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Loop-mediated isothermal amplification test as an alternative for molecular diagnosis of natural infection by *Anaplasma marginale*

Uso do teste de amplificação isotérmica por loop como alternativa para diagnóstico molecular de infecção natural por *Anaplasma marginale*

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ABSTRACT: Bovine anaplasmosis is one of the most important tick-borne diseases caused by *Anaplasma marginale*, an intraerythrocytic parasite of cattle. Anaplasmosis is considered endemic in tropical and subtropical regions and results in significant economic losses in the livestock industry. The disease begins with a high rate of parasite multiplication in the host but is often asymptomatic, making it difficult to implement control measures. Reducing uncertainty of infection through early detection, even in conditions of low parasitemia, can assist producers in management decisions and reduce the economic impact of anaplasmosis. Thus, the aim of this study was to evaluate the Loop-Mediated Isothermal Amplification (LAMP) test as a rapid and cost-effective alternative for the molecular diagnosis of natural infection by *A. marginale.* Assessing the quality of anaplasmosis diagnostic assays allows for the appropriate selection of tests for clinical and epidemiological interest. To evaluate the LAMP test, 150 field samples of blood from buffaloes and cattle from farms in Amapá, Brazilian Amazon, were comparatively examined by LAMP, PCR, and real-time PCR. The prevalence of anaplasmosis detected by LAMP (56%) was higher than real-time PCR (28.66%) and conventional PCR (3.33%). Although the kappa agreement between the techniques was not satisfactory, the results suggest that LAMP is a rapid, sensitive, and specific technique for detecting *A. marginale* and can be an additional diagnostic option in the clinical routine for anaplasmosis in cattle and buffaloes.

Keywords: PCR; real-time PCR; LAMP; bovine anaplasmosis; buffalo.

RESUMO: A anaplasmose bovina é uma das mais importantes doenças transmitidas por carrapatos causada por *Anaplasma marginale*, parasita intraeritrocitário de bovinos. A anaplasmose é considerada endêmica em regiões tropicais e subtropicais e acarreta grandes perdas econômicas na indústria pecuária. A doença tem início com alta taxa de multiplicação do parasita no hospedeiro, mas frequentemente assintomático o que dificulta a adoção de medidas de controle. Reduzir a incerteza da infecção por meio da detecção precoce e em condições de baixa parasitemia pode ajudar nas decisões de manejo do produtor e reduzir o impacto econômico da anaplasmose. Assim, o objetivo deste estudo foi avaliar o teste de amplificação isotérmica LAMP como alternativa rápida e econômica para diagnóstico molecular de infecção natural por *A. marginale*. A avaliação na qualidade de ensaios de diagnóstico de anaplasmose permite a escolha adequada do teste para interesse clínico e epidemiológico. Para avaliar o teste LAMP, 150 amostras de campo, de sangue de búfalos e bovinos provenientes de fazendas do Amapá, Amazônia brasileira, foram comparativamente examinadas por LAMP, PCR e PCR tempo real. A prevalência de anaplasmose obtida por LAMP (56%) foi maior que PCR tempo real (28,66%) e PCR convencional (3,33%). Apesar da concordância kappa entre as técnicas não ter sido satisfatória, os resultados sugerem LAMP como técnica rápida, sensível e específica na detecção de *A. marginale* e pode ser uma opção a mais de diagnóstico na rotina clínica para anaplasmose em bovinos e bubalinos.

Palavras-chave: PCR; PCR tempo real; LAMP; anaplasmose bovina; búfalo.

INTRODUCTION

Tick-borne diseases (TBD) represent a significant challenge for livestock farming worldwide, causing substantial economic losses, particularly in tropical and subtropical regions (Ogata *et al.*, 2021). In Brazil, TBD result in annual economic loss of approximately 3.24 billion dollars (Grisi *et al.*, 2014).

Bovine anaplasmosis is one of the most important TBD, with its main etiological agent being the rickettsia *Anaplasma marginale*, an obligate intracellular bacterium of bovine erythrocytes (Kovalchuk; Babii; Arkhipova, 2020). The disease is typically transmitted by biological vectors, primarily through various species of ticks, with *Rhipicephalus microplus* being the main vector, or by mechanical vectors such as blood-feeding insects and veterinary instruments (Spare *et al.*, 2020).

Cattle of all breeds and ages can be infected; however, the severity of the disease depends on nutritional status, management, and age, with more severe cases occurring in animals older than two years. Bovine anaplasmosis is a hemolytic disease that can cause anemia, persistent fever, lethargy, jaundice,

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abortion, rapid loss of milk production and weight, and adult mortality. Cattle that survive the infection become persistently infected carriers, serving as reservoirs for other animals (Aubry; Geale, 2011; OIE, 2018).

Historically, this immunity is geographically and seasonally related to warm and humid climates, where the disease is considered endemic. However, the widespread movement of animals and climate change have contributed to a broader incidence in areas previously free of the disease (Railey; Marsh, 2021).

Although cattle are the natural hosts of *A. marginale*, it is a multi-host pathogen that can infect various species of ruminants, including buffaloes (*Bubalus bubalis*) (Kocan *et al.*, 2010). These animals are considered more resistant to many parasitic diseases and are regarded as potential reservoirs for *Anaplasma marginale* in livestock ecosystems of tropical countries. However, their role in the epidemiological process of bovine anaplasmosis in endemic areas remains uncertain (Obregón *et al.*, 2018).

The subclinical nature of persistently infected animals increases costs for producers due to the lack of awareness of infection occurrence within the herd, hindering control and treatment actions for anaplasmosis (Railey; Marsh, 2021). Notably, carrier animals are the primary source of infection for competent vectors in endemic areas (Aubry; Geale, 2011).

Even in clinically affected animals, clinical diagnosis can only be confirmed through pathogen identification. Microscopic, serological, and PCR-based tests are employed for this purpose. Giemsa-stained blood smears are a conventional diagnostic method in animals exhibiting clinical signs (Obregón *et al.*, 2018). However, it is not reliable in cases of low parasitemia levels (Kocan *et al*., 2010). Serology, on the other hand, suffers from reduced specificity due to cross-reactivity among *Anaplasma* species (Kovalchuk; Babii; Arkhipova, 2020).

Carrier animals exhibit very low levels of parasitemia ranging from 10^3 to 10^7 infected erythrocytes/ml of blood (Obregón *et al*., 2018). Molecular methods based on PCR and real-time PCR are efficient and represent a valid alternative to serological analysis and direct microscopy for the diagnosis of bovine anaplasmosis (Kovalchuk; Babii; Arkhipova, 2020). However, these methods require expensive experimental equipment and a high degree of laboratory expertise in molecular biology (Ma *et al.*, 2011).

Therefore, loop-mediated isothermal amplification (LAMP) has emerged as an additional option to traditional molecular methods, combining sensitivity, specificity, and greater simplicity of execution (Giglioti *et al.*, 2019). Being isothermal, the LAMP test does not require cycles with temperature variation and time, allowing it to be performed on simpler equipment such as a heat block or water bath (Notomi *et al.*, 2000), making its application more practical and accessible. Hence, considering the simpler and earlier detection possibility, the aim was to evaluate the loop-mediated isothermal amplification technique as a viable alternative to traditional DNA replication techniques for the molecular diagnosis of natural infection by *A. marginale*, applicable to both clinical and epidemiological investigations.

MATERIAL AND METHODS

The investigation was conducted in the state of Amapá, far north of Brazil, where the Amazon rainforest covers approximately 73% of the state's area. Environmental heterogeneity is characterized by vegetation including mangrove areas, floodplain fields, cerrado enclaves, várzea, and terra firme forests. The state's climate is classified as tropical super-humid, with a seasonal rainfall regime. From December to June, there are more rainy days with high monthly rainfall indices, while the drier days occur from July to November.

These characteristics make the region a habitat for numerous vectors of infectious and parasitic diseases in Amazonian livestock. The state has the second largest buffalo herd in the Americas, which sometimes shares pastures with cattle, exposing both species to the same ectoparasites.

A cross-sectional study was conducted on three rural properties (Farm A, B, and C) in the municipalities of Santana and Mazagão, all of them with a history of ectoparasite infestation. Farm A exclusively raises buffaloes, while Farm B has both cattle and buffaloes. Both farms operate under a semi-intensive system for meat production and are located in floodplain areas prone to seasonal flooding. Due to geoclimatic conditions, we were unable to access the cattle on Farm B, which were isolated by flooded areas. Farm C operates a semi-intensive production system for buffaloes intended for meat production and cattle for dairy purposes. It is situated on terra firme (non-flooded land) and includes a dairy facility supplying the state market.

For the collection of biological samples, a total of 114 buffaloes and 36 cattle, regardless of breed, sex, and age, were selected, with preference given to those visually identified with ectoparasite infestation (Table 1).

Whole blood samples were collected from each selected animal by jugular venipuncture into vacuum tubes containing EDTA, properly identified by the animal's ear tag number, and kept refrigerated. The samples were collected at the end of the rainy season when vector proliferation is typically high. The tubes were kept frozen and transported by air to the serology and molecular biology laboratory at UFRA in Belém, Pará, for processing.

The DNA was extracted from 200 µl of each collected blood sample using the commercial kit (Wizard® Genomic DNA Purification Kit - Promega), following the manufacturer's protocol.

PCR assays were performed using the primers LAMP B3 and F3 (Gigliotiet *et al*., 2019), which amplify a 193 bp fragment based on sequences from the msp1b gene of *A. marginale* (Access: GenBank No. M59845.1) (Table 2).

The reactions were carried out in a conventional thermocycler using the Ampliqon Taq DNA Pol Master Mix Red 2x kit (Neobio), with 0.2 µM of each primer and 3 µL of extracted DNA, made up to a final volume of 25 µL with water. Two samples of purified and sequenced DNA for *A. marginale* were used as positive controls, and ultrapure water replaced DNA in the negative control. The cycling protocol consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 58°C for 40s, and extension at 72°C for 30s. A final extension step was performed at 72°C for 5 min. Following

amplification, aliquots of the PCR products were subjected to agarose gel electrophoresis (1.5%) for 45 min and visualized using a UV transilluminator.

The real-time PCR reactions were performed on a CFX96™ Real-Time System (BioRad) with a final volume of 10 µL, containing 1x GreenMaster qPCR (Cellco Biotec do Brasil Ltda), 5 pmol of each primer, and 1 µL of sample DNA. The primer oligonucleotides used amplify a 104 bp fragment of the msp5 gene specific to *Anaplasma marginale* (Picoloto *et al.*, 2010).

The thermal cycling stages were standardized as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing and extension at 60°C for 30 seconds, and a melt curve from 65°C to 95°C with a 1°C/s ramp rate. A synthetic DNA fragment, gBlocks® (IDT, Integrated DNA Technologies®), containing the target sequence for *A. marginale*, was initially diluted to a concentration of 18 ng/µL and subsequently used to create a standard curve from serial dilutions ranging from 10 (10^{-1} a 10^{-10}) (KE *et al.*, 2006), for determination of the cutoff point. The dilution series and number of copies were determined according to the manufacturer's instructions for the synthetic DNA. All reactions included positive controls (gBlock) and negative controls (RNAse DNAse free water).

For the LAMP assay, a set of primers designed by Giglioti et al. (2019) from specific sequences of the msp1b gene of the bacterium *A. marginale* (Access: GenBank M59845.1) was selected. The reactions were optimized to a final volume of 25 µL, containing 40 pmol of each internal primer (FIB and BIP), 5 pmol of each external primer (F3 and B3), 20 pmol of Loop primers LF and LB, 1 µL of Bst 2.0 turbo polymerase (8U/µL), 1 mM extra MgSO4, 1.4 mM of mix dNTP (10 mM), 1x EvaGreen DNA Stain (50x), and 3 µL of sample DNA. The reaction mixtures were incubated at 65°C for 90 minutes followed by a 5-minute incubation at 90°C to terminate the reaction.

Subsequently, for result interpretation, aliquots of the amplicons were subjected to 2% agarose gel electrophoresis for 45 minutes at 100V and visualized using a UV transilluminator. Following amplification in the real-time thermocycler, LAMP reactions were also

Table 1 – Distribution of selected buffaloes and cattle to participate in the study.

Farm	City	Geographical feature	Buffaloes tested	Cattle tested	Total tested
A	Mazagão	Várzea	45	0	45
B	Mazagão	Várzea	45	0	45
C	Santana	Terra Firme	24	36	60
Total			114	36	150

Table 2 – Primers used in PCR, real-time PCR, and LAMP assays for detection of Anaplasma marginale.

Assay	Primer	Sequel 5'-3'	Gene	Reference
PCR	F	GCACTACCGTTCATGGATGA	*msp1b*	GIGLIOTI *et al.*, 2019.
	R	TCCCCTGTGATATCTGTGCC		
Real Time PCR	F	CTACTGCCTCACAAGGACGA	*msp5*	PICOLOTO *et al.*, 2010.
	R	AAGGCGAGGAGCTGTTTAAG		
	F3	GCACTACCGTTCATGGATGA		
	B3	TCCCCTGTGATATCTGTGCC		
	FIP	TGCCTTGCCAAATTCTTGCTCCAAACAC CTGACACTGGTGAGAAG		
LAMP	BIP	AGCAGGCTTCAAGCGTACAGTAAATCCG CGAGCATGTGCA	*msp1b*	GIGLIOTI *et al.*, 2019.
	LF	TCACCCGCTGGTACTTCAA		
	LB	GCCTGGAGATGTTAGACCGA		

monitored for double-stranded DNA fluorescence to confirm the target. Dissociation curves were acquired in 1°C/min increments, ranging from 63°C to 96°C. The sensitivity of each technique was calculated as the ratio of positive tests by the evaluated method to the total number of positive tests from any of the applied techniques (Branier *et al.*, 1995).

The R statistical software was used to compare the diagnostic results of natural infection by *A. marginale*, establishing the level of agreement between the applied techniques using the kappa test. A *Kappa* coefficient less than 0, 0 – 0.2, 0.21 - 0.4, 0.41 – 0.6, 0.61 – 0.8, and 0.81-1 denotes insignificant, slight, fair, moderate, substantial, and almost perfect agreement, respectively, as interpreted by Landis and Koch (1977).

All procedures in this research were approved by the Ethics Committee on Animal Use of the Federal Rural University of Amazonia (CEUA-UFRA) and comply with ethical principles and guidelines for animal experimentation adopted by the Brazilian College of Animal Experimentation (Process No. 7397070621).

RESULTS

For all tested techniques, results from all positive controls (gBlock and samples sequenced for *A. marginale*) showed amplification, while negative controls (DNA replaced by ultrapure water) did not amplify, indicating no contamination in the reactions. In conventional PCR, samples that amplified a 193 bp fragment similar to the positive control were considered positive (Figure 1).

For real-time PCR, the cutoff point considered in the reactions was 10^{-7} dilution of the gBlock, corresponding to 4.56 x 10^3 copies/µL. The average melting temperature (Tm) of the positive control was 82°C (Figure 2), and the positive samples ranged from 81 to 82.5°C. The reaction efficiency, calculated by the BioRad CFX96 platform software, was 100%.

Out of the two purified and sequenced samples, one showed 91.49% similarity with the Brazilian isolate Jaboticabal strain (GenBank accession number CP023731.1) and the other 95.19% similarity with the South African isolate C57c msp1b (Access: GenBank number KU647718.1).

The electrophoresis of the LAMP reactions resulted in weak amplifications of fragments of varying sizes according to the LAMP pattern. In the fluorescence analyses, the LAMP products showed reproducible melting curves with a temperature of ~88°C (Figure 3).

Out of the 150 blood samples analyzed, 96 tested positive in at least one of the applied tests. However, according to kappa statistics, the techniques showed poor agreement in their results. The results from PCR and real-time PCR did not show agreement (Table 3).

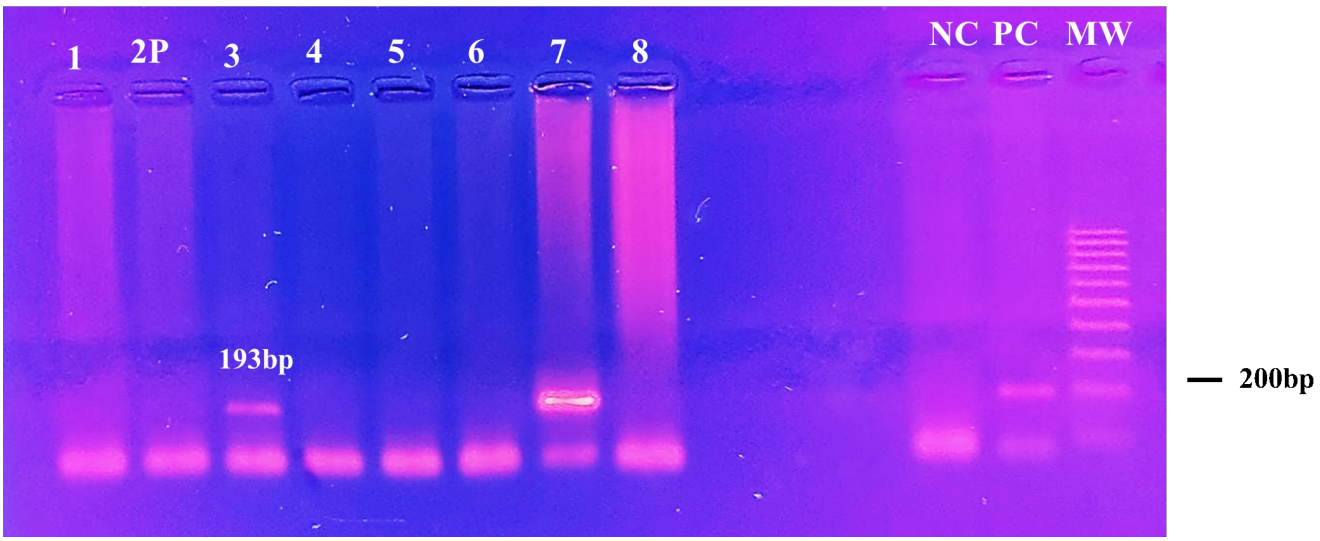
The sensitivity of the evaluated techniques according to Breniere *et al.* (1995) was 5.2% for PCR, 44.8% for real-time PCR, and 87.5% for the LAMP test. Estimates of the prevalence of bovine anaplasmosis detected by each diagnostic method are summarized in Tables 4, 5, and 6.

DISCUSSION

Anaplasmosis is an intraerythrocytic disease caused by *A. marginale* infection, which can lead to considerable economic losses in the livestock industry and is endemic in Brazil (Kocan *et al.*, 2010). The nonspecificity of symptoms does not ensure accuracy in clinical diagnosis and can easily be confused with other hemoparasitoses. Although stained blood smear examination for direct detection is considered the gold standard, it has limitations in detecting low levels of parasitemia (Kumar *et al.*, 2019). Additionally, due to the morphological similarity among Anaplasma species and other pathogens, distinguishing these organisms microscopically can be challenging (Ganguly *et al.*, 2023).

In recent years, molecular tools have been essential for epidemiological and clinical research on parasitic diseases. Several PCR assays for detecting parasite DNA have been established, but few studies have explored the use of LAMP technique for diagnosing

Figure 1 – 1.5% agarose gel electrophoresis of PCR products for *A. marginale* detection.



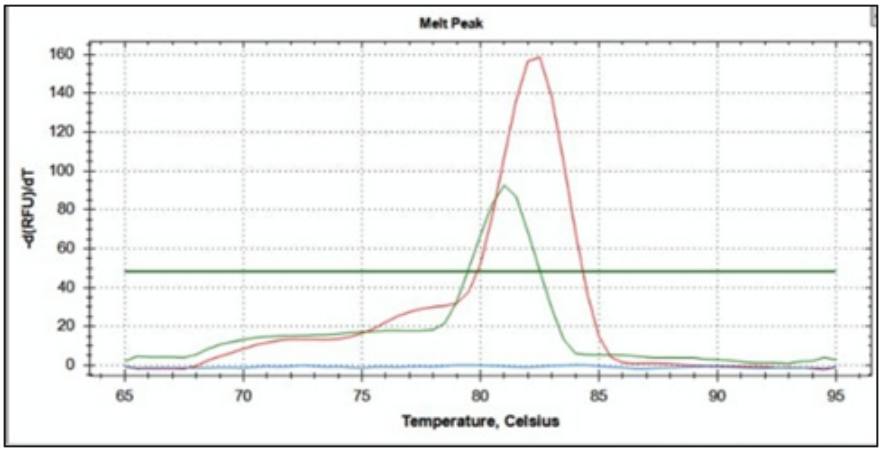
MW: Molecular Weight reference (100 bp ladder). Samples 1-8: DNA samples (3 and 7 with amplification). NC: Negative Control. PC: Positive Control.
Source: Author's collection.

Anaplasma marginale. The LAMP test has emerged as the most effective, applicable, and practical tool for detecting pathogens in humans or animals, especially in developing countries (Wen *et al.*, 2016).

In addition, most research involving LAMP targeted at *Anaplasma marginale* is limited to tests with experimental infections in controlled environments. However, geographical location, climatic conditions, and region is phylogenetically associated with strains isolated in Brazil and China (Ybanez *et al.*, 2016).

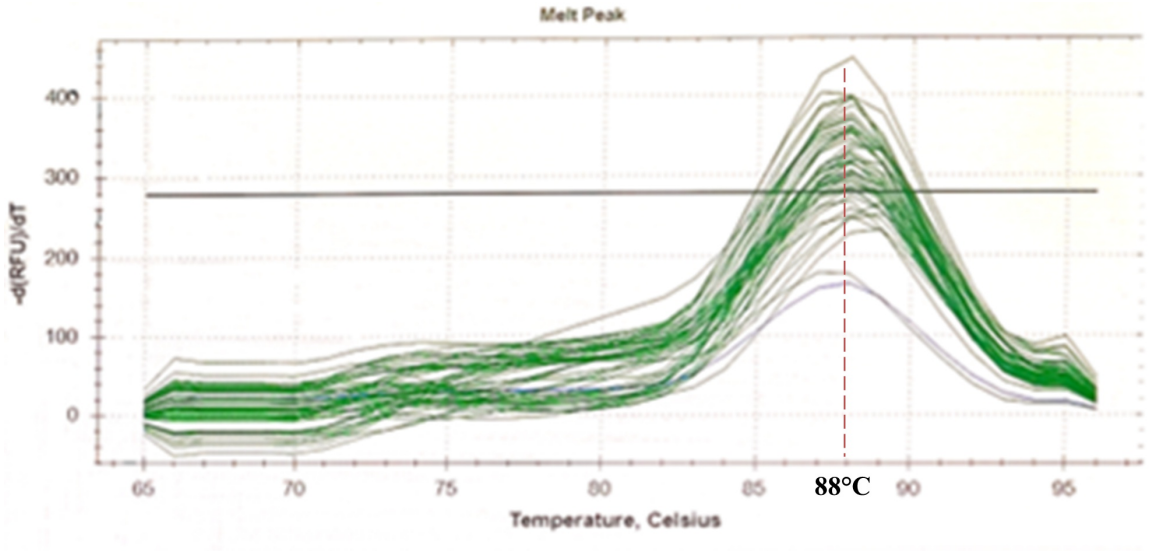
Similarly, the primers used in conventional PCR and LAMP reactions, based on the msp1 gene region, were designed and validated by Giglioti *et al.* (2019). Positive samples for *Anaplasma marginale* were detected by all applied techniques, confirming the presence of this parasite in herds in Amapá. However,

Figure 2 – Melting temperature in real-time PCR with DNA from buffalo infected with *Anaplasma marginale.*



Source: Author's collection.

Figure 3 – Melting temperature in Real-time LAMP.



Source: Author's collection.

animal health and management practices impact the prevalence of infection and the level of *A. marginale* parasitemia in herds. In contrast, this study aimed to evaluate LAMP, PCR, and real-time PCR techniques for detecting *A. marginale* in the blood of cattle and buffalo with natural infections, raised under the geoclimatic conditions of the Amazon region.

For real-time PCR, primers based on the MSP5 protein gene were used, which Wen *et al.* (2016) mention it as a potential molecular target due to its highly conserved region in *A. marginale*, allowing detection in both acute and chronic infections. Additionally, gene-based analyses of msp5 in A. marginale isolates in the Philippines revealed that this conventional PCR identified positive animals only in farm C and only in cattle. This reinforces the theory of Obregón *et al.* (2018), who state that buffalo are more resistant to parasitic infections and primarily act as reservoirs of the pathogen in livestock ecosystems in tropical countries. Additionally, the levels of parasitemia in cattle appear to be higher than in buffaloes.

On the other hand, the LAMP test detected more positive samples than the other assays, demonstrating higher diagnostic sensitivity. LAMP also confirmed the presence of *A. marginale* in samples from all three farms, similar to real-time PCR. Similarly, Moura *et al.* (2024), studying T. vivax in buffalo from

the Amazon region, confirmed LAMP as a highly sensitive technique compared to conventional PCR and real-time PCR. This sensitivity was also validated by Wen *et al.* (2016), who found that LAMP was 100 times more sensitive than conventional PCR in *A. marginale* research. The same result was reported by Ganguly *et al.* (2023) in similar research. As for real-time PCR, the findings contrasted with Giglioti *et al.* (2019), who reported results 10 times more sensitive for qPCR compared to LAMP.

Despite the dissociation curve obtained from fluorescence observation not showing nonspecific amplifications, LAMP's main limitation is the risk of contamination during nucleic acid extraction and after amplification, which can lead to false positives (Bari *et al.*, 2024). Therefore, despite its high sensitivity, which ensures its use as a screening test, positive results should be confirmed by real-time PCR.

Meanwhile, in the kappa analysis, the LAMP assay showed weak agreement with the results

Table 3 – Analysis of statistical agreement using kappa test among PCR, real-time PCR, and LAMP techniques. R software.

Statistical indices	PCR and Real time PCR	Real time PCR and LAMP	PCR and LAMP
k [1]	- 0.019	0.201	0.029
Observed agreement	0.693	0.580	0.460
IC95%	4.466 – 30.413	0.196-0.510	0.011-0.070
Probability	0.332	0.002	0.136

[1] The kappa value, k, where k < 0, 0 – 0.2, 0.21 - 0.4, 0.41 – 0.6, 0.61 – 0.8 and 0.81-1 denotes insignificant, slight, fair, moderate, strong, and almost perfect agreement, respectively, according to the interpretation by Landis and Koch (1977).

Table 4 – Percentage of positive samples detected by PCR and real-time PCR techniques.

Farm	Species	PCR		Real time PCR	
Farm A	Buffalo	0%	(0/45)	57.77%	(26/45)
Farm B	Buffalo	0%	(0/45)	22.22%	(10/45)
Farm C	Cattle	13.88%	(5/36)	2.77%	(1/36)
	Buffalo	0%	(0/24)	25%	(6/24)
	Cattle/ Buffalo	8.33%	(5/60)	11.66%	(7/60)
Total	Cattle/ Buffalo	3.33%	(5/150)	28.66%	(43/150)

Table 5 – Percentage of positive samples detected by LAMP and PCR techniques.

Farm	Species	PCR		LAMP	
Farm A	Buffalo	0%	(0/45)	57.77%	(26/45)
Farm B	Buffalo	0%	(0/45)	57.77%	(26/45)
Farm C	Cattle	13.88%	(5/36)	36.11%	(13/36)
	Buffalo	0%	(0/24)	79.16%	(19/24)
	Cattle/ Buffalo	8.33%	(5/60)	53.33%	(32/60)
Total	Cattle/ Buffalo	3.33%	(5/150)	56%	(84/150)

Table 6 – Percentage of positive samples detected by LAMP and real-time PCR techniques.

Farm	Species	Real time PCR		LAMP	
Farm A	Buffalo	57.77%	(26/45)	57.77%	(26/45)
Farm B	Buffalo	22.22%	(10/45)	57.77%	(26/45)
Farm C	Cattle	2.77%	(1/36)	36.11%	(13/36)
	Buffalo	25%	(6/24)	79.16%	(19/24)
	Cattle/ Buffalo	11.66%	(7/60)	53.33%	(32/60)
Total	Cattle/ Buffalo	28.66%	(43/150)	56%	(84/150)

obtained by PCR and real-time PCR. The PCR and real-time PCR tests did not show agreement, with diagnoses attributed to chance (k: -0.019). However, it should be considered that low prevalences tend to be associated with low levels of agreement, as the value of "k" depends on chance agreement.

Moreover, the presence of variable sequences within conserved regions of the msp1 gene may justify the lower sensitivity observed in conventional PCR. Similarly, Chaisi *et al.* (2017) found discordant results when comparing nPCR and qPCR techniques in the detection of *A. marginale.* The difference in results was attributed to sequence variation identified in one of the nPCR primers among msp1β sequences of *A. marginale* from South Africa, resulting in false negatives.

In the most, the different molecular techniques evaluated showed good diagnostic performance for bovine anaplasmosis. The specificity and high sensitivity demonstrated by the LAMP assay can be an advantage for screening tests in endemic areas compared to traditional molecular methods. This can be particularly useful for buffalo farming in the Amazon, given the region's stable enzootic condition and the species' natural resistance. Additionally, LAMP offers greater simplicity in execution due to isothermal amplification and various result interpretation options that can adapt to different settings.

CONCLUSION

This study revealed the occurrence of Anaplasma marginale in buffalo and cattle herds from farms in Amapá. The prevalence estimate of infection varied among the evaluated techniques, with the LAMP assay standing out for its high negative predictive value, which can be particularly relevant as a screening method. Although further studies are needed, the ease of execution, speed, and high sensitivity make LAMP a valuable alternative method for molecular diagnosis of *Anaplasma marginale*, suitable for clinical routines or epidemiological investigations.

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