



Occurrence of *Mycoplasma bovis* and *Ureaplasma diversum* in dairy cattle from Pernambuco state, Brazil

[Ocorrência de *Mycoplasma bovis* e *Ureaplasma diversum* em bovinos leiteiros do estado de Pernambuco, Brasil]

A.A.M. Macêdo, J.M.B. Oliveira, B.P. Silva, J.M. Borges, L.B.F. Soares,
G.M. Silva, S.B. Santos*, R.A. Mota, J.W. Pinheiro-Júnior

Universidade Federal Rural de Pernambuco - Recife-PE

ABSTRACT

The objective of this study was to conduct an investigation of *Mycoplasma bovis* and *Ureaplasma diversum* infections in cattle in the microregion of the Ipanema Valley, state of Pernambuco, Brazil. Vaginal swabs were collected from 355 breeding cows in reproductive age and were analyzed by multiplex PCR (mPCR) and culture. An epidemiological investigation of risk factors was performed for Mollicutes. mPCR analysis showed that, 9.29% (33/355) of the cows were positive for *M. bovis* and 21.69% (77/355) for *U. diversum*; coinfection was observed in 2.81% (10/355) of the cows. The microbiological isolation showed, 81.81% (27/33) of *Mycoplasma* spp. and 24.67% (19/77) of *Ureaplasma* spp.. The risk factors related to Mollicutes infection identified were semi-intensive breeding system (OR= 4.6), pasture rent (OR= 3.6), non-isolation of animals with reproductive disorders (OR= 3.2), and natural mounting and artificial insemination (OR= 3.5). There was a significant association between Mollicutes infection and abortions in the first gestational third (P= 0.001). This is the first record of *M. bovis* and *U. diversum* infection in cows in the semiarid region of the state of Pernambuco, Brazil. Preventive measures directed to the identified risk factors can decrease the occurrence of Mollicutes in these herds.

Keywords: mPCR, mollicutes, cows, risk factors, ipanema valley

RESUMO

O objetivo deste estudo foi realizar uma investigação de *Mycoplasma bovis* e *Ureaplasma diversum* em bovinos leiteiros da microrregião do Vale do Ipanema, estado de Pernambuco, Brasil. Foram coletados swabs vaginais de 355 vacas em idade reprodutiva. As amostras foram analisadas por multiplex PCR (mPCR) e cultura. Foi realizada uma investigação dos fatores de risco para Mollicutes. Na mPCR, 9,29% (33/355) das vacas foram positivas para *M. bovis* e 21,69% (77/355) para *U. diversum*; coinfeção foi observada em 2,81% (10/355) das vacas. O isolamento microbiológico mostrou crescimento de *Mycoplasma* spp. em 81,81% (27/33) das amostras e em 24,67% (19/77) para *Ureaplasma* spp. Os fatores de risco relacionados à infecção por Mollicutes identificados foram sistema de produção semi-intensivo (OR= 4,6), aluguel de pastagem (OR= 3,6), não isolamento de animais com desordens reprodutivas (OR= 3,2) e monta natural e inseminação artificial (OR= 3,5). Houve uma associação significativa entre a infecção por Mollicutes e abortos no primeiro terço gestacional (P=0,001). Este é o primeiro relato da infecção por *M. bovis* e *U. diversum* em vacas na região semiárida do estado de Pernambuco, Brasil. As medidas preventivas direcionadas aos fatores de risco identificados podem diminuir a ocorrência de Mollicutes nesses rebanhos.

Palavras-chave: mPCR, Mollicutes, vacas, fatores de risco, Vale do Ipanema

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*Autor para correspondência (corresponding author)

E-mail: sanbsantos@gmail.com

INTRODUCTION

Animal mycoplasmosis is a disease caused by prokaryotes without a cell wall, which belong to the Mollicutes class. *Mycoplasma bovis* and *Ureaplasma diversum* have been associated with reproductive problems in cattle (Sanderson *et al.*, 2000; Ghanem *et al.*, 2013), but other species of *Mycoplasma* spp., such as *Mycoplasma bovirhinis*, *Mycoplasma canadense* and *Mycoplasma bovis*, were also identified in reproductive disorders (Pfützner and Sachse, 1996; Lysnyansky *et al.*, 2009). *M. bovis* causes mastitis, infertility, endometritis, salpingitis, pneumonia, arthritis, and abortions (Pfützner and Sachse, 1996).

Genital infections caused by *Mycoplasma bovis* in females is characterized by granular vulvovaginitis presenting with mucopurulent vaginal discharge, which may cause infertility, and in some cases, necrotizing endometritis (Cardoso and Vasconcellos, 2004). On the other hand, *Ureaplasma diversum* the infection is associated with abortion, recurrence of estrus, granular vulvovaginitis and infertility in infected herds (Miller *et al.*, 1994).

Previous studies indicated that the occurrence of Mollicutes infection associated with reproductive problems in cattle in different countries such as Australia (Argue *et al.*, 2013), Israel (Lysnyansky *et al.*, 2009), and the United States of America (Sanderson *et al.*, 2000). In Brazil, it was reported in the states of Paraíba (Santos *et al.*, 2013), Alagoas (Oliveira Filho *et al.*, 2005), São Paulo (Buzinhani *et al.*, 2007), and Mato Grosso (Gaeti *et al.*, 2014). The economic losses are associated with infertility and low reproductive performance (Junqueira *et al.*, 2006; Cardoso and Vasconcelos, 2004).

The risk factors associated with Mollicutes infection in cattle herds are related to dietary stress, such as high milk production, excessive movement of animals (exhibition and trade cattle) (Aebi *et al.*, 2015), and heifers and cows with lower pregnancy rate (León *et al.*, 1995).

Considering that Mollicute infection in cattle was already diagnosed in herds from to Brazil and that there is commercial transport of these animals between Brazilian states, this study aimed to conduct an epidemiological investigation of *Mycoplasma bovis* and *Ureaplasma diversum* infections in cattle in the microregion of the Ipanema Valley, state of Pernambuco.

MATERIALS AND METHODS

The present study analyzed cattle from 18 properties distributed among the six districts of the Ipanema Valley microregion in the Pernambuco semi-arid region: Águas Belas (n= 3), Buíque (n= 3), Venturosa (n= 3), Pedra (n= 3), Itaíba (n= 3) and Tupanatinga (n= 3) (Figure 1). A total of 336.221 cattle (IBGE, 2007) and an expected prevalence of 65.6% were considered (Santos *et al.*, 2013). At a significance level of $P < 0.05$, these parameters provided a minimum sample size (n) of 355 cows (Thrusfield, 2013). The number of animals evaluated in each herd was calculated using the formula of Astudillo (1979), based on the afore mentioned values. The properties were chosen by convenience, and the inclusion criteria was females of reproductive age group.

Vaginal mucous samples were collected after vulvar cleaning and asepsis with alcohol (70°GL), by using sterile cotton swab, and transported in 3mL of phosphate buffered saline (pH 7.2) (Nascimento *et al.*, 2005). The samples were placed in an isothermal box containing recyclable ice and sent to the laboratory for processing.

An aliquot of 600µL was used for DNA extraction with a commercial kit (Promega® Wizard Genomic DNA Purification Kit Catalog A1125). Aliquots for microbiological isolation were stored in 2:1 ratio glycerol in microtubes, vortexed and stored in a freezer at -16°C until use.

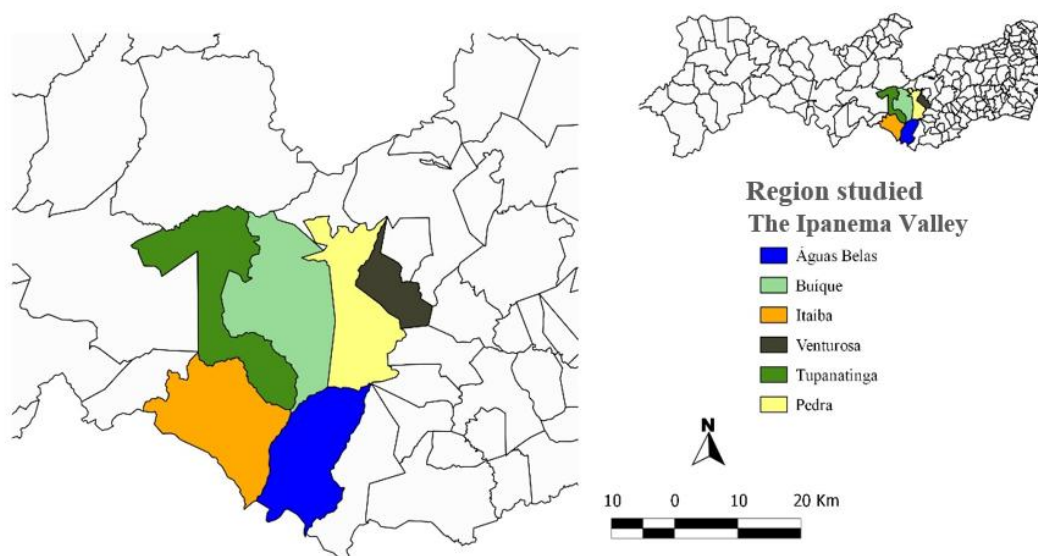


Figure 1. Area of investigation for Mollicutes in reproductive tract of cattle, microregion of the Ipanema Valley – Pernambuco-PE, Brazil.

The extracted DNA was analyzed by multiplex polymerase chain reaction (mPCR) technique for *Mycoplasma bovis* and *Ureaplasma diversum* species identification according to the thermal profile and oligonucleotides as described by Tramuta *et al.* (2011). The following primers were used for mPCR: *Mycoplasma bovis* (16S rRNA), 5'GTT TGA TCC TGG CTC AGG AT 3' and 5'AAG GTA CAT TCA ATA TAG TGG 3', with amplicon 476bp; *Ureaplasma diversum* (16S rRNA), 5'GTT TGA TCC TGG CTC AGG AT 3' and 5'CTC ATA AGC GAG CC GAC ATT 3', with amplicon 831bp. The reaction mixtures comprised a total of 25µL containing 5µL DNA template, 30pmol of each oligonucleotide and 6.25µL GoTaq®Green Master Mix (Promega®Corporation, Madison, WI, USA, Ref. M7122), and ultrapure water. The *Ureaplasma diversum* strain (GM U132 VUP6, ATCC) and *Mycoplasma bovis* strain (PG11, ATCC) were used as positive control. For the negative control, the DNA template was replaced by water. For electrophoresis, 1.5% agarose gel (100V/60min), 9µLPCR reaction mixtures plus 0.3µL Blue Green Loading Dye I (LGC®Biotecnologia, Cotia, SP, Brazil) and 5µL of molecular size marker (100bp DNA Ladder®, LGC®Biotecnologia, Cotia, SP, Brazil) were used. To confirm the species studied, sequencing of PCR positive samples was performed.

All the samples were screened for mycoplasma and ureaplasma by mPCR. Only mPCR confirmed isolates were further tested for recovery of pathogens using microbial culture method. Samples were cultured for *Mycoplasma* spp. isolation in modified Hayflick medium broth and plate, as described by Whitford *et al.* (1994), and for *Ureaplasma* spp. in UB specific medium broth and plate. The samples were observed at stereoscopic microscope and the isolation was considered positive for *Mycoplasma* spp. when the samples resembling “fried-egg-shaped” colonies, and the small, dark brown granular colonies, for *Ureaplasma* spp. All samples were monitored for 21 days.

Epidemiological questionnaires were designed and applied to obtain information about the type of farming and management practices and identify possible risk factors of each herd. The questionnaires were applied by a single trained investigator, and answered by people who could provide more about the animals. The variables investigated and the respective categories were: consortium creation (yes/no); (herds of Milk / milk and meat); Wetlands on the property (yes/no); Flooded areas on surrounding properties (yes/no); Presence of hematophagous insects (yes/no); Insect control (yes/no); Herd type (open/closed); Performs quarantine (yes/no); Reproductive management (natural breeding/artificial insemination/both).

Univariate analysis of infection-associated risk factor was performed, by using the Pearson chi-square test (χ^2) or Fisher's exact test, followed by a logistic regression analysis, which considered the "Gold standard" the mPCR result for Mollicutes as the dependent variable. The independent or explanatory variables considered in the model were those with a statistical significance of <0.20 . This probability was stipulated so that possible event risk factors were not excluded from the analysis (Hosmer and Lemeshow, 1989). The EPIINFO™ 3.5.1 program was used to perform the statistical calculations, considering as a risk factor in the final model the variables that presented statistical significance $P < 0.05$.

The present study was approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal Rural University of Pernambuco, under license N° 042/2015. All authors gave their consent for inclusion in this study.

RESULTS AND DISCUSSION

From the 355 samples analyzed using PCR, 9.3% (33/355) were positive for *Mycoplasma bovis* and 21.7% (77/355) for *Ureaplasma diversum*; coinfection was present in 2.8% (10/355). The positive result distribution is shown in Table 1. *Mycoplasma* spp. growth in the Hayflick medium was 81.8% (27/33) and *Ureaplasma* spp. growth in the UB medium was 24.7% (19/77).

Table 1. Results of PCR for the *M. bovis* and *U. diversum* in dairy cows in the Ipanema Valley microregion, Pernambuco state, Brazil

Property	N	Positive mPCR	<i>M. bovis</i>	<i>U. diversum</i>	Coinfection
A	35	23 (65.7%)	7	13	3
B	33	24 (72.7%)	7	15	2
C	58	23 (39.7%)	4	17	2
D	8	5 (62.5%)	-	4	1
E	20	1 (5.0%)	-	1	-
F	20	2 (10.0%)	-	2	-
G	13	1 (7.7%)	1	-	-
H	8	6 (75.0%)	-	6	-
I	14	5 (35.7%)	-	4	1
J	14	3 (21.4%)	1	1	1
K	8	0 (0.0%)	-	-	-
L	11	0 (0.0%)	-	-	-
M	13	2 (15.4%)	1	1	-
N	17	2 (11.7%)	-	2	-
O	28	1 (3.6%)	1	-	-
P	17	0 (0.0%)	-	-	-
Q	12	1 (8.3%)	-	1	-
R	26	1 (3.8%)	1	-	-
TOTAL	355	100 (28.2%)	23	67	10

N - number of samples gathered at the property.

The risk factors associated with Mollicutes infection in the studied herds were semi-intensive breeding system (OR= 4.6, $P = 0.040$), (OR= 3.2, $P < 0.001$), pasture rental performance (OR= 3.6, $P < 0.001$), and simultaneous use of artificial insemination and natural breeding in reproductive management (OR= 3.5, $P < 0.001$) (Table 2).

After analysis, the reproductive history of the studied herds, showed a significant association between the gestational stage in which abortion occurred ($P = 0.001$) and Mollicutes infection (Table 3). In addition, 85 cows had a history of granular vulvovaginitis, 3.5% (3/85) were positive for *Mycoplasma bovis* and 5.9% (5/85) were positive for *Ureaplasma diversum*.

Table 2. Analysis of risk factors associated with Mollicutes infection in dairy cattle from Ipanema Valley, Pernambuco state, Brazil

Variables	N	mPCR Positive	Valor P	Logistic regression OR (I.C. 95%)	Valor P
<i>Creation system</i>					
Semi-intensive	331	98 (29.6%)	0.031 ^{(A)*}	4.6 (1.0-20.0)	0.040*
Extensive	24	2 (8.3%)			
<i>Animal isolation</i>					
Yes	194	34 (17.5%)	<0.001 ^{(B)*}		
No	161	66 (41.0%)		3.2 (2.0-5.3)	<0.001*
<i>Pasture rent</i>					
Yes	209	79 (37.8%)	<0.001 ^{(B)*}	3.6 (2.1-6.2)	<0.001*
No	146	21 (14.4%)			
<i>Destination of sick animals</i>					
Discard	184	44 (23.9%)	0.064 ^(A)		
Remains	171	56 (32.7%)		1.5 (0.9-2.4)	0.065
<i>Reproductive management</i>					
Natural mount	243	54 (22.2%)	<0.001 ^{(A)*}	-	
Artificial insemination	66	23 (34.8%)		1.8 (1.0-3.3)	0.037*
Both	46	23 (50.0%)		3.5 (1.8-6.7)	<0.001*

^(A) Fisher's Exact Test; ^(B) χ^2 Test; ¹ different database (N= 296); N - Total Number of Samples; OR - Odds Ratio; I.C. - Confidence Interval; *Significant association at the P< 0.05 level.

This is the first record of *Mycoplasma bovis* and *Ureaplasma diversum* infections in dairy cattle in the semiarid region of the state of Pernambuco, Brazil. Mollicutes identification in the reproductive tract of bovine females was reported in Brazil (Nascimento et al., 2005; Buzinhani et al., 2007; Marques et al., 2013) and different countries (Lysnyansky et al., 2009; Argue et al., 2013; Ghanem et al., 2013).

An occurrence of 9.3% for *Mycoplasma bovis* infection was observed in the present study. These values are similar to those found in Ghanem et al. (2013) study, in Japan, which found 7.4% occurrence of *Mycoplasma bovis* in cervical vaginal mucus samples from post-partum period of Holstein cows.

Table 3. Reproductive history associated with Mollicutes (*M. bovis* and *U. diversum* or both) infection in dairy cows in Ipanema Valley, Pernambuco state, Brazil

Variable	N	mPCR Positive	Valor P
<i>Animals with mastitis</i>			
Yes	329	94 (28.6%)	0.549 ^(A)
No	26	6 (23.1%)	
<i>Placental retention history</i>			
Yes	322	92 (28.6%)	0.599 ^(A)
No	33	8 (24.2%)	
<i>Recurrence of estrus</i>			
Yes	343	99 (28.9%)	0.190 ^(A)
No	12	1 (8.3%)	
<i>Gestation period of abortion ¹</i>			
1/3	55	24 (43.6%)	0.001 ^{(A)*}
2/3	149	29 (19.5%)	
3/3	73	17 (23.3%)	

χ^2 Test; N - Total Number of Samples; ¹ different database (N= 277); * Significant association at the P< 0.05 level.

The low occurrence of Mollicutes infection may be related to the low amount of DNA present in the biological material. Husted (2003) reported that the low amount of DNA in the analyzed samples may result in a low frequency of positive samples. Furthermore, the animals did not present suggestive clinical signs during the gathering, unlike the studies conducted by Santos *et al.* (2013; 2015) which investigated the occurrence of Mollicutes in herds with reproductive disorders.

Historically, *M. bovis genitalium* and *Ureaplasma diversum* stand out as agents involved in outbreaks of reproductive diseases in cattle, the main species being diagnosed in mycoplasmosis clinical. *M. bovis genitalium* have been isolated from the vaginal secretions of cows, this agent has been associated with natural cases of granular vulvovaginitis (Afshar, 1967; Lysnyansky *et al.*, 2009).

In the present study, *Ureaplasma diversum* was detected in 21.7% (77/355) of the samples. The higher occurrence of *Ureaplasma diversum* infection compared to *Mycoplasma bovis genitalium* may be due to the agent colonization characteristic, for the reason that *Ureaplasma diversum* remains longer in the organism of asymptomatic animals depending on the urea concentrations in the urogenital tract excreta, which is an important nutrient for these bacteria (Sanderson *et al.*, 2000). Although, the silent disease can manifest itself with decreases in the reproductive performance of the cattle (Argue *et al.*, 2013; Santos *et al.*, 2015).

In the seven properties ("A," "B," "C," "D," "H," "I," and "J"), the occurrence of positive animals was high for the agents in question. In these properties, the milk production was higher, which corroborates with Gábor *et al.* (2008) that reported the Mollicutes occurrence in a herd with higher milk production. Moreover, the use of natural breeding and/or artificial insemination as reproductive management may have contributed for these agents spread between the herd. According to (Nicholas and Ayling, 2003), contaminated semen is an important transmission route in bovine specie.

It was observed that 81.8% (27/33) of the mPCR-positive samples grew *Mycoplasma* spp. in the specific medium. This percentage is higher

than that reported by other authors in Brazil (Nascimento *et al.*, 2005; Buziniani *et al.*, 2007) who also isolated only *Mycoplasma* spp. The results of the present study were higher than what was reported in previous studies that isolated *Mycoplasma* spp. species in cattle in other countries (Petit *et al.*, 2008; Lysnyansky *et al.*, 2009). In addition, the isolation of the genus is more practical than that of different *Mycoplasma* spp. species. About this, Nagatomo *et al.* (2001) inoculated several species of *Mycoplasma* spp. in liquid medium and found different isolation rates compared to the culture media and a sensitivity to temperature variations.

The isolation rate of *Ureaplasma* spp. was reported as 24.67% (19/77). In previous studies, higher results have been described (Buziniani *et al.*, 2007; Petit *et al.*, 2008; Marques *et al.*, 2013); however, in the afore mentioned research, the isolation was performed from herds with clinical signs of mycoplasmosis.

The isolation rate of *Ureaplasma* spp. was lower than that for *Mycoplasma* spp. It is possible that the presence of dead cells (*Ureaplasma* spp.) in the samples accounted for the lower rate of growth in the specific medium.

Natural breeding and artificial insemination were identified as risk factors associated with Mollicutes infection in the present study. Some authors confirm the presence of *Ureaplasma diversum* (Hobson *et al.*, 2013) and *Mycoplasma* (Cardoso and Vasconcelos, 2004) in semen and indicate the importance of this transmission route for the agent's spread in cattle (Petit *et al.*, 2008).

Animals submitted to the semi-intensive system were 4.6 times more likely to be infected by Mollicutes than those submitted to the extensive system. Santos *et al.* (2013) detected Mollicutes (65.6% - 21/32) and *Ureaplasma diversum* (15.6% - 5/32) in a reproductive disease outbreak where animals were reared in a semi-intensive system. In this system, the transmission of the agent by direct contact is favored (Gambarini *et al.*, 2009) mainly at the time of milking, because the cows have the habit of smelling and licking the external genitalia of each other, which facilitates the agent's dissemination (Rocha, 2009).

In properties with pasture rental practice, the animals are more likely to become infected by Mollicutes (OR= 3.6), which is also true for properties where animals with reproductive problems are not isolated (OR= 3.2). Thus, both the pasture rental practice and not isolating animals with reproductive disorders can lead to the contact of cattle with secretions and excretions from infected animals (Nicholas and Ayling, 2003).

There was an association between abortion in the first gestational third (P= 0.001) and Mollicutes infection, and history of abortion was observed in 45.6% of the positive cows. Petit *et al.* (2008) indicated that the detection of *Mycoplasma bovis* is greater in animals with a history of abortion. Another study implicated the occurrence of abortion in the first (25%) and third (75%) third of gestation. Given that there are divergences between the studies related to the timing of abortion, it is suggested that experimental or observational studies be conducted with the purpose of establishing the time of pregnancy at which the cows have a higher risk of aborting when infected with Mollicutes.

It was observed that 9.41% (8/85) of the positive cows (3/8 for *Mycoplasma bovis* and 5/8 for *Ureaplasma diversum*) showed signs of granular vulvovaginitis during the gathering of samples. Gambarini *et al.* (2009) and Cardoso *et al.* (1997) reported that granular vulvovaginitis may be associated with the presence of *Ureaplasma diversum*. Oliveira-Filho *et al.* (2005) affirmed that the presence of *Ureaplasma diversum* is associated with the occurrence of granular vulvovaginitis syndrome, and it is independent of the injury extension observed in the vulvovaginal mucosa. Some animals had granules in the vulva and were negative for Mollicutes, representing 90.5% (77/85). The clinical condition of VVG is not pathognomonic for Mollicutes infection.

CONCLUSION

This is the first record of the occurrence of *M. bovis* and *U. diversum* infection in dairy cattle in the semiarid region of Pernambuco. The semi-intensive breeding system, pasture rent, non-isolation of animals, natural mounting and artificial insemination are a risk for the

occurrence of *M. bovis* and *U. diversum* in the cattle studied. Mollicutes are associated with abortions in the in the first gestational third. Preventive measures directed at these identified risk factors may contribute to a decrease in the occurrence of these agents in the herds.

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