

In vitro Culture and Morphology of Fish Trypanosomes from South American Wetland Areas

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Received: June 16, 2016; Accepted: July 21, 2016; Published: July 27, 2016

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Abstract

Fish trypanosomes are a group of parasites with taxonomic issues and can demonstrate potential pathogenic effects to the host. Nowadays, producing *in vitro* isolates of fish trypanosomes has become a key point to better understand their biology and taxonomy. Despite of that fact, to date only one species of fish trypanosome was isolated in South America. In addition, there is little information about trypanosomes that occur in Pantanal, an important World Conservation Complex. The purpose of this study was to investigate trypanosome infections in fish from hyperseasonal Savanna in the Brazilian Pantanal wetlands by producing *in vitro* isolates. During the dry period, 74 fish representing 4 species were collected from 5 lagoons, and blood was sampled by cardiac or caudal vein puncture. Analysis of blood smears and *in vitro* cultures showed that 7.31% of hosts were infected by trypanosomes. The *in vitro* isolation and maintenance were made by using Ponselle blood agar base without NaCl and 60% Eagle Basal Medium and Novy-MacNeal-Nicolle medium mixed with fish Ringer's solution. Here, we have highlighted the importance of combining different diagnostic techniques to adequately identify trypanosome infections in fish and have provided a brief morphological description and morphometric features of 20 blood trypomastigotes. In addition, this is the first report of trypanosomes isolated from fish of the Pantanal wetlands, which can contribute to future studies of ultrastructural characteristics, host-parasite interactions, or molecular biology.

Keywords: Epimastigote; *In vitro* culture; Morphology; Pantanal; *Trypanosoma*; Trypomastigote

Introduction

The first record of a trypanosome was likely made in 1841, when parasites were observed in the blood of *Salmo fario* [1]. Since this observation, more than 200 trypanosome species

have been identified in freshwater and marine fish worldwide usually based on morphology of blood trypomastigotes and the hypothesis of parasite-host specificity [2-7]. However, certain fish trypanosome species are pleomorphic and are not specific to the vertebrate host [8-10]. Thus, the access to molecular data information is growing in an attempt to supplement current morphological and morphometric data, and to clarify the species identification [9,11-13].

Although the characterization and taxonomy of fish trypanosomes has been improved through the use of molecular approaches [8,11-13], mixed infections that normally occur in nature can limit its accuracy [9,14]. Therefore, the achievement of *in vitro* isolates and the establishment of laboratory clonal lineages are becoming increasingly important [9,14]. Besides this application, culturing can provide a great number of parasites that can be used in different studies, providing important information regarding their biology and ultrastructural features [9], in addition to the antigenic characteristics of parasites and their relationship with the host immune system [15,16].

In fact, the pathogenic effects of fish trypanosomes species are not completely known. Some experimental infections studies have demonstrated that *Trypanosoma danilewskyi* (synonym *Trypanosoma carassii*) infections can lead the host to develop anemia and anorexia [17,18]. In addition, the investigation of natural infections with other trypanosomes reveal their potential role as pathogen inducing alterations of hematological parameters [19,20] and on total body weight of fish [21]. However, other investigations suggested there is a delicate balance between fish trypanosomes and host immune system, which can lead to the persistence of the parasite in low intensity and host survival [22].

In Brazil, studies on fish trypanosomes have focused on their occurrence and the identification of species. Traditionally, species identification has been based upon morphological features of blood trypomastigotes, along with the host specificity hypothesis [4,6,23,24]. At least 64 nominal species of fish trypanosomes have been recorded to date, with most of them identified using morphological parameters exclusively [3,9]. Only recently, trypanosomes from armored catfish were isolated and maintained *in vitro* using 9 different culture media and one new nominal species could be identified as *Trypanosoma abeli* [9,26]. Altogether, those data shown the scarcity of knowledge regarding fish trypanosomes species that really occur in Brazil [9] and their role as pathogens of fish. Therefore, cultivating *in vitro* isolates represents a key point in studies of those parasites, which can allow new studies regarding their molecular taxonomy as well as other investigations under controlled laboratory conditions, such as their role as pathogens of fish.

The hyperseasonal Savanna of the Brazilian Pantanal wetlands is an important ecosystem in the Brazilian Midwest that is considered a World Heritage of Humanity, and it contains more than 260 species of fish [27]. The present knowledge of the various trypanosomes that occur in these fish species is limited to infections described in two fish species: *Trypanosoma* sp. in *Gymnotus* aff. *inaequilabiatus* [28] and *Trypanosoma azoubeli* in *Pterodoras granulosus* [29]. In our study, we investigated trypanosome infections in fish of the Pantanal wetlands. We examined the morphological features of trypomastigotes in blood and conducted *in vitro* isolation of epimastigotes and trypomastigotes.

Methods

Fish collection and study area

Seventy-four fish were collected during the dry period and were identified as *Gymnotus* sp. (n = 2), *Hoplias malabaricus* (n = 8), *Hoplosternum littorale* (n = 23) and *Pterygoplichthys* sp. (n = 41). The sampling area was the Poconé sub-region of the Pantanal wetlands in the municipality of Nossa Senhora do Livramento (MT, Brazil). Fish were collected from 5 seasonal natural lagoons: Lagoa Funda (56° 18' 49" W 16° 20' 22" S); Tank 2 (56° 19' 7" W 16° 21' 19" S); Tank 3 (56° 19' 1" W 16° 21' 31" S); Lagoa da Fazenda Nossa Senhora Aparecida (56° 19' 20" W 16° 22' 18" S); and Baía das Pedras (56° 21' 7" W 16° 24' 36" S). Our study was conducted in accordance with the guidelines of the Brazilian Institute of Environment and Renewable Natural Resources' (authorization number 26305-1).

Fish were anaesthetized using eugenol diluted in water (50 mg/ L) [26] and were examined for the presence of leeches. Blood samples were taken by cardiac or caudal vein puncture following the recommendations of the Ethics Committee for Animal Experimentation (protocol number 025/ 2011) of the Federal University of Juiz de Fora (MG, Brazil).

Prevalence and intensity of infection

Blood smears were prepared using 20 µL of blood, stained with 9% Giemsa solution, and screened by light microscopy

(1,000× magnification) to detect trypanosomes. To estimate the intensity of infection (expressed in parasites/ mL), all parasites found in 1 cm² of the smear (250 microscopic fields in 1,000× magnification) were recorded and calculated as follows:

$$12 \text{ cm}^2 \text{ ----- } 20 \text{ } \mu\text{L of blood} \quad n^\circ \text{ of parasites ---- } 0.75 \mu\text{L}$$

$$1 \text{ cm}^2 \text{ ----- } 0.75 \text{ } \mu\text{L of blood} \quad x \text{ ---- } 1000 \text{ } \mu\text{L}$$

"n° of parasites" represents the number recorded in 1 cm² of smears

"x" represents the estimated number of parasites in 1 mL of blood

The overall prevalence of trypanosomes was calculated as described previously [30] by using blood smears analysis and *in vitro* culture isolation.

Morphology and morphometry of blood trypomastigotes

Parasites were photographed and measured using Image-Pro Plus® 5.0 (Media Cybernetics, Rockville, MD, USA). The measurements recorded included total body length with flagellum (TL), body length along the cell midline (BL), body width at the center of the nucleus (BW), length of the free flagellum (F), Nucleus Length (NL), nucleus width at the center of the nucleus (NW), distance from the center of the nucleus to the anterior of the cell (NA), distance from the center of the nucleus to the posterior of the cell (NP), distance from the center of the kinetoplast to the center of the nucleus (KN), Kinetoplast Length (KL), Kinetoplast Width (KW), and distance from the center of the kinetoplast to the posterior of the cell (KP). The nuclear index (NI = NP/ NA) and kinetoplast index (KI = NP/ KN) were also calculated (Figure 1).

In vitro cultivation of trypanosomes

The trypanosomes were isolated using three biphasic culture media: Ponselle blood agar base without NaCl (PO), mixed with 60% Eagle Basal Medium (BME) and supplemented with 20 µg/mL hemin and 10% heat-inactivated Fetal Calf Serum (FCS); Blood Agar Base (BAB) and 50% BME, supplemented with 20 µg/

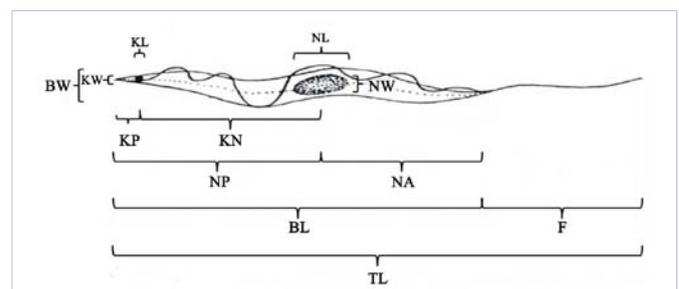


Figure 1: Morphological parameters measured in blood trypomastigotes for morphological characterization [adapted from 31]. Total Body Length With Flagellum (TL), Body Length Along the Cell Midline (BL), Body Width at the Center of the Nucleus (BW), length of the free flagellum (F), Nucleus Length (NL), Nucleus Width at the Center of the Nucleus (NW), Distance from the center of the nucleus to the anterior of the cell (NA), Distance from the center of the nucleus to the posterior of the cell (NP), Distance from the center of the kinetoplast to the center of the nucleus (KN), Kinetoplast Length (KL), Kinetoplast Width (KW), and Distance from the center of the kinetoplast to the posterior of the cell (KP).

mL hemin and 10% heat-inactivated FCS; and Novy-MacNeal-Nicolle (NNN) medium mixed with fish Ringer's solution as described previously [26]. Following inoculation with blood samples, cultures were kept in room temperature for 5 days and then transferred to a BOD incubator (Eletrolab) at 22°C. For positive cultures, trypanosomes were transferred to new tubes containing the same medium every 7 days.

Results

Prevalence and intensity of infection

The total prevalence of trypanosome infections in *Pterygoplichthys* sp. was 7.31% (3/ 41) and one of these had the infection detected only by *in vitro* isolation. Analysis of blood smears showed that *Pterygoplichthys* sp. was infected with trypanosomes, with an intensity of 9×10^{-4} parasites/ mL

Blood smear analysis and culture isolation failed to reveal the presence of trypanosomes in any other fish species. In addition, all fish were inspected for the presence of leeches, but none were found.

Morphological aspects of blood trypomastigotes

The trypanosomes observed showed deeply Giemsa staining of the cytoplasm with some small vacuoles along the body. The nucleus was oval and displaced toward to anterior region of the body (NI > 1). The kinetoplast was circular or oval and located at the posterior extremity of the body (KI < 2). The flagellum emerged from the posterior end of the body and followed the outline causing ripples, finishing in a long free portion (Figure 2A–C). Some of the parasites had striations along their body length (Figure 2C). The morphometric analysis results for 20 trypomastigotes are shown in Table 1.

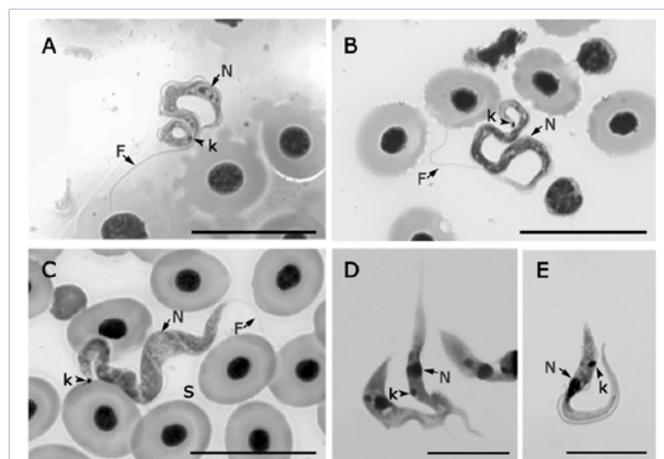


Figure 2: Morphological features of *Trypanosoma* sp. from Pantanal wetland fish. (A, B) Elongated trypomastigotes with nucleus (N), Kinetoplast (k), and Flagellum (F) indicated. (C) Trypomastigote with striations (S) along the body, and a short flagellum. (D) Various epimastigote forms observed in cultures. (E) Short and slender trypomastigote with a rod-like kinetoplast observed in Ponselle blood agar base without NaCl mixed with 60% Eagle Basal Medium. The scale bars (A–E) indicate 10 µm.

Table 1: Morphometric features of *Trypanosoma* sp. in the blood of *Pterygoplichthys* sp. from Brazilian Pantanal. The measures are expressed in µm.

Measurements*	Mean	SD	Minimum	Maximum
TL	54.99	11.29	35.11	63.45
BL	44.85	7.29	29.85	52.76
BW	2.06	0.70	1.17	4.46
F	12.85	7.48	3.61	21.39
NL	3.72	0.73	2.36	5.23
NW	1.63	0.52	1.07	3.41
NA	18.89	6.76	6.17	27.53
NP	26.34	2.58	21.60	29.63
KN	25.64	2.48	21.21	29.44
KL	0.87	0.14	0.61	1.07
KW	0.78	0.13	0.56	1.14
KP	0.48	0.10	0.33	0.73
NI	1.7	0.90	0.91	4.00
KI	1.0	0.02	0.99	1.06

* The codes are presented in the methods section

In vitro cultivation and maintenance of trypanosomes

Following the inoculation of PO/ 60% BME and NNN/ fish Ringer's solution cultures with trypomastigotes from blood, we observed epimastigotes (Figure 2D) and trypomastigotes (Figure 2E) *in vitro*. The period required for trypomastigotes to differentiate into epimastigotes could not be recorded in this study because of the field conditions. *In vitro* epimastigotes had an elongated body with a round kinetoplast (Figure 2D), whereas trypomastigotes had a slender and shorter body when compared with the blood forms (Figure 2E). Trypomastigotes and epimastigotes divided by binary fission (not shown) and were usually clustered with the anterior or posterior extremities in the center (not shown).

Discussion

We have demonstrated a low prevalence of trypanosome infection among *Pterygoplichthys* sp. This genus of fish belongs to the Loricariidae family and is representative of the hosts with the greatest number of trypanosome infections in Brazil. It was previously shown that the prevalence of trypanosomes in *Hypostomus punctatus* was 100% [32], whereas trypanosome prevalence ranged from 22.6-100% in 6 other species of armored catfish [19]. These differences in prevalence among fish species could be related to the behavior of the host [19], the abundance of the vector in the environment [5,33], and the diagnostic method used [28,32].

It has been confirmed that the examination of fresh blood is more sensitive than the microhematocrit method and blood smears [28,32]. Although recently a prevalence of 100% has been recorded in armored catfish through analysis of blood smears

[9], this technique is considered of low sensitivity. Polymerase Chain Reaction (PCR) assays represents other efficient method for fish trypanosomes diagnose [8]. However, PCR amplification of the trypanosomes DNA were not achieved for the positive hosts in this study, which can be related to the low intensity of trypanosomes in total blood or the quality of the material stored. In fact, the sensitivity of the various diagnostic methods available for use could be affected by the intensity of the infection, which is known to vary according to the stage of infection [18,34]. Indeed, we used the same blood smear screening techniques described in a previous study [9], but the prevalence and intensity were much lower (9×10^{-4} parasites/ mL in this study compared with 1×10^2 parasites/ mL [9]).

In vitro isolation of fish trypanosomes is laborious and time-consuming, especially when the field conditions for primary isolations are not propitious, such as the lack of laboratory structure, the geographical isolation of collection sites and the high temperatures exhibited. Furthermore, various media components, temperature, and pH can affect the growth of the parasites [35,36]. Initial attempts to isolate fish trypanosomes in Brazil were unsuccessful [37,38]; however, *T. abeli* was recently isolated from armored catfish [26]. We were able to isolate trypanosomes from fish of the Brazilian Pantanal wetlands for the first time, providing new information regarding the diversity of fish trypanosomes and enabling future studies of their biology. The biphasic media used were found to be more effective than monophasic media with respect to the maintenance of fish trypanosomes [26]. Previous findings suggest that diffusion of nutrients from the blood agar base is necessary for the cultivation of trypanosomes [39]. The first trypomastigotes observed in culture resemble the blood forms and were replaced by epimastigotes, and then shorter trypomastigotes grew, which have been previously observed by other groups [21,26].

In the present study, we have provided a brief morphological description and presented morphometric features of trypanosomes from *Pterygoplichthys* sp. We also successfully isolated trypanosomes from Pantanal fish for the first time and demonstrated the importance of combining diagnostic techniques for the identification of trypanosome infections. The low number of parasites found in blood and the lack of molecular data prevented the morphotype classification; however, the present findings will lay an impact on future studies on Pantanal fish trypanosomes, showing that is possible to achieve their culturing besides the field adversities, which can promote other investigations regarding not only their taxonomy as well as their ultrastructural features and metabolic requirements.

Acknowledgment

The authors are grateful to Dr. João Batista de Pinho and to field technician Chico Bill from Universidade Federal do Mato Grosso. This work was supported by FAPEMIG and CNPq grants to Dr. Marta D'Agosto, Dr. Thaïs Souto-Pradrón and MSc. Alyssa Rossi Borges.

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