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ORIGINAL ARTICLE

Morphological and Morphometric Characterization of Trypanosomes in *Leptodactylus lineatus* and *Osteocephalus* sp. (Anura) from Brazilian Midwest

André Flávio Soares Ferreira Rodrigues[1], Drausio Honório Morais[2], Vinícius Tadeu Carvalho[3], Marta D'Agosto[4] & Moara Lemos[5]*

[1] *Departamento de Ciências Naturais - CDB, Universidade Federal de São João del-Rei. Pça D. Helvécio n. 74, Fábricas, São João del-Rei, MG, Brazil.*
[2] *Universidade Federal Rural da Amazônia (UFRA), PA 275, Km 13, Zona Rural. Parauapebas, Pará, Brazil.*
[3] *Programa de Pós-Graduação em Bioprospecção Molecular, Universidade Regional do Cariri, Campus do Pimenta, Rua Cel. Antônio Luiz, Crato, CE, Brazil.*
[4] *Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n - Campus Universitário Martelos, Bairro São Pedro, Juiz de Fora, MG, Brazil.*
[5] *Département de Parasites et Insectes Vecteurs, Institut Pasteur, Rue Dr. Roux 25-28, Paris, France.*

**E-mail para correspondência: moara.lemos@pasteur.fr*

RESUMO

Caracterização morfológica e morfométrica dos tripanossomas de anuros do Centro-Oeste brasileiro. Este estudo teve por objetivo avaliar o parasitismo causado por tripanossomas em anuros naturalmente infectados do Centro-Oeste brasileiro e realizar a caracterização morfológica e morfométrica dos tripanossomas. Os anuros foram capturados às margens do Rio Guaporé, na transição entre os biomas Cerrado e Floresta Amazônica. Amostras de sangue de 16 espécies de anuros foram obtidas por punção cardíaca e analisadas para a presença de hemoparasitos. Tripanossomas foram encontrados infectando duas espécies de anuros, *Leptodactylus lineatus* (Leptodactylidae) e *Osteocephalus* sp. (Hylidae). Foi observada alta prevalência dos tripanossomas (100%; N = 3 hospedeiros) em *L. lineatus*, com intensidade da infecção calculada em 9.9 x 10^2 parasitos/ml. Em *Osteocephalus* sp., a prevalência foi comparativamente menor (36%; N = 4 hospedeiros infectados de 11 analisados), no entanto, a intensidade de infecção foi maior 2.16 x 10^3 parasitos/ml. Variações morfológicas relacionadas ao comprimento e largura do corpo foram observadas entre as diferentes espécies de hospedeiros e também em indivíduos da mesma espécie. Os tripanossomas polimórficos foram separados em morfotipos classificados como corpo alongado ou curto. Os tripanossomas que infectam *L. lineatus* possuem o corpo alongado que pode ser delgado ou largo e aqueles que infectam *Osteocephalus* sp. apresentam o corpo curto com a extremidade posterior alargada. A análise parasitológica e a caracterização morfológica apresentada neste estudo contribuem para o conhecimento da diversidade dos tripanossomas de anuros neotropicais.
Palavras-chave: Anuros, Cerrado, Floresta Amazônica, Trypanosoma.

ABSTRACT

The objective of this study was to evaluate parasitism caused by trypanosomes in naturally infected anurans from Brazilian Midwest and characterize trypanosomes by morphology and morphometry. Anurans were captured from margins of the Guaporé River in transition areas between Cerrado and Rain Forest. Blood samples of 16 anurans species were obtained by cardiac puncture and analyzed for the presence of hemoparasites. Trypanosomes were found infecting two anuran species, Leptodactylus lineatus (Leptodactylidae) and *Osteocephalus* sp. (Hylidae). It was observed high prevalence of trypanosomes (100%; N = 3) in *L. lineatus*, with intensity of infection of 9.9 × 10^2 parasites/ml. In *Osteocephalus* sp. the prevalence of trypanosomes was comparatively lower (36%; N = 4 from 11), however, the intensity of infection was higher 2.16 × 10^3 parasites/ml. Morphological variation related to body length and width of bloodstream trypomastigotes were observed in

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different host species and between individual hosts of the same species. Polymorphic trypanosomes were separated into morphotypes and classified as elongated or short trypanosomes. Trypanosomes infecting *L. lineatus* are elongated with slender or broad body and trypanosomes infecting *Osteocephalus* sp. presented a short body with broad posterior extremity. The parasitological analyses and morphological characterizations presented in this study contribute to the knowledge of diversity of trypanosomes from neotropical anurans.
Keywords: Amazon Forest, Anurans, Cerrado, Trypanosoma.

INTRODUCTION

Trypanosomes are hemoparasites flagellates belonging to the Family Trypanosomatidae which are transmitted by hematophagous invertebrates. These exclusively parasitic organisms infect all the vertebrate classes (Maslov et al., 1996; Hughes & Piontkivska, 2003). Trypanosomes are recorded in anurans from all continents (Bardsley & Harmsen, 1973), and are transmitted by hematophagous leeches, sand flies, or culicids (Anderson & Ayala, 1968; Desser et al., 1973; Martin & Desser, 1990; 1991; Siddal & Desser, 1992; Desser, 2001; Ferreira et al., 2008). Bloodstream trypomastigotes are highly polymorfic parasites (Bardsley & Harmsen, 1973; Martin et al., 2002; Ferreira et al., 2007). The general body morphology includes elongated and slender species as *Trypanosoma mega* Mathis & Leger, 1911, which infects African toads, and spherical body shaped with short flagellum as *Trypanosoma chatoni* Mathis & Leger, 1911, which infects Leptodactylids and Ranids (Jones & Woo, 1986; Desser, 2001; Ferreira et al., 2007; Lemos et al., 2008; Netherlands et al., 2015). *Trypanosoma rotatorium* (Mayer, 1843), retain highest diversity of morphotypes including trypanosomes with slender, leaf-shaped, and spherical body shapes, and can be considered as a complex of species (Bardsley & Harmsen, 1973).

Brazil harbor the highest anuran diversity of the world with more than 1000 species (Segalla et al., 2016) and the studies of anuran trypanosomes reported in Brazil include the description of few species infecting hosts from different families: *Trypanosoma borreli* Marchoux & Salimbeni, 1907 from hylids, *Trypanosoma leptodactyli* Carini, 1907 and *Trypanosoma herthameyeri* Attias et al., 2016 from leptodactylids (Carini, 1907; Marchoux & Salimbeni, 1907; Attias, et al., 2016). Phylogenetical studies revealed great diversity of Brazilian anuran trypanosomes reflecting remarkable morphological variation of bloodstream trypomastigotes, and ultrastructural differences of *in vitro* developmental stages (Ferreira et al., 2007; 2008; Leal et al., 2009; Lemos et al., 2008; 2013; Ferreira et al., 2015; Attias et al., 2016). The objective of this study was to characterize anuran trypanosomes in naturally infected hosts from the Brazilian Midwest, including the transition areas between Cerrado and Rain Forest.

MATERIALS AND METHODS

During a wildlife rescue a total of 46 specimens from 16 anuran species belonging to the families Bufonidae, Craugastoridae, Hylidae, Leptodactylidae, Microhylidae and Odontophrynidae (Table 1) were captured from the Guaporé River margins (15°07'32"S, 58°57'16"W) in transition areas between Cerrado and the Rain Forest, near Vale de São Domingos, and Pontes and Lacerda, state of Mato Grosso, Brazil. Blood samples were obtained by cardiac puncture and blood smears were fixed in methanol and stained with Giemsa. The prevalence of trypanosomes was calculated according to Bush et al. (1997) and the intensity of infection was estimated according Borges et al. (2016). Morphological and morphometrical characterization of trypanosomes were performed by the analysis of 20 trypanosomes from each infected host. Measurements were performed using AnalySIS® soft images and morphometrical parameters used according to Lemos et al. (2015): body length along the cell midline (BL), body width at the region of nucleus center (BW), nucleus length (NL), nucleus width at region of nucleus center (NW), distance from nucleus center to cell anterior end (NAE), distance from nucleus center to posterior cell end (NPE), kinetoplast length (KL), kinetoplast width (KW), distance from kinetoplast center to anterior cell end (KAE), and distance from kinetoplast center to posterior cell end (KPE). The nuclear index (NI = NPE/NAE) and the kinetoplast index (KI = PNE/KN) were also calculated. Morphometric data were distributed in class intervals following the Yule's formula ($2{,}5.\sqrt[4]{n}$) (Sampaio, 2002). The classes obtained was compared by Kruskall-Wallis test. *P*-values were considered significant when equal to, or less than 0.05. Anuran species were identified based on morphology according Duellman (1978; 2005) and Lima et al. (2012) and nomenclature. Taxonomy of the species of anurans followed the Brazilian Society of Herpetology (SBH), according to Segalla et al. (2016), Dubois (2017) and Frost (2019). Sample collection was authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA – Brazilian Environmental and Renewable Natural Resources Institute), Fauna Management Executive – Mato Grosso, under license protocol Nº 001 / UHE Guaporé.

Anurans were deposited in the Coleção de Vertebrados da Universidade Federal de Mato Grosso (UFMT) under the numbers: *R. diptycha* (UFMT-A 3448 – 3451); *R*. gr. *margaritifera* (UFMT-A 3130, 3133, 3138, 3139, 3142, 3143, 3145, 3146 and 3151); *Pristimantis* sp. (UFMT-A 3134 and 12450); *Dendropsophus* sp. (UFMT-A 17625); *B. raniceps* (UFMT-A 3441); *S. ruber* (UFMT-A 1923); *Osteocephalus* sp. (UFMT-A 1822, 1823, 1917,1918 and UFMT-A 2754 – 2760); *P. boliviana* (UFMT-A 1909 and 6221); *P. albonatus* (UFMT-A 1847); *L. elenae* (UFMT - A 1946) *L. labyrinthicus* (UFMT-A 3584 and 9621); *L. lineatus* (UFMT-A 1960 – 1962); *L. rhodomystax* (UFMT-A 13108); *E. ovalis* (UFMT-A 1828); *C. egeayi* (UFMT-A 8024 – 8026) and *P. strussmannae* (UFMT-A 1833 – 1835). Blood smears were deposited in the Coleção de Protozoários

do Instituto Oswald Cruz (COLPROT - Protozoological Collection of Institute Oswaldo Cruz) under the numbers: serie B 2007-052 and serie B 2007-053.

RESULTS

From the four anuran families analyzed, trypanosomes were found infecting hylids and leptodactylids (Table 1), and of the 16 anuran species analyzed, trypanosomes were observed in two species: *Leptodactylus lineatus* (Schneider, 1799), with a prevalence of 100% (N = 3) and intensity of infection of 9.9×10^2 parasites/ml; and in *Osteocephalus* sp. Steindachner, 1862, with a prevalence of 36% (N = 4 from 11), and intensity of infection of 2.16×10^3 parasites/ml (Table 1).

Table 1. List of hosts, prevalence and parasitaemia of trypanosomes from Brazilian Midwest.

Family	Number of individuals	Prevalence	Parasitemia (parasites/ml)
Bufonidae			
Rhinella diptycha (Cope, 1862)	4	-	-
Rhinella gr. *margaritifera* (Laurenti, 1768)	9	-	-
Craugastoridae			
Pristimantis sp.	2	-	-
Hylidae		-	-
Dendropsophus sp.	1	-	-
Boana raniceps (Cope, 1862)	1	-	-
Scinax ruber (Laurenti, 1768)	1	-	-
Osteocephalus sp.	11	36%	9.9×10^2
Phyllomedusa boliviana Boulenger, 1902	2	-	-
Leptodactylidae			
Physalaemus albonatus (Steindachner, 1864)	1	-	-
Leptodactylus elenae Heyer, 1978	1	-	-
Leptodactylus labyrinthicus (Spix, 1824)	2	-	-
Leptodactylus lineatus (Schneider, 1799)	3	100%	2.16×10^3
Leptodactylus rhodomystax Boulenger, 1884	1	-	-
Microhylidae			
Elachistocleis ovalis (Schneider, 1799)	1	-	-
Ctenophryne geayi Mocquard, 1904	3	-	-
Odontophrynidae			
Proceratophrys strussmannae Avila, Kawashita-Ribeiro & Morais 2011	3	-	-

Trypanosomes were characterized considering their morphological variations and were grouped into two different morphotypes (Figure 1A – D). Data from morphometrical analysis are shown in Table 2.

Morphortype 1 was found in *Osteocephalus* sp. (Figure 1A and B). Trypanosomes infecting *L. lineatus,* Morphotype 2 (Figure 1C and D), were classified according to the Yule´s formula and separated in two groups which were defined by widths according to Kruskall-Wallis two-sample tests – $P < 0.05$ analysis in slender and broad trypomastigotes (Figure 1 C and D). Morphotype 1 trypanosomes was observed infecting only *Osteocephalus* sp. (Figures 1 A and B; Table 2) and present a short body with broad posterior end, a dark stained cytoplasm and a short free portion of the flagellum which is pale stained. The kinetoplast is located near the oval nucleus (IK > 2) and the cytoplasm may contain small vacuoles. Morphotype 2 trypanosomes which infect *L. lineatus* were classified into slender and broad forms (Figures 1C and 1D; Table 2) and differs from trypanosomes infecting *Osteocephalus* sp. Trypanosomes have elongated bodies, shaped in C or S with a narrow anterior extremity. The flagellum is 20 µm long, about 20% of the body length with a free portion pale stained. The nucleus is oval and situated in the posterior region of the body (NI < 1), a rod-like kinetoplast is positioned close to the nucleus (KI > 2) (Figure 1D) and the cytoplasm may contain few vacuoles. Longitudinal striations were visualized in both slender and broad trypanosomes of Morphotype 2 (not shown in Figure 1).

Figure 1. Photomicrographs of trypanosomes infecting *Osteocephalus* sp. and *Leptodactylus lineatus*. (A) Morphotype 1 a short body trypanosome with (B) a broad posterior region infecting *Osteocephalus* sp. (C) Morphotype 2 an elongated trypanosome and (D) a broad trypanosome infecting *L. lineatus*. Scale bar = 10 µm.

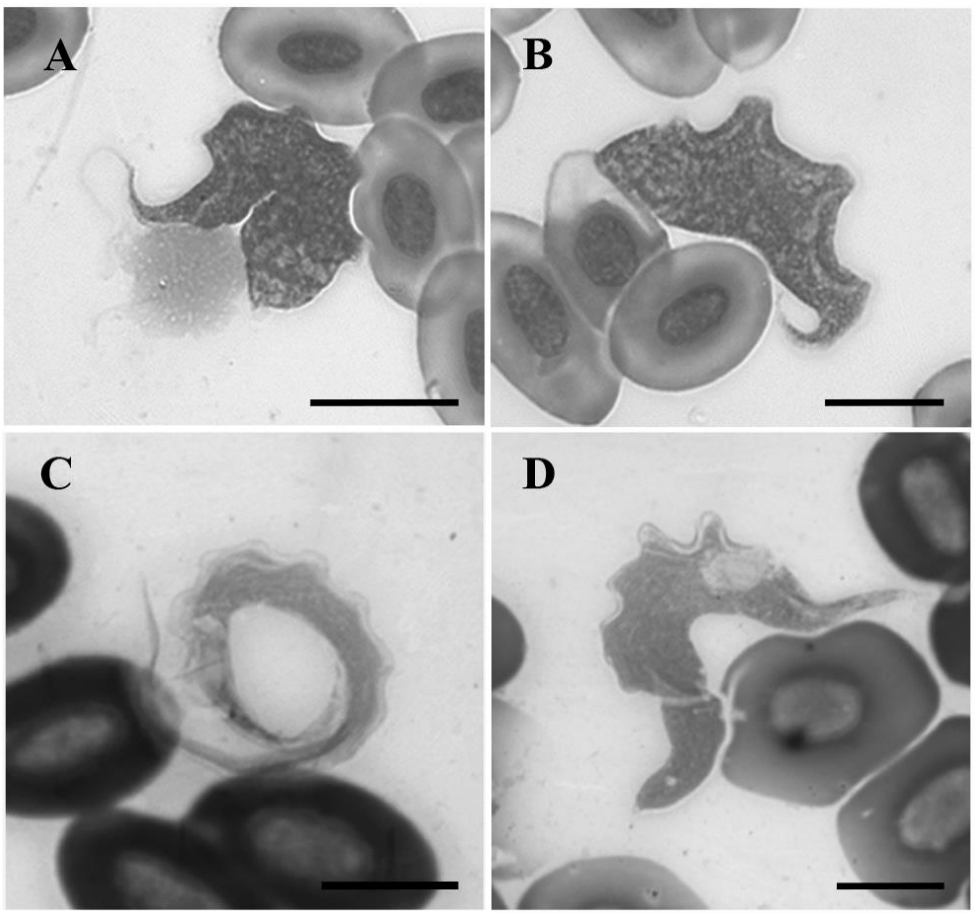

Table 2. Morphometric analysis of bloodstream trypomastigotes.

Hosts	Morphotypes	BL	BW	NL	NW	NAE	NPE	KL	KW	KAE	PK
***Osteocephalus* sp.**	Morphotype 1	28.6	18.8	9.5	9.5	14.2	15.4	1.7	1.0	19.1	11.4
Leptodactylus lineatus	Morphotype 2 Slender	102.2	5	6.3	4.4	73.3	29.8	1.1	0.9	77.3	23.8
	Morphotype 2 Broad	105.1	10.4	6.9	4.8	71	34.1	1.8	0.9	73.9	24

*Body length (BL) taken along the cell midline, body width (BW) measured at the region of nucleus center, nucleus length (NL), nucleus width (NW) measured at the center of the nucleus, distance from the center of the nucleus to the anterior end (NAE), distance from the center of the nucleus to the posterior end (NPE), kinetoplast length (KL), kinetoplast width (KW), distance from the kinetoplast center to the anterior cell end (KAE), and distance from kinetoplast center to the posterior end (KPE).

DISCUSSION

In this study, we characterized the morphology and morphometry of trypanosomes infecting anurans from the Guaporé river margins in transitional areas between Cerrado and Rain Forest. Trypanosomes were separated into morphotypes based on length and width variations attributed to the polymorphism broadly related for bloodstream trypomastigotes previously reported (Diamond, 1965; Bardsley & Harmsen, 1973; Barta et al., 1989; Martin & Desser, 1990; Martin et al., 1992; Desser, 2001; Ferreira et al., 2007). Here, trypanosomes shared morphological similarities with previous described species. For example, morphotype 1 is similar to the leaf-shaped group 2 probably associated to *T. rotatorium*-like group characterized in Ferreira et al. (2007), which includes widely distributed and highly pleomorphic parasites of anurans, and also to *Trypanosoma loricatum* (Mayer, 1843), reported to occur in ranids (Diamond 1965; Barta et al., 1989) and to a small trypanosome of African frogs (Netherlands et al., 2015). Morphotype 2, comprehend the slender and broad forms, which were similar to trypanosomes associated with *Trypanosoma bocagei* França, 1911 in Asia (Feng & Chung, 1940) and *Trypanosoma bocagei*-like group 1 in Brazil (Ferreira et al., 2007). Trypomastigotes were also morphologically similar to *Trypanosoma bufophlebotomi* Ayala, 1968 and *Trypanosoma fallisi* Martin & Desser, 1990 from bufonids (Ayala, 1970; Martin & Desser, 1990).

Identification of anuran trypanosomes was traditionally based on morphological characteristics of bloodstreams trypomastigotes and the infected host species (Bardsley & Harmsen, 1973). Recent studies demonstrated that blood samples contain different species, indicating mixed trypanosome infections (Ferreira et al., 2007; Spodareva et al., 2018). The pleomorfism is related to the morphological changes during the life-cycle of anuran trypanosomes and could reflect the occurrence of recurrent infections in natural environment (Feng & Chung, 1940) or the result of immunological responses of the hosts acting on a single trypanosome species (Martin & Desser, 1990).

Our findings revealed the presence of different trypanosomes morphotypes infecting *L. lineatus* and *Osteocephalus* sp. The precision of species determination requires criteria such as *in vitro* isolation, observation of different developmental stages, and/or tools for molecular approaches. In this study, we used blood smears obtained during a wildlife rescue and other approaches were not achieved due the in fields difficulties. Based on morphological data the species identification was prevented. Nevertheless, we believe the information reported here contributes to the knowledge about diversity of trypanosomes in anuran.

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