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Short Communication

Genetic diversity and molecular survey of *Trypanosoma* (*Megatrypanum*) *theileri* in cattle in Brazil's western Amazon region

Diversidade genética e levantamento molecular de *Trypanosoma* (*Megatrypanum*) *theileri* em bovinos na região da Amazônia Ocidental do Brasil

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Abstract

Trypanosoma (*Megatrypanum*) *theileri* is a flagellated protozoan that infects ruminants and it displays high genetic diversity. In this study, we investigated the prevalence rates of this protozoan based on hemoculture and molecular diagnosis. The isolates of *T. theileri* thus obtained were characterized by molecular markers SSU rDNA and gGAPDH and molecular diagnosis based on Cathepsin L-like gene (PCR-TthCATL). The PCR-TthCATL and hemoculture indicated an overall prevalence rate of 8.13%, and the CATL derived sequence named IB was identified for the first time in cattle in the western Amazon region, as well as IF in Brazil. We also describe a possible new PCR-TthCATL derived sequence in cattle, designated IL.

Keywords: Molecular diagnosis, phylogenetic analysis, hemoculture, Trypanosomatids.

Resumo

Trypanosoma (*Megatrypanum*) *theileri* é um protozoário flagelado que infecta ruminantes e apresenta alta diversidade genética. Neste estudo, investigamos as taxas de prevalência deste protozoário com base na hemocultura e no diagnóstico molecular. Os isolados de *T. theileri* obtidos foram caracterizados pelos marcadores moleculares SSU rDNA e gGAPDH e o diagnóstico molecular foi baseado no gene do tipo Catepsina L (PCR-TthCATL). O PCR-TthCATL e a hemocultura indicaram uma taxa de prevalência total de 8,13% e a sequência derivada do gene Catepsina L denominada IB de *T. theileri* foi identificada pela primeira vez em bovinos da Amazônia Ocidental, bem como a IF no Brasil. Também descrevemos uma possível nova sequência derivada da PCR-TthCATL em bovinos, designada IL.

Palavras-chave: Diagnóstico molecular, análise filogenética, hemocultura, Tripanossomatídeos.

The *Trypanosoma theileri* Clade, a group of phylogenetically related trypanosomes isolated from ruminants and classified into the subgenus *T.* (*Megatrypanum*) (RODRIGUES et al., 2006, 2010a,b), is a complex group of parasites segregated in two lineages (TthI and TthII) that harbor 14 distinct genotypes validated using at least two different molecular markers, SSU and ITS rDNA (HAMILTON et al., 2009; LEE et al., 2010; RODRIGUES et al., 2010a,b; GARCIA et al., 2011a,b; FISHER et al., 2013).

In Brazil, the extensive geographical area used for animal husbandry and the intense internal flow of animals favor the contact and transmission of *T. theileri* and allows genetic events that increase the parasites diversity. Thus, the presence of *T. theileri* in the Rondônia state (western Amazon region) through hemoculture and molecular diagnosis based on cathepsin L gene were evaluated and the genotypic diversity of *T. theileri* in cattle herds.

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From 2012 and 2013, blood samples were collected from dairy cows (≥24 months old) kept by farms in Ji-Paraná (Rondônia state) from six different milk production regions, based on data from the Association of Technical Assistance and Rural Extension of the State of Rondônia – EMATER/RO.

The population size of the study was 34,527 cows from 856 farms; the prevalence rate was estimated to be 50% (given that there were no previous studies and that the maximum number of occurrences with normal distribution would be at the 50% mark); maximum error of 6%; 95% confidence interval (CI); and design effect (*deff*) of 1.2. The sample size was weighted according to the sampling design, taking into consideration the probabilities of selection among the farms and animals resulting in five animals from each of 64 farms from six different regions (Table 1).

Blood samples were preserved in ethanol (1:1) for molecular analysis. Additional five blood samples were collected from different cows in same farms for trypanosomes isolation (MARCILI et al., 2009a,b) and the isolates were deposited in the Brazilian Trypanosomatid Collection (CBT) at the Department of Preventive Veterinary Medicine and Animal Health, University of São Paulo, São Paulo, Brazil.

DNA from samples of blood preserved in ethanol was purified using the Wizard DNA Clean-Up System (Promega, Madison, USA), while the phenol-chloroform method was to extract DNA from the trypanosome culture samples. DNA from samples of blood preserved in ethanol was subjected to a *T. theileri*-specific PCR assay, based on the Cathepsin L-like (CATL) gene, to screen the DNA samples (RODRIGUES et al., 2010b). Five positive samples that generated the most intense amplified DNA bands were sequenced.

For molecular characterization and phylogenetic studies of *T. theileri* isolates, the trypanosomatid barcode (V7V8 region of SSU rDNA) was analyzed (SILVA et al., 2004), and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) (HAMILTON et al., 2004). DNA was purified and sequenced in an automated sequencer (ABI-PRISM 3500 Genetic Analyzer, Foster City, CA).

All the sequences thus obtained were aligned with sequences that had previously been determined for other isolates of *T. theileri* available in GenBank, using ClustalX (THOMPSON et al., 1997), and were adjusted manually using GeneDoc (NICHOLAS et al., 1997). The V7V8 SSU rDNA sequences retrieved from GenBank were AY773674-AY773693, HQ664895-HQ664909, AB007814, GQ176155, and GQ176160, while the sequences for gGAPDH were HQ664784-HQ664806, AJ620282, and FM164792.

The V7V8 SSU rDNA, gGAPDH and CATL sequences were used to build a phylogenetic tree using maximum parsimony, as implemented in PAUP version 4.0 b10 (SWOFFORD, 2002) with 500 bootstrap replicates.

Prevalence on the farms and in dairy cows was calculated with a 95% CI, using the R statistical package (R DEVELOPMENT CORE TEAM, 2013).

This study was approved by the Bioethical Committee for Animal Research of Federal University of Mato Grosso, under Protocol No. 23108.015662/12-5.

This study resulted in a prevalence of 12.19% (95% CI: 8.91-16.40) among cows tested positive for *T. theileri* using PCR-TthCATL, while prevalence per herd was 42.19% (95% CI: 29.94-55.18%) (Table 1). Previous studies have reported prevalence rates ranging from 5% to 26% by PCR-TthCATL (GARCIA et al., 2011a; SIVAKUMAR et al., 2013; YBAÑEZ et al., 2013; YOKOYAMA et al., 2015). Moreover, it should be noted that the farms in the study area were small, with 56 (87.6%) of them covering less than 100 ha, and their proximity to each other may increase the risk of contact with blood-sucking arthropods, which may explain the high inter-herd prevalence rate (42.19%).

Thirteen isolates were established from the blood culture (CBT 111, CBT 112, CBT 113, CBT 114, CBT 115, CBT 119, CBT 120, CBT 121, CBT 122, CBT 123, CBT 136, CBT 137, and CBT 155), showing a prevalence of 4.06% (95% CI: 2.27-7.02%) and 12.5% (95% CI: 5.55-23.15%) among animals and herd, respectively, and 39 cows tested positive for *T. theileri* using PCR-TthCATL (Table 1). The small number of animals testing positive by hemoculture compared to PCR-TthCATL can be explained by the low parasitemia of *T. theileri* in blood and the higher sensitivity of PCR. This was demonstrated by Rodrigues et al. (2010b), who reported that 34 out of 72 blood samples tested positive by PCR-TthCATL, but only 22 of the same 72 blood samples tested positive by hemoculture.

Therefore, considering the PCR-TthCATL and hemoculture together, the overall prevalence was 8.13% (95% CI: 6.18%-10.59%), with 52 positive animals, while the herd prevalence rate was

Table 1. Total number of farms, sampled farms, farms testing positive by PCR-TthCATL and hemoculture, total number of cows, sampled cows and cows testing positive by PCR-TthCATL and hemoculture per rural region in the municipality of Ji-Paraná, Rondônia, Brazil.

Region	No. of farms	No. of sampled farms	No. of positives farms		N. of cows	No. of sampled cows	No. of positives cows	
			PCR-TthCATL	Hemoculture			PCR-TthCATL	Hemoculture
			Positive (%)	Positive (%)			Positive (%)	Positive (%)
1	122	9	2 (22.22)	2 (4.44)	5246	45	3 (6.67)	2 (4.44)
2	124	9	7 (77.77)	0 (0)	5952	45	10 (22.22)	0 (0)
3	54	4	0 (0)	0 (0)	2268	20	0 (0)	0 (0)
4	176	13	8 (61.54)	6 (45.15)	8096	65	15 (23.08)	11 (16.92)
5	143	11	3 (27.27)	0 (0)	4433	55	3 (5.45)	0 (0)
6	237	18	7 (38.89)	0 (0)	8532	90	8 (8.89)	0(0)
Total	856	64	27 (42.19)	8 (12.5)	34527	320	39 (12.19)	13 (4.06)

46.88% (95% CI: 34.28-59.77%), with 30 farms testing positive for *T. theileri*.

A phylogenetic analysis based on traditional markers (SSU rDNA and gGAPDH genes) allowed the *T. theileri* isolates from western Amazon to be divided into two genotypes, namely TthIB (86% and 83% bootstrap to SSU rDNA and gGAPDH, respectively) and TthIIB (100% and 93% bootstrap to SSU rDNA and gGAPDH, respectively) (Figure 1 and Table 2).

This is the first report of genotype TthIB in Brazil's western Amazon region. Previous studies in the Amazon have described

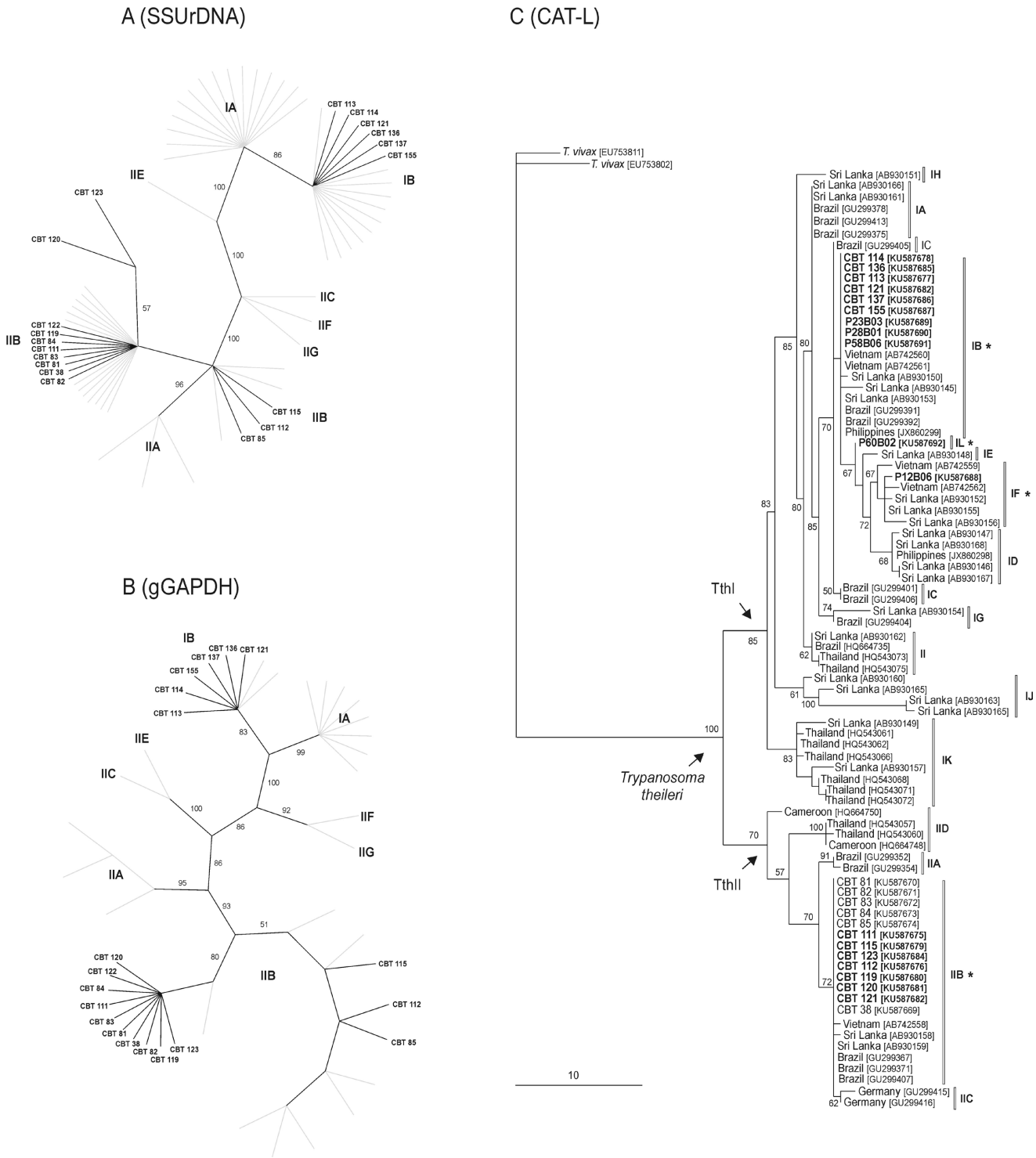

Figure 1. Phylogenetic trees inferred by maximum parsimony. Numbers at nodes are the support values for the major branches (bootstrap or posterior probability) derived, respectively, from 500 replicates for MP. A, V7V8 SSU rDNA gene (712 characters; 18 parsimony-informative sites); B, gGAPDH gene (655 characters; 71 parsimony-informative sites); C, Cathepsin L-like (CAT-L) gene (347 characters; 203 parsimony-informative sites). *Trypanosoma vivax* was used as outgroup of cathepsin L-like gene analysis.

Table 2. *Trypanosoma* isolates, blood samples, genotypes identification and GenBank accession numbers for sequences of CATL, SSU rDNA and gGAPDH genes generated in the present study.

Trypanosoma theileri isolates and blood samples	Genotypes and CATL derived sequence based on DNA sequences			GenBank accession numbers		
	CATL	SSU rDNA	gGAPDH	CATL	SSU rDNA	gGAPDH
CBT 111	IIB	IIB	IIB	KU587675	KU587637	KU587656
CBT 112	IIB	IIB	IIB	KU587676	KU587638	KU587657
CBT 113	IB	IB	IB	KU587677	KU587639	KU587658
CBT 114	IB	IB	IB	KU587678	KU587640	KU587659
CBT 115	IIB	IIB	IIB	KU587679	KU587641	KU587660
CBT 119	IIB	IIB	IIB	KU587680	KU587642	KU587661
CBT 120	IIB	IIB	IIB	KU587681	KU587643	KU587662
CBT 121	IIB	IIB	IIB	KU587682	KU587644	KU587663
CBT 122	IB	IB	IB	KU587683	KU587645	KU587664
CBT 123	IIB	IIB	IIB	KU587684	KU587646	KU587665
CBT 136	IB	IB	IB	KU587685	KU587647	KU587666
CBT 137	IB	IB	IB	KU587686	KU587648	KU587667
CBT 155	IB	IB	IB	KU587687	KU587649	KU587668
P12B06	IF	-	-	KU587688	-	-
P23B01	IB	-	-	KU587689	-	-
P23B03	IB	-	-	KU587690	-	-
P58B06	IB	-	-	KU587691	-	-
P60B02	IL	-	-	KU587692	-	-

the occurrence of isolates belonging to lineage II in Venezuela (Yarucuy and Cojedes States) and Brazil, where genotype TthIIB was detected in the state of Rondônia, while genotypes TthIIA and TthIIB were observed in the state of Pará (RODRIGUES et al., 2010a; GARCIA et al., 2011b).

Five CATL DNA derived sequences from blood samples (P12B06, P23B01, P23B03, P58B06 and P60B02) were allocated to IB genotype. However, the cattle blood sample P12B06 also disclosed a CATL derived sequence that clustered with Asiatic cattle samples as IF (0.18% of divergence). The blood sample P60B02 disclosed a single CATL derived sequence that might represent a new group of sequence and was herein identified as IL (Figure 1 and Table 2). Only direct samples were grouped with CATL derived sequences IF and IL presenting low divergence to IB, 3.7% and 0.88%, respectively. All sequences of *T. theileri* obtained in this study were deposited in GenBank and the accession numbers are listed in Table 2.

The genotype IF, identified in this study for the first time in Brazil, had previously been detected in blood samples from cattle in Sri Lanka and Vietnam (SIVAKUMAR et al., 2013; YOKOYAMA et al., 2015). Besides that, a possible new group of CATL derived sequences was identified (IL). The inclusion of sequences in unexpected genotypes IF and IL warn for new studies in poorly studied areas in Brazil. The sequences with the greatest differences are related to sequences obtained from the direct products of the diagnostic PCR and it was not possible to obtain the other markers of these animals.

The genotypic diversity of *T. theileri* is more effectively evidenced when using polymorphic markers such as ITS, SL or CATL. Traditional markers (gGAPDH and SSU rDNA) are more conserved and do not evidence all *T. theileri* genotypes (RODRIGUES et al., 2003, 2006). In addition, blood culture can select genotypes of parasites with better growth in axenic culture media (RODRIGUES et al., 2003; MARCILI et al., 2013).

Concatenated analysis of one or more markers increases the reliability in phylogenetic trees (MARCILI et al., 2009c; GARCIA et al., 2011b), but the topologies generated by isolated markers such as SL or CATL are congruent to analyzes based on up to six markers. Another important fact is the number of CATL gene sequences deposited in the GenBank that do not have the other markers for the same animals or isolates. In this way, the absence of other markers for the sequences positioned in the IF and IL genotypes with the CATL gene does not disqualify the existence of such genotypes in Brazil, but aim that new studies related to the isolation and characterization of *T. theileri*.

In other *Trypanosoma* species with high genetic diversity, such as *T. cruzi*, the concatenated analyzes of several genes make the analyzes more robust, but the phylogeny generated through traditional markers (V7V8 SSU rDNA) is able to segregate all strains and evidence a new Lineage (TCbat) (MARCILI et al., 2009a,b,c).

In this way, the diversity of *T. theileri* has not been fully described, even in countries such as Brazil that has several studies carried out. The inclusion of new sequences from regions not studied in the already known panel of *T. theileri* will contribute to the description of the diversity of this parasite.

In conclusion, the present study describes the IB and IF group of sequences for the first time in cattle in Brazil's western Amazon region, expanding the known geographic distribution and genetic diversity of the agent. Furthermore, the discovery of a possible new group of CATL derived sequence of *T. theileri*, called IL, was described in cattle.

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