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Original Article
Livestock Diseases

Bacteria isolated from the lower respiratory tract of sheep and their relationship to clinical signs of sheep respiratory disease[1]

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ABSTRACT.- Franco M.F., Gaeta N.C., Alemán M.A.R., Mellville P.A., Timenetsky J., Balaro M.F.A. & Gregory L. 2019. **Bacteria detected from the lower respiratory tract of sheep and their relationship to clinical signs of sheep respiratory disease.** *Pesquisa Veterinária Brasileira 39(10):796-801.* Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: natalia.gaeta@hotmail.com

Respiratory diseases are among the most important diseases in sheep flocks. Herein was studied the bacterial etiology of respiratory disease and the clinical signs of 99 female and male sheep breed in the states of São Paulo (SP) and Rio de Janeiro (RJ), Brazil. After physical examination of animals, tracheobronchial flushing samples were obtained. The usual bacteria and *Mycoplasma* spp. were searched, as well as their association with the clinical status and clinical signs of sheep with respiratory disease. The main observed signs were: tachypnea (75%), increase of rectal temperature (09.4%), mucopurulent/purulent nasal discharge (21.9%), cough (25%), dyspnea (31.2%), changes of lung sounds at auscultation (87.5%) and chest percussion (28.1%) in pneumonic sheep. Non-fermenting gram-negative bacteria and *Bacillus* sp. were the most isolated bacteria. Microorganisms of the Mollicutes class were molecularly (PCR) detected in 33.3% of the animals. In addition, the specific detection of *M. mycoides* subsp. *capri* was described for the first time in sheep from the state of São Paulo, Brazil.

INDEX TERMS: Bacteria, lower respiratory tract, sheep, clinics, sheep respiratory disease, *Mycoplasma* spp., pneumonia, respiratory disease, bacterioses.

RESUMO.- [**Bactérias detectadas no trato respiratório inferior de ovinos e a relação com as manifestações clínicas da doença respiratória ovina.**] A doença respiratória é uma das doenças mais importantes em rebanhos ovinos. Esta pesquisa teve como objetivo determinar a etiologia bacteriana da doença respiratória e sua relação com sinais clínicos em ovinos criados nos estados de São Paulo e Rio de Janeiro, Brasil. Noventa e nove ovelhas machos e fêmeas dos Estados de São Paulo (SP) e Rio de Janeiro (RJ) foram estudadas. Após o exame físico, amostras de lavagem traqueobrônquica foram obtidas. A presença de bactérias aeróbias e *Mycoplasma* spp. foram estudados, assim como a associação entre os microrganismos e estado clínico e sinais clínicos de doença respiratória em ovinos. As principais manifestações clínicas observadas foram: taquipneia (75%), alta temperatura retal (09,4%), secreção nasal mucopurulenta/purulenta (21,9%), tosse (25%), dispneia (31,2%), sons pulmonares alterados na ausculta (87,5%) e na percussão torácica (28,1%) em ovelhas pneumônicas. Bactérias gram-negativas não fermentadoras e *Bacillus* sp. foram as bactérias mais isoladas. Microrganismos da classe Mollicutes foram detectados molecularmente (PCR) em 33,3% dos ovinos. Além disso, descreve-se pela primeira vez no estado de São Paulo, Brasil, a detecção do

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M. mycoides subsp. *capri* na espécie ovina utilizando a reação de polimerase em cadeia.

TERMOS DE INDEXAÇÃO: Bactérias, trato respiratório inferior, ovinos, ovelhas, clínica, doença respiratória ovina, *Mycoplasma* spp., pneumonia, bacterioses.

INTRODUCTION

Sheep respiratory disease is one of the most important diseases in sheep flocks (Marcondes et al. 2011, Radostits et al. 2017) due to its economic losses. In New Zealand, pneumonia in lambs cost NZ$28.1 million in 2008 (Goodwin-Ray et al. 2008). From 1996 to 2010, 10% of diagnosed diseases in lambs in the "Universidade Federal do Mato Grosso do Sul" were pneumonic related (Almeida et al. 2013). The incidence of pneumonia in sheep and other respiratory diseases in Argentina was 57.9% (Suárez & Busetti 2009). In Tanzania, 31.4% of lungs had pneumonic lesions in slaughter (Mellau et al. 2010). Recently, McRae et al. (2016) detected pneumonic lesions in 28% of lambs at slaughter in New Zealand.

Important risk factors related to respiratory disease of ruminants are the environmental high temperature and humidity, increased animal density, stress, dust, poor ventilation, and parasites (Goodwin-Ray et al. 2008, Taylor et al. 2010, Scott 2011). Some viruses are considered primary pathogens, changing the epithelial cells, and interfering in the immune response (Bosch et al. 2013), leading to secondary bacterial infections. According to Sharp & Nettleton (2007) acute respiratory disease is not caused by virus infections alone.

Mannheimia (*M.*) *haemolytica* and *Pasteurella* (*P.*) *multocida* are important respiratory pathogens in sheep flocks (Radostits et al. 2017). Both bacteria belong to family Pasteurellaceae, order Pasteurellales (Griffin 2010). These bacteria are part of the respiratory tract microbiota in sheep (Viana et al. 2007) and as stated before, immunosuppression and viral infections predispose to respiratory disease caused by both species (Bosch et al. 2013, Viana et al. 2007). Almost 10% of pneumonic sheep were positive for *P. multocida* in lungs in Nigeria (Odugbo et al. 2006). Ten sheep died during an outbreak of *M. haemolytica* associated pneumonia in the State of Minas Gerais in 2009 (Araujo et al. 2009).

Mycoplasma spp. belongs to class Mollicutes, order Mycoplasmatales and family Mycoplasmataceae (Razin & Hayflick 2010). They are the smallest organisms with self-replication (Razin & Hayflick 2010) and some are detected in healthy and diseased ruminants (Ayling et al. 2004, Kumar et al. 2011, Oliveira et al. 2016, Tortorelli et al. 2017, Gaeta et al. 2018). *M. mycoides* subsp. *capri* (Srivastava et al. 2000, Al-Momani et al. 2011, Kumar et al. 2013), *M. agalactiae* (Kumar et al. 2014), *M. capricolum* subsp. *capricolum* (Fischer et al. 2012), *M. capripneumoniae* (Igbal et al. 2019) and *M. ovipneumoniae* (George & Carmichael 1975, Ayling et al. 2004, Dassanayake et al. 2010) are described as important respiratory mycoplasmas for small ruminants.

Sheep with respiratory disease often show depression, inappetence, high rectal temperature, dyspnea, coughing, mucopurulent or purulent nasal discharge and increased cardiac and respiratory rates (Scott 2011). Only a few studies aimed detect the bacterial etiology of pneumonic sheep in Brazil (Coutinho et al. 2009, Marcondes et al. 2011). Therefore, herein it was detected the bacterial etiology of respiratory disease and their relationship to clinical signs of sheep respiratory disease.

MATERIALS AND METHODS

All procedures were carried out in agreement with the guidelines of the Committee of Ethics on Animal Use (Protocol no. 3498060716).

In this cross-sectional research, 99 male and female sheep from 12 flocks from the States of São Paulo (SP=6) and Rio de Janeiro (RJ=6) were enrolled in this study, according to a non-probabilistic sampling for convenience.

Animals were classified as healthy and unhealthy (showing clinical signs of respiratory disease) after physical exam (Benesi et al. 2013, Gaeta et al. 2018). Sheep were considered unhealthy if show at least two of the following parameters: respiratory rate more than 30 respiratory movements per minute, coughing, rectal temperature higher than 40°C and abnormal sound on thoracic auscultation.

Tracheobronchial wash samples were obtained from both health and pneumonic sheep (Anton & Mayayo 2007). Briefly, trichotomy and antisepsis using 70% alcohol, chlorhexidine and iodopovidone were performed in the third part of the trachea. An Intracath® (BD, USA) was introduced by tracheocentesis and 20 to 50mL of sterile saline 0.9% was instilled, recovering up to 5mL. An aliquot (500µL) was added to Stuart transport media and stored at 4°C until the isolation of bacteria. Another aliquot was added to transport media for *Mycoplasma* spp. containing glycerol and stored in liquid nitrogen.

Tracheal suspensions were inoculated in Brain Heart Infusion broth and the cultures in agar plates were performed by plating 10µL of these samples in MacConkey and Blood agar (5%). The cultures were incubated for 24 to 72 hours at 37°C in aerobic atmosphere. The obtained colonies were noted for their morphology, Gram stained and screened for biochemical tests (*Staphylococcus* sp.: catalase test, sensitivity to bacitracin and furazolidone and oxidase test; *Streptococcus* sp.: catalase test, sensitivity to bacitracin, growth at 65% NaCl, bile-esculin test and growth at 45°C; *Bacillus* sp.: catalase test; *Klebsiella* sp. and non-fermenting bacteria: triple sugar iron growth, production of indole, urease and fenilalanine desaminase, oxidation-fermentation test, motility test, lysine descarboxylation test, use of citrate and malonate, lactose and glucose fermentation, nitrate reduction, Voges-Proskauer test and Methyl-red test) (Murray et al. 1999).

Mycoplasma spp. isolation was performed using solid and liquid SP-4 media (Tully 1995). Agar plates and broths were incubated in aerobiosis at 37°C for 15 days. *Mycoplasma* spp. were screened for producing "fried-egg"-like colonies and differentiated for glucose fermentation and/or arginine hydrolysis without turbiding the broths. Polymerase chain reaction (PCR) was performed to confirm the DNA of Mollicutes in samples (Van Kuppeveld et al. 1992). Then, the PCR positive samples were tested again for *M. bovis* and *M. agalactiae* (Chávez González et al. 1995) and *M. mycoides* subsp. *capri* (Monnerat et al. 1999).

The association between health status and clinical signs and microbiologic findings were calculated using Chi-square test or Fisher's Exact test. Variables with a *P*-value <0,05 were considered significant. All calculations were performed using Software Statistical Package for Social Sciences 16.0 (SPSS, IBM).

RESULTS

After clinical examination, the sheep were classified as healthy (67.7%, 67/99) and pneumonic (32.32%, 32/99). Clinical signs such as tachypnea, high rectal temperature, mucopurulent/purulent nasal discharge, coughing, dyspnea, abnormal pulmonary sounds on auscultation and thoracic percussion were increased in pneumonic sheep compared to health sheep (Table 1).

Forty-five bacterial isolates were obtained. *Bacillus* sp., non-fermentative gram-negative bacteria, *Streptococcus* sp. and *Staphylococcus* sp. were the main species. *P. multocida* and *M. haemolytica* were not isolated (Table 2). Fried-egg-like colonies were observed in 23.3% (17/73) of samples. The PCR methodology with generic primers for *Mollicutes* spp. confirmed all isolates of Mollicutes. In addition, PCR was also performed directly in tracheobronchial wash samples, and the DNA of *Mollicutes* spp. was detected in 45.2% (33/73) of samples (38% in pneumonic sheep and 49% in healthy sheep). *M. mycoides* subsp. *capri* (01/33) was detected by PCR. The used specific primers in this study did not allow detect other Mollicute species. No association between species detected and health status were detected.

Regarding to clinical manifestation, only *Bacillus* sp. was associated to respiratory rate (P=0.005) and submassive/massive sound on thoracic percussion (P=0.024).

Table 1. Clinical signs detected in healthy and pneumonic sheep from the States of São Paulo and Rio de Janeiro, Brazil

Clinical signs	Healthy % (N/T)	Pneumonic % (N/T)	P-valor
Body Condition Score			
<2	73.1 (49/67)	42.9 (15/32)	0,037*
>2	26.9 (18/67)	53.1 (17/32)	
Heart rate (bpm)			
<120	67.7 (46/67)	50.0 (16/32)	0.073
>120	31.3 (21/67)	50.0 (16/32)	
Respiratory rate (breaths/min)			
<30	59.7 (70/67)	25.0 (08/32)	0.001*
>30	40.3 (27/67)	75.0 (24/32)	
Rectal temperature (ºC)			
38.5 - 40.0	97.0 (65/67)	90.6 (29/32)	0,011*
>40.0	03.0 (02/67)	09.4 (03/32)	
Nasal discharge			
Normal	98.5 (66/67)	79.1 (25/32)	0,001*
Altered[a]	01.5 (01/67)	21.9 (07/32)	
Cough			
Absent	97.0 (65/67)	75.0 (24/32)	0,001*
Present	03.0 (02/67)	25.0 (08/32)	
Dyspnea			
Absent	94.0 (63/67)	68.8 (22/32)	0,001*
Present	06.0 (04/67)	31.2 (10/32)	
Breathing Pattern			
Costoabdominal	98.5 (66/67)	98.6 (29/32)	0,098
Costal/Abdominal	01.5 (01/67)	09.4 (03/32)	
Laringotracheal sound			
Normal	98.5 (66/67)	84.4 (27/32)	0,013*
Loud	01.5 (01/67)	15.6 (05/32)	
Percussion			
Clear	94.0 (63/67)	71.9 (23/32)	0,002*
Submassive/massive	06.0 (04/67)	28.1 (09/32)	
Auscultation			
Normal	86.6 (58/67)	12.5 (04/32)	<0,001*
Altered	13.4 (09/67)	87.5 (28/32)	
Fine Crackles	06.0 (04/67)	53.1 (17/32)	<0,001*
Thick Crackles	01.5 (01/67)	09.4 (03/32)	0,098
Snoring	03.0 (03/67)	18.8 (06/32)	0,005*
Whistle	03.0 (03/67)	03.1 (01/32)	1,000

[a] Mucopurulent/purulent; * means P<0.05 (Chi-Square Test or Fisher's Exact Test); N = number of observations, T = total of sheep.

Table 2. Aerobic bacteria detected in tracheobronchial wash samples of sheep in relation to respiratory health status

Bacteria	Healthy % (N/T)	Pneumonic % (N/T)	Total % (N/T)	*P*-value
Bacillus sp.	27.6 (08/29)	43.7 (07/16)	33.3 (15/45)	0.131
Streptococcus sp.	24.1 (07/29)	-	15.5 (07/45)	0.096
Staphylococcus sp.	20.7 (06/29)	25.0 (04/16)	22.2 (10/45)	0.484
NFGN	24.1 (07/29)	18.7 (03/16)	22.2 (10/45)	0.980
Klebsiella oxytoca	-	06.2 (01/16)	02.2 (01/45)	0.303
Klebsiella pneumoniae	-	06.2 (01/16)	02.2 (01/45)	0.303

N = number of isolates of the specific bacterium, T = total number of isolates.

DISCUSSION

The present study helps to better understand the main bacteria involved in the respiratory disease of Brazilian sheep from the States of São Paulo and Rio de Janeiro, Brazil. Bronchopneumonia was detected in 32.3% of sheep as detected in other studies in Brazil (Viana et al. 2007) and Ethiopia (Garedew et al. 2010).

Respiratory rate, rectal temperature, nasal discharge, cough, dyspnea, percussion and altered sounds in pulmonary auscultation were increased in pneumonic sheep ($P<0.05$). These findings confirm the importance of the observed clinical signs of sheep to determine the respiratory health during physical examination (Viana et al. 2007, Legesse et al. 2018).

Opportunistic agents may have a controversy importance due their detection in healthy hosts. *Bacillus* sp. was the most isolated bacteria, especially in pneumonic sheep. This microbial genera was also detected in other studies with the same propose (Asaye et al. 2015, Megra et al. 2006, Gebremeskel et al. 2017, Yegoraw et al. 2017, Yimer & Asseged 2007). In fact, *Bacillus* sp. is an opportunistic infectious agent that belongs to the ovine respiratory tract microbiota (Rajivkumar & Ghaar 2000, Garedew et al. 2010). The presence of non-anthracis *Bacillus* microorganism in clinical samples was considered a paradigm for a long time. As mentioned in literature, many infectious agents were previously considered not be pathogenic and became be accepted as pathogenic (Farrar Jr. 1963) as happened with *Bacillus* sp. considered a non-contaminant in clinical samples (Shimoyama et al. 2017). Otherwise *Bacillus cereus* is known to be responsible for nosocomial pneumonia in human, and in immunocompromised or immunocompetent human patients (Gray et al. 1999, Miyata et al. 2013, Shimoyama et al. 2017). *Bacillus* sp. has been isolated from lung tissue (Elshafee 2003) and tracheobronchial flushing samples of cattle (Oliveira et al. 2016, Gaeta et al. 2018). The present study stated the association of clinical signs of sheep, the respiratory disease and the isolation of *Bacillus* sp. (high respiratory rate and the presence of abnormal sound on thoracic percussion).

Klebsiella oxytoca and *K. pneumoniae* are virulent bacteria that are often associated with pneumonia in sheep (Ajuwape & Aregbesola 2002, Patel et al. 2017). Herein, both species were detected, as well was in other studies in Ethiopia et al. (2010) and Iraqi (Al-Sultan 1995).

Mycoplasmas are frequently related to respiratory disease in sheep, and are responsible for important economic losses (Kumar et al. 2012). Mollicutes were detected by PCR in 45.2% of studied sheep tracheal samples. Al-Momani et al. (2006) obtained similar results in Jordan (35% samples). In addition, we detected Mollicutes in 38% of pneumonic and 49% in healthy sheep. It is well stablished that Mollicutes are part of the respiratory tract microbiota of ruminants, and after a stress, they may become pathogenic. These findings are also in agreement with other studies Swedish flocks (Tauni 2017), and India (Kumar et al. 2012). Mollicutes have also been detected in the lower respiratory tract of calves (Oliveira et al. 2016, Gaeta et al. 2018) in Brazil. *M. mycoides* subsp. *capri* was detected by PCR in present study in a healthy sheep. Opportunistic bacteria are easily spread into the flocks, particularly in animals in close proximity. As stated before, any situation that compromises the immune system favors the disease development. During a respiratory outbreak by *M. mycoides* subsp. *capri*, Hernandez et al. (2006) described a 60% mortality in a Mexican goat flock. Animals showed abundant nasal discharge, fever, dyspnea, prostration, ear drop, and decrease in milk production. Depending on the number of affected animals, economic losses might be severe. Finally, most of Mollicutes detected by PCR in studied samples was not specifically identified. This questioned the role of other species of Mollicutes in respiratory disease of sheep, such as *M. bovis* (Kumar et al. 2012), *Acholeplasma* sp., and *M. arginine* (DaMassa et al. 1992).

CONCLUSIONS

In the present study, *Bacillus* sp. was the main bacteria detected in both healthy and pneumonic sheep. Important pathogens such as *Klebsiella oxytoca* and *K. pneumoniae* were both isolated in pneumonic sheep, confirming their importance in the etiology of the disease in the southeastern region of Brazil.

Mollicutes is part of the ruminant's microbiota, but they are also important to ovine pneumonia.

This seems to be the first study that detected *Mycoplasma mycoides* subsp. *capri* in the State of São Paulo.

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Conflict of interest statement.- The authors have no competing interests.

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