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RESEARCH ARTICLE

Comparative Analysis of PCR, Real-time PCR and LAMP Techniques in the Diagnosis of *Trypanosoma vivax* Infection in Naturally Infected Buffaloes and Cattle in the Brazilian Amazon

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A B S T R A C T

Bovine trypanosomiasis requires, even in situations of low parasitemia, a rapid, sensitive and specific diagnosis in order to take the correct therapeutic measures and avoid the dissemination in herds and significant economic losses. Therefore, the study was designed to evaluate the comparative performance of molecular assays PCR, real-time PCR and LAMP in the detection of *Trypanosoma vivax* in blood samples from cattle and buffalo naturally infected in the Brazilian Amazon. PCR indicated a 4% (06/150) prevalence of the protozoan parasite in the tested herds while real-time PCR and LAMP estimated 38% (57/150) and 83.33% (125/150) of animals with trypanosomiasis, respectively. The kappa analysis denoted reasonable agreement between the results of real-time PCR and LAMP and poor agreement between conventional PCR and the other techniques, as interpreted by Landis and koch, 1977. Despite this, the higher percentage of parasite detection obtained by the LAMP assay coincides with its reputation as a highly sensitive method, suggesting that it is a viable alternative to traditional molecular techniques with potential use in epidemiological screening and clinical diagnostic efficiency. However, further studies are required to adjust LAMP protocols to facilitate the implementation and reading of the results, ensuring their applicability in the laboratorial routine.

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INTRODUCTION

The protozoan *Trypanosoma vivax* is the main cause of bovine trypanosomiasis in South America, being mechanically transmitted by tabanids and Stomoxyine flies, resulting in asymptomatic animals with a gradual decrease in productivity, causing losses to livestock (Garcia Pérez *et al*., 2020).

Over the last decades, outbreaks have been observed in different Brazilian states, causing a significant economic impact on livestock farming (Bastos *et al*., 2020). In the state of Amapá, located in the far north of Brazil, where a significant portion of the territory is occupied by the Amazon rainforest, one of the first outbreaks of *T. vivax* in the country occurred, identified by Serra-Freire in 1981. However, since then, there is an epidemiological silence regarding the herds in the region, which is home to the second-largest buffalo population in the Americas.

Due to the dearth of research on the prevalence of naturally infected animals with *T. vivax* in the unique geoclimatic and management conditions of this region, combined with the nonspecific symptoms and fluctuating parasitemia of the disease, the adoption of molecular methods can offer a more accurate diagnosis, especially in the early stages of infection. These fluctuations in parasitemia are related to the host's immune response and the antigenic variation of the surface variant glycoproteins of trypanosomes (Pereira *et al*., 2020). Therefore, a reliable and comprehensive diagnosis serves as the foundation for clinical and epidemiological investigations, control measures, and appropriate treatment (Desquesnes *et al*., 2022a).

Direct parasitological examinations such as blood smears, although simple and inexpensive, have low

specificity and sensitivity, especially in chronic infections (Alcindo *et al*., 2022), while serological tests do not differentiate between animals with active infections, those that have been treated and cured, and they also do not detect newly infected animals (Cadioli *et al*., 2015).

Polymerase Chain Reaction (PCR) methods have high specificity, contributing to more accurate results. However, false-negative results can still occur when parasitemia is very low, such as in chronic infections, when too much DNA is used in the PCR reaction, or due to the presence of reaction inhibitors. False-positive results can occur due to sample contamination from another positive sample (Desquesnes *et al*., 2022b). Late or incorrect diagnosis allows for the rapid proliferation of the hemoparasite in the herd, favoring the occurrence of outbreaks that lead to the death of animals and severe economic losses (Pereira *et al*., 2018). Real-time PCR may be applied to hosts and vectors with high sensitivity and specificity, but its use is still limited due to the requirement for high technical skills, equipment, and cost (Desquesnes *et al*., 2022b).

Research using nucleic acid isothermal amplification assays are still limited in pathogenic trypanosomatids, but the tested techniques have shown robust amplification capacity, with Loop-Mediated Isothermal Amplification (LAMP) being the standout methodology (Sereno *et al*., 2022). In LAMP, unlike PCR, isothermal amplification allows diagnosis to be performed with simple equipment, such as a dry block heater or water bath, enabling its application for field-level diagnosis or in resource-limited settings (Soroka *et al*., 2021).

However, the complexity in primer design and the inability of its products to be used for additional analyses, such as cloning or sequencing, are limitations in the implementation of LAMP. Despite the early stage of development of this technology for the diagnosis of trypanosomatids (Sereno *et al*., 2022), the increasing popularity of LAMP in a wide range of scientific fields suggests that it may become a gold standard in the future, alongside PCR (Soroka *et al*., 2021). For this purpose, constant evaluations of the technique must be conducted to validate its application and diagnostic inference in various environmental, economic, and epidemiological contexts.

MATERIALS AND METHODS

Study of area and animal selection: 126 water buffaloes and 24 cattle were selected regardless of breed, sex and age from three farms in Amapá state, Brazilian Amazon. Two farms are in a floodplain area and the other in a plain area. Farms with a history of ectoparasite infestation were selected, and animals detected with ectoparasites through visual inspection were given priority. No animal showed apparent symptoms of bovine trypanosomiasis.

DNA extraction: DNA was extracted from whole blood samples using the Promega Wizard® Genomic DNA Purification kit, following the manufacturer's protocol.

PCR: Reactions were conducted for 25µL using the Ampliqon Taq DNA Pol Master Mix Red kit, 0.2µM of each primer and 3µL of genomic DNA. A sample sequenced for *T. vivax* was used as positive control and ultrapure water as negative control. The primer set selected was Tvi2 and DTO156, which amplifies a 177bp fragment of the CatL-Like gene (Table 1). The cycling conditions included, 1 cycle of 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 59.6°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 5 minutes. Aliquots of the amplicon were subjected to 1.5% agarose gel electrophoresis for 40 minutes and displayed by UV transilluminator.

Real-time PCR: Reactions were performed with 10µL containing 5µL of GreenMaster qPCR (Cellco Biotec do Brasil Ltda), 5pmol of each primer and 1µL of sample DNA. The designed primer set flanked a 90bp fragment based on sequences of 18S rRNA, ITS1 and 5.8S rRNA genes of *T. vivax* (GenBank: LC589626.1) (Table 1). Specificity was confirmed using the BLAST tool and testing samples positive for *Babesia* spp and *T. cruzi*. Amplifications occurred in BioRad CFX96 at: 1 cycle 95°C for 2 mins, followed by 40 cycles of 95°C for 15 secs, 60°C for 30 secs and dissociation from 65-95°C with 1°C/s reduction. A synthetic DNA, gBlocks® (IDT, Integrated DNA Technologies®), containing the target sequence for *T. vivax*, was diluted to an initial concentration of 18ng/µL, in sequence, used for the preparation of a standard curve from serial dilutions in ratio of 10 ((10^{-1} to 10^{-10}) (Ke *et al*., 2006), to define the cut-off point. The initial dilution and copy number were determined as instructed by the gBlock manufacturer. All reactions were followed by positive (gBlock) and negative (ultrapure water) controls.

LAMP: A set of six primers designed by Njiru *et al*. (2011) from the *T. vivax* satellite DNA sequence (GenBank: J03989) was used. Reactions were optimized to 25 µl, containing FIP and BIP (40 pmol), F3 and B3 (5 pmol), Loop LF and LB primers (20 pmol) (Table 1), 8U/µl Bst 2.0 turbo polymerase and 10x buffer solution (Cellco Biotec do Brasil Ltda), 1 mM extra MgSO4, 1.4 mM dNTPs mix (10 mM), 1x EvaGreen DNA Stain (50x) and 3 µl of sample DNA. Assays were conducted in a conventional thermal cycler at 63°C for 45 min, followed by 5 minutes at 80°C to finish. Controls were subjected to reactions using the same protocol, in thermoblock and thermal cycler and the results compared. To interpret the results, the amplicons were submitted to electrophoresis in a 2% agarose gel, as mentioned in the PCR. After amplification, the reactions were monitored in real time to confirm the correct target. Dissociation curves were acquired in 1°C steps for 30 seconds from 63°C to 96°C (Njiru *et al*., 2011).

Statistical Analysis: The kappa test was used to analyze the agreement between the applied techniques. The results were interpreted as established by Landis and Koch (1977).

This research was approved by the Ethics Committee on Animal Use -UFRA, number 7397070621.

RESULTS

The negative controls did not amplify in any of the techniques, and the positive controls amplified in all the applied techniques. For conventional PCR, samples were

Table 1: *Trypanosoma vivax* specific primer set used in each molecular technique applied.

Technique	Primer	Sequence 5" – 3"	Gene	Reference
PCR	F	GCCATCGCCAAGTACCTCGCCGA	*CatL-Like*	(Cortez *et al.*, 2009)
	R	TTAGAATTCCCAGGAGTTCTTGATCCAGTA		
PCR real time	F	GAACCTGCAGCTGGATCATT	*18S rRNA, ITS1, 5.8S rRNA*	*
	R	AGCTGCGTGGTTTGCTGT		
LAMP	F3	TGTTCTGGTGGCCTGTTGC	Satellite DNA	(Nijiru *et al.*, 2011)
	B3	GGCCGGAGCGAGAGGTGC		
	FIP	GTGGAGCGTGCCAACGTGGCACCCGCTCCCAGACCATA		
	BIP	TGTCTAGCGTGACGCGATGGAAGAGGGAGTGGGGAAGG		
	LF	CACATGGAGCATCAGGAC		
	LB	CCGTGCACTGTCCCGCAC		

* Primers were designed using the Primer3Plus software (https://www.primer3plus.com/).

Table 2: Statistical agreement analysis by kappa test between conventional PCR, real-time PCR and LAMP techniques. Software R.

Technique	PCR	LAMP	Real-Time PCR
PCR	_	p-value 0.5 [2]	p-value 0.010 [2]
LAMP	*k* 0.000[1] IC95% (0.003-0.021)	_	p-value 0.0001 [2]
Real-time PCR	*k* 0.093[1] IC95% (6,097-35,489)	*k* 0.218[1] IC95% (0.071-0.211)	_

[1]*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 represent insignificant, weak, fair, moderate, strong, and almost perfect agreement, respectively, according to the interpretation by Landis and Koch, 1977.
[2]p-value < 0.05: Significant agreement; p-value ≥ 0.05: Non-significant agreement.

Table 3: Results of PCR, real-time PCR, and LAMP tests of samples submitted for genome sequencing.

Samples	Sequencing Result	Conventional PCR	Real-time PCR	LAMP
70	*T. vivax*	Positive	Positive	Positive
130	Buffalo Genome	Positive	Negative	Negative
134	*T. vivax*	Positive	Positive	Positive
138	*T. vivax*	Positive	Positive	Positive

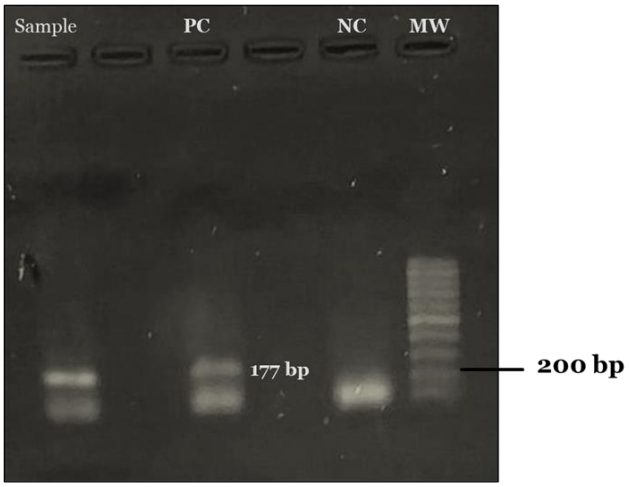


Fig. 1: PCR reaction products analyzed by 1.5% agarose gel electrophoresis: Sample: *Trypanosoma vivax* positive sample; PC: Positive Control ; NC: Negative Control; MW: Molecular Weight

considered positive if, after reaction with 1.5% agarose gel electrophoresis, they presented a 177bp band similar to the positive control (Fig. 1).

The detection limit of real-time PCR was a dilution of 10^{-7} of the gBlock, corresponding to 4.56 x 10^3copies/µL. The average melting temperature (Tm) of the positive control was 87°C, and the Tm of the positive samples ranged from 85 to 88°C (Fig. 2). The efficiency of the reactions was calculated using the CFX96 software from BioRad and ranged from 98 to 110%. There was no amplification for the other tested hemoparasites in real-time PCR. In the LAMP assay, the positive and negative controls tested under the same temperature and time conditions in the heat block and the thermal cycler yielded the same results in gel electrophoresis. The prevalence obtained by the LAMP test was considerably higher than the other techniques, with 83.4% of positive samples, in contrast to only 4% of prevalence detected by conventional PCR. Real-time PCR showed that 38% of the animals were infected with T. vivax (Fig. 3).

The statistical analysis computed by the kappa test showed a significant but moderate agreement between the real-time PCR and LAMP techniques, while conventional PCR did not agree with any of the applied techniques (Table 2). Four products from real-time PCR (samples 70, 134 and 138- GenBank: MT586222.1, amplifying with the positive control, and sample 130- GenBank: XM_055553675.1 with nonspecific amplification) were purified and submitted to gene sequencing by the Sanger method. The three amplicons that amplified along with the positive control corresponded to *T. vivax* DNA based on the sequencing analysis, with identity compatible with the Nigerian isolate TvY486. However, in sample 130, the sequencing corresponded to fragments of the host genome (Table 3). In the post-amplification analysis of real-time LAMP, dissociation curves were observed, with Tm of ∼90°C for the samples considered positive (Fig. 4).

DISCUSSION

Molecular approaches for the diagnosis of animal trypanosomiasis have emerged as a breakthrough in detecting active infections during the chronic phase, where low levels of parasitemia pose a limitation for the use of conventional parasitological methods. However, despite the generally high sensitivity of molecular techniques based on DNA amplification, several factors need to be considered, such as resistant breeds and species, disease phase, sample quality, DNA extraction method, primers, and the presence of PCR inhibitors, which can influence the results (Ferreira *et al.*, 2023).

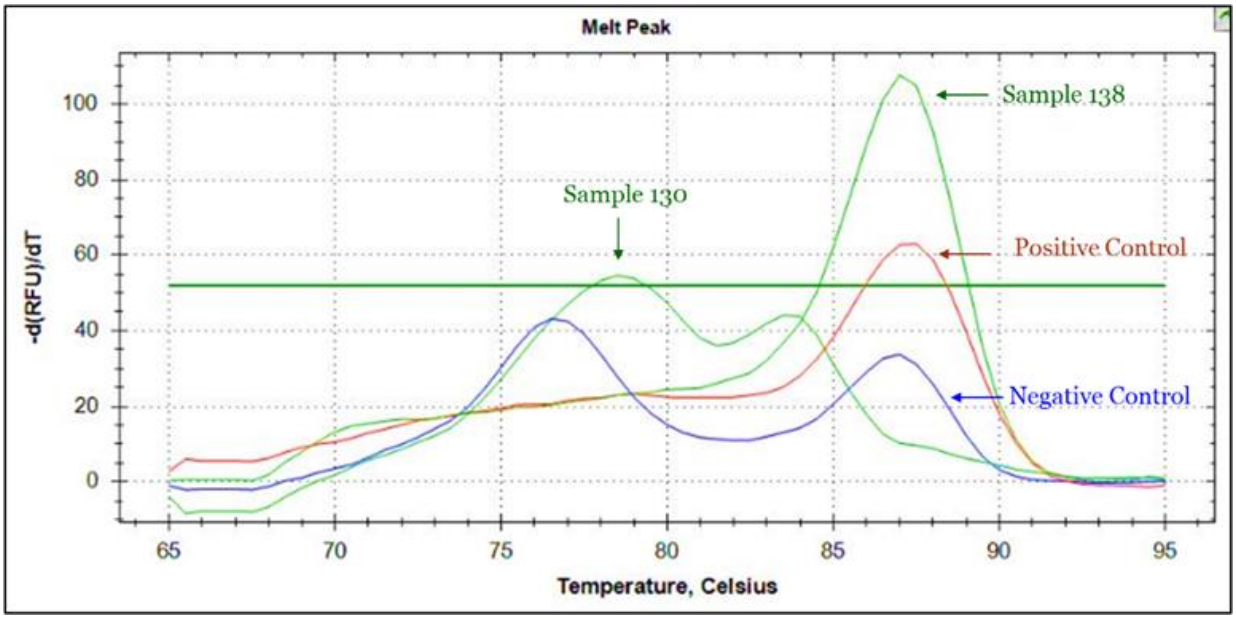
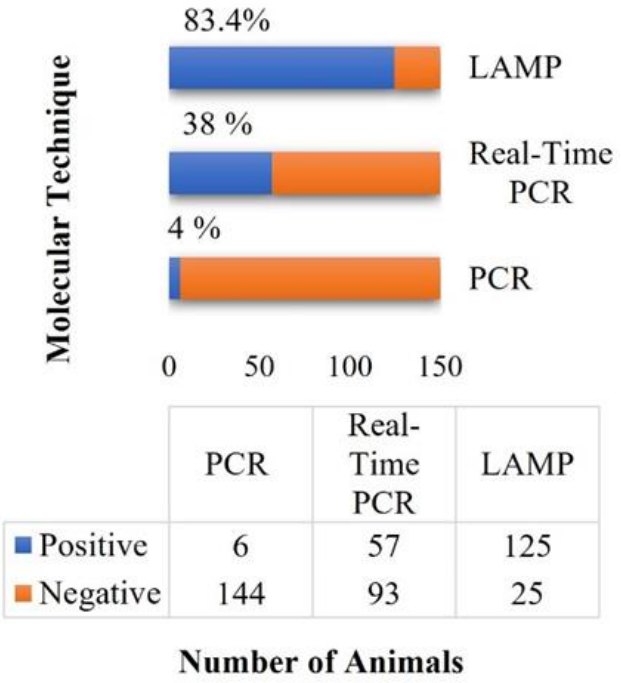


Fig. 2: Real-time PCR assay for *Trypanosoma vivax* diagnosis



	PCR	Real-Time PCR	LAMP
Positive	6	57	125
Negative	144	93	25

Fig. 3: Percentage of animals with bovine trypanosomiasis detected by PCR, real-time PCR and LAMP.

To ensure the specificity of the tests, various primer designs have been used in the diagnosis of *T. vivax*, which is a critical point for molecular assays (Ferreira *et al*., 2023). In this study, LAMP testing employed primers designed from the satellite DNA of the protozoan, following the approach of Njiru *et al*. (2011), Cadioli *et al.* (2015) and Silva *et al.* (2023). According to Gonzales *et al.* (2006), amplification of satellite DNA may have higher diagnostic potential even when using samples with low levels of parasitemia, as satellite regions can have thousands of copies, thus improving diagnostic performance.

In real-time PCR, the primer set was designed from fragments that encompass regions of the 18S rRNA, ITS1, and 5.8S rRNA genes of *T. vivax*. A study conducted by Pimentel *et al*. (2012) has shown that the primers amplifying the 18S rRNA subunit showed high performance in the diagnosis of protozoan infection, which was also confirmed by Bastos *et al*. (2020) in an investigation of *T. vivax* outbreaks in central Brazil. This same genetic region was utilized by McLaughlin *et al*. (1996), who argued that it is an extremely conserved sequence with multiple copies in the locus, making it an efficient alternative for the diagnosis of coinfections. The ITS1 region was also tested by Fidelis *et al*. (2019) and Takeet *et al*. (2017), identifying sequences from South American strains of the protozoan. Laohasinnarong *et al*. (2015) stated that primers targeting the ITS1 region of rDNA are promising for routine diagnosis of pathogenic trypanosomes in clinical specimens from infected animals.

According to Cortez *et al*. (2009), the region of the CatL-Like gene used to design the Tvi2 and DTO156 primers is highly conserved among *T. vivax* isolates from West Africa, East Africa, and South America. This primer design has been extensively validated in the literature (Laohasinnarong *et al*., 2015; Garcia *et al*., 2016; Fidelis *et al*., 2019; Alcindo *et al*., 2020). Thus, although the use of the same target gene for all the techniques is more judicious, the possible variation in the sensitivity of the primers does not invalidate the results of the performance of the techniques observed, since all the amplified regions are based on highly conserved regions that have been proven in the literature to be a reference molecular target in the diagnosis of T. *vivax*.

The evaluated techniques did not show agreement in the estimation of *T. vivax* detection in natural infection, unlike the findings of Fidelis *et al*. (2019) who reported a kappa agreement of 0.87 between PCR and qPCR in experimental *T. vivax* infection. In naturally infected herds, which are often asymptomatic, the level of parasitemia may be influenced by factors such as environment, management practices, genetic resistance, age, disease stage, overall health condition, and physiological state of the host, which can affect the

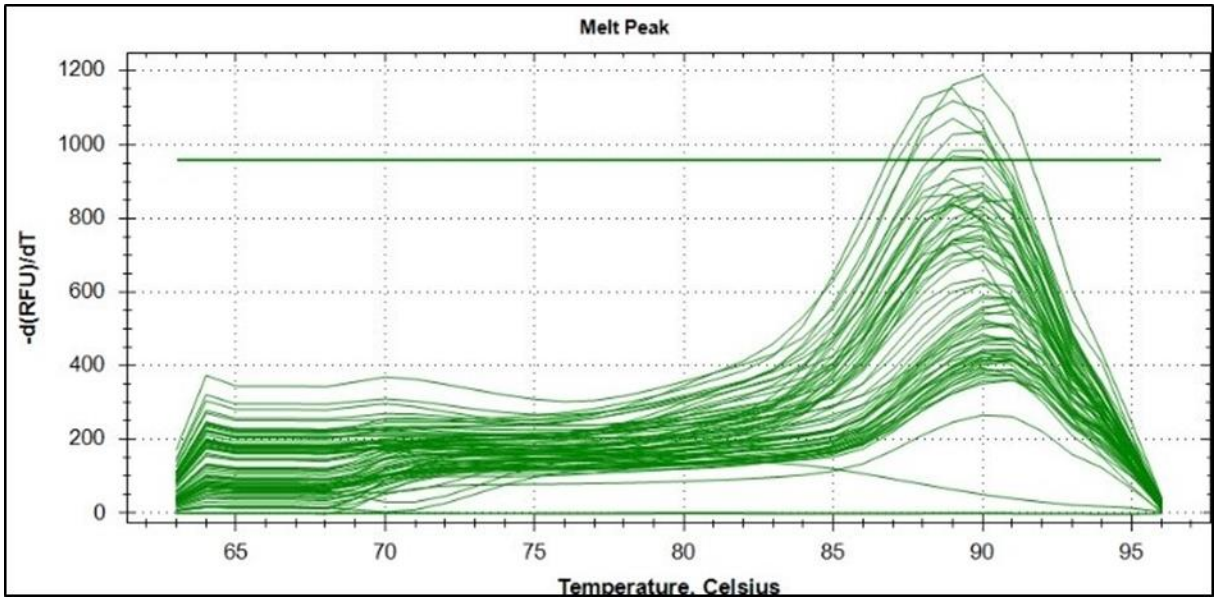


Fig. 4: Melting temperature in real-time LAMP assay for *Trypanosoma vivax* diagnosis.

inference of diagnosis. In experimental conditions, some variables must be better controlled, which is why research with experimental infection does not always reflect the same applicability as clinical results.

Under the circumstances of this research, the prevalence of trypanosomiasis in the herd revealed by the LAMP test was higher than those obtained by polymerase chain reactions. This finding is close to studies reporting that LAMP assays are more sensitive than PCR-based methodologies (Liu *et al*. 2012; Wang *et al*. 2014; Noden *et al*., 2018). Njiru *et al*. (2011) using the same LAMP primer design, achieved 10 times more sensitive results when compared to PCR. In Laohasinnarong *et al*.'s (2015) investigation in cattle and goats, all samples positive for TviCatL-PCR were also positive for LAMP, but with a lower detection rate, suggesting that conventional PCR is not an appropriate test for epidemiological surveillance of *T. vivax* in endemic areas.

Although conventional PCR and real-time PCR are excellent diagnostic tools, they are subject to false negative results when parasitemia is below the detection limit (Cadioli *et al*., 2015). In contrast to Laohasinnarong *et al*. (2015), Fikru *et al*. (2016) further state that the ITS1 region, which was targeted by the real-time assay, has a high CG content, which may interfere with diagnostic accuracy and justify lower infection rates compared to those identified by the LAMP technique. Another point that may explain the higher prevalence detected by LAMP test is that PCR-based techniques are more affected by inhibitors found in the blood and in the extraction process, interfering with the sensitivity of the diagnosis (Ferreira *et al*., 2023).

Despite the validation of the Tvi2 and DTO156 primers, one important point to highlight is that, in this study, conventional PCR showed false-positive amplification of a genomic DNA fragment with a similar molecular weight to the target. However, real-time PCR and LAMP did not reproduce this non-specificity.

Another issue is the influence of the abundant and constant presence of vectors on the level of parasitemia and, consequently, on the test results. The geoclimatic conditions of the Amazon region provide a suitable environment for vector adaptation throughout the year, predicting a state of enzootic stability in the region, which explains the observation of hematophagous flies in the investigated farms. This scenario favors the circulation of the protozoan in livestock, maintaining low levels of parasitemia in the hosts due to the immune response to constant pathogen exposure, as described by Cadioli *et al*. (2012). The absence of evident symptoms in the studied animals and the detection of the parasite, confirming the circulation of the protozoan in the herd, reinforces the suspicion of an endemic condition of animal trypanosomiasis in the region.

On the other hand, the climate changes observed in recent years can disrupt this enzootic equilibrium and lead to outbreaks in endemic areas, as occurred in buffaloes in Venezuela (Garcia *et al*., 2016). Dyonisio *et al*. (2021) found, in their investigations in the Amazon region, several species of ticks, as well as the buffalo louse (H. *tuberculatus*), hosting *T. vivax* and, therefore, increasing the possibilities of transmission of trypanosomiasis in buffalo and cattle. In this case, despite the resistance to parasitic infestations attributed to the buffalo species, the adaptive capacity of the ectoparasite as a vector may have been greater.

Due to the absence of a laboratory for these purposes in the region, although well preserved, the samples needed to be transported for processing by air to another state. Therefore, we cannot guarantee that the quality of the samples was not compromised. However, it should be noted that the difficulty of accessing properly equipped laboratories is a common situation in many regions of the Amazon and in countries with limited resources.

The good sensitivity of the LAMP assay and the possibility of performing it using simpler equipment such as a thermal block can be a viable option for obtaining faster and reliable results in remote areas away from major centers. However, it is important to note that, unlike PCR technology, which allows for confirmation of the obtained amplicon, the inability to confirm the product make it essential to ensure the amplification of the correct target. In this study, the LAMP product was confirmed

post-amplification by analyzing the dissociation curves, which showed a consistent Tm of ~90°C, indicating the amplification of a single fragment of the same size as the sequenced samples.

The observed melting curve behavior in this study was consistent with the results obtained by Njiru *et al*. (2011) using the same protocol. However, Quyen *et al*. (2019) argues that green dyes like Evagreen may have a moderate inhibitory effect on the LAMP assay when the concentration is increased by 0.5x to 5x, but still lower than SYBR Green dye, which was classified as having a high inhibitory effect on LAMP efficiency. Other dyes, such as SYTO-9, showed no inhibitory effect and therefore may be considered for future investigations to simplify result interpretation through visually detectable colorimetric changes or under ultraviolet light.

Conclusions: This research confirmed the circulation of the *T. vivax* protozoan in cattle and buffalo herds in Amapá, predominantly in asymptomatic animals. The results also suggested that the LAMP technique has high sensitivity in detecting *T. vivax* in bovines and buffalo from endemic regions, demonstrating its potential as an alternative for clinical and epidemiological molecular diagnosis of animal trypanosomiasis. Further studies should be conducted to adjust the LAMP protocols for their adaptation to simpler laboratories closer to the rural environment.

Authors contribution: ACMB, ESF and WLAP developed the project. ACBM and EMB executed the experiment and analyzed the blood samples. ACBM, EMB and ESF performed data analysis. ESF performed the statistical analyzes on the variable data. All authors read, reviewed and approved the structure and writing of the manuscript. There is no conflict of interest among authors.

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