

Ocurrence and risk factors associated with *Mycoplasma agalactiae* infection in dairy goat herds of Paraíba State, Brazil¹

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ABSTRACT.- Matos R.A.T., Santos S.B., Alves R.V., Silva E.J., Marinho M.L., Júnior J.W.P., Mota R.A. & Garino Júnior F. 2019. **Ocurrence and risk factors associated with *Mycoplasma agalactiae* infection in dairy goat herds of Paraíba State, Brazil.** *Pesquisa Veterinária Brasileira* 39(2):93-98. Graduate Program in Veterinary Medicine, Centro Universitário Cesmac, Rod. Divaldo Suruagy s/n, Marechal Deodoro, AL 57160-000, Brazil. E-mail: rodrigoatmatos@gmail.com

Mycoplasmosis is a disease that may cause severe economical losses in goat and sheep herds, and it is associated with mastitis, polyarthritis, agalactia, conjunctivitis, pneumonia and reproductive failure. The objective of this study was to determine the occurrence of *Mycoplasma agalactiae* in milk samples and investigate the main risk factors associated with infection in goats from farms of the state of Paraíba, Brazil. For *Mycoplasma agalactiae* diagnosis, 251 milk samples were submitted to DNA extraction using a commercially available kit, following the manufacturer's instructions and Polymerase Chain Reaction (PCR) was performed. In addition, questionnaires were applied to identify the main risk factors associated with contagious agalactia. Out of the two hundred fifty-one samples analyzed, 50 (19.9%, I.C. 15.1-25.4%) were PCR positive for *M. agalactiae*. In the risk factors analysis, some associations were observed for the following variables: size of the herd ($P < 0.001$, OR=7.1, I.C. 2.4-20.6), replacement of farm animals ($P < 0.001$, OR=4.7, I.C. 1.8-12.2) and participation of animals in fairs and exhibitions ($P = 0.029$, OR=2.0, I.C. 1.0-3.9). The results allowed confirming the occurrence of *Mycoplasma agalactiae* in milk samples of goats from Paraíba. Therefore, it is strictly necessary to monitor dairy goat flocks and to raise the awareness of farmers about the economic importance of the disease, since it causes severe economic losses for producers of the state. Identification of risk factors is essential for adoption of control measures and for the correction of the management factors in farms where there are animals with positive diagnosis, avoiding, so, pathogen dissemination.

INDEX TERMS: *Mycoplasma agalactiae*, infection, mycoplasmosis, polymerase chain reaction, milk, goats, caprine, bacterioses.

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RESUMO.- [Ocorrência e fatores de risco associados à infecção por *Mycoplasma agalactiae* em rebanhos caprinos leiteiros do estado da Paraíba, Brasil.] As micoplasmoses ocasionam prejuízos econômicos nas criações de ovinos e caprinos, e estão associadas com quadros de mastite, poliartrite, agalaxia, conjuntivite, pneumonia e falhas reprodutivas. Objetivou-se neste estudo determinar a ocorrência de *Mycoplasma agalactiae* em amostras de leite e investigar os principais fatores de risco associados à infecção em caprinos provenientes de propriedades rurais do estado da Paraíba, Brasil. Para o diagnóstico de *Mycoplasma agalactiae*, foram analisadas 251 amostras de leite, que foram submetidas à

extração do DNA genômico usando um *kit* comercial, seguindo as recomendações do fabricante. Para diagnóstico da infecção utilizou-se a Reação em Cadeia da Polimerase (PCR). Além disso, foram aplicados questionários para identificar os principais fatores de risco associados à infecção por *M. agalactiae*. Das 251 amostras analisadas, 50 (19,9%; I.C. 15,1-25,4%) foram positivas na PCR para *M. agalactiae*. Observaram-se na análise dos fatores de risco, algumas associações para as seguintes variáveis: tamanho do rebanho ($P < 0,001$; OR 7,1), reposição de animais da propriedade ($P < 0,001$; OR 4,7) e participação dos animais em feiras e exposições ($P = 0,029$; OR 2,0). Os resultados permitiram confirmar a ocorrência do *Mycoplasma agalactiae* em amostras de leite de caprinos da Paraíba. Portanto, é necessário o monitoramento dos rebanhos caprinos leiteiros e a conscientização dos produtores rurais para a importância econômica da doença, visto que a mesma acarreta severos prejuízos econômicos para os produtores do estado. A identificação dos fatores de risco são imprescindíveis para a adoção de medidas de controle e para a correção dos fatores de manejo em propriedades que tenham animais com diagnóstico positivo, evitando assim, a disseminação do patógeno.

TERMOS DE INDEXAÇÃO: *Mycoplasma agalactiae*, infecção, micoplasmose, reação de cadeia de polimerase, leite, caprinos, bacterioses.

INTRODUCTION

Mycoplasmosis are diseases caused by bacteria of the class *Mollicutes*, which are considered the smallest self-replicating prokaryotes without cellular walls (Chazel et al. 2010). In goats, the main mycoplasmosis are contagious caprine pleuropneumonia, CCPP (OIE 2014) and contagious agalactia of ovine and caprine animals, CAOC (OIE 2013). This disease can cause mastitis, agalactia, polyarthritis, keratoconjunctivitis and, occasionally, abortion and pneumonia (Gómez-Martín et al. 2013). The main agent of CAOC is *Mycoplasma agalactiae*, although other species, such as: *M. capricolum* subsp. *capricolum*, *M. putrefaciens* and *M. mycoides* subsp. *capri* can also cause the disease (Madanat et al. 2002, Gil et al. 2003).

Infection takes place orally, via respiratory tract or at the mammary area, and, after a period of bacteremia, it disseminates to the eyeball, mammary gland, joints, tendons, uterus and lymph nodes. Among the early signals, the most important are the birth of infeasible offspring and abortions. Transplacental infection has been described in goatlings (Azevedo et al. 2012). Infection by *M. agalactiae* spreads across the herd by direct contact with infected animals and in the environment via contaminated milk and milking equipment, ocular discharge and the hands of milkers. There are also reports of venereal transmission (Madanat et al. 2001).

CAOC is geographically distributed throughout Europe, Western Asia, USA and Northern Africa, and is endemic in most Mediterranean countries (Gil et al. 2003). In Brazil, *M. agalactiae* was isolated from sick goats in 2001, in the state of Paraíba (Azevedo et al. 2006). Reports of the disease in the states of Pernambuco and Rio Grande do Norte have been published after that (Azevedo 2005), and it has also been diagnosed in the microregion of Cariri Paraibano (Bandeira et al. 2008). However, there is neither recent study nor data available on the current situation of the disease in the State of Paraíba,

nor are there are not even studies about the risk factors in Paraíba which may be associated with the infection by *M. agalactiae* in dairy goats. The aim of this research was to verify the occurrence of *M. agalactiae* in milk samples and to investigate the major risk factors associated with the infection in goats coming from farms in microregions of the State of Paraíba, in Brazil.

MATERIALS AND METHODS

Area of study and sampling. In the period from March 2016 to April 2017, 13 dairy goat farms, located in the Western and Eastern Paraíba Cariri, Western Curimataú and Eastern Seridó microregions and in the municipalities of Gurjão (07° 14' 48" S and 36° 29' 22" W), Juazeirinho (07° 04' 06" S and 36° 34' 40" W), Monteiro (07° 53' 22" S and 37° 07' 12" W), Nova Floresta (06° 27' 19" S and 36° 12' 12" W), Prata (07° 41' 27" S and 37° 04' 49" W), São Sebastião do Umbuzeiro (08° 09' 08" S and 37° 00' 37" W) and Zabelê (08° 04' 32" S and 37° 05' 54" W) were selected and visited using a convenience-oriented sampling. The selection took into account the occurrence of clinical signs suggesting infection by *Mycoplasma agalactiae*.

The size of the sample for the prevalence study was determined considering an anticipated prevalence of 10% for the infection in goats (Santos et al. 2014), which determined a minimum sampling of 138 goats, considering a level of significance of 95% and a statistical error of 5% (Thrusfield 2004).

The following formulas were used to calculate the number of animals:

$$n = 1,96^2 \cdot P_{esp} \cdot (1 - P_{esp}) / d^2; n_{ajust} = (N \times n) / (N + n)$$

Where: n = minimum number of goats to be sampled, N = total number of goats in the herd, P_{esp} = anticipated prevalence, d^2 = absolute precision.

As the criterion for inclusion of goats in the sample, all females in lactation coming from farms with animals with signals suggesting infection were selected. A total of 251 samples were collected with a safety margin at the discretion of the authors. It was not possible to standardize the number of samples and goats per farm due to the significant differences in herd sizes and to the fact that some animals were not in the lactation period.

Collection of samples. In the 13 farms, 251 samples of milk from goats of the races Alpine American, British Alpine, Anglo-Nubian, Saanen, Alpine Parda, Toggenburg and mixed-race were collected. All the farms had animals with a history of sharp decrease in the production of milk. In four farms goats with a history of reproductive disorders were observed. For the collection of samples, udders were washed with a solution of sodium hypochlorite and soaked in iodized alcohol. A pool of both udders was then made, and a sample collected from each animal in sterile Falcon-type tubes containing 50% glycerinated saline solution added with Penicillin (2.000UI/ml) was stored in freezers at -20°C and -80°C up to the moment of the molecular diagnosis.

Molecular diagnosis. Milk samples were aliquoted in volume of 300µl and genomic DNA was extracted using the "Wizard® Genomic DNA Purification Kit" (Promega) commercial kit, following the manufacturer's protocol. The quality and amount of DNA extracted were evaluated using an automatic quantifier

(Multiscan Go, [®]ThermoScientific). The polymerase chain reaction (PCR) was done with the oligonucleotides described by Tola et al. (1997), *Ma* (FS1 5'-AAAGGTGCTTGAGAAATGCC-3' and FS2 5'-GTTGCAGAAGAAAGTCCAATCA-3'), which amplify a fragment of 375 base pairs of the gene 16SRNA of *Mycoplasma agalactiae*. For the reaction, 8µL of PCR Mix LGC[®] 2X (LGC Biotecnologia[®], Code 13-11250, Cotia/SP, Brazil) were used, containing: 200mM of each dNTP; 1.5mM of MgCl₂; *Taq* DNA Polimerase (0.5U) in appropriate reaction concentration and buffer (Tris-HCl pH: 8.5, KCl); oligonucleotides at 30 pmol; 8µL genomic DNA and PCR water, totalizing a final volume of 25 µL of reaction. The positive reaction control was the one isolated from Pernambuco Ma62 (BrPE62), and PCR water was used as negative control. The thermal profile used was the same described by Tola et al. (1997). The reactions were carried out using a Bioer XP Thermal Cycler[®] thermocycler (Bioer Technology Corporation LTDA, Hangzhou, China). PCR products were stained with *Blue Green Loading Dye I*[®] (LGC Biotecnologia[®], Code 13-15009.06, Cotia/SP, Brazil) and submitted to 1.5% agarose gel electrophoresis. Amplicons were displayed on transilluminator ([®]TransiluminatorLoccusBiotecnologia L-Pix photodocumented).

Analysis of risk factors and statistics. For the study of risk factors, epidemiological questionnaires were applied containing objective questions to the producers about the general characteristics of farms and their productive, reproductive and sanitary management.

Data were expressed using absolute and relative frequencies. Interpretation of prevalence (high, average and low) was based on percentiles 25 and 75 of the percentages of goats which were positive in the herds. The analysis of herds evaluated the association between the history of clinical manifestations suggestive of CAOC by *Mycoplasma agalactiae* in the herds and the positive results for *Mycoplasma agalactiae* in PCR using Pearson's chi-square (X^2) statistical test (Sampaio 1998). Regarding the analysis of animal risk factors, an univariate analysis of the variables of interest was first carried out using Fisher's exact X^2 test, whenever necessary, and after that a logistic regression analysis was done using as dependent variable the result obtained in PCR (positive or negative). The independent variables considered in the model were those which showed statistical significance below 0.20. This probability was stipulated so that possible risk factors of the event were not excluded from the analysis (Hosmer & Lemeshow 1989). EpiInfo[™] 7 was used for statistical calculations and the level of significance adopted was 5.0%.

Ethical aspects. The research was approved by the Research Ethics Committee (CEP) of Federal University of Campina Grande (UFCG) under protocol no. 296-2015.

RESULTS AND DISCUSSION

Among the 251 samples analyzed, 50 (19.9%, I.C. 15.1-25.4%) were positive in the PCR for *Mycoplasma agalactiae*, with eight positive farms (61.5%) distributed among the following municipalities: Gurjão, Juazeirinho, Prata, São Sebastião do Umbuzeiro and Zabelê. The results per farm are shown in Table 1.

Farms A, F and M had high prevalences; the remainder showed average and low prevalence of CAOC, considering the percentiles of 25 and 75. In the distribution by municipalities, prevalences were observed in the municipalities of Gurjão

(16.7%), Juazeirinho (64.3%), Prata (18.3%), São Sebastião do Umbuzeiro (40.0%) and Zabelê (12.5%). In the municipalities of Monteiro and Nova Floresta, all the goats evaluated were negative for infection by *M. agalactiae*. The results of the analysis of the association of PCR with the history of clinical signs in the herds and of the risk factors associated with infection by *Mycoplasma agalactiae* in goats are shown in Table 2 and 3.

The prevalence of CAOC by *Mycoplasma agalactiae* in this study (19.9%) was lower than that found by Azevedo et al. (2006) in Paraíba. These authors reported a prevalence of 100.0% in small ruminants after an outbreak of contagious agalactia in farms located in Paraíba, Brazil. The values were possibly different due to the occurrence of outbreaks in several farms studied by the authors. However, when compared with a study done by Bandeira et al. (2008), also in the state of Paraíba, a higher prevalence was found in goat milk than that observed by these authors, which was of 7.5% (9/120). This difference may be due to the number of animals and herds used in the survey, to the acquisition of animals from other regions of Brazil and to differences in handling between farms, since the studies were done in different farms, although some municipalities were the same. In the present study, a higher prevalence was observed in goat milk rather than that found by Alves et al. (2013) in the State of Pernambuco, which was of 3.7% (3/81). These same authors explain that the difference between the prevalence values is due to the different stages of mycoplasmosis in the herds studied. These same authors state that factors such as chronic or subclinical character of the disease and treatment of mastitis in herds with antibiotics for long periods may also have contributed to the differences between prevalences.

As for the clinical signs associated with the positive results of PCR for infection by *Mycoplasma agalactiae*, most variables presented a significant association with the diagnosis of CAOC, except for the variable regarding reproductive disorders, infertility and infertility associated with abortion ($P>0.05$). Although, there have been association of cases of mastitis unresponsive to treatment and polyarthritis with the diagnosis of infection by *M. agalactiae*, it is not possible

Table 1. Frequency of positive dairy goats per property

Property	N	Positivity
A	14	9 (64.3%)
B	45	12 (26.7%)
C	16	2 (12.5%)
D	11	0 (0.0%)
E	13	2 (15.4%)
F	20	7 (35.0%)
G	20	1 (5.0%)
H	26	0 (0.0%)
I	12	0 (0.0%)
J	13	0 (0.0%)
L	6	1 (16.7%)
M	40	16 (40.0%)
N	15	0 (0.0%)
Total	251	50 (19.9%)

N = number of samples collected on the property.

Table 2. Analysis of the association of the PCR results with the history of clinical manifestations in the goat herds

Variables	N	PCR	Value P ^A
		Positive	
History of mastitis that does not respond to antibiotic therapy			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
History of mastitis and polyarthritis at the same time			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
Birth history of offspring with polyarthritis			
Yes	20	10 (50.0%)	<0.001*
No	231	40 (17.3%)	
History of pneumonias, polyarthritis, conjunctivitis, sudden death			
Yes	40	17 (42.5%)	<0.001*
No	211	33 (15.6%)	
History of reproductive disorders			
Yes	85	29 (34.1%)	<0.001*
No	166	21 (12.6%)	
What reproductive disorders			
Infertility	65	19 (29.2%)	0.088
Infertility + abortion	20	10 (50.0%)	

^A Test X²; N = total of analyzed samples, 1 different database (N = 85); * Significant association at the 5.0% level.

Table 3. Analysis of risk factors associated with *Mycoplasma agalactiae* infection

Variables	N	PCR	Value P	Regression logistic	Value P
		Positive		OR (I.C. 95%)	
Racial characteristics					
Pure	226	46 (20.3%)	0.794 ^A		
Mixed race	25	4 (16.0%)			
Size of the goat herd					
Up to 50 animals	65	4 (6.1%)	<0.001 ^{B*}	-	
Between 51 and 100 animals	145	46 (31.7%)		7.1 (2.4 - 20.6)	<0.001*
Between 101 and 200 animals	15	0 (0.0%)		**	
Over 200 animals	26	0 (0.0%)		**	
Feeders and drinking troughs for young and adult goats					
Yes	60	11 (18.3%)	0.724 ^B		
No	191	39 (20.4%)			
Insect control					
Yes	191	39 (20.4%)	0.724 ^B		
No	60	11 (18.3%)			
Cleaning of premises					
Yes	219	43 (19.6%)	0.767 ^B		
Weekly	32	7 (21.9%)			
Spare goats are owned by					
Yes	231	40 (17.3%)	<0.001 ^{B*}		
No	20	10 (50.0%)		4.7 (1.8 - 12.2)	0.001*
Milking line					
Yes	196	34 (17.3%)	0.054 ^B		
No	55	16 (29.1%)		1.9 (0.9 - 3.9)	0.056
Treatment of clinical mastitis					
Yes	80	21 (26.2%)	0.086 ^B		0.088
No	171	29 (16.9%)		1.7 (0.9 - 3.3)	
Destination of diseased goats					
Slaughter	20	10 (50.0%)	0.088 ^B		0.091
Antimicrobial treatment	65	19 (29.2%)		2.4 (0.8 - 6.7)	
Goats participate in fairs and/or exhibitions					
Yes	65	19 (29.2%)	0.029 ^{B*}		0.031*
No	186	31 (16.7%)		2.0 (1.0 - 3.9)	

^A Fisher's Exact Test, ^B Test X², 1 different database (N = 85); N = total of samples, OR = odds ratio, I.C. = confidence interval; * Significant association at the 5.0% level, ** the OR could not be calculated.

to state that the presence of these clinical signals is decisive for the occurrence of infection by *Mycoplasma agalactiae*, although other agents may cause these same clinical signals, just as the presence of the agent in goat milk samples does not indicate the occurrence of infection. An association can often occur with other diseases such as Brucellosis, Leptospirosis and Toxoplasmosis, which exhibit similar clinical signals. Even when there is no association between the clinical signs of reproductive disease with the infection by *M. agalactiae*, an investigation should be undertaken to eliminate the possibility of infection by the agent.

In the analysis of risk factors, an association with the positive effects of PCR for infection by *Mycoplasma agalactiae* was observed for the following variables: size of the herd ($P < 0.001$, $OR = 7.1$, I.C. 2.4-20.6), replenishment of animals of the farm ($P < 0.001$, $OR = 4.7$, I.C. 1.8-12.2) and participation of the animals in fairs and exhibitions ($P = 0.029$, $OR = 2.0$, I.C. 1.0-3.9). As for the size of the herd, the herds which had between 51 and 100 animals showed greater chances of having positive animals than the herds with up to 50 animals ($OR = 7.1$, I.C. 2.4-20.6). In herds in which there is acquisition of animals for breeding stock replenishment there is an increased chance of animals being positive for infection by *M. agalactiae* when compared with closed herds ($OR = 4.7$, I.C. 1.8-12.2). This is evidence that when replenishment animals do not come from the farms there is a greater possibility of positive animals being introduced in the herds, favoring the dissemination of the agent. In a study done in Jordan, Al-Momani et al. (2008) found that the agent can be introduced by disease-carrying animals from other herds used for reproduction. These same authors reported that other factors can contribute to the occurrence of the agent in the herds, such as: inadequate cleaning of milking equipment and separation of young animals from their mothers. The participation of animals in fairs and exhibitions increased the chances of animals getting infected by *M. agalactiae* when compared with animals coming from herds which did not take part in these events ($OR = 2.0$, I.C. 1.0-3.9). This result corroborates what was reported by Bandeira et al. (2008), who stated that mycoplasmosis outbreaks in goats in Paraíba and Pernambuco are caused by the participation of animals in fairs and exhibitions and to the introduction of animals coming from the country's Southeastern and Center-Western regions. These authors suggested that the rapid spread of *M. agalactiae* and probably of other infections is favored by the intense trade and transit of animals from the States of Paraíba, Pernambuco and Rio Grande do Norte. It should be stressed that there are few studies on the risk factors associated with the occurrence of infection by *Mycoplasma agalactiae*, and further research is needed to investigate others risk factors which may be associated with the infection by this agent.

CONCLUSIONS

The results of the study allowed the authors to confirm the occurrence of *Mycoplasma agalactiae* in milk samples from goats coming from Paraíba. However, it is necessary to monitor the dairy goat herds and to raise the awareness of rural producers about the economic relevance of this disease, since it entails severe economic losses for the producers.

The identification of risk factors is indispensable for the adoption of control measures and for the correction of the handling factors in farms which have animals with positive diagnosis, thus avoiding dissemination of the pathogen.

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

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