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Bacterial pathogens of the lower respiratory tract of calves from Brazilian rural settlement herds and their association with clinical signs of bovine respiratory disease[1]

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ABSTRACT.- Gaeta N.C., Ribeiro B.L.M., Alemán M.A.R., Yoshihara E., Nassar A.F.C., Marques L.M., Timenetsky J. & Gregory L. 2018. **Bacterial pathogens of the lower respiratory tract of calves from Brazilian rural settlement herds and their association with clinical signs of bovine respiratory disease.** *Pesquisa Veterinária Brasileira 38(3):374-381.* Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Prof. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: lgregory@usp.br

Bovine respiratory disease (BRD) is considered the major cause of economic losses in dairy and beef cattle production. The study aimed to detect the most important bacteria related to respiratory disease in tracheobronchial fluid samples of healthy and dairy calves with clinical signs of BRD in Brazilian rural settlements. Hundred and forty-one mongrel dairy calves were randomly selected from 42 family farm dairy herds from Brazilian settlements. Physical examination was performed and calves were classified as healthy (n=100) and BRD (n=41). Tracheobronchial fluid samples were collected. Isolation and molecular detection of *Mycoplasma dispar*, *M. bovis* and *M. mycoides* subsp. *mycoides* SC besides isolation of other aerobic bacteria were performed. Abnormal lung sounds (crackle/snoring/whistle), mucopurulent/purulent nasal discharge, body temperature >39.5°C and respiratory rate >40 breaths/min were higher in BRD calves compared to healthy calves (P<0.05). *Bacillus* sp., *Staphylococcus intermedius* and non-fermentative Gram-negative were the most prevalent bacteria isolated. Non-identified species from Enterobacteriaceae family was higher in BRD calves compared to healthy calves (P<0.05). *Mollicutes* were isolated in 7.4% of samples and only *M. dispar* was detected. *Mollicutes* was associated with purulent/mucopurulent nasal discharge (P=0.017). *Pantoea agglomerans* was associated to tachypnea (P=0.020), and *Streptococcus* spp. was associated with hyperthermia. Statistical tendencies were observed to *M. dispar* and tachypnea (P=0.066), and *P. agglomerans* and tachycardia (P=0.066). The obtained results describe the microorganisms found in tracheobronchial fluid of calves with BRD in some herds of Brazilian family farming and their relation to clinical signs of BRD.

INDEX TERMS: Bacterial pathogens, respiratory tract, calves, Brazil, rural settlement, bovine respiratory disease, BRD, *Mycoplasma* spp., aerobic bacteria, cattle, clinics.

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RESUMO.- [**Patógenos bacterianos do trato respiratório inferior de bezerros criados em rebanhos de assentamentos brasileiros e sua associação com os sinais clínicos da doença respiratória dos bovinos.**] A doença respiratória dos bovinos (DRB) é considerada a principal causa de perdas econômicas nas produções de leite e carne. O objetivo deste estudo foi detectar as mais importantes bactérias relacionadas a doença respiratória presentes em amostras de lavado traqueobrônquico de bezerros sadios e com sinais clínicos da DRB de assentamentos brasileiros. Cento e quarenta e um bezerros leiteiros sem raça definida foram randomicamente selecionados de 42 rebanhos leiteiros de assentamentos brasileiros. Exame físico foi realizado e os animais foram classificados em sadios (n=100) e com DRB (n=41). Amostras de lavado traqueobrônquico foram coletadas. Foram realizados o isolamento e a detecção molecular de *Mycoplasma dispar*, *M. bovis* e *M. mycoides* subsp. *mycoides* SC além de isolamento de outras bactérias aeróbias. Ruídos pulmonares anormais (crepitação/ ronco/sibilo), secreção nasal mucopurulenta/ purulenta, temperatura corporal >39.5°C e frequência respiratória >40 movimentos respiratórios/min foram observados com maior frequência em bezerros com DRB comparado aos animais sadios (P<0.05). *Bacillus* sp, *Staphylococcus intermedius* e bactérias Gram-negativas não fermentadoras foram as bactérias mais prevalentes. Bactérias da família Enterobacteriaceae cuja espécie não fora identificada foram mais frequentes em bezerros com DRB comparado aos bezerros sadios (P<0.05). *Mollicutes* foram isolados em 7,4% das amostras e somente *M. dispar* foi detectado. *Mollicutes* foi associado à secreção nasal purulenta/mucopurulenta (P=0.017). *Pantoea agglomerans* foi associada a taquipneia (P=0.020), e *Streptococcus* spp. Foi associado a hipertermia. Tendência estatística foi observada para *M. dispar* e taquipneia (P=0.066), e *P. agglomerans* e taquicardia (P=0.066). Os resultados obtidos descrevem os micro-organismos encontrados no lavado traqueobrônquico de bezerros com DRB em rebanhos de agricultura familiar brasileira e sua relação com as manifestações clínicas da DRB.

TERMOS DE INDEXAÇÃO: Patógenos bacterianos, trato respiratório inferior, bezerros, assentamento, doença respiratória dos bovinos, *Mycoplasma* spp., bactéria aeróbica, bovinos, clínica.

INTRODUCTION

Brazilian rural settlements are composed of small milk producers, which supply local dairies. They are important to local milk industry and social development, generating jobs in Brazilian rural area. However, this production is usually characterized as extensive, with mongrel dairy cattle, inadequate environment and sanitary management (Lima 2010).

Bovine respiratory disease (BRD) is considered the major cause of economic losses in dairy and beef cattle production due to its high morbidity and mortality rates (Griffin 1997, Miles 2009, Hilton 2014), especially in less technology farms, such as family farms. BRD is the second major cause of losses in calf raising (Panciera & Confer 2010). Opportunistic bacteria are factors for the development of BRD (Caswell & Archambault 2007, Angen et al. 2009, Griffin et al. 2010, Holman et al. 2015). Stress conditions favor the immune response decay and the development of some bacteria in respiratory tract may cause a respiratory infection. (Panciera & Confer 2010).

Pasteurella multocida, *Mannheimia haemolytica* and *Mycoplasma bovis,* are the major bacterial pathogens of BRD (Caswell & Archambault 2007, Dabo et al. 2007, Rice et al. 2007, Griffin et al. 2010). *Mycoplasma mycoides* subsp. *mycoides* SC is also an important microbe due to its role in Contagious Bovine Pleuropneumonia (OIE 2014), as well as *M. dispar* which is standing out as an important pathogen of BRD (Thomas et al. 2002, Marques et al. 2007, Angen et al. 2009, Šiugždaitė et al. 2015, Oliveira et al. 2016). In Brazil, there are few studies for the bacterial components of respiratory tract of healthy and BRD cattle (Gonçalves 1987, Barros et al. 1994, Benesi et al. 2013, Oliveira et al. 2016). In addition, there is a lack of studies of microbes in the respiratory tract of calves of small producers such as those from family farms.

Animals diagnosed with BRD often show depression signals, weight loss, cough, mucopurulent or purulent nasal discharge, fever, increased respiratory rate, and abnormal pulmonary sound in auscultation (Radostits 2002, Dabo et al. 2007, Griffin et al. 2010). Because the similarities of clinical signs and variation of possible bacteria the presumptive diagnosis after physical examination remains difficult.

Because of the low knowledge of bacterial agents and microbial diagnosis for bovine respiratory disease in Brazilian rural settlements, the physical examination is the only way to help this activity. Thus, the aim of this study was to detect the most important bacteria related to respiratory disease in tracheobronchial fluid samples of healthy and dairy calves with clinical signs of BRD in Brazilian rural settlements.

MATERIALS AND METHODS

Ethical statement. The present study was conducted at the "Laboratory of General Bacteriology" from Biological Institute, and at the Laboratory of Mycoplasmas and at the School of Veterinary Medicine and Animal Science, from University of São Paulo, Brazil. Samples were collected from August 2014 until March 2015. All procedures were carried out in agreement with the guidelines of the Committee of Ethics on Animal Use (Protocol number: 7973040214).

Area characterization and case definition. Pontal do Paranapanema is located at the extreme west region of the state of São Paulo, Brazil. The study was carried out at Caiuá, Presidente Epitácio and Mirante do Paranapanema, important cities from Pontal do Paranapanema. Hundred and forty-one bovine males and females were studied. The animals aged from one to twelve months, were mongrel dairy calves and randomly selected from 42 rural settlement dairy herds. Calves received colostrum directly from their mothers as confirmed by the owners. After weaning, calves received a diet based on pasture and mineral salt.

Physical examination was performed in all randomly selected calves. Heart and respiratory rates, hydration level, color of mucous and specific physical examination to evaluate respiratory tract were included. Calves that showed at least two of the following parameters were considered unhealthy: mucopurulent or purulent nasal discharge, cough, crackle, snoring, respiratory rate above 40 breaths per minute and rectal temperature above 39,5°C (Benesi et al. 2013, Lima et al. 2016, Gaeta et al. 2017). Two experienced veterinarians performed the physical examination in all calves, that were classified as healthy (n=100) and calves showing clinical signs of bovine respiratory disease (n=41).

Clinical sample collection and microbiology identification. Tracheobronchial fluid samples were collected after antisepsis of the trachea. An Intracath® (BD, New Jersey, USA) was introduced by traqueocentesis, and 20mL of sterile saline 0.9% were instilled, recovering 1mL-5mL. An aliquote was added to a cryogenic tube with a transport solution for *Mycoplasma* spp. and glycerol, and stored in liquid nitrogen. Another aliquote was added to Brain Heart Infusion medium and stored at -4°C until further analysis.

Mycoplasma spp. culture and isolation was performed in SP-4 broth and agar (Tully 1995). Plates were incubated in aerobiosis at 37°C for fifteen days. The agar plates were daily observed for the production of "fried-egg" colonies. In broth, the glucose fermentation or arginine hydrolysis and the lack of turbidity, were confirmed. Molecular detection of *Mycoplasma* spp. was performed using a sub-culture in broth and the clinical samples. The DNA extraction followed the method described by Fan et al. (1995). Polymerase chain reaction (PCR) was initially performed to detect *Mollicutes* (Van Kuppeveld et al. 1992). Then the positive samples were used to detect *M. bovis* (Chávez González et al. 1995), *M. dispar* (Marques et al. 2007), *M. mycoides* subsp. *mycoides* SC (Dedieu et al. 1994) and *Ureaplasma diversum* (Cardoso et al. 2002).

Regarding to samples in BHI medium, 10μL of this suspension were seeded on 5% sheep blood agar (Muller Hinton) and incubated for 48h at 37°C. The obtained colonies were gram stained and observed for hemolysis production. The colonies identification was performed for biochemical tests (Winn Jr et al. 2005).

Statistical analysis. All the statistical results were obtained on the Statistical Package of Social Science 16.0 (Chicago, NY) and a 95% confidence interval (CI). Descriptive analysis was performed to determine absolute and relative frequencies. Associations between categorical variables of health status (BRD and health), microorganisms (sheep blood agar bacteria and *Mollicutes*) and clinical signs (behavior, ocular mucosa, heart rate, respiratory rate, nasal discharge, cough, breathing pattern, percussion and auscultation) were analyzed by Pearson's chi-square test or Fisher's exact test in the form of univariate analysis (Hosmer & Lemeshow 1989). Microbiological findings were considered the independent variables. Healthy status (healthy and BRD calves) and clinical signs were considered dependent variables. Odds Ratio was also calculated. Variables with $P<0.05$ were considered significant. Variables with $0.05<P<0.10$ were considered statistical tendencies.

RESULTS

Physical examination

Physical examination results are presented in Table 1. All clinical signs were detected in healthy calves, except tachycardia (heart rate >100bpm) and cough. In BRD calves, lethargy was not detected. Abnormal lung sounds (crackle/snoring/whistle) ($P<0.001$), mucopurulent/purulent nasal discharge ($P=0.002$), body temperature >39.5°C ($P<0.001$) and respiratory rate >40 breaths/min ($P<0.001$) were higher in BRD calves compared to healthy calves.

Bacterial cultures in blood agar

Hundred and seventy-six bacterial isolates were obtained (77% Gram positive and 23% Gram negative). *Bacillus* sp. (56%), *Staphylococcus intermedius* (32.6%) and non-fermentative Gram-negative (9.2%) were the most prevalent bacteria isolated in samples of tracheobronchial fluid. Regarding to non-fermentative Gram-negative bacteria, coccobacilli positive for catalase and oxidase tests (38%; 05/13), bacilli positive for catalase and oxidase tests (31%; 04/13) and bacilli negative for catalase and oxidase tests (31%; 04/13) were detected. In lower prevalence, *P. agglomerans, Staphylococcus aureus, Streptococcus* sp., *Serratia rubidae, Proteus* spp., *Pseudomonas* spp., *Escherichia coli, Enterobacter gergoviae, Enterobacter aerogenes, Stenotrophomonas maltophilia, Enterobacter cloacae* and non-identified species from Enterobacteriaceae Family were detected (Table 1). *Pasteurella multocida* and *Mannheimia haemolytica* were not isolated.

Comparisons by the health status showed that the frequency of non-identified species from Enterobacteriaceae family was higher in BRD calves compared to healthy calves ($P=0.028$). *Bacillus* sp. was numerically higher in BRD calves (59.5%) compared to healthy (54.5%) calves ($P=0.586$). The frequency of *Staphylococcus intermedius* was numerically higher in BRD (38.1%) calves compared to healthy (30.3%) ones ($P=0.367$). Non-fermentative Gram-negative bacteria showed a numerical higher frequency in BRD (11.9%) compared to healthy calves (9.1%) ($P=0.473$). As the fourth most prevalent bacteria, the finding of *Pantoea agglomerans* was numerically higher in healthy calves (9.1%) compared to BRD calves (2.4%) ($P=0.281$) (Table 2). Fungal colonies were detected in healthy calves only (8.1%).

Pure cultures were observed in 77 samples. *Bacillus* spp. was the most prevalent (31.2%), followed by *S. intermedius* (6.4%), *P. agglomerans* (5.0%) and non-fermentative Gram-negative bacteria (4.3%) in BRD calves. Non-identified species from Enterobacteriaceae family was only, that were classified detected in BRD calves. On the other hand, *Serratia rubidae, Proteus* spp., *Pseudomonas* sp, *E. coli, E. aerogenes* and *S. maltophilia* were detected in healthy calves only (Table 3).

Cutures to *Mollicutes*

"Fried-egg" colonies were obtained in healthy (7.1%; 07/99) and BRD calves (7.1%; 03/42) calves. All colonies in SP4 agar were identified as *Mollicutes.* After specific PCR, mostly colonies did not have the species determined by the primers used. *M. dispar* was isolated in 1.4% of samples. *M. bovis* and *Mmm*SC were not isolated.

Regarding to the detection of *Mollicutes* in the direct material (tracheobronchial fluid samples) twenty-nine samples (20.6%) were positive for *Mollicutes* in healthy (20.2%; 20/99) and BRD (21.4%; 09/42) calves. Mostly samples of non-targeted molicutes were higher in BRD calves than in healthy calves ($P = 0.013$). Only *M. dispar* was detected (2.1%) by the primers used in this study.

Detected or isolated microorganisms and clinical signs of BRD

Comparison between the searched microorganisms and clinical signs of BRD revealed *Mollicutes* associated with purulent/mucopurulent nasal discharge ($P=0.017$) (Table 4). Regarding to the bacteria obtained in sheep blood agar plates, the absence of *P. agglomerans* was associated to tachypnea ($P=0.020$). On the other hand, the presence of *Streptococcus* spp. was associated with hyperthermia ($P=0.025$) (Table 4). Statistical tendencies were observed to *M. dispar* and tachypnea ($P=0.066$), and *P. agglomerans* and tachycardia ($P=0.066$).

Table 1. Clinical signs of healthy and bovine respiratory disease calves observed during the physical examination

Clinical signs	Healthy	BRD	Total	P-value
	% (N/T)	% (N/T)	% (N/T)	OR (95% CI)
Behavior				
Alert	97 (89/92)	100 (40/40)	98 (129/132)	0.553
Depression	03 (03/92)	00 (00/40)	02 (03/132)	(--)
Ocular mucosa				
Normal	86 (72/84)	69 (29/37)	83 (101/121)	0.317
Pale	14 (12/84)	19 (08/37)	14 (20/121)	1.655(0.613-4.468)
Heart rate				
<100 bpm	86 (39/96)	69 (15/42)	39 (38/138)	0.587
>100 bpm	14 (59/96)	19 (27/42)	69 (22/138)	1.232(0.581-2.610)
Respiratory rate				
<40 breaths/min	100 (90/90)	69 (21/42)	39 (111/132)	**P<0.001**
>40 breaths/min	00 (00/90)	19 (21/42)	62 (21/132)	(--)
Body temperature				
< 39.5 °C	99 (92/93)	49 (20/41)	84 (112/134)	**P<0.001**
> 39.5 °C	01 (01/93)	51 (21/41)	16 (22/134)	96.600(12.268-60.611)
Nasal discharge				
Normal	92 (89/97)	49 (28/39)	84 (117/136)	**0.002**
Purulent/Mucopurulent	08 (08/97)	51 (11/39)	16 (19/136)	4.371(1.600-11.938)
Cough				
Absent	100 (97/97)	95 (40/42)	99 (137/139)	0.090 (--) *
Present	00 (00/97)	05 (02/42)	01 (02/139)	
Breathing Pattern				
Costoabdominal	93 (83/89)	92 (33/36)	93 (116/125)	0.716
Costal/abdominal	07 (06/89)	08(03/36)	07 (09/125)	1.258(0.297-5.326)
Percussion				
Clear	85 (68/80)	79 (22/28)	83 (90/108)	0.432
Submassive/massive	15 (12/80)	21 (06/28)	17 (18/108)	1.545(0.519-4.604)
Auscultation				
Normal	38 (37/98)	05 (02/40)	28 (39/138)	**P<0.001**
Crackle/Snoring/Whistle	62 (61/98)	95 (38/40)	72 (99/138)	11.525(2.625-50.596)

* Statistical tendencies, $P<0.05$. Bold values mean statistical significances.

Table 2. Cultures in sheep blood agar of samples from tracheobronchial fluid samples of healthy and bovine respiratory disease calves according health status

Aerobic bacteria	Healthy % (Positive/total)	BRD % (Positive/total)	Total % (Positive/total)	OR (95% CI)	P-value
Bacillus spp	54.5 (54/99)	59.5 (25/42)	56.0 (79/141)	1.225 (0.589-2.549)	0.586
S. intermedius	30.3 (30/99)	38.1 (16/42)	32.6 (46/141)	1.415 (0.665-3.014)	0.367
NFGN	08.1 (08/99)	11.9 (05/42)	09.2 (13/141)	1.537 (0.472-5.007)	0.473
P. agglomerans	09.1 (09/99)	02.4 (01/42)	07.1 (10/141)	0.244 (0.030-1.989)	0.281
S. aureus	02.0 (02/99)	-- (--)	01.4 (02/141)	-- (--)	1.000
Streptococcus spp.	05.0 (05/99)	09.5 (04/42)	06.4 (09/141)	1.979 (0.504-7.770)	0.451
Serratia rubidae	02.0 (02/99)	-- (--)	01.4 (02/141)	-- (--)	1.000
Proteus spp.	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
Pseudomonas spp.	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
E. coli	03.0 (03/99)	02.4 (01/42)	02.8 (04/141)	0.780 (0.079-7.727)	1.000
E. gergoviae	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
E. aerogenes	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
S. maltophilia	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
E. cloacae	01.0 (01/99)	02.4 (01/42)	01.4 (02/141)	2.390 (0.146-39.137)	0.509
Enterobacteriaceae family	01.0 (01/99)	09.5 (04/42)	03.5 (05/141)	10.316 (1.117-95.274)	**0.028**

* Statistical tendencies, $P<0.05$. Bold values mean statistical significances.

Table 3. Pure cultures in sheep blood agar plates from tracheobronchial fluid samples of healthy and bovine respiratory disease dairy calves according health status

Aerobic bacteria	Healthy % (Positive/total)	BRD % (Positive/total)	Total % (Positive/total)	OR (95% CI)	P-value
Bacillus spp	32.3 (32/99)	28.6 (12/42)	31.2 (44/141)	0.838 (0.380-1.847)	0.660
S. intermedius	07.1 (07/99)	04.8 (02/42)	06.4 (09/141)	0.657 (0.131-3.303)	0.725
P. agglomerans	06.1 (06/99)	02.4 (01/42)	05.0 (07/141)	0.378 (0.044-3.241)	0.674
NFGN	05.1 (05/99)	02.4 (01/42)	04.3 (06/141)	0.459 (0.052-4.049)	0.669
Streptococcus spp.	02.0 (02/99)	02.4 (01/42)	2.1 (03/141)	1.183 (0.104-13.411)	1.000
Serratia rubidae	01.0 (01/99)	-- (--)	0.07 (01/141)	-- (--)	1.000
Proteus spp.	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
Pseudomonas spp.	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
E. coli	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
E. aerogenes	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
S. maltophilia	01.0 (01/99)	-- (--)	00.7 (01/141)	-- (--)	1.000
Enterobacteria	-- (--)	04.8 (02/42)	01.4 (02/141)	-- (--)	0.087*

* Statistical tendencies, $P<0.05$. Bold values mean statistical significances.

Table 4. Microorganisms detected in the lower respiratory tract of calves associated with clinical signs of bovine respiratory disease

Clinical sign	*Mollicutes*		OR (95% CI)	*P*-value
	Absent % (N)	Present % (N)		
Nasal discharge				
Normal	89.7 (96/107)	72.4 (21/29)	3.325 (1.192-9.274)	**0.017**
Purulent/Mucopurulent	**10.3 (11/107)**	27.6 (08/29)		
Clinical sign	*M. dispar*		OR (95% CI)	P-value
	Absent % (N)	Present % (N)		
Respiratory Rate				
<40 breaths/min	85.3 (110/129)	33.3 (01/03)	11.579 (1.000-134.093)	0.066*
>40 breaths/min	14.7 (19/129)	66.7 (02/03)		
Clinical sign	*P. agglomerans*		OR (95% CI)	P-value
	Absent % (N)	Present % (N)		
Heart Rate				
<100bpm	25 (08/32)	66.7 (04/06)	0.167 (0.026-1.088)	0.066*
>100bpm	75 (24/32)	33.3 (02/06)		
Respiratory Rate				
<40 breaths/min	40.6 (13/32)	100 (06/06)	--(--)	**0.020**
>40 breaths/min	59.4 (19/32)	00 (00/06)		
Clinical sign	*Streptococcus* spp.		OR (95% CI)	P-value
	Absent % (N)	Present % (N)		
Rectal Temperature				
<39.5 °C	85.7 (108/126)	50 (04/08)	6.000 (1.375-26.174)	**0.025**
>39.5 °C	14.3 (18/126)	50 (04/08)		

* Statistical tendencies, $P<0.05$. Bold values mean statistical significances.

DISCUSSION

The association between the studied bacteria and clinical signs of BRD in family farms with limited care was evaluated. The finding of *Mycoplasma dispar* confirms in part its regular presence as microbiota in respiratory tract. However the detection of this molicute in BRD may also confirm the opportunist role of some *Mollicutes* to cause a disease (Tegtmeier et al. 1999). In addition, our results suggest the potential role of other mycoplasma besides *M. bovis, Mmm*SC and *M. dispar* in the development of BRD. *Bacillus* spp., *Staphylococcus intermedius*, non-fermentative Gram-negative bacteria, and *Pantoea agglomerans* were the most isolated regular bacteria. It was also detected a relation between of some clinical signs of BRD and the finding of *Mollicutes*, *M. dispar, P. agglomerans* and *Streptococcus* spp.

Tachypnea, hyperthermia, mucopurulent/purulent nasal discharge and auscultation were more intense in BRD calves ($P<0.05$). These observations are in agreement with the mention of clinical diagnosis of BRD (Radostits 2002, Dabo et al. 2007, Griffin et al. 2010) and show again the importance of this procedure.

P. multocida and *M. haemolytica* are important pathogens of BRD (Griffin 2010, Griffin et al. 2010), but in the present study, these microorganisms were not isolated. Similar results

were obtained by Benesi et al. (2013). However these species were isolated in other studies (Härtel et al. 2004, Autio et al. 2007, Angen et al. 2009, Oliveira et al. 2016). The high occurrence of *Bacillus* spp. in the studied samples may justify their inhibition of other bacteria due to the secretion of bacteriocins (Cherif et al. 2001, Shelburne et al. 2007). In fact, *Bacillus* spp. inhibit the growth of *P. multocida, M. haemolytica* and *H. somni* (Xie et al. (2009). Other hypothesis is the faster growth of opportunistic bacteria such as *Bacillus* sp., which prevent the growth of *M. haemolytica* and *P. multocida. Bacillus* sp., *S. intermedius*, and non-fermentative Gram-negative were highly isolated from BRD calves. The sheep blood agar also allowed the isolation of *Streptococcus* spp., *E. coli,* and *Pseudomonas* spp. from tracheobronchial fluid samples. Moreover, non-identified species from Enterobacteriaceae family isolates were recovered more frequently from BRD calves ($P<0.05$) compared to the healthy calves. Similar results were obtained in two Brazilian studies. Benesi et al. (2013) detected *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *P. aeruginosa* and enterobacteria. Evaluating calves from an intensive production type, Oliveira et al. (2016) also detected *Bacillus* spp., *Staphylococcus* spp., *P. aeruginosa* and *E. coli,* but in lower prevalence. In other countries, Elshafee (2003) detected *Staphylococcus* spp., *Bacillus* spp., *Enterobacter* spp., *Escherichia* spp., *Pseudomonas* spp. and *Serratia* spp. in bovine pneumonic lungs. Many of mentioned microorganisms are present in the environment and could be inhaled by calves and detected in both upper and lower respiratory tracts. In particular situations, *Bacillus cereus* (Miller et al. 1997), and *S. intermedius* (Gerstadt et al. 1999) were responsible for human pneumonia. Non-identified species from Enterobacteriaceae family obtained in pure cultures, herein, were recovered from BRD calves ($P<0.05$). In fact, the species are not considered important pathogens related to BRD (Loneragan et al. 2001, Griffin et al. 2010), however their opportunistic role should be considered.

Mollicutes were isolated in BRD and healthy calves. These microorganisms were also detected by PCR in 20.6% of the direct material (tracheobronchial fluid), and this result were lower in percentage compared to those reported by Oliveira et al. (2016) and Marques et al. (2007). Only *M. dispar* was detected in samples. Oliveira et al. (2016) reported a higher frequency of *M. dispar* in trachoebronchial lavage samples, particularly in healthy calves. However, Autio et al. (2007), Marques et al. (2007), and Angen et al. (2009) reported higher prevalence in BRD animals. *M. bovis* is a well-known pathogen related to BRD (Griffin et al. 2010) that was not detected in this study. Same results were observed by Nikunen et al. (2007) and Angen et al. (2009). *M. mycoides* subsp. *mycoides* SC were not detected in this study and is in agreement with other Brazilian researches (Marques et al. 2007, Oliveira et al. 2016). The targeted *Mollicutes* such as *M. bovis* and *M. mycoides* subsp. *mycoides* SC, herein, to BRD with used primers were not detected. This may suggest that other mycoplasmas may have a role to BRD. *M. bovirhinis* (Angen et al. 2009), *Acholeplasma* spp. (Zinka & Maid 2012), *M. alkalensis* and *M. arginini* (Thomas et al. 1986) were species that have been described in both upper and lower respiratory tract of cattle

The present data revealed relations between microorganisms and clinical signs of BRD. *Mollicutes* microorganisms were associated with mucopurulent/purulent nasal discharge. Maeda et al. (2003) reported an association between *M. bovis* and a non-characterized nasal discharge. Griffin et al. (2010) referred *M. bovis* pneumonia with or without nasal discharge. Oliveira et al. (2016) reported the association between submassive sound on acoustic percussion of the thorax and the absence of *Mollicutes*. In the present research, important clinical signs of BRD, such as tachypnea and hyperthermia were associated with *P. agglomerans* and *Streptococcus* spp., while both microorganisms were described as the etiologic agent of human pneumonia (Kays et al. 2002, Shubov et al. 2011). Statistical tendency was observed between *M. dispar* and tachypnea. In an experimental infection of *M. dispar* in calves, Ribeiro (1979) reported that only one calf showed clinical signs of BRD and that *M. dispar* pneumonia might be a mild infection.

CONCLUSIONS

The obtained results described the microorganisms detected in tracheobronchial fluid of healthy and calve with BRD in Brazilian rural settlements. *Bacillus* sp., *Staphylococcus intermedius* and non-fermentative Gram-negative bacteria were the most prevalent.

Besides, important bacteria such as *Pasteurella multocida*, *Mannheimia haemolytica* and *Mycoplasma bovis* were not detected. *Mycoplasma dispar* was found out, but mollicutes that did not have the species confirmed were more prevalent, suggesting the potential role of other species in BRD.

In addition, the association between clinical signs and microorganisms, such as *Mollicutes* X purulent/mucopurulent nasal discharge, could help clinicians during the diagnosis of the etiological agent of BRD.

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