Determination of bacterial aetiologic factor on tracheobronchial lavage in relation to clinical signs of bovine respiratory disease

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This study aimed to determine the occurrence of *Mannheimia haemolytica*, *Pasteurella multocida* and *Mycoplasma* spp., in relation to clinical signs of respiratory disease. Tracheobronchial lavage samples were collected from 96 (healthy and unhealthy) cattle in the State of São Paulo, Brazil. *Mycoplasma* spp. (12.5 %) and *Pasteurella multocida* (15.50 %) were the most prevalent species. *Bacillus* sp., *Staphylococcus* sp., *Escherichia coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were also isolated. *Mollicutes* (70.83 %), *Mycoplasma bovis* (2.94 %) and *Mycoplasma dispar* (38.23 %) were identified using conventional PCR. Submassive sound on acoustic percussion of the thorax was associated with the absence of *Mollicutes* (*P*=0.025). Whistling (*P*=0.076) and coarse crackle (*P*=0.046) were associated with the absence of *Mycoplasma dispar*. Clear sound on acoustic percussion of the thorax was associated with the absence of *Mycoplasma bovis* (*P*=0.007). Coughing was associated with the presence of *Pasteurellamultocida*[*P*=0.035; confidence interval (CI), 1.12–26.89], but its absence was associated with mucopurulent (*P*=0.0215; CI, 1.55–34.5) and mucoid nasal discharge (*P*=0.068; CI, 19–28.5), submassive sound (*P*=0.031; CI, 1.23–75.5), fine crackle (*P*=0.058; CI, 1.23–20.1) and coarse crackle (*P*=0.046; CI, 2.38–70.8). The high prevalence of *Pasteurella multocida* and *Mycoplasma* spp. in unhealthy calves increases the importance of these micro-organisms in the pathogenesis of respiratory diseases. This study increases the information about the role of *Mycoplasma dispar* in respiratory diseases. Differences in some species in relation to clinical signs can be applied as a presumptive diagnosis.

INTRODUCTION

Bronchopneumonia belongs to bovine respiratory disease complex, and is responsible for economic losses (Miles, 2009; USDA, 2013) due to its high morbidity and mortality rates (Nanduri *et al.*, 2015). It is associated with several

Abbreviations: CI, confidence interval; MmmSC, *Mycoplasma mycoides* subsp. *mycoides* SC.

pathogens, although the majorly reported are *Mannheimia haemolytica*, *Pasteurella multocida* and *Mycoplasma* spp. (Holman *et al.*, 2015), particularly *Mycoplasma bovis* (Aebi *et al.*, 2015), *Mycoplasma dispar* (Marques *et al.*, 2007a, b; Angen *et al.*, 2009; Šiugždaitė *et al.*, 2015) and *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) (Séry *et al.*, 2014). Since these bacteria colonize the respiratory tract of healthy and sick animals, these species are considered opportunistic micro-organisms (de Jong *et al.*, 2014). Changes in respiratory mucosa by viral infections favours bacterial colonization and proliferation, creating secondary infections (Horwood *et al.*, 2014). Literature often highlights infection associated with viruses and bacteria (Griffin *et al.*, 2010; Moore *et al.*, 2015) and with more than one bacteria (Angen *et al.*, 2009; Griffin *et al.*, 2010; Soehnlen *et al.*, 2012).

Determining the aetiology of bronchopneumonia is a challenge to the clinicians. A quick and precise diagnostic method is necessary to prevent bronchopneumonia dissemination in the herd. Animals with respiratory diseases often have depression, anorexia, fever, coughing, increased respiratory and cardiac rates, abnormal respiratory noise and bilateral nasal discharge (Radostits *et al.*, 2007; Benesi *et al.*, 2013). There are few studies that evaluated the respiratory microbiota of healthy calves and calves with respiratory disease in Brazil (Gonçalves, 1987; Barros *et al.*, 1994; Benesi *et al.*, 2013).

Therefore, the aim of this study was to investigate the bacteria involved in bronchopneumonia in relation to clinical signs of calves raised in the State of São Paulo, Brazil.

METHODS

A cross-sectional study was conducted between August and November 2014, using 96 male and female, 1 to 24 months of age, dairy cattle from semi-intensive production farms in Bragança Paulista city and Mococa city, State of São Paulo, Brazil. All procedures were carried out in agreement with the guidelines of the Ethical Principles in Animal Research adopted by the Ethics Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of the University of São Paulo (protocol no. 2929/2013).

In Bragança Paulista, calves were removed from the maternity pen and placed into a newborn pen bedded with slatted floors. Calves were allocated in one pen (approximately 60 calves) and remained in the same pen until fully weaned (approximately 120 days). All calves received colostrum immediately after birth (4 litres) and milk two times a day (4 litres each) during 4 months. Grains were immediately available on the first day of life. After weaning, calves were moved to another pen and received a grass–grain-based diet. In Mococa, calves remained with cows after birth in pasture. A farm employee confirmed that all calves received colostrums from their mothers. After weaning, calves received grass-based diet during summer and grass–silage-based diet during winter.

Physical examination was performed according to Rosenberger (1993) in order to evaluate respiratory and heart rates, temperature, mucosa hydration (hydrated, low dehydration, medium dehydration, high dehydration); colour of the mucosa (pink, pale, reddish, yellowish, bluish); nasal discharge features (serous, mucopurulent, purulent or reddish); presence of nasal lesions; coughing, respiratory pattern (thoracoabdominal, thoracic or abdominal); noise after rib cage percussion (clear, submassive or massive) and pulmonary noise on auscultation (no alterations, fine crackle, coarse crackle, snoring and whistling).

After physical examination, animals were divided into healthy calves and calves with respiratory diseases, considering sick those that presented with at least two of the following parameters: temperature higher than 39.5 °C, mucopurulent or purulent nasal discharge, coughing, crackles on pulmonary auscultation and at least 40 breaths (Benesi *et al.*, 2013).

Ninety-six tracheobronchial washing samples were collected after trichotomy and antisepsis of the trachea. An Intracath® (BD) was introduced by tracheocentesis, and 20 ml of sterile saline 0.9 % was instilled, recovering up to 5 ml. A fraction was added to a specific transport media for *Mycoplasma* spp. containing glycerol and stored in nitrogen. Another fraction was added to Stuart transport media (Laborclin) to isolate other aerobic bacteria and stored at 4 °C.

Mycoplasma spp. isolation was performed using SP-4 media (Tully, 1995). Plates were incubated at 37 °C for 15 days, and they were analysed using optical microscope on a daily basis. Plates with 'fried-egg'-like colonies, as well as arginine hydrolysis and/or glucose fermentation, were considered positive. PCR was accomplished to confirm the presence of *Mollicutes* in all samples according to van Kuppeveld *et al.* (1992). Positive samples in the first PCR were tested for *Mycoplasma dispar*, *Mycoplasma bovis* and MmmSC, according to Marques *et al.* (2007b), Chávez González *et al.* (1995) and Dedieu *et al.* (1994), respectively.

Isolation and identification of aerobic bacteria were performed by plating 10 µl of each sample in blood agar plates, incubating them at 37 °C for 48 h. Species were identified after colony analysis and biochemical tests. In particular, *Pasteurella multocida* and *Mannheimia haemolytica* isolation was performed according to Viana *et al.* (2007).

Association between bronchopneumonia and bacteria, as well as bacteria and clinical signs, was studied using chi-square test. Significant variables were selected using multivariate logistic regression by forward method. Statistical Package for Social Sciences 19.0 (IBM) was used to perform all statistical tests. Independent variables with statistical significance lower or equal to 10 % were selected to the model.

RESULTS

Mycoplasma spp. were isolated in 12.5 % samples (12/96). After specific PCR, *Mycoplasma dispar* (16.7 %), *Mycoplasma bovis* (25 %) and both species (1.3 %) were identified. MmmSC was not found. PCR was performed using all samples. The presence of *Mollicutes* was confirmed in 70.83 % (68/96) of samples from which 69 % (47/68) are calves with respiratory diseases and 31 % (21/68) are healthy calves. After specific PCR, *Mycoplasma dispar* was the most identified species, followed by *Mycoplasma bovis*. MmmSC was not detected. *Mycoplasma* spp. could not be identified in 56 % of samples with the primers used (Table 1). Co-infection between *Mycoplasma dispar* and *Mycoplasma bovis* was observed in only one healthy calf.

Mannheimia haemolytica was not isolated. *Pasteurella multocida* was identified in five healthy calves and six calves with bronchopneumonia (11/71; 15.50 %). Besides *Pasteurella multocida*, *Staphylococcus* sp., *Bacillus* sp., *Escherichia coli*, *Klebsiella oxytoca* and *Pseudomonas aeruginosa* were isolated. No growth was observed in 33.82 % (23/68) (Table 2).

Pasteurella multocida was associated with bronchopneumonia (P=0.076). Friesian Holstein calves were more susceptible to

Table 1. Occurrence of *Mycoplasma dispar*, *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides* by PCR of tracheobronchial washing, according to health status, State of São Paulo, Brazil, 2014

Micro-organisms	Healthy	With bronchopneumonia	Total
Mycoplasma dispar	18/68 (26.47 %)	8/68 (11.76 %)	26/68 (38.24 %)
Mycoplasma bovis	1/68 (1.40 %)	1/68 (1.40 %)	2/68 (2.84 %)
MmmSC	0/68 (0.0 %)	0/68 (0.0 %)	0/68 (0.0 %)
No identified species	28/68 (27.94 %)	12/68 (13.23 %)	41/68 (29.41 %)
Total	47/68 (45.59 %)	21/68 (26.47 %)	68/68 (100.0 %)

bronchopneumonia than mixed breeding calves (P=0.095). Although any association between age and respiratory disease was not observed (P=0.138), the first variable was related to the presence of *Mollicutes* (P=0.0177), demonstrating that younger calves were more susceptible to the micro-organism (Table 3).

Because of the absence of some information in the medical records, it was not possible to include all calves in the study of the association between clinical signs and micro-organisms. Multivariate analysis showed an association between *Mollicutes* and submassive sound during rib cage percussion (P=0.025). *Mycoplasma dispar* was associated with whistling (P=0.076) and coarse crackle (P=0.046). The absence of *Mycoplasma bovis* was associated with clear sound in rib cage percussion (P=0.007). Logistic regression was also used to study the association between *Pasteurella multocida* and clinical signs. While its presence was associated with coughing, its absence was associated with mucoid and mucopurulent nasal discharge, as well as submassive sound, fine crackle and coarse crackle during pulmonary auscultation (Table 4).

DISCUSSION

To understand the importance of some bacteria in bovine respiratory diseases in Brazil, the present study aimed to estimate the occurrence of *Pasteurella multocida*, *Mannheimia haemolytica* and *Mycoplasma* spp. in tracheobronchial lavage samples of healthy calves and calves with respiratory diseases in relation to clinical signs of bovine respiratory disease. Our data revealed that *Pasteurella multocida*, Mycoplasma bovis and *Mycoplasma dispar*, species well known to be associated with respiratory diseases in cattle, were detected in both healthy and unhealthy calves, confirming their position as part of the respiratory tract microbiota of calves. *Staphylococcus* sp., *Bacillus* sp., *E. coli*, *K. oxytoca* and *Pseudomonas aeruginosa* were also isolated. A relation between *Pasteurella multocida*, *Mollicutes*, *Mycoplasma bovis* and *Mycoplasma dispar* and the presence or absence of clinical signs of respiratory diseases in calves was detected.

Mycoplasmas were isolated (12.5 %) and identified by PCR (70.83 %). *Mycoplasma dispar* and *Mycoplasma bovis* were identified, although *Mycoplasma mycoides* subsp. *mycoides* was not detected. A few studies evaluated the presence of these bacteria in the respiratory tract of Brazilian cattle. Marques *et al.* (2007a) also detected high frequency of *Mollicutes* in sick animals. Foreign studies also highlight their significance to pneumonia pathogenesis, especially *Mycoplasma bovis* and *Mycoplasma dispar* (Pardon *et al.*, 2011; Gabinaitiene *et al.*, 2011; Castillo-Alcala *et al.*, 2012; Akan *et al.*, 2014; Šiugždaitė *et al.*, 2015). The detection of these bacteria in healthy calves in the present and other studies (Marques *et al.*, 2007a; Šiugždaitė *et al.*, 2015) suggests that they are part of respiratory tract microbiome. Although MmmSC is greatly important in this scenario, as this species is responsible for bovine contagious pleuropneumonia (Srivastava *et al.*, 2000; OIE, 2014; Alhaji &

Table 2. Isolation of aerobic bacteria from tracheobronchial washing samples of healthy and sick bovines, State of São Paulo, 2014

Micro-organisms	Healthy	With bronchopneumonia	Total
Pasteurella multocida	5/71 (7.04 %)	6/71 (11.27 %)	11/71 (15.5 %)
Mannheimia haemolytica	0/71 (0.00 %)	0/71 (0.00 %)	0/71 (0.00 %)
Staphylococcus sp.	6/71 (8.45 %)	4/71 (5.63 %)	10/71 (14.08 %)
Bacillus sp.	5/71 (7.04 %)	2/71 (2.82 %)	7/71 (9.86 %)
E. coli	2/71 (2.82 %)	1/71 (1.41 %)	3/71 (4.22 %)
K. oxytoca	3/71 (4.22 %)	0/71 (0.00 %)	3/71 (4.22 %)
Pseudomonas aeruginosa	3/71 (4.22 %)	0/71 (0.00 %)	3/71 (4.22 %)
Streptococcus spp.	2/71(2.82 %)	0/71 (0.00 %)	2/71 (2.82 %)
Contamination	8/71 (11.27 %)	1/71 (1.41 %)	9/71 (12.67 %)
No growth	10/71 (14.08 %)	13/71 (18.31 %)	23/71 (32.39 %)
Total	44/71 (47.89 %)	27/71 (18.31 %)	71/71 (100 %)

Table 3. Association between age and the presence of *Mollicutes* in tracheobronchial washing of calves, State of São Paulo, 2014

Age	*Mollicutes*				Total		*P*-value
	Presence		Absence				
	n	%	*n*	%	*n*	%	
>12 months	8	8.33	6	6.25	14	14.58	0.0177*
<12 months	20	20.83	62	64.58	82	85.42	
Total	28	29.16	68	70.83	96	100	

Significance level: 10 %.
*Significant association.

Babalobi, 2015; Alemayehu *et al.*, 2015), there are no reports about the isolation and identification of MmmSC in Brazil.

An important micro-organism related to pneumonia, *Pasteurella multocida* was isolated in 5.5 % samples in both studied herds. Although Benesi *et al.* (2013) had not isolated this species, other studies highlighted the importance of *Pasteurella multocida* to the pathogenesis of bronchopneumonia (Angen *et al.*, 2009; Griffin, 2010; Holman *et al.*, 2015; Francoz *et al.*, 2015). *Pasteurella multocida* is also considered a commensal bacterium and part of the respiratory tract microbiome (Griffin *et al.*, 2010).

Pasteurella multocida and *Mycoplasma* spp. are the most reported bacteria in bovine respiratory disease outbreaks. However, the present study has detected other commensal bacteria that can be responsible for bronchopneumonia, hence their opportunistic characteristic. Benesi *et al.* (2013) has found similar microbiological profile in calves with bronchopneumonia, reporting the presence of *Staphylococcus* sp., *Bacillus* sp., *Streptococcus* sp., *Pseudomonas*

Table 4. Association between *Pasteurella multocida* and the absence of some respiratory clinical signs of bovines with bronchopneumonia, State of São Paulo, 2014

Variable	*Pasteurella multocida*				Total		*P*-value (CI)
	Presence		Absence				
	n	%	*n*	%	*n*	%	
Mucopurulent nasal discharge							
Presence	9	10.84	4	4.8	13	15.64	
Absence	66	79.5	4	4.8	70	84.3	0.0215 (1.55–34.58)
Total	75	90.34	8	9.6	83	100	
Mucoid nasal discharge							
Presence	4	4.88	2	2.44	6	15.64	
Absence	70	85.73	6	7.32	76	84.3	0.068 (1.19–28.52)
Total	74	90.61	8	9.76	82	100	
Submassive sound							
Presence	3	4.48	2	2.99	5	7.47	
Absence	58	86.57	4	5.97	62	92.54	0.031 (1.23–75.5)
Total	61	91.05	6	8.96	67	100	
Fine crackle							
Presence	30	34.09	6	6.82	36	40.91	
Absence	50	56.82	2	2.27	52	59.09	0.058 (1.23–20.1)
Total	80	90.91	8	9.09	88	100	
Coarse crackle							
Presence	15	17.04	6	6.81	21	23.85	
Absence	65	73.86	2	2.27	67	76.13	0.003 (2.38–70.8)
Total	80	90.9	8	9.08	88	100	

CI, confidence interval.
Significance level: 10 %.

aeruginosa, *Klebsiella* sp. and *E. coli*. The present study obtained tracheobronchial lavage samples after antisepsis of the trachea, avoiding any contamination. Although the association between these bacteria and bronchopneumonia was not observed, their presence cannot be neglected, since co-infections can occur.

The association between microbiological findings and clinical signs was studied. While *Mollicutes* and *Pasteurella multocida* were associated with submassive sound, the absence of *Mycoplasma bovis* was associated with clear sound. In pneumonia, pulmonary parenchyma is filled with solid tissue, decreasing air volume; then, submassive or massive sound is obtained depending on the organ compromised (Gonçalves, 2014). *Mycoplasma dispar* was associated with whistling and coarse crackle, as well as *Pasteurella multocida* that was also associated with coughing, mucopurulent nasal discharge and fine crackle. Griffin (2010) referred to low correlation between clinical signs and morbidity. The author observed similar clinical signs during all pneumonias, such as depression, loss of appetite, change in respiratory pattern and increase in body temperature, which might make it difficult to act on a specific pathogen. In the present study, however, an association between some specific clinical signs and bacteria, such as coughing and mucopurulent nasal discharge and the presence of *Pasteurella multocida* and whistling and *Mycoplasma dispar*, were observed.

In conclusion, the identification of *Pasteurella multocida* and *Mycoplasma* spp. in both healthy calves and calves with respiratory diseases also confirms their position as part of the respiratory tract microbiota of calves. Their high prevalence in unhealthy calves also increases the importance of these micro-organisms in the pathogenesis of the respiratory diseases. In this context, this study increases the information about the possible role of *Mycoplasma dispar* in respiratory diseases in cattle. Differences detected in the presence or absence of some species in relation to clinical signs can be applied as a presumptive diagnosis of the micro-organism involved in respiratory disease.

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