DOI: 10.5965/223811712332024537

Revista de Ciências Agroveterinárias 23 (3): 2024
Universidade do Estado de Santa Catarina

Prevalence and risk factors of hemoparasites in cattle in León, Nicaragua

Detecção molecular e fator de risco de hemoparasitas em bovinos no município de León, Nicarágua

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Submission: 19/12/2023 | *Acceptance: 13/04/2024*

ABSTRACT

Hemoparasite infections are a substantial challenge to agriculture, worldwide. Infected cattle suffer malnutrition, stunting, decreased milk production, and reproductive loss, which can result in great economic loss. The aim of this study was to characterize the predominant hemoparasites affecting cattle in Nicaragua, whose economy is largely dependent on agriculture, and define associated epidemiological factors. Blood from 68 bovines in the municipality of León were analyzed by microscopy and molecular techniques. PCR revealed *Anaplasma marginale* in 33.82% (IC 95%: 21.84-45.80), 19.11% (IC 95%: 9.03-29.19) of animals were infected with *Babesia* spp, and 8.82% (95% CI: 1.34-16.30) were simultaneously co-infected with *Babesia* spp and *A. marginale*. However, *A. phagocytophilum*, *Leishmania* spp., or *Trypanosoma* spp were not detected (0%, 95% CI: 0.0-5.20). *A. marginale* was more frequently detected in males (p=0.041) and calves (p=0.041). This is the first study to report the prevalence of hemoparasites in cattle using molecular diagnosis in western Nicaragua.

KEYWORDS: hemoparasites, cattle; Nicaragua; *Anaplasma* spp.; *Babesia* spp.

RESUMO

Agentes infecciosos chamados hemoparasitas destroem as hemácias em bovinos. O objetivo deste estudo foi detectar os principais hemoparasitas em bovinos no município de León, Nicarágua, e os fatores epidemiológicos associados à sua presença. Foram coletadas 68 amostras de sangue bovino em EDTA e realizada PCR a partir de uma amostra conglomerada selecionada para representar os bovinos do município de León. A análise de PCR revelou que 33,82% (IC 95%: 21,84-45,80) dos animais foram positivos para *A. marginale*, 19,11% (IC 95%: 9.03-29,19) dos animais estavam infectados com *Babesia* spp, 8,82% (95% de IC: 1,34-16,30) estavam infectados com *Babesia* spp. e *A. marginale*, no entanto, nenhum animal foi considerado positivo para *A. phagocytophilum*, *Leishmania* spp. ou *Trypanosoma* spp (0%, IC 95%: 0,0-5,20). A. marginale foi detectada com maior frequência em machos (p=0,041) e bezerros (p=0,041). Este é o primeiro estudo que relata a prevalência de hemoparasitas em bovinos, aplicando diagnóstico molecular no oeste da Nicarágua.

PALAVRAS-CHAVE: hemoparasitas, bovinos; Nicarágua; *Anaplasma* spp.; *Babesia* spp.

Blood-borne parasites constitute a substantial cause of concern in the agricultural environment, from subsistence farming to commercial production. Hemoparasites in cattle have a major impact on the economy and public health. In addition to challenges to animal health and welfare, many of these pathogens pose risks to human health through zoonotic spread. Agents such as *Anaplasma* spp, *Leihsmania* spp, *Trypanosoma* spp, *Babesia* spp and *Ehrlichia spp. are among the most prevalent, disproportionately affecting communities in tropical* and subtropical *regions*.

Greater and more granular epidemiological clarity could translate into better control efforts. An important aspect of hematoparasites is that they can be transmitted by various vectors, including ticks, hematophagous flies, and mosquitoes. The dynamics of the vector population depend greatly on the specific demographic and environmental characteristics of an area, with different factors favoring population growth and dispersal to facilitate the spread of the pathogen (JAIMES-DUEÑEZ et al. 2018b, ROBI et al. 2021, JAIMES-DUEÑEZ et al. 2018a) and transmission between herds and between different species of animals, including between cattle and humans. (SALAMANCA-CARREÑO et al. 2018). It is well established that the tropics bear a disproportionate burden of parasitic diseases and can make the most of efficient and effective control measures. Vector populations are also more concentrated in tropical regions than in other regions. In South America alone, the economic impact of controlling babesiosis and anaplasmosis is estimated to be 875 million dollars. (HOVE et al. 2018). It is important to emphasize that the characteristics of tropical regions are not homogeneous throughout the world, making it important to establish local epidemiology as a basis for more strategic disease control efforts.

During the infectious cycle of blood-borne parasites, erythrocytes are destroyed, creating a cascade of health problems and dwarfism. Infected cattle suffer emaciation, losing up to 30% of their body weight. Moderate production falls to an average of 1.16 L/day for each infected cow (RASHID et al. 2019), and malnutrition is another concern, as has already been described with trypanosomiasis, attributed to hepatocellular damage and the resulting albumin deficiency (HOTA et al. 2019). Bovine hemoparasitosis can have serious reproductive implications, causing abortions or even being transmitted through semen (COUTO et al. 2022).

The hemoparasites that affect cattle can also be transmitted to other mammals, including humans. *The zoonotic spread of rickettsial parasites transmitted by ticks, such as Anaplasma* spp, and protozoan parasites transmitted by hematophagous flies, such as *Trypanosoma* spp. and Leishmania spp, are serious and long-standing problems for human and veterinary public health.

Nicaragua is an agricultural country with more than 6 million heads cattle, and livestock farming has been one of its main economic endeavors for many years. More than 90% of livestock is in the hands of small producers (VERGARA-CAMUS & KAY 2017), who can suffer the most significant costs when their animals suffer from hemoparasitic infections. It is estimated that 60% of cattle mortality in Nicaragua is associated with hemoparasitosis, mainly in dry areas where the low availability of pasture decreases the grazing area and increases vulnerability (INTA NICARAGUA 2021).

Despite this, no study has been conducted to characterize the load of specific hemoparasites affecting cattle in Nicaragua or to determine related epidemiological factors. This information is crucial for informing evidence-based prevention and control measures, which would ultimately improve the health and well-being of human and animal populations. Therefore, this study aimed to use molecular techniques to identify and describe the most important bovine hemoparasites in the municipality of León, Nicaragua, and to identify risk factors for infection. The overall objective is to provide an evidence base for partnerships that unite human and animal health domains to reduce the burden of vector-borne parasites in Nicaragua.

A descriptive pilot study was carried out during the rainy season, from August to October 2022, among cattle in rural communities in Leon, Nicaragua. A sample of 68 cattle selected from the estimated total cattle population of 27295 (ANUARIO ESTADÍSTICO 2019 2021) was determined to be sufficient for a prevalence pilot, with a confidence level of 90% and precision of 10%. An agnostic prevalence (50%) was assumed, as the study aimed to detect various hemoparasites, and the calculation was carried out in EPIDAT 4.1 (PÉREZ & BRAYANA 2020). A cluster sampling technique (rural community) was employed,

first randomly selecting 4 of the 14 known rural communities in the study area: Abangasca (n= 15), Chacaraseca (n=34), El Convento (n=4) and Las Chacaras (n=15). Within each community, the animals were randomly selected.

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Blood was collected from the ear vein for blood smear preparation and from the jugular vein for complete blood count (CBC) and DNA extraction. Data were collected from each animal (breed, age, sex and tick control) at the same time as sample collection using a standardized instrument developed for this study.

For all patients, CBC was performed as described by Verbrugge et al. (VERBRUGGE & HUISMAN 2015). Blood smears were prepared using the rapid panoptic staining method and examined microscopically with an OLYMPUS CX31 optical microscope (100x) to identify *Anaplasma, Babesia, Ehrlichia, Leishmania, and Trypanosoma spp*.

QIAamp DNA kits (Qiagen, Hilden, Germany) were used to extract and purify DNA from 200 µl of whole blood with a final elution volume of 50 µl and a negative control (water without nuclease). *Conventional PCR was performed to detect Leishmania* spp, *T. vivax, T. cruzi, T. brucei evansi, Anaplasma* spp., A. phagocytophilum, and *A. marginale* using the primers described in Table 1.

Table 1. PCR primers to detect hemoparasites in cattle.

Hemoparasites	Sequences 5´- 3´	Gen	Products (pb)	Reference
Leishmania spp	CCTATTTTACACCAACCCCCAGT GGGTAGGGGCGTTCTGCGAAA	kinetoplast	120	(SHANG et al. 2011)
Trypanosoma vivax	CGCAAGTGGACCGTTCGCCT ACGCGGGGCGAACAGAAGTG	Proline racemase	239	(FIKRU et al. 2014)
Trypanosoma brucei evansi	ACAGTCCGAGAGATAGAG CTGTACTCTACATCTACCTC	minicircle	436	(GETAHUN et al. 2022)
Trypanosoma cruzi	CGAGCTCTTGCCCACACGGGTGCT CCTCCAAGCAGCGGATAGTTCAGG	kinetoplast	188	(MATEUS et al. 2019)
Anaplasma phagocytophilum	AACGGATTATTCTTTATAGCTTGCT GGCAGTATTAAAAGCAGCTCCAGG	16S rRNA	546	(PANTHAWONG et al. 2020)
Anaplasma spp	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC	16S rRNA	932	(PANTHAWONG et al. 2020)
Anaplasma marginale	TAAGAATTAAGCATGTGACCGCTGAC GTGTTCGTTGGGGTGTGATAGATGAG	msp5	652	(CORONA et al. 2001)

For a reaction volume of 50 µl, we combined 25 µl of Master Mix 2X (Promega, USA), 12 µl of nuclease-free water, 4 µl of each primer (1x10 3 nM) and 5 µl of DNA sample. We added a negative control (nuclease-free water) and a positive control (DNA from positive samples deposited for each agent, provided by the Veterinary Diagnostic and Research Center of Nicaragua). PCR was performed using an Applied Biosystem 2720 thermal cycler, raising the temperature to 94°C for 10 min, followed by 40 cycles (95°C for 50 seconds, 55°C for one minute, 72°C for one minute) and a final extension for 7 min at 72°C. To visualize the PCR products, electrophoresis was performed on a 1.3% agarose gel stained with ethidium bromide, applying 10 µl of the amplification product to each well and visualizing the result using an ultraviolet light transilluminator.

No animal experiments were performed. *All the cattle studied were handled in accordance with the "Law for the Protection and Welfare of Domestic and Wild Domesticated Animals (747)" (NORMAS JURÍDICAS DE NICARAGUA 2011)*. Blood sampling is a routine laboratory diagnosis performed by experienced veterinarians. Informed consent was obtained from each participant.

For statistical analysis, the numerical variables were analyzed using the Shapiro–Wilk test and presented as means with standard deviations. Categorical variables are presented as proportions with 95% confidence intervals (95% CI). Inferential statistics were used to determine bivariate associations between independent and dependent variables using Fisher's exact test. In the case of numerical variables, Student's t-test was applied.

As a result of the direct smear, 73.52% (95% CI: 62.30-84.75) animals had structures compatible with *Anaplasma* spp., and 19.11% (95% CI: *9.03-29.19) were infected with Babesia spp.* Co-infection with *Anaplasma* spp and *Babesia* spp was observed simultaneously in 10.76% (95% CI: 3.37-20.15). *Trypanosoma*, *Ehrlichia*, and *Leishmania* spp. were not observed in any of the cattle (0%, 95% CI: 0.0-5.2).

PCR analysis revealed that 33.82% (95% CI: 21.84-45.80) of the cattle were positive for *A. marginale* (Figure 1) and 19.11% (95% CI: *9.03-29.19) were infected with Babesia spp.* Co-infections with *Babesia* spp and *A. marginale* were observed in 8.82% (95% CI: 1.34-16.30). *None were found to be positive for A. phagocytophilum*, *Leishmania* spp., or Trypanosoma spp (0%, 95% CI: 0.0-5.20).

In the analysis to identify factors associated with hemoparasite infection, we focused on A. marginale and *Babesia* spp (Table 2). A. marginale *was statistically more common among male cattle (p = 0.041) and calves (p* = 0.041). No epidemiological risk factors for *Babesia* spp. were identified by this analysis. *In the clinical analysis, a higher basophil fraction was observed in cattle infected with Babesia* spp*(p* = 0.038), whereas no characteristic was associated with A. marginale infection (Table 3).

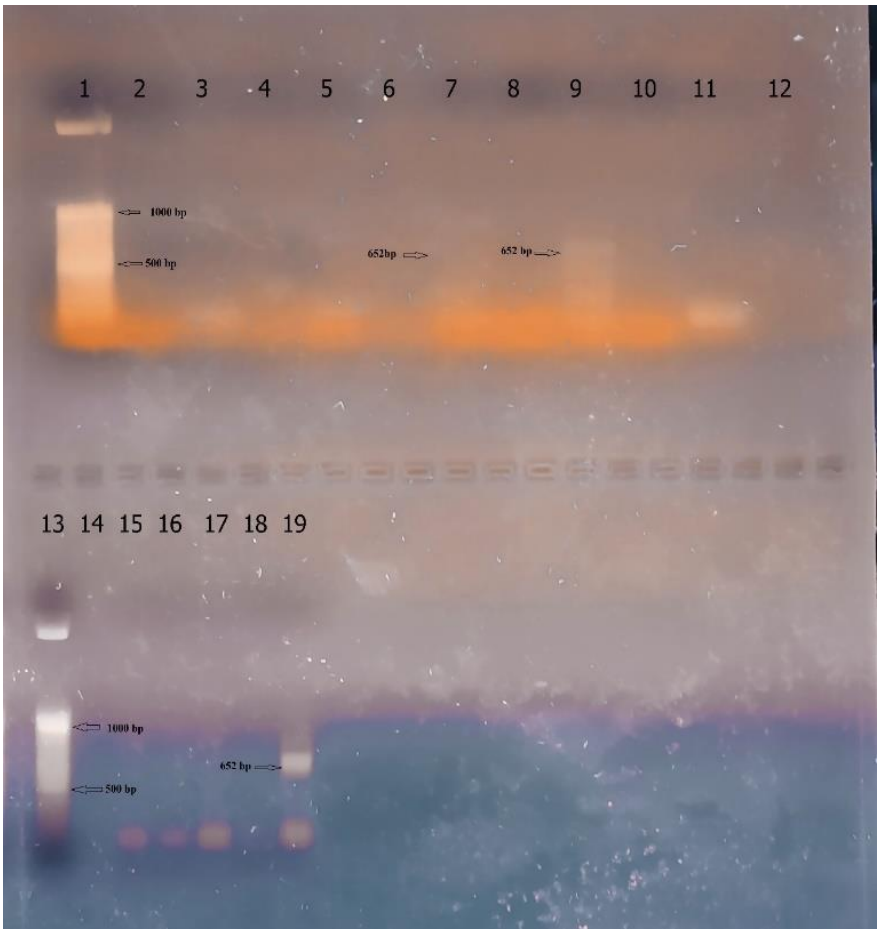

Figure 1. PCR analysis of *Anaplasma marginale*. Lines 1 and 13: molecular weight marker (100 bp); lines 7 and 9: samples positive for *Anaplasma marginale*; line 18: negative control; line 19: positive control.

Table 2. Epidemiological factors associated with Anaplasma marginale and Babesia infection in cattle in León.

Characteristic		PCR analysis of *Anaplasma marginal*		*Babesia* spp	
		Negative	Positive	Negative	Positive
Breed	Brahman	10	5	14	1
	Brown	13	3	15	1
	Pardo/Brahman mix	22	15	26	11
	Significance*	0.316		0.080	
Gender	Female	44	19	51	12
	Male	1	4	4	1
	Significance	0.041		0.665	
Age Category	Calf	2	5	5	2
	Cow	42	17	48	11
	Heifer	1	1	2	0
	Significance	0.041		0.750	
Tick control	No	6	17	17	6
	yes	12	33	38	7

	Significance	0.600		0.339	
Community	Abangasca	6	6	10	3
	Chácaraseca	25	10	27	8
	The Convent	4	1	4	1
	Las Chácaras	10	6	14	1
	Significance	0.554		0.549	

Table 3. Association between hematological parameters and infection by *A. marginale* and *Babesia* spp. in cattle in León.

Blood parameter	*A. marginale PCR*				*Babesia* spp		
	Results	Mean	SD	p-value	Mean	SD	p-value
Hematocrit%	Negative	30.42	4.26	0.442	29.84	5.18	0.393
	Positive	29.43	6.16		31.15	3.89	
Protein g/dL	Negative	7.56	0,52	0.338	7.52	0.67	0.759
	Positive	7.41	0,76		7.46	0.28	
White blood cells/ml (x103)	Negative	10.78	2.92	0.696	10.52	3.23	0.459
	Positive	10.44	4.11		11.29	3.86	
Red blood cells/ml (x106)	Negative	8.26	10,62	0.392	7.86	9.68	0.722
	Positive	6.53	2.14		6.89	1.72	
Lymphocytes %	Negative	30.84	10.85	0.996	32.11	12.63	0.092
	Positive	30.83	16.23		25.46	12.57	
N. Segmented %	Negative	41.64	17.56	0.918	40.71	18.09	0.461
	Positive	41.17	18.31		44.77	16.05	
Eosinophiles %	Negative	5.98	4.71	0.379	5.69	4.81	0.795
	Positive	4.91	4.58		5.31	4.13	
Monocytes%	Negative	20.44	15.70	0.824	20.49	15.58	0.784
	Positive	21.30	13.71		21.77	12.49	
Basophiles %	Negative	1.40	2.50	0.933	1.09	2.21	0.038
	Positive	1.35	2.23		2.62	2.81	

We found a high prevalence of *A. marginale*, *Babesia* spp and their co-infection. This is similar to what was reported for Latin America as a region by a meta-analysis for *A. marginale* (48.9% [95% CI: 30.3–67.8%]), *Babesia* spp. (39.8% [95% CI: 24.6- 57.2%]), and their co-infection (26.1% [95% CI: 9.1–55.8%]) (FERREIRA et al. 2022). In this study, a high prevalence of *A. marginale* was observed in Mexico (67.1%) and northern Brazil (71.9%). Overall, Brazil had a prevalence of 36.6% for *A. marginale*, 62.6% for *Babesia* spp., and 8.2% for their co-infections, which is more similar to what we found in Nicaragua. *Babesia* spp. prevalence was considerably different between regions, with the highest values observed in the North (97.4%) and the lowest in the South (9.5%).

We found that male cattle may be at greater risk of infection by *A. marginalis*. This study is similar to a report from Pakistan on the higher prevalence of *Anaplasma* in men (26.25%) than in women (16.62%) (FAROOQI et al. 2018). The higher prevalence in men can be attributed to the fact that most of them are destined for meat, and farmers are negligent and neglect health care. However, this differs from the higher prevalence reported in female cattle in Pakistan (ASHRAF et al. 2021) and a study in Kenya, which reported no gender difference (OKAL et al. 2020). One hypothesis is that women experience a higher prevalence of parasites with depressed immunity during pregnancy due to stress and altered hormone levels (KAMANI et al. 2010). There is also a potential for increased exposure to infection through contaminated needles through the practice of injecting hormones to release milk (ATIF et al. 2012). Another risk factor for *A. marginals* infection was young age, as reported in Pakistan (KHAN et al. 2019). Young animals may have less robust or underdeveloped immune systems than older animals (SHAKAR et al. 2018). A limitation of our analysis was the small number of male individuals available for our analysis, although this was not considered to be any different from the sexual distribution of the herds.

The hematological parameters did not provide any additional information about infected and non-infected animals in relation to *A. marginalis*. However, the infected animals exhibited hematocrit anemia and slightly depleted CBC. This is consistent with the course of hemoparasite infection and has also been reported elsewhere, in addition to lower packed cell volume (PCV) and hemoglobin concentration (ESMAEILNEJAD et al. 2018). In addition, these same researchers attributed the anemia they observed to oxidative stress, which correlated with the load of the *Anaplasma* parasite in the blood, reducing the symmetry of the membrane and increasing the osmotic fragility of the erythrocytes, which also makes them susceptible to erythrophagocytosis. We also observed that *Babesia* infection was associated with a basophilic presentation, which was hypothesized by others as a function of the immune response to tick infestation (UNIGWE et al. 2022).

We observed a higher prevalence of *Anaplasma* spp. via microscopy than detected via PCR. As molecular techniques are more sensitive and specific than direct observations of blood smears, this was an unexpected discovery. We expected to identify only a subset of true infections by microscopy because erythrocyte inclusion bodies are only found when the infection is acute with a high parasite load, making direct observation of this feature unlikely during early infection or with a low parasite load (PARODI et al. 2021). Microscopy is inherently subjective and requires trained personnel. False positives can also occur with microscopy because the stain used can produce artifacts, and inclusion bodies that resemble *A. marginale* can appear in the smear. However, we are not the first to report this phenomenon with *Anaplasma* in cattle, as a group in the Philippines also reported a higher frequency of blood smear detection (73.7%) than PCR (63.7%) (AQUINO et al. 2018). In our study, the laboratory staff have a lot of experience in the laboratory identification of veterinary pathogens using a wide range of techniques, including microscopy and molecular methods, and we believe that this is unlikely to be explained by user error or subjectivity alone.

This is the first report on the prevalence of hemoparasites in cattle that applies molecular diagnostics in the country and the first attempt to define the epidemiological factors of bovine infection by *A. marginale* in western Nicaragua. This fundamental work will help develop strategies for the prevention and control of bloodborne parasites in Nicaragua. Other regions with similar demographic and ecological characteristics can also benefit from our experience. Finally, our study demonstrates the feasibility of further research into these and other parasitic and vector-borne diseases, which can ultimately inform veterinary and vector programs that benefit public health and improve the profitability of livestock farming for small producers.

FUNDING

This study was supported by the Fondos Concursables de Investigación 2021, REF:STCNU/187/2021, of the Consejo Nacional de Universidades (CNU).

ACKNOWLEDGMENTS

We would like to thank the small livestock farmers in the municipality of León who agreed to participate in the study on a voluntary basis.

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