First report of *Anaplasma marginale* infection in goats, Brazil

Nayara B. da Silva¹, Naomi S. Taus²,³, Wendell C. Johnson², Anabela Mira⁴, Leonhard Schnittger⁴, Jessica D. M. Valente¹, Odilon Vidotto⁵, Hayley E. Masterson³, Tháílitha S. W. J. Vieira¹, Massaro W. Ueti²,³*, Rafael F. C. Vieira¹*,

¹ Department of Veterinary Medicine, Universidade Federal do Paraná, Curitiba, Paraná, Brazil, ² Animal Diseases Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Pullman, Washington, United States of America, ³ Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, United States of America, ⁴ Institute of Pathobiology, Center of Research in Veterinary and Agronomic Sciences, INTA-Castelar, Argentina, ⁵ Department of Preventive Veterinary Medicine, Universidade Estadual de Londrina, Londrina, Paraná, Brazil

☯ These authors contributed equally to this work.

* massaro_ueti@wsu.edu (MWU); rvieira@ufpr.br (RFCV)

Abstract

*Anaplasma marginale*, the causative agent of bovine anaplasmosis, is a tick-borne bacterium that causes significant economic losses for cattle industries and is increasingly being detected in other animal species. *Rhipicephalus microplus* is the main vector of this bacterium and may be found parasitizing small ruminants. In northeastern Brazil, multispecies grazing is a common family subsistence practice on smallholder farms possibly facilitating interspecies transmission of pathogens. Considering that *A. marginale* infection has been previously molecularly described in sheep, this study has aimed to estimate the prevalence of *A. marginale* and factors associated with the infection in goats from northeastern Brazil. A total of 403 goat blood samples were included in the study. An epidemiological questionnaire was applied to each farm owner addressing age, gender, presence of ticks and multispecies grazing. All samples were screened for *A. marginale*- and *A. ovis*-infection using primers targeting the *Anaplasma* spp. msp4 gene. The identity of *A. marginale* in the blood was confirmed by PCR amplification of msp5 followed by sequencing. *Anaplasma* spp. were differentiated by sequencing of the repeat region of the msp1α gene. For the statistical analysis the Chi-square or the Fisher’s exact test was used to verify association of the individual factors (age, gender, presence of ticks, and multispecies grazing) with *Anaplasma* spp. infection. We report the first molecular detection of *A. marginale* in goats from northeastern Brazil, based on msp1α, msp4 and msp5 gene sequencing analysis. Sequencing of the detected *A. marginale* msp1α gene revealed the F repeat. *Amblyomma parvum* and *R. microplus* were found feeding on animals.
Introduction

*Anaplasma marginale*, the causative agent of bovine anaplasmosis, is a tick-borne bacterium that causes significant economic losses for cattle industries [1]. *A. marginale* infects cattle through tick transmission worldwide. The bacterium has been detected in non-bovine species, but the relevance of these other species in the ecology of bovine anaplasmosis is unknown. At least 20 ixodid tick species have been implicated in the transmission of *A. marginale*, including *Dermacentor* spp. and *Rhipicephalus* spp. [1].

*Anaplasma marginale* is found in regions where tick vectors are endemic. In tropical and subtropical regions, *Rhipicephalus microplus* is the vector of bovine anaplasmosis. Despite host specificity of *R. microplus* for cattle [2], this tick species may be found parasitizing small ruminants [3]. In Brazil, *R. microplus* is endemic [4] and hampers livestock production resulting in annual economic losses estimated at US$ 3.24 billion [5].

In northeastern Brazil, multispecies grazing is a common family subsistence practice on smallholder farms possibly facilitating interspecies transmission of pathogens. A study of co-grazing ruminants has shown a single *A. marginale* strain infecting coexisting cattle, buffalo and ticks [6]. *A. marginale* infection has been previously molecularly described in sheep from Iran [7]. However, to the best of our knowledge, *A. marginale* has never been detected in goats. This study aimed to estimate the prevalence of *A. marginale* and factors associated with the infection in goats from the State of Paraíba, northeastern Brazil.

Material and methods

With the approval from the Ethics Committee for Animal Experimentation and Animal Welfare of the Universidade Federal da Paraíba (protocol 3305/14), a total of 403 blood samples from goats (368 females and 35 males), previously surveyed for other pathogens [8], were included in this study. All samples were collected in an anticoagulant tube containing ethylenediaminetetraacetic acid and stored at -80°C. An epidemiological questionnaire was given to each farm owner addressing age, gender, presence of ticks and multispecies grazing. The age of goats was stratified into groups of ≤ one year and > one year.

Genomic DNA was extracted from 200 μL of whole blood using a commercial kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer’s instructions. Negative controls using ultra-pure water were performed in parallel to monitor cross-contamination in each batch of 30 samples.

PCR amplification of the caprine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was done to verify successful DNA extraction, as previously described [9]. Samples were screened for *A. marginale* and *A. ovis*-infection using previously described primers targeting the *Anaplasma* spp. *msp4* gene (~870 bp) [10]. Amplified DNA fragments of the *msp4* gene from two *Anaplasma* spp. isolates were directly sequenced using the Sanger method, and analyzed sequences compared by BLASTn with those present in the GenBank database.

The identity of *A. marginale* in the blood was confirmed by PCR amplification of *msp5* followed by sequencing (GenBank accession numbers: MH037254-MH037263). *Anaplasma* spp. were differentiated by amplifying, cloning and sequencing of the repeat region of the *msp1α* gene as previously described [11]. The *msp1α* gene sequences obtained herein have been submitted to GenBank under the following accession numbers: MH590661-MH590670.

Either the Chi-square or the Fisher’s exact test was used to assess association of the individual factors such as age, gender, presence of ticks, and multispecies grazing including sheep and cattle with *Anaplasma* spp. infection. P-values were calculated and considered significantly
different when p < 0.05. Data was compiled and analyzed by Epi Info™ Software (version 7.1.5, CDC).

Results

All samples have successfully amplified the GAPDH gene. Eleven out of 403 goats (2.73%; CI 95%: 1.53–4.82%) were positive for the *Anaplasma msp4* gene. All analyzed *Anaplasma* spp. *msp4* gene sequences showed ≥99% identity to multiple *A. marginale msp4* gene sequences deposited in GenBank (KX989533, AY283196, EU283844, AY702919, CP001079). Ten out of eleven samples were positive for the *Anaplasma msp5* gene by nested PCR (Fig 1A), and sequencing of the *msp5* gene confirmed the presence of *A. marginale* sensu stricto (Fig 1C).

![Fig 1. Detection of *A. marginale msp1a* and msp5 genes in infected goats. A) 2% agarose gel detecting *msp1a* and msp5, B) MSP1a tandem repeats and C) alignment of MSP5 between *Anaplasma ovis* (Ao Jintai), *Anaplasma marginale* Florida (Am-Fl) and *Anaplasma marginale* from a Brazilian goat (Am-Goat). Red indicates variation in the *A. ovis* MSP5 protein and blue highlights identical amino acids at the same location between Am-Fl and Am-Goat.](https://doi.org/10.1371/journal.pone.0202140.g001)
The rate of *A. marginale*-infection and corresponding estimated parameters for each of the evaluated potential risk factor is shown in Table 1.

Tick-infested goats were six times more likely to be infected with *A. marginale* (*p* = 0.02788).

The tick species feeding on the studied goats were identified as *Amblyomma parvum* (49/52, 94.23%) and *R. microplus* (3/52, 5.77%). All *A. marginale*-positive goats were found on farms with multispecies (sheep and cattle) grazing (*p* = 0.04300). To determine the *A. marginale* tandem repeats as previously described [12], we performed *msp1α* PCR (Fig 1A) and the amplicons were cloned and sequenced. The sequence results revealed MSP1α tandem repeat F in Brazilian goats (Fig 1B).

**Discussion**

Although some goat *Anaplasma* MSP4 and MSP5 sequences previously reported in GenBank database have greater similarity to *A. marginale* than *A. ovis* (e.g. JN572928 and NZ_PKOE 00000000), this study describes the first molecular report of *A. marginale* in goats based on *msp1α* gene sequence analysis. Anti-*Anaplasma* spp. antibodies have been identified in goats from northeastern Brazil [13]. However, direct molecular detection of *A. marginale* in small ruminants has been only reported in sheep from Iran [7]. *R. microplus* has been described as the main vector of *A. marginale* [1], yet no study has tested the vector competence of *A. parvum* for *A. marginale*. Previous studies have suggested that *Amblyomma* ticks may be involved in the transmission of *A. marginale* [6,14]. Further studies are necessary to evaluate if *A. parvum* is a competent vector of *A. marginale* in Brazil.

Co-grazing of goats, sheep and cattle is the most common practice of northeastern Brazil, since it allows a wider diversification of products for commercialization. A previous study in Rio de Janeiro State, southeastern Brazil, has shown that *A. marginale* strains identified in water buffaloes were closely related to *A. marginale* from cattle as determined by sequencing *msp1α* [6]. In the Brazilian goats, the detected *A. marginale* was also closely related to cattle species and all detected MSP1α displayed the F repeat. Due to the different number of tandemly repeated 29 amino acid units, the molecular weight of MSP1α varied between *A. marginale* isolates [15]. A previous report demonstrated individual animals had the same tandem repeat but different numbers of repeats ranging from 2 to 6 repeats [12]. In another study, molecular weight variation was used to differentiate and characterize geographic genotypes [15]. However, in both cases, sequences were used to determine *A. marginale* genotypes.

### Table 1. Prevalence of *Anaplasma marginale* in goats within each variable studied, Paraíba State, northeastern Brazil.

|                          | +/n   | (%)  | OR   | 95% CI  | p-value |
|--------------------------|-------|------|------|---------|---------|
| **Age**                  |       |      |      |         |         |
| >1                       | 10/337| 2.97 | 1.987 | 0.25–15.79 | 0.43814 |
| ≤1                       | 1/66  | 1.52 |       |         |         |
| **Gender**               |       |      |      |         |         |
| Female                   | 10/368| 2.72 | 0.949 | 0.12–7.64 | 0.63673 |
| Male                     | 1/35  | 2.86 |       |         |         |
| **Presence of ticks**    |       |      |      |         |         |
| Yes                      | 3/26  | 11.54| 6.0163| 1.49–24.21 | 0.02788 |
| No                       | 8/377 | 2.12 |       |         |         |
| **Multispecies grazing** |       |      |      |         |         |
| Yes                      | 11/304| 3.62 | †     | †       | 0.04300 |
| No                       | 0/99  | 0.00 |       |         |         |

Abbreviations: *msp4*, major surface protein 4; +, Number of positive animals; n, number of samples; OR, odds ratio; 95% CI, 95% confidence interval
†, not applicable
*, reference.

https://doi.org/10.1371/journal.pone.0202140.t001
Brazil, six microsatellite genotypes have been reported, including repeats B, C, D, E, G and H, with genotype E being the most common in cattle [16,17]. Unfortunately, the nucleotide distance between the Shine-Dalgarno (SD) sequence and the translation initiation codon (ATG) could not be determined [18]. Thus, it is unknown whether other A. marginale genotypes infect goats. Additional studies are necessary to elucidate this question.

Conclusions

This study provides the first insight into A. marginale infection in goats from Paraíba State, northeastern Brazil and demonstrates that goats may play a role in the epidemiology of A. marginale as a yet unrecognized reservoir. Competent ticks feeding on goats and cattle may transfer the pathogen between the two livestock species.

Acknowledgments

We thank Iago Barbosa for his assistance during blood sampling.

Author Contributions

Conceptualization: Leonhard Schnittger, Massaro W. Ueti, Rafael F. C. Vieira.
Data curation: Nayara B. da Silva, Jessica D. M. Valente.
Formal analysis: Naomi S. Taus, Wendell C. Johnson, Anabela Mira, Jessica D. M. Valente, Hayley E. Masterson, Thállitha S. W. J. Vieira, Massaro W. Ueti.
Investigation: Nayara B. da Silva, Naomi S. Taus, Leonhard Schnittger, Odilon Vidotto, Hayley E. Masterson, Thállitha S. W. J. Vieira.
Methodology: Wendell C. Johnson, Leonhard Schnittger, Odilon Vidotto, Thállitha S. W. J. Vieira, Massaro W. Ueti, Rafael F. C. Vieira.
Project administration: Rafael F. C. Vieira.
Resources: Leonhard Schnittger, Odilon Vidotto, Thállitha S. W. J. Vieira, Massaro W. Ueti, Rafael F. C. Vieira.
Supervision: Rafael F. C. Vieira.
Writing – original draft: Nayara B. da Silva, Leonhard Schnittger, Jessica D. M. Valente, Thállitha S. W. J. Vieira, Massaro W. Ueti, Rafael F. C. Vieira.
Writing – review & editing: Leonhard Schnittger, Odilon Vidotto, Thállitha S. W. J. Vieira, Massaro W. Ueti, Rafael F. C. Vieira.

References

1. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA. The natural history of Anaplasma marginale. Vet Parasitol. 2010; 167: 95–107. https://doi.org/10.1016/j.vetpar.2009.09.012 PMID: 19811876
2. Ma M, Chen Z, Liu A, Ren Q, Liu J, Liu Z, et al. Biological parameters of Rhipicephalus (Boophilus) microplus (Acari: Ixodidae) fed on rabbits, sheep, and cattle. Korean J Parasitol. 2016; 54: 301–305. https://doi.org/10.3347/kjp.2016.54.3.301 PMID: 27417084
3. Brito DRB, Santos ACG, Guerra RMSNC. Ectoparasitos em rebanhos de caprinos e ovinos na micro-região do Alto Mearim e Grajaú, Estado do Maranhão. Rev Bras Parasitol Veterinária. 2005; 14: 59–63.
4. Dantas-Torres F, Onofrio VC, Barros-Battesti DM. The ticks (Acari: Ixodida: Argasidae, Ixodidae) of Brazil. Syst Appl Acarol. 2009; 14: 30–46.
5. Grisi L, Leite RC, Martins JR de S, Barros ATM de, Andreotti R, Cançado PHD, et al. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev Bras Parasitol Veterinária. 2014; 23: 150–156. http://dx.doi.org/10.1590/S1984-29612014042 PMID: 25054492

6. Silva JB da, Cabezas-Cruz A, Fonseca AH, Barbosa JD, de la Fuente J. Infection of water buffalo in Rio de Janeiro Brazil with Anaplasma marginale strains also reported in cattle. Vet Parasitol. 2014; 205: 730–734. https://doi.org/10.1016/j.vetpar.2014.09.009 PMID: 25260335

7. Yousefi A, Rahbari S, Shayan P, Sadeghi-dehkordi Z, Bahonar A. Molecular detection of Anaplasma marginale and Anaplasma ovis in sheep and goat in west highland pasture of Iran. Asian Pac J Trop Biomed. 2017; 7: 455–459. http://dx.doi.org/10.1016/j.apjtb.2017.01.017

8. Machado CAL, Vidotto O, Conrado FO, Santos NJR, Valente JDM, Barbosa IC, et al. Mycoplasma ovis infection in goat farms from northeastern Brazil. Comp Immunol Microbiol Infect Dis. Pergamon; 2017; 55: 1–5. https://doi.org/10.1016/j.cimid.2017.08.004 PMID: 29127986

9. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of Babesia gibsoni (Asian genotype) and B. canis DNA in canine blood samples. J Clin Microbiol. 2003; 41: 4172–4177. https://doi.org/10.1128/JCM.41.9.4172-4177.2003 PMID: 12958243

10. de la Fuente J, Atkinson MW, Naranjo V, Mera IGF de, Mangold AJ, Keating KA, et al. Sequence analysis of the msp4 gene of Anaplasma ovis strains. Vet Microbiol. 2007; 119: 375–381. https://doi.org/10.1016/j.vetmic.2006.09.011 PMID: 17052866

11. Castañeda-Ortiz EJ, Veit MW, Camacho-Nuez M, Mosqueda JJ, Mousel MR, Johnson WC, et al. Association of Anaplasma marginale strain superinfection with infection prevalence within tropical regions. PLoS One. 2015; 10. https://doi.org/10.1371/journal.pone.0120745 PMID: 25793966

12. Palmer GH, Knowles DP, Rodriguez JL, Gnad DP, Hollis LC, Marston T, et al. Stochastic transmission of multiple genotypically distinct Anaplasma marginale strains in a herd with high prevalence of Anaplasma infection. J Clin Microbiol. 2004; 42: 5381–5384. https://doi.org/10.1128/JCM.42.11.5381-5384.2004 PMID: 15528749

13. Ramos RN, Ramos CAN, Araújo FR, Melo ESP, Tembue AASM, Faustino MAG, et al. Deteccão de anticorpos para Anaplasma sp. em pequenos ruminantes no semi-árido do estado de Pernambuco, Brasil. Rev Bras Parasitol Veterinária. 2008; 17: 115–117. http://dx.doi.org/10.1590/S1984-29612008000200011 PMID: 18823582

14. Silva JB da, Fonseca AH da, Barbosa JD. Molecular characterization of Anaplasma marginale in ticks naturally feeding on buffaloes. Infect Genet Evol. 2015; 35: 38–41. https://doi.org/10.1016/j.meegid.2015.07.027 PMID: 26209411

15. Cabezas-Cruz A, Passos LMF, Lis K, Kennell R, Valdés JJ, Ferrolho J, et al. Functional and immunological relevance of Anaplasma marginale major surface protein 1a sequence and structural analysis. PLoS One. 2013; 8: 1–13. https://doi.org/10.1371/journal.pone.0065243 PMID: 23776486

16. Machado RZ, Silva JB da, André MR, Gonçalves LR, Matos CA, Obregón D. Outbreak of anaplasmosis associated with the presence of different Anaplasma marginale strains in dairy cattle in the states of São Paulo and Goiás, Brazil. Brazilian J Vet Parasitol. 2015; 24: 438–446. https://doi.org/10.1590/S1984-29612015078 PMID: 26648009

17. Pohl AE, Cabezas-Cruz A, Ribeiro MFB, Silveira JAG da, Silaghi C, Pfister K, et al. Detection of genetic diversity of Anaplasma marginale isolates in Minas Gerais, Brazil. Rev Bras Parasitol Veterinária. 2013; 22: 129–35. http://dx.doi.org/10.1590/S1984-29612013000100024 PMID: 24252959

18. Estrada-Peña A, Naranjo V, Acevedo-Whitehouse K, Mangold AJ, Kocan KM, de la Fuente J. Phylogeographic analysis reveals association of tick-borne pathogen, Anaplasma marginale, MSP1a sequences with ecological traits affecting tick vector performance. BMC Biol. 2009; 7: 57. https://doi.org/10.1186/1741-7007-7-57 PMID: 19723295