying these microorganisms to better understand the importance of mycoplasmas in the development of clinical signs of BRD. In addition, it is important to note that other microorganisms could contribute to BRD.

Conclusion

Our study confirmed the importance of mycoplasmas as etiologic agents of BRD. Although *M. dispar* has been detected in healthy cattle, the increased occurrence of this bacterium and the detection of *M. bovis* in BRD calves confirm their roles in the pathogenesis of BRD. The increased frequency of undetermined mycoplasma species in samples indicates the complexity of the respiratory tract microbiome and the possible role of other mycoplasmas in BRD. This new information about the association between some clinical signs of BRD and *Mycoplasma* spp. infection will be useful in the presumptive identification of the microorganisms involved in BRD infection.

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Conflict of Interest Declaration: Authors declare no conflict of interest

Off-label Antimicrobial declaration: Authors declare no off-label use of antimicrobials

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