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Trypanosoma amblyommi sp. nov. (Protozoa: Kinetoplastida) isolated from *Amblyomma brasiliense* (Acari: Ixodidae) ticks in Rio de Janeiro, Brazil

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Summary

Parasites of the genus *Trypanosoma* are microorganisms that display wide morphological, biological and genetic variability. Here we present the first description of an isolate of the genus *Trypanosoma* naturally infecting the tick *Amblyomma brasiliense*. The ticks were collected from a specimen of *Tayassu pecari* (Queixada, white-lipped peccary) from the Itatiaia National Park, Itatiaia, Rio de Janeiro, Brazil. The isolate was characterised by molecular, morphometric and biological analyses. A *Trypanosoma* culture was isolated from crushed nymphal and adult ticks, propagated in the tick cell line IDE8 and maintained in L15B culture medium, incubated at 32 °C. The isolate grew well in L15B medium at 30 °C, 32 °C and 34 °C but not at lower or higher temperatures. The culture remained stable in axenic L15B medium at 30 °C. Cryopreserved cultures retained viability after cryopreservation in liquid nitrogen. Growth in axenic medium and developmental forms of the trypanosomes were analysed. Analysis of the 18S rDNA region confirmed the authenticity of this new species and the nucleotide sequence was deposited in Genbank. The species was named *Trypanosoma amblyommi* sp. nov. strain C1RJ. Characteristics related to pathogenicity, involvement with vertebrate hosts, epidemiology, developmental cycle and transmission mechanisms are still unknown. Therefore, further studies are necessary to understand aspects of the biological cycle of *Trypanosoma amblyommi* sp. nov.

Keywords

Trypanosoma amblyommi sp. nov.; *Amblyomma brasiliense* ticks; tick cell line

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Conflict of Interest
None

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Introduction

Members of the genus *Trypanosoma* belong to the family *Trypanosomatidae* and present a complex taxonomic classification due to their wide morphological, biological and molecular variation. Haematophagous arthropods act as biological or mechanical vectors for different species of this family, infecting a wide range of vertebrate hosts. Several species of the genus *Trypanosoma* are aetiological agents of diseases transmitted to humans and animals, stimulating interest in these protozoans (Haag et al., 1998; Hoare, 1972).

While most *Trypanosoma* species are transmitted by blood-sucking insects, ticks are also likely to be vectors of some members of this genus (Morzaria et al., 1986; Thekisoe et al., 2007). We recently reported isolation of a novel trypanosome, *Trypanosoma rhipicephalis* sp. nov, from Brazilian *Rhipicephalus microplus* ticks removed from cattle (Marotta et al., 2018).

This study describes an isolate of the genus *Trypanosoma* naturally infecting *Amblyomma brasiliense* ticks parasitising the white-lipped peccary *Tayassu pecari*, characterised through molecular, morphological and biological analyses. Although *A. brasiliense* are aggressive toward humans, their vector capacity for aetiologic agents of diseases transmitted to humans or animals is unknown (Aragao, 1936; Sanches et al., 2008).

Materials and Methods

Origin of Amblyomma brasiliense ticks

A specimen of adult *T. pecari* was found dead in the Itaporani Waterfall, Itatiaia National Park, Itatiaia, Rio de Janeiro, Brazil (located between the coordinates 22º 19' and 22º45' S, and 44º15' and 44º50' W). The animal was kept refrigerated (2-8 °C) for approximately 24 hours and was sent to the Federal Rural University of Rio de Janeiro (UFRRJ) municipality of Seropedica, state of Rio de Janeiro for post mortem investigation. The necropsy report confirmed that the macroscopic findings were consistent with vertebral fractures and cavitary haemorrhages, consistent with trauma, probably due to a fight between animals and fall into a waterfall.

Eight live ticks were collected from the peccary and identified according to Barros-Battesti et al. (2006) for adults and Martins et al. (2010) for nymphs. Nymphs and adults were identified as belonging to the species *A. brasiliense*. It was not possible to identify the larvae to the species level.

Isolation of trypanosomes

In a laminar flow cabinet, live ticks were surface-sterilised by immersion in 70% ethanol for one minute, 0.05% sodium hypochlorite solution for 30 seconds, 70% ethanol again for 1 minute, detergent based on 2% chlorhexidine (Riohex® Rioquimica, Brazil) for 30 seconds, a third wash in 70% ethanol for one minute and finally sterile ultrapure water with penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (250 µg/ml) for one minute. The ticks were then dried on sterile gauze.

After surface-sterilisation, the ticks were separated into two pools, consisting of one pool of four *Amblyomma* sp. larvae and one pool of 2 nymphal and 2 adult *A. brasiliense*. The pooled ticks were crushed in a beaker with the aid of a glass syringe plunger (piston). The crushed ticks were resuspended in 5 ml of L15B medium (Munderloh and Kurtti, 1989) supplemented with 10% heat-inactivated foetal calf serum (FCS), 10% tryptose phosphate broth, 0.1% bovine lipoprotein concentrate (MP Biomedicals), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, pH adjusted to 6.6-6.8 with 1N NaOH.

Each tick suspension, containing all parts of the ticks without prior centrifugation, was transferred to a 25 cm^2 flask containing a monolayer of cells of the *Ixodes scapularis* embryo-derived cell line IDE8 (Munderloh et al., 1994) 4 days after seeding at passage 112, grown in complete L-15B medium as described above, and incubated at 32 °C.

Maintenance and monitoring of trypanosome cultures

The inoculated cultures were monitored by examination of Giemsa-stained smears, prepared by spreading a drop of culture supernatant on a slide and air-drying, every three days post-infection (DPI). Following initial isolation in IDE8 cells, trypanosome cultures were maintained in two ways: co-cultivation with IDE8 cells and axenic culture in complete L15B medium, both incubated in sealed 25 cm^2 culture flasks in a bacteriological incubator at 30 °C. Renewal of the medium from cultures with IDE8 cells was performed weekly by removal and replacement of approximately 2/3 of the medium.

To obtain a pure, tick cell-free trypanosome culture after four passages in IDE8 cultures, the isolated trypanosomes were resuspended, collected by rinsing and transferred to a sterile 15 ml tube for centrifugation at 700 X *g* for 10 minutes. The supernatant was transferred to a new sterile 15 ml tube and centrifuged at 200 X *g* for 10 minutes. Then the supernatant was discarded and the pellet was resuspended in 8 ml of PBS and examined by inverted microscope to rule out the presence of tick cells. After further centrifugation at 200 X *g* for 10 minutes, the resultant pellet was resuspended in 5 ml of complete L15B medium, transferred to a 25 cm^2 culture flask and incubated at 30 °C. The culture medium was renewed weekly, with removal and replacement of approximately 2/3 of the medium after mixing of the culture. Cultures were monitored with an inverted phase contrast microscope and by examination of Giemsa-stained smears of culture supernatant prepared as above.

The isolated trypanosomes were passaged by transfer of 1 ml of culture supernatant (1:5 dilution) to three replicates each of both IDE8 cultures and axenic cultures in 25 cm^2 flasks. Aliquots of the axenically-cultured trypanosomes were cryopreserved with 10% DMSO in liquid nitrogen at -196 °C at monthly intervals following isolation. For freezing, a culture was resuspended and transferred to a 15 ml sterile centrifuge tube and centrifuged at 500 X *g* for 5 minutes. Thereafter, the supernatant was removed and the pellet resuspended in 2 ml of complete L15B culture medium. An equal volume of ice-cold culture medium with 20% filtered DMSO was added dropwise and gently mixed. The cell suspension was divided between 4 labelled cryotubes, placed in a Nalgene ™ Cryo 1°C isopropanol freezing container, and held at –80 °C for at least 90 minutes. Subsequently the cryotubes were transferred to a liquid nitrogen storage container (-196 °C). The cryopreserved trypanosomes

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were resuscitated two months after freezing by removal from liquid nitrogen, thawed slowly in a water bath at 32 °C, diluted in 5 ml complete L-15B medium and incubated at 30 °C.

Propagation in different culture conditions

Axenic propagation of the isolated trypanosomes was tested in the following culture media: MEM and DMEM (supplemented with 2 mM L-glutamine and 10% foetal bovine serum), M199 (supplemented with 10% foetal bovine serum), BHI, BHI supplemented with blood agar, and Schneider's insect medium (supplemented with 10% foetal bovine serum). Axenic propagation was tested at the following incubation temperatures over 15 days: 26 °C, 28 °C, 30 °C, 32 °C, 34 °C and 37 °C. Three replicate cultures in 25 cm^2 flasks were evaluated for each condition.

Growth profile and developmental forms in axenic culture

The developmental profile of the isolated trypanosomes in axenic culture was evaluated at an early passage, 4 days after subculture. Initially, the viable (highly motile) trypanosomes were counted in a Neubauer chamber to prepare the inoculum concentration of 1 x 10^4 parasites/ml and were subsequently transferred to axenic cultures in 25cm^2 culture flasks with complete L15B medium. The growth curve was performed in triplicate at 30 °C. Aliquots of 10 µl were collected at intervals of 48 hours until the 30th DPI for quantification as above and morphological analysis until the 16th DPI. Developmental forms were analysed in Giemsa-stained smears by examination of 50-100 trypanosomes per sample, based on published descriptions of Barros et al. (2014). No medium change was performed during this 30-day period.

Morphometric analysis

Morphometry was performed on randomly-selected stained trypanosomes from axenic cultures, evaluated with a light microscope (Olympus BX45®) coupled with a photo documentation system (D'Cell®software). The measurements were performed according to Hoare (1972), by evaluating the total length of the parasite (from the anterior end to the posterior end), the free flagellum length, nucleus diameter, kinetoplast diameter, distance from the posterior end to the middle of the nucleus, distance from the posterior end to the middle of the kinetoplast, distance from the middle of the nucleus to the middle of the kinetoplast, distance from the middle of the nucleus to the anterior end.

DNA extraction and polymerase chain reaction (PCR)

DNA extraction was performed on trypanosomes at the second passage in axenic culture, using a Qiagen® Qiamp kit according to the manufacturer's recommendations.

Nested-PCR was performed for amplification of a partial region of the 18S rDNA gene specific to the family *Trypanosomatidae* using TRY927F (5'-GAAACAAGAAACACGGGAG-3') and TRY927R (5'-CTACTGGGCAGCTTGGA-3') external primers, that amplify a fragment of approximately 900 bp, and SSU561F (5'-TGGGATAACAAAGGAGCA-3') and SSU561R (5'-CTGAGACTGTAACCTCAAAGC-3') internal primers that amplify a fragment of approximately 700 bp, according to the protocol of Smith et al. (2008).

To amplify the partially-conserved sequence of the largest ribosomal subunit gene (24Sα rDNA) of members of the family *Trypanosomatidae*, PCR was performed using D75 (5'-GCAGATCTTGGTTGGCGTAG-3') and D76 (5'-GGTTCTCTGTTGCCCCTTTT-3') primers that amplify a fragment of approximately 270 bp, according to Souto et al. (1999).

PCR products were subjected to 2% agarose gel electrophoresis at 90 W for 30 minutes. The gels were stained with ethidium bromide and visualised with a UV light transilluminator.

Sequencing and phylogenetic analysis

PCR amplification products were purified using the QIAquick® PCR Purification Kit (Qiagen), according to the manufacturer's recommendations. After purification, the DNA was sequenced using a capillary-type Sanger platform in an ABI 3730 DNA Analyser (Applied Biosystems, Life Technologies®). The resultant sequences were compared to published sequences using the NCBI Nucleotide BLAST program.

Phylogenetic trees were built from the partial sequences of the 18S rRNAgene using the Mega 6 program, the Maximum Likelihood test and the Tajima-Nei model.

Results

Trypanosome isolation and culture

The crushed tick suspension did not have any adverse effect on the IDE8 cells detectable by microscopic examination. No microorganisms were detected in the culture inoculated with material from the four larval ticks. From the pool of four *A. brasiliense* nymphal and adult ticks inoculated into IDE8 cell culture, typical forms of *Trypanosoma* were seen from the 7th DPI onwards. The isolated trypanosomes, designated strain C1RJ, grew well axenically in complete L15B medium at temperatures of 30 °C, 32 °C and 34 °C but not at higher or lower temperatures. However, no parasite growth and no viable trypanosomes were seen after the 7th DPI in the other culture media tested (MEM, DMEM, M199, BHI, BHI supplemented with blood agar, Schneider's Insect Medium) at all temperatures examined.

The trypanosome culture remained viable over 24 passages in axenic culture in L15B medium, and was co-cultured with IDE8 cells through ten passages, both at 30 °C. Co-cultivation of the trypanosomes with IDE8 cells resulted in detachment and subsequent death of the tick cells, commencing at 7 DPI. Trypanosomes cryopreserved at the third axenic passage were viable when resuscitated after 60 days of storage in liquid nitrogen. Figure 1A shows the growth curve obtained in axenic culture in L15B at 30 °C over a 30-day period. The growth curve was initiated with an inoculum (day 0) of 1 x 10^4 parasites/ml that comprised 87% typical epimastigote forms with elongated bodies, well-tapered posterior and anterior ends, 12% trypomastigotes with an average body length smaller than the epimastigote form and kinetoplast posterior to the nucleus and 1% spheromastigote forms (Fig. 1B). Epimastigote forms predominated during the first fortnight, with a fall to below 50% and a concurrent increase in spheromastigote forms seen on the 16th DPI. Peak growth was seen on the 16th DPI with 910 x 10^4 parasites/ml. By the 30th DPI, degenerating and dead forms predominated (data not shown).

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Morphometric analysis

Morphometric variations between different developmental forms in axenic culture were observed (Fig. 2). The morphometric measurements of trypanosome strain C1RJ trypomastigote, epimastigote and spheromastigote developmental forms are presented in Table 1.

The epimastigote form showed the greatest average body length (41.72 μm) and the spheromastigote form had the lowest mean total body length (19.44 μm). The mean length of the free flagellum was similar between the epimastigote and trypomastigote forms, with the spheromastigote form having the largest free flagellum length (11.61 μm). The diameters of the nucleus and kinetoplast of the epimastigote, trypomastigote and spheromastigote forms were not significantly different. The mean distance between the posterior end and middle of the nucleus was similar between the epimastigote and trypomastigote forms, but much shorter in the spheromastigote form (3.32 μm). The mean distance from the middle of the nucleus to the middle of the kinetoplast was similar in the epimastigote and spheromastigote forms. The epimastigote form presented the greatest mean values for the distance from the posterior extremity to the middle of the kinetoplast (15.47 μm) and the distance from the anterior end to the middle of the nucleus (15.61 μm). The diameter of the nucleus was smallest in the spheromastigote form (1.71 μm).

Molecular analysis

In the nested-PCR carried out on axenically-cultured trypanosomes, the 18S rDNA PCR fragment was approximately 900 bp in the first round of amplification. In the second round the size of the amplified fragment was approximately 700 bp. The partial sequence of the 18S rDNA gene showed 89% similarity with *T. rhipicephalis* (accession number KX711901) matching 99% of the query sequence, and 88% similarity with *Trypanosoma* KG1 (accession number AB281091) matching 99% of the query sequence. It also presented 90% similarity (with e-value 2 e-133) and 60% alignment with *Trypanosoma caninum* (accession number JF951431, JF9075537). In the 24Sα rDNA PCR reaction using primers D75 and D76 the size of the amplified fragment was 270 bp for the isolate. For the 24Sα rDNA gene there was 96% similarity with *Trypanosoma rangeli (*query coverage 63% GenBank KJ742907), *Trypanosoma grosi* AKHA (query coverage 65% GenBank AB175624). The phylogenetic analysis targeting the 18S rDNA gene (sequence conserved within the family *Trypanosomatidae*) confirmed that the trypanosome isolated from *A. brasiliense* belongs to the family *Trypanosomatidae*. The phylogenetic tree showed that the species is within the same clade as *T. rhipicephalis* and in a clade close to *T. caninum* and *Trypanosoma* KG1 (Fig. 3). The analysis of the 18S rDNA region confirmed the authenticity of this new species. Molecular analysis showed that our trypanosome isolate, strain C1RJ, was clearly separated from other species of the genus *Trypanosoma*, regardless of the molecular target used, with bootstrap values of 85 for the tree built using the target 18S rDNA sequence (Fig. 3). The nucleotide sequences described were deposited in GenBank under the access number KX711902.

Discussion

Here we describe the first isolation, molecular characterisation, morphological and biological analyses of a member of the genus *Trypanosoma* infecting ticks of the species *A. brasiliense* parasitising a specimen of *Tayassu pecari*, from the municipality of Itatiaia, RJ, Brazil. The new species was named *Trypanosoma amblyommi* sp. nov.

The *A. brasiliense* tick is endemic in South America, with reports in Argentina, Paraguay and Brazil (Guglielmone et al., 2003; Sanches et al., 2008). Humans are often parasitised by this species of tick in Brazil (Aragao, 1936), but their vectorial capacity for bioagents infecting humans or animals is still unknown (Sanches et al., 2008). The trypanosome isolated in the present study was found in a pool of nymphs and adults of *A. brasiliense,* which are among the most aggressive ticks attacking humans in Brazil (Aragao, 1936).

Despite the isolation of this *Trypanosoma* from crushed ticks, it is not possible to determine whether the origin of the protozoan was from a vertebrate or invertebrate host.

The *Tayassu pecari* specimen presented a good body score and high infestation by ticks. The autopsy report confirmed trauma with macroscopic findings consistent with vertebral fractures and internal bleeding. There was no evidence of injury caused by trypanosomatid infection. In Brazil, wild pigs were identified as important reservoirs of *Trypanosoma evansi* and *Trypanosoma cruzi* (Herrera et al., 2008)

Some species of the genus *Trypanosoma* multiply intracellularly in the vertebrate host in the amastigote form e.g. *T. cruzi*, in blood in the trypomastigote form e.g. *Trypanosoma brucei* or in the epimastigote form e.g. species of the subgenus *Megatrypanum* (Hoare, 1972). In the present study, *in vitro* replication of *T. amblyommi* was in the epimastigote form.

An interesting observation was the isolation using the tick cell line IDE8 and the inability of *T. amblyommi* to grow in conventional trypanosome culture media and at conventional incubation temperatures. *T. amblyommi* was propagated in L15B medium, which is used for culturing tick cells (Munderloh and Kurtti., 1989) and only at temperatures between 30 and 34 °C. Similar behaviour was observed for the recently-described *T. rhipicephalis*, also isolated into IDE8 cell culture from ticks in the state of Rio de Janeiro (Marotta et al., 2018). In the present study, although no adverse effect of the initial crushed tick suspension on the IDE8 cells was detected, subsequent co-cultivation of *T. amblyommi* with tick cells resulted in detachment and death of the latter. Further studies are required to establish whether the tick cell death resulted simply from competition with the faster-growing trypanosomes for nutrients in the culture medium, from release of toxic substances by the trypanosomes, or because trypanosomes were internalised by the tick cells and somehow compromised the latters' viability. This phenomenon was not reported to occur with *T. rhipicephalis* in IDE8 cells (Marotta et al., 2018).

The morphometric evaluation revealed wide morphological diversity. *T. amblyommi* presented large dimensions, as seen by the total length of the trypomastigote form with a mean of 35.81 μm, being larger than *Trypanosoma vivax* (Ramírez et al., 1997), *T. cruzi* (Madeira et al., 2009), *Trypanosoma evansi* (Elshafie et al., 2013) and *T. rangeli* (Madeira et

al., 2009), but smaller than *Trypanosoma theileri* (Wink, 1979) and *T. caninum* (Madeira et al., 2009). Wide variation was also observed in other measurements, such as the distance between the posterior end and the kinetoplast, the distance between the nucleus and anterior end and the posterior end to the nucleus.

Sequencing analysis of the 18S rDNA *and* 24Sα rDNA regions confirmed the authenticity of this new species. In the phylogenetic 18S rDNA analysis, *T. amblyommi* appears within the same clade as *T. rhipicephalis*, *T. caninum* and *Trypanosoma* KG1. *Trypanosoma* KG1 was described after isolation from naturally infected *Haemaphysalis hystricis* ticks in Japan (Thekisoe et al., 2007), while *T. caninum* was isolated from a dog in Brazil (Madeira et al., 2009) and its vector is unknown.

Our results indicate that *T. amblyommi* is a new species of the genus *Trypanosoma*. However, aspects related to ultrastructure, pathogenicity, involvement with vertebrate hosts, epidemiology, cycle, transmission mechanisms, classification and taxonomy are still unknown. Further studies are needed to determine these aspects of the biological cycle of the newly-identified *T. amblyommi*. Isolation in the present study of *T. amblyommi* and, using similar techniques, of *T. rhipicephalis* (Marotta et al., 2018) from small samples of two unrelated tick species removed from very different hosts (peccary and cattle) suggests that ticks in Brazil may frequently harbour trypanosomes. Further studies are likely to reveal even more novel species of this haemoparasite.

Description

Name: *Trypanosoma amblyommi* sp. nov.

Mammalian host: Unknown.

Location: Itatiaia National Park, Itatiaia, State of Rio de Janeiro, Brazil.

Vector: Possibly the tick *Amblyomma brasiliense.*

Biology and morphology: This species was isolated in co-cultivation with IDE8 tick cells and grown in L15B medium supplemented with foetal calf serum (FCS). The developmental stages found in axenic cultures were epimastigotes (predominantly), trypomastigotes and spheromastigotes. The mean total body length in the epimastigote form was 41.72 μm, free flagellum 10.74 μm and longitudinal axis of the kinetoplast 1.23 μm. Measurement of total body length in the trypomastigote form was on average 35.81 μm, free flagellum 10.76 μm and longitudinal axis of the kinetoplast 1.09 μm. The mean total body length in the spheromastigote form was 19.44 μm, free flagellum 11.61 μm and longitudinal axis of the kinetoplast was 1.05 μm.

Molecular characteristics: The trypanosome presents amplified products for the 24Sα rDNA gene of about 270 bp using D75/D76 primers. In the first nested-PCR reaction for the 18S rDNA gene using the primers TRY927F and TRY927R, the amplified fragment was 900 bp. In the second reaction, using the SSU561F and SSU561R primers, the amplified

fragment was 700 bp. In the phylogenetic analysis of ribosomal genes, this trypanosome is close to *Trypanosoma* sp. KG1 and *T. caninum*.

Storage: Axenic cultures of these trypanosomes are cryopreserved in 10% DMSO, stored in liquid nitrogen at –196°C and deposited in the Parasitic Diseases Laboratory (LDP), located in Annex I of the Veterinary Institute, Department of Epidemiology and Public Health, Federal Rural University of Rio de Janeiro (UFRRJ), municipality of Seropedica, state of Rio de Janeiro, Brazil.

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Key Findings

- Novel trypanosome species harboured by *Amblyomma brasiliense* ticks in Brazil
- Parasites grew axenically in L15B medium, but not in classic trypanosome culture media
- Parasites multiplied at 30–34 °C, but not at higher or lower temperatures
- The species described presented wide morphological variation

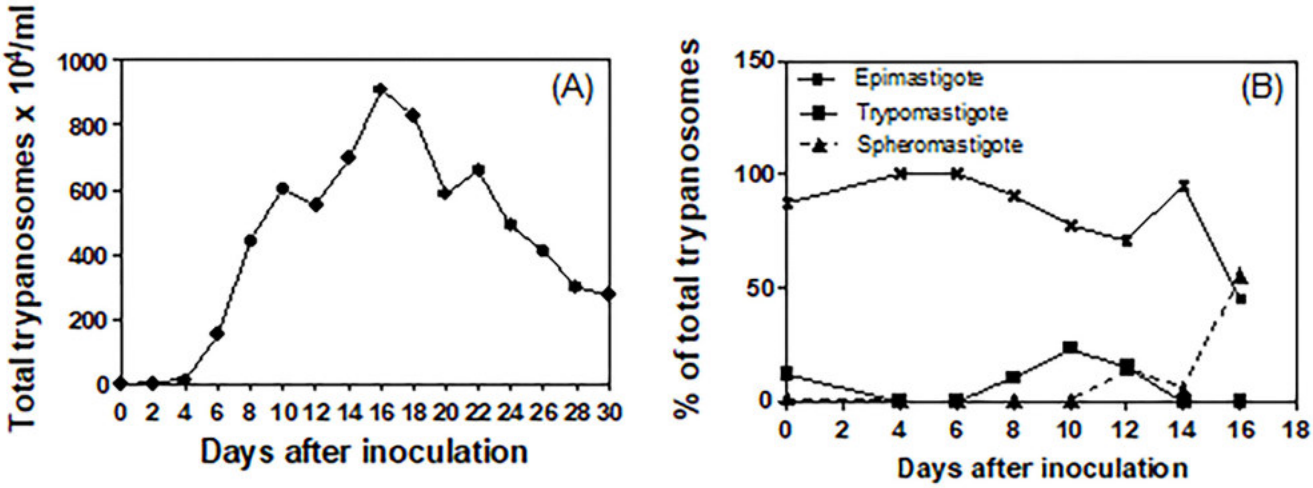

Fig. 1.

Growth of *Trypanosoma amblyommi* sp. nov. in axenic culture in complete L-15B medium. **A.** Growth curve determined by counting total numbers of trypanosomes at 2-day intervals over 30 days; mean of three replicate cultures. **B.** Proportions of different developmental forms determined by examination of Giemsa-stained smears prepared at 2-day intervals over 16 days.

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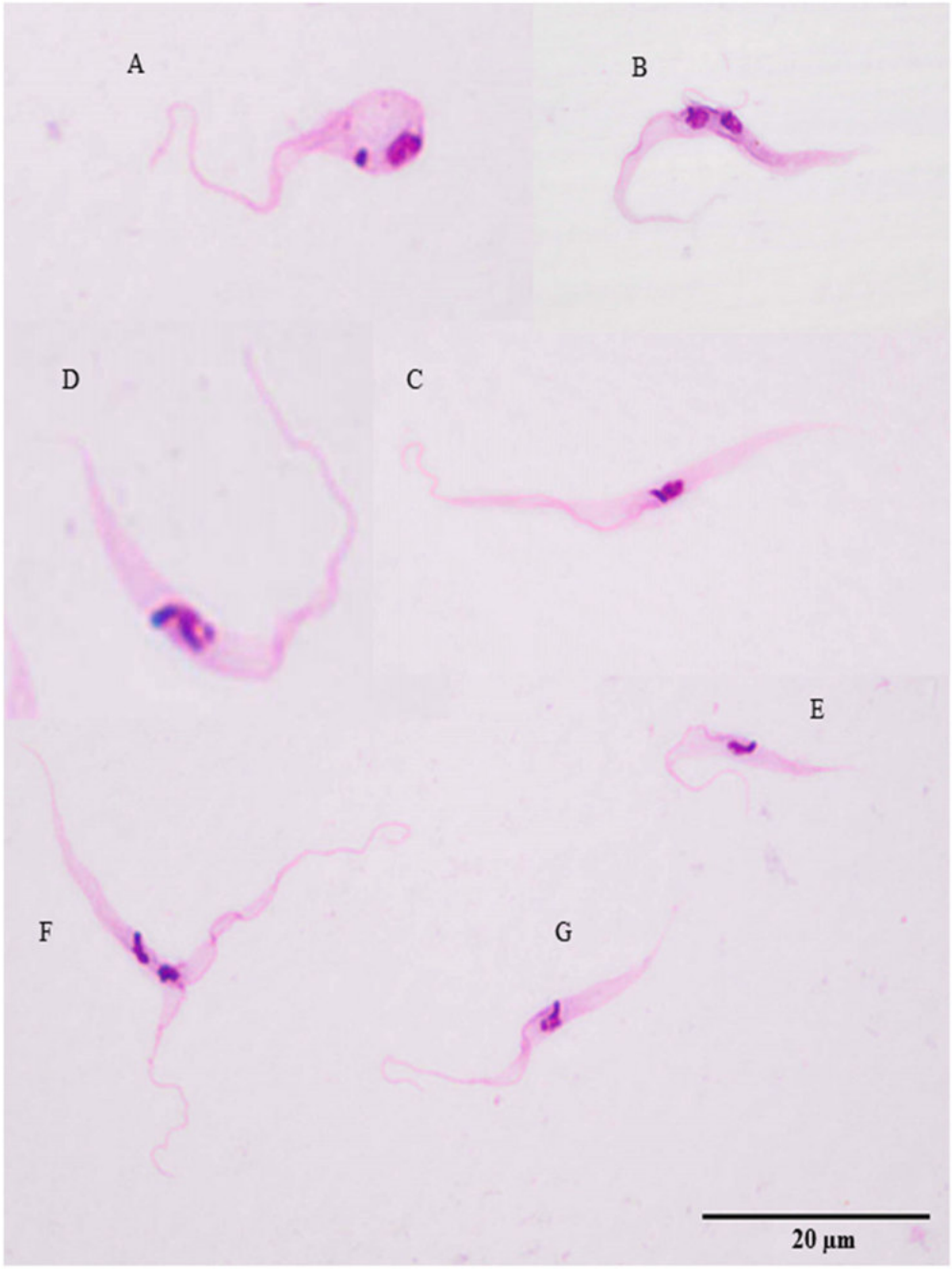

Fig. 2.

Photomicrographs showing morphological diversity of *Trypanosoma amblyommi* sp. nov. in axenic culture in complete L-15B medium at 30 °C. A. spheromastigote; D, F. dividing form; E. epimastigote; B, G, C. forms in transition to trypomastigote Giemsa-stained smears; scale bar = 20 µm.

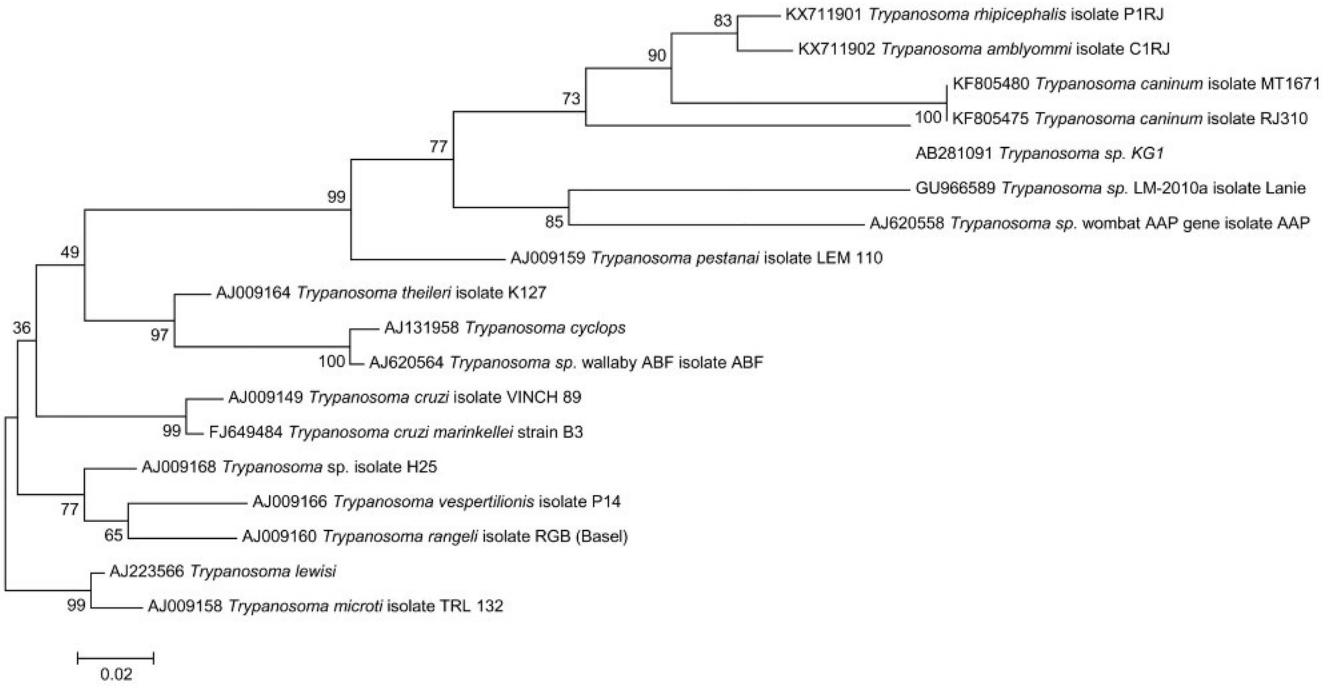

Fig. 3.

Phylogenetic analysis of *Trypanosoma amblyommi* sp. nov. and other trypanosome species. Phylogenetic tree based on 18S rDNA sequences analysis. Statistical method Maximum Likelihood - Kimura 2-parameter model. Bootstrap: 1000.

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Table 1

Morphometric data (μm) obtained from trypomastigote, epimastigote and spheromastigote developmental forms of *Trypanosoma amblyommi* sp. nov.

	TL	PK	KN	NA	FF	PN	NL	K
Trypomastigote	35.81≠7.60	12.30± 3.56	1.39±0.47	11.06±4.10	10.76±2.92	13.28±3.61	1.75±0.40	1.09 ±0.25
	(56.16-25.42)	(21.82-7.17)	(3.92-0.94)	(26.03-5.68)	(19.34-4.06)	(22.52-8.19)	(3.33-1.21)	(1.86-0.64)
Epimastigote	41.72±8.85	15.47±4.26	1.18±0.38	15,61±5.12	10.74±2.90	14.59±4.09	1.84±0.37	1.23±0.39
	(60.87-24.20)	(27.74-7.38)	(2.08-0.67)	(29.18-8.06)	(17.59-5.81)	(26.99-8.03)	(2.50-111)	(2.37-0.52)
Spheromastigote	19.44± 5.92	3.26±1.48	1.45±0.08	5.55±4.61	11.61±4.88	3.32±2.18	1.71±0.49	1.05±0.31
	(36.82-7.98)	(6.40-1.01)	(3.89-0.28)	(15.83-0.23)	(22.18-3.31)	(13.80-0.12)	(2.82-0.73)	(1.89-0.35)

PK: posterior end to kinetoplast, KN: kinetoplast to middle of nucleus, PN: posterior end to middle of nucleus, NA: middle of nucleus to anterior end, FF: free flagellum, TL: total length, NL: nucleus diameter, K: Kinetoplast diameter. Average ± Standard deviation (minimum - maximum value).