

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

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33 SUMMARY

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

53 Key words: *Trypanosoma amblyommi* sp. nov., *Amblyomma brasiliense* ticks, tick cell line

54

55 KEY FINDINGS

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

61 INTRODUCTION

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

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

74 This study describes an isolate of the genus *Trypanosoma* naturally infecting
75 *Amblyomma brasiliense* ticks parasitising the white-lipped peccary *Tayassu pecari*,
76 characterised through molecular, morphological and biological analyses. Although *A.*
77 *brasiliense* are aggressive toward humans, their vector capacity for aetiologic agents of
78 diseases transmitted to humans or animals is unknown ([Aragao, 1936](#); [Sanches et al.,
79 2008](#)).

80

81 MATERIALS AND METHODS

82 *Origin of Amblyomma brasiliense* ticks

83 A specimen of adult *T. pecari* was found dead in the Itaporani Waterfall, Itatiaia
84 National Park, Itatiaia, Rio de Janeiro, Brazil (located between the coordinates 22° 19'
85 and 22°45' S, and 44°15' and 44°50' W). The animal was kept refrigerated (2-8 °C) for
86 approximately 24 hours and was sent to the Federal Rural University of Rio de Janeiro
87 (UFRRJ) municipality of Seropedica, state of Rio de Janeiro for post mortem
88 investigation. The necropsy report confirmed that the macroscopic findings were

89 consistent with vertebral fractures and cavitary haemorrhages, consistent with trauma,
90 probably due to a fight between animals and fall into a waterfall.

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

95 *Isolation of trypanosomes*

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

102 After surface-sterilisation, the ticks were separated into two pools, consisting of one
103 pool of four *Amblyomma* sp. larvae and one pool of 2 nymphal and 2 adult *A. brasiliense*.
104 The pooled ticks were crushed in a beaker with the aid of a glass syringe plunger
105 (piston). The crushed ticks were resuspended in 5 ml of L15B medium (Munderloh and
106 Kurtti, 1989) supplemented with 10% heat-inactivated foetal calf serum (FCS), 10%
107 tryptose phosphate broth, 0.1% bovine lipoprotein concentrate (MP Biomedicals), 2
108 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, pH adjusted to
109 6.6-6.8 with 1N NaOH.

110 Each tick suspension, containing all parts of the ticks without prior centrifugation, was
111 transferred to a 25 cm² flask containing a monolayer of cells of the *Ixodes scapularis*
112 embryo-derived cell line IDE8 ([Munderloh et al., 1994](#)) 4 days after seeding at passage
113 112, grown in complete L-15B medium as described above, and incubated at 32 °C.

114

115 *Maintenance and monitoring of trypanosome cultures*

116 The inoculated cultures were monitored by examination of Giemsa-stained smears,
117 prepared by spreading a drop of culture supernatant on a slide and air-drying, every
118 three days post-infection (DPI). Following initial isolation in IDE8 cells, trypanosome
119 cultures were maintained in two ways: co-cultivation with IDE8 cells and axenic
120 culture in complete L15B medium, both incubated in sealed 25 cm² culture flasks in a
121 bacteriological incubator at 30 °C. Renewal of the medium from cultures with IDE8
122 cells was performed weekly by removal and replacement of approximately 2/3 of the
123 medium.

124 To obtain a pure, tick cell-free trypanosome culture after four passages in IDE8
125 cultures, the isolated trypanosomes were resuspended, collected by rinsing and
126 transferred to a sterile 15 ml tube for centrifugation at 700 X g for 10 minutes. The
127 supernatant was transferred to a new sterile 15 ml tube and centrifuged at 200 X g for
128 10 minutes. Then the supernatant was discarded and the pellet was resuspended in 8
129 ml of PBS and examined by inverted microscope to rule out the presence of tick cells.
130 After further centrifugation at 200 X g for 10 minutes, the resultant pellet was
131 resuspended in 5 ml of complete L15B medium, transferred to a 25 cm² culture flask
132 and incubated at 30 °C. The culture medium was renewed weekly, with removal and
133 replacement of approximately 2/3 of the medium after mixing of the culture. Cultures
134 were monitored with an inverted phase contrast microscope and by examination of
135 Giemsa-stained smears of culture supernatant prepared as above.

136 The isolated trypanosomes were passaged by transfer of 1 ml of culture supernatant
137 (1:5 dilution) to three replicates each of both IDE8 cultures and axenic cultures in 25
138 cm² flasks. Aliquots of the axenically-cultured trypanosomes were cryopreserved with
139 10% DMSO in liquid nitrogen at -196 °C at monthly intervals following isolation. For
140 freezing, a culture was resuspended and transferred to a 15 ml sterile centrifuge tube
141 and centrifuged at 500 X g for 5 minutes. Thereafter, the supernatant was removed and
142 the pellet resuspended in 2 ml of complete L15B culture medium. An equal volume of
143 ice-cold culture medium with 20% filtered DMSO was added dropwise and gently
144 mixed. The cell suspension was divided between 4 labelled cryotubes, placed in a
145 Nalgene™ Cryo 1°C isopropanol freezing container, and held at -80 °C for at least 90
146 minutes. Subsequently the cryotubes were transferred to a liquid nitrogen storage

147 container (-196 °C). The cryopreserved trypanosomes were resuscitated **two months**
148 **after freezing** by removal from liquid nitrogen, thawed slowly in a water bath at 32 °C,
149 diluted in 5 ml complete L-15B medium and incubated at 30 °C.

150

151 *Propagation in different culture conditions*

152 **Axenic propagation** of the isolated trypanosomes was tested in the following culture
153 media: MEM and DMEM (supplemented with 2 mM L-glutamine and 10% foetal
154 bovine serum), M199 (supplemented with 10% foetal bovine serum), BHI, BHI
155 supplemented with blood agar, and Schneider's insect medium (supplemented with
156 10% foetal bovine serum). **Axenic propagation** was tested at the following incubation
157 temperatures over 15 days: 26 °C, 28 °C, 30 °C, 32 °C, 34 °C and 37 °C. **Three replicate**
158 **cultures in 25 cm² flasks were evaluated for each condition.**

159

160 *Growth profile and developmental forms in axenic culture*

161 The developmental profile of the isolated trypanosomes **in axenic culture** was
162 evaluated **at** an early passage, 4 days after subculture. Initially, the viable (**highly**
163 **motile**) trypanosomes were counted in a Neubauer chamber to prepare the inoculum
164 concentration of 1 x 10⁴ parasites/ml and were subsequently transferred **to** axenic
165 cultures in 25cm² culture flasks with complete L15B medium. The growth curve was
166 performed in triplicate at 30 °C. Aliquots of 10 µl were collected at intervals of 48 hours
167 until the 30th DPI for quantification as above and morphological analysis until the 16th
168 DPI. Developmental forms were analysed in Giemsa-stained smears by examination of
169 50-100 trypanosomes per sample, based on published descriptions of [Barros et al.](#)
170 [\(2014\)](#). No medium change was performed during this 30-day period.

171

172 *Morphometric analysis*

173 Morphometry was performed on randomly-selected stained trypanosomes **from axenic**
174 **cultures**, evaluated with a light microscope (Olympus BX45®) coupled with a photo

175 documentation system (D'Cell®software). The measurements were performed
176 according to Hoare (1972), by evaluating the total length of the parasite (from the
177 anterior end to the posterior end), the free flagellum length, nucleus diameter,
178 kinetoplast diameter, distance from the posterior end to the middle of the nucleus,
179 distance from the posterior end to the middle of the kinetoplast, distance from the
180 middle of the nucleus to the middle of the kinetoplast, distance from the **middle of** the
181 nucleus to the anterior end.

182

183 *DNA extraction and polymerase chain reaction (PCR)*

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

186 Nested-PCR was performed for amplification of a partial region of the 18S rDNA gene
187 specific to the family *Trypanosomatidae* using TRY927F (5'-
188 GAAACAAGAAACACGGGAG-3') and TRY927R (5'-CTACTGGGCAGCTTGGA-3')
189 external primers, that amplify a fragment of approximately 900 bp, and SSU561F (5'-
190 TGGGATAACAAAGGAGCA-3') and SSU561R (5'-CTGAGACTGTAACCTCAAAGC-
191 3') internal primers that amplify a fragment of approximately 700 bp, according to the
192 protocol of [Smith et al. \(2008\)](#).

193 To amplify the partially-conserved sequence of the largest ribosomal subunit gene
194 (24Sα rDNA) of members of the family *Trypanosomatidae*, PCR was performed using
195 D75 (5'-GCAGATCTTGTTGGCGTAG-3') and D76 (5'-GGTTCTCTGTGCCCCCTTTT-
196 3') primers that amplify a fragment of approximately 270 bp, according to [Souto et al.](#)
197 [\(1999\)](#).

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

201

202 *Sequencing and phylogenetic analysis*

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

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

210

211

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213

214 RESULTS

215 *Trypanosome isolation and culture*

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

226 The trypanosome culture remained viable over 24 passages in axenic culture in L15B
227 medium, and was co-cultured with IDE8 cells through ten passages, both at 30 °C. Co-
228 cultivation of the trypanosomes with IDE8 cells resulted in detachment and subsequent
229 death of the tick cells, commencing at 7 DPI. Trypanosomes cryopreserved at the third
230 axenic passage were viable when resuscitated after 60 days of storage in liquid

231 nitrogen. Figure 1A shows the growth curve obtained in axenic culture in L15B at 30 °C
232 over a 30-day period. The growth curve was initiated with an inoculum (day 0) of $1 \times$
233 10^4 parasites/ml that comprised 87% typical epimastigote forms with elongated bodies,
234 well-tapered posterior and anterior ends, 12% trypomastigotes with an average body
235 length smaller than the epimastigote form and kinetoplast posterior to the nucleus and
236 1% spheromastigote forms (Fig. 1B). Epimastigote forms predominated during the first
237 fortnight, with a fall to below 50% and a concurrent increase in spheromastigote forms
238 seen on the 16th DPI. Peak growth was seen on the 16th DPI with 910×10^4 parasites/ml.
239 By the 30th DPI, degenerating and dead forms predominated (data not shown).

240

241 *Morphometric analysis*

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

246 The epimastigote form showed the greatest average body length (41.72 μm) and the
247 spheromastigote form had the lowest mean total body length (19.44 μm). The mean
248 length of the free flagellum was similar between the epimastigote and trypomastigote
249 forms, with the spheromastigote form having the largest free flagellum length (11.61
250 μm). The diameters of the nucleus and kinetoplast of the epimastigote, trypomastigote
251 and spheromastigote forms were not significantly different. The mean distance
252 between the posterior end and middle of the nucleus was similar between the
253 epimastigote and trypomastigote forms, but much shorter in the spheromastigote form
254 (3.32 μm). The mean distance from the middle of the nucleus to the middle of the
255 kinetoplast was similar in the epimastigote and spheromastigote forms. The
256 epimastigote form presented the greatest mean values for the distance from the
257 posterior extremity to the middle of the kinetoplast (15.47 μm) and the distance from
258 the anterior end to the middle of the nucleus (15.61 μm). The diameter of the nucleus
259 was smallest in the spheromastigote form (1.71 μm).

260

261 *Molecular analysis*

262 In the nested-PCR carried out on axenically-cultured trypanosomes, the 18S rDNA
263 PCR fragment was approximately 900 bp in the first round of amplification. In the
264 second round the size of the amplified fragment was approximately 700 bp. The partial
265 sequence of the 18S rDNA gene showed 89% similarity with *T. rhipicephalis* (accession
266 number KX711901) matching 99% of the query sequence, and 88% similarity with
267 *Trypanosoma* KG1 (accession number AB281091) matching 99% of the query sequence.
268 It also presented 90% similarity (with e-value 2 e-133) and 60% alignment with
269 *Trypanosoma caninum* (accession number JF951431, JF9075537). In the 24S α rDNA PCR
270 reaction using primers D75 and D76 the size of the amplified fragment was 270 bp for
271 the isolate. For the 24S α rDNA gene there was 96% similarity with *Trypanosoma rangeli*
272 (query coverage 63% GenBank KJ742907), *Trypanosoma grosi* AKHA (query coverage
273 65% GenBank AB175624). The phylogenetic analysis targeting the 18S rDNA gene
274 (sequence conserved within the family *Trypanosomatidae*) confirmed that the
275 trypanosome isolated from *A. brasiliense* belongs to the family *Trypanosomatidae*. The
276 phylogenetic tree showed that the species is within the same clade as *T. rhipicephalis*
277 and in a clade close to *T. caninum* and *Trypanosoma* KG1 (Fig. 3). The analysis of the 18S
278 rDNA region confirmed the authenticity of this new species. Molecular analysis
279 showed that our trypanosome isolate, strain C1RJ, was clearly separated from other
280 species of the genus *Trypanosoma*, regardless of the molecular target used, with
281 bootstrap values of 85 for the tree built using the target 18S rDNA sequence (Fig. 3).
282 The nucleotide sequences described were deposited in GenBank under the access
283 number KX711902.

284

285 DISCUSSION

286 Here we describe the first isolation, molecular characterisation, morphological and
287 biological analyses of a member of the genus *Trypanosoma* infecting ticks of the species

288 *A. brasiliense* parasitising a specimen of *Tayassu pecari*, from the municipality of Itatiaia,
289 RJ, Brazil. The new species was named *Trypanosoma amblyommi* sp. nov.

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

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

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

305 Some species of the genus *Trypanosoma* multiply intracellularly in the vertebrate host in
306 the amastigote form e.g. *T. cruzi*, in blood in the trypomastigote form e.g. *Trypanosoma*
307 *brucei* or in the epimastigote form e.g. species of the subgenus *Megatrypanum* ([Hoare,](#)
308 [1972](#)). In the present study, *in vitro* replication of *T. amblyommi* was in the epimastigote
309 form.

310 An interesting observation was the isolation using the tick cell line IDE8 and the
311 inability of *T. amblyommi* to grow in conventional trypanosome culture media and at
312 conventional incubation temperatures. *T. amblyommi* was propagated in L15B medium,
313 which is used for culturing tick cells (Munderloh and Kurtti., 1989) and only at
314 temperatures between 30 and 34 °C. Similar behaviour was observed for the recently-
315 described *T. rhipicephalis*, also isolated into IDE8 cell culture from ticks in the state of
316 Rio de Janeiro (Marotta et al., 2018). In the present study, although no adverse effect of

317 the initial crushed tick suspension on the IDE8 cells was detected, subsequent co-
318 cultivation of *T. amblyommi* with tick cells resulted in detachment and death of the
319 latter. Further studies are required to establish whether the tick cell death resulted
320 simply from competition with the faster-growing trypanosomes for nutrients in the
321 culture medium, from release of toxic substances by the trypanosomes, or because
322 trypanosomes were internalised by the tick cells and somehow compromised the
323 latters' viability. This phenomenon was not reported to occur with *T. rhipicephalis* in
324 IDE8 cells (Marotta et al., 2018).

325 The morphometric evaluation revealed wide morphological diversity. *T. amblyommi*
326 presented large dimensions, as seen by the total length of the trypomastigote form with
327 a mean of 35.81 μm , being larger than *Trypanosoma vivax* (Ramírez et al., 1997), *T. cruzi*
328 (Madeira et al., 2009), *Trypanosoma evansi* (Elshafie et al., 2013) and *T. rangeli* (Madeira
329 et al., 2009), but smaller than *Trypanosoma theileri* (Wink, 1979) and *T. caninum* (Madeira
330 et al., 2009). Wide variation was also observed in other measurements, such as the
331 distance between the posterior end and the kinetoplast, the distance between the
332 nucleus and anterior end and the posterior end to the nucleus.

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

339 Our results indicate that *T. amblyommi* is a new species of the genus *Trypanosoma*.
340 However, aspects related to ultrastructure, pathogenicity, involvement with vertebrate
341 hosts, epidemiology, cycle, transmission mechanisms, classification and taxonomy are
342 still unknown. Further studies are needed to determine these aspects of the biological
343 cycle of the newly-identified *T. amblyommi*. Isolation in the present study of *T.*
344 *amblyommi* and, using similar techniques, of *T. rhipicephalis* (Marotta et al., 2018) from
345 small samples of two unrelated tick species removed from very different hosts (peccary

346 and cattle) suggests that ticks in Brazil may frequently harbour trypanosomes. Further
347 studies are likely to reveal even more novel species of this haemoparasite.

348

349 DESCRIPTION

350 **Name:** *Trypanosoma amblyommi* sp. nov.

351

352 **Mammalian host:** Unknown.

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

354 **Vector:** Possibly the tick *Amblyomma brasiliense*.

355

356 **Biology and morphology:** This species was isolated in co-cultivation with IDE8 tick
357 cells and grown in L15B medium supplemented with foetal calf serum (FCS). The
358 developmental stages found in axenic cultures were epimastigotes (predominantly),
359 trypomastigotes and spheromastigotes. The mean total body length in the epimastigote
360 form was 41.72 μm , free flagellum 10.74 μm and longitudinal axis of the kinetoplast
361 1.23 μm . Measurement of total body length in the trypomastigote form was on average
362 35.81 μm , free flagellum 10.76 μm and longitudinal axis of the kinetoplast 1.09 μm . The
363 mean total body length in the spheromastigote form was 19.44 μm , free flagellum 11.61
364 μm and longitudinal axis of the kinetoplast was 1.05 μm .

365

366 **Molecular characteristics:** The trypanosome presents amplified products for the 24S α
367 rDNA gene of about 270 bp using D75/D76 primers. In the first nested-PCR reaction
368 for the 18S rDNA gene using the primers TRY927F and TRY927R, the amplified
369 fragment was 900 bp. In the second reaction, using the SSU561F and SSU561R primers,
370 the amplified fragment was 700 bp. In the phylogenetic analysis of ribosomal genes,
371 this trypanosome is close to *Trypanosoma* sp. KG1 and *T. caninum*.

372

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

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380

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385

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393

394 CONFLICT OF INTEREST

395 None

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464 FIGURE AND TABLE LEGENDS.

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466

467 **Fig. 1.** Growth of *Trypanosoma amblyommi* sp. nov. in axenic culture in complete L-15B
468 medium. **A.** Growth curve determined by counting total numbers of trypanosomes at
469 2-day intervals over 30 days; mean of three replicate cultures. **B.** Proportions of
470 different developmental forms determined by examination of Giemsa-stained smears
471 prepared at 2-day intervals over 16 days.

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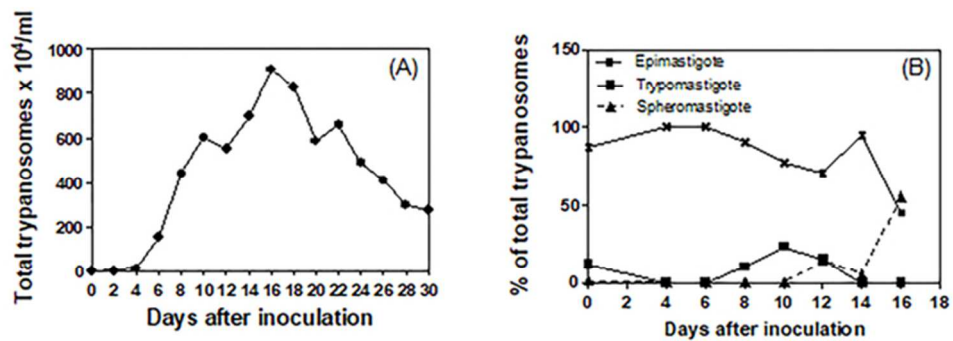
473 **Fig. 2.** Photomicrographs showing morphological diversity of *Trypanosoma amblyommi*
474 sp. nov. in axenic culture in complete L-15B medium at 30 °C. A. spheromastigote; D,
475 F. dividing form; E. epimastigote; B, G, C. forms in transition to trypomastigote
476 Giemsa-stained smears; scale bar = 20 µm.

477

478 **Fig. 3.** Phylogenetic analysis of *Trypanosoma amblyommi* sp. nov. and other
479 trypanosome species. Phylogenetic tree based on 18S rDNA sequences analysis.
480 Statistical method Maximum Likelihood - Kimura 2-parameter model. Bootstrap: 1000.

481

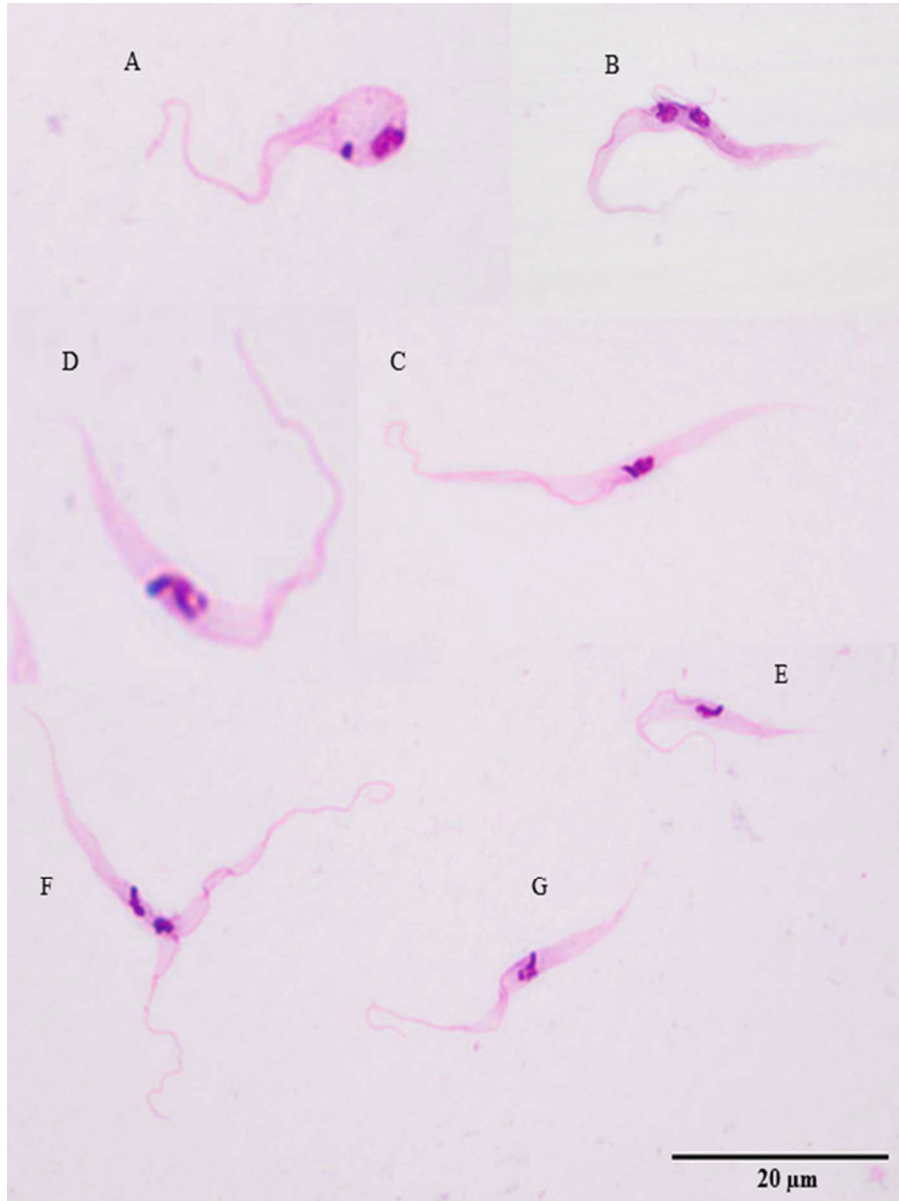
482 **Table 1.** Morphometric data (µm) obtained from trypomastigote, epimastigote and
483 spheromastigote developmental forms of *Trypanosoma amblyommi* sp. nov.



Growth of *Trypanosoma amblyommi* sp. nov. in axenic culture in complete L-15B medium.

28x10mm (600 x 600 DPI)

ur Peer Review



Photomicrographs showing morphological diversity of *Trypanosoma amblyommi* sp. nov. in axenic culture in complete L-15B medium at 30 °C.

190x254mm (96 x 96 DPI)

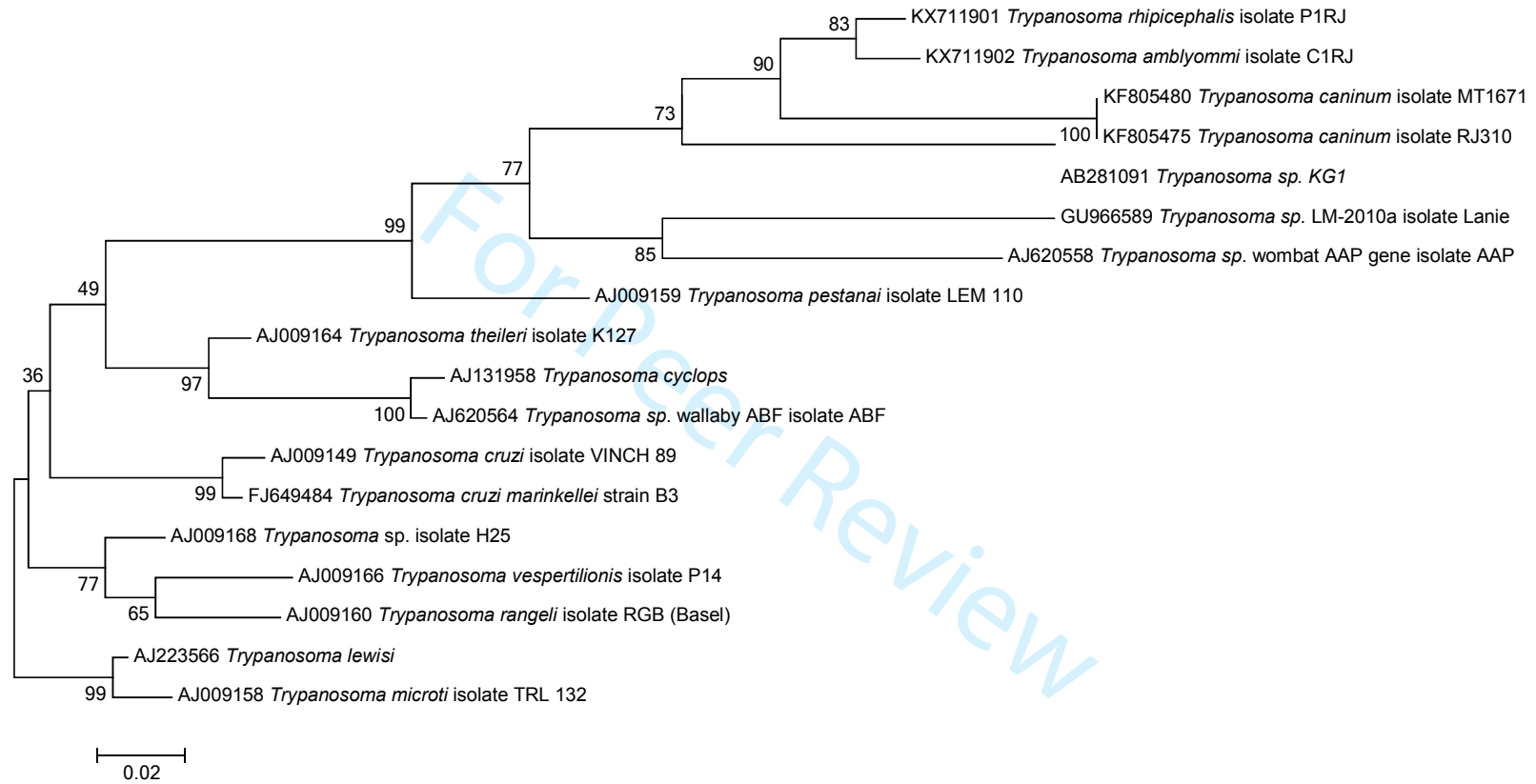


Table 1. Morphometric data (μm) obtained from trypomastigote, epimastigote and spheromastigote developmental forms of *Trypanosoma amblyommi* sp. nov.

	TL	PK	KN	NA	FF	PN	NL	K
Trypomastigote	35.81 \pm 7.60 (56.16-25.42)	12.30 \pm 3.56 (21.82-7.17)	1.39 \pm 0.47 (3.92-0.94)	11.06 \pm 4.10 (26.03-5.68)	10.76 \pm 2.92 (19.34-4.06)	13.28 \pm 3.61 (22.52-8.19)	1.75 \pm 0.40 (3.33-1.21)	1.09 \pm 0.25 (1.86-0.64)
Epimastigote	41.72 \pm 8.85 (60.87-24.20)	15.47 \pm 4.26 (27.74-7.38)	1.18 \pm 0.38 (2.08-0.67)	15.61 \pm 5.12 (29.18-8.06)	10.74 \pm 2.90 (17.59-5.81)	14.59 \pm 4.09 (26.99-8.03)	1.84 \pm 0.37 (2.50-1.11)	1.23 \pm 0.39 (2.37-0.52)
Spheromastigote	19.44 \pm 5.92 (36.82-7.98)	3.26 \pm 1.48 (6.40-1.01)	1.45 \pm 0.08 (3.89-0.28)	5.55 \pm 4.61 (15.83-0.23)	11.61 \pm 4.88 (22.18-3.31)	3.32 \pm 2.18 (13.80-0.12)	1.71 \pm 0.49 (2.82-0.73)	1.05 \pm 0.31 (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 \pm Standard deviation (minimum - maximum value).