

Peculiarities of *Trypanosoma rangeli* KP1(–) Strains Isolated from the Wild Rodent *Phyllomys dasythrix* (Santa Catarina, Brazil): Comparisons with *T. rangeli* KP1(+) strains and *Trypanosoma lewisi* (Kinetoplastea, Trypanosomatidae)

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Abstract. Two KP1(–) strains of *Trypanosoma rangeli* (SC-58, SC-61) isolated from the wild rodent *Phyllomys dasythrix* from Santa Catarina (Brazil) were compared with some KP1(+) reference stocks from different Latin America countries, and also with *Trypanosoma lewisi*. The strains were analyzed by some morphological and biological features, and by biochemical and molecular techniques. The mean total length (TL) of the bloodstream trypomastigotes of *T. rangeli* varied between 31.3–33.0 μm , and those of *T. lewisi* (adult forms) was 28.2 μm , values within the variation known for each species. In *T. rangeli* KP1(+) and *T. lewisi*, the nucleus was located in the anterior portion of the body, with nuclear indexes (NI) ≥ 1.2 , as typically described for both species. Differently, most trypomastigotes of the KP1(–) stocks presented NI ≤ 1.0 . Another striking feature of the KP1(–) strains was their very fastidiously growth in axenic cultures when compared with the KP1(+) stocks and *T. lewisi*. Three isoenzyme loci (MDH, IDH and PGM) clearly distinguished *T. rangeli* and *T. lewisi*, and the distinction between the KP1(+) and KP1(–) strains was possible at MDH, PGM and GPI loci. All *T. rangeli* strains presented the typical 760 bp amplicon derived from their KP2 minicircles. However, the KP3 products of the KP1(+) strains were a single large band (~330bp), whereas those of the KP1(–) had two distinct bands (350 and 300 bp). *T. lewisi* presented 700 and 400 bp amplicons, as previously reported. The peculiarities of *T. rangeli* isolates from *P. dasythrix* corroborate a possible speciation process within this taxon.

Key words: KP1(+) and KP1(–) *Trypanosoma rangeli* strains, *Trypanosoma lewisi*, morphology, biology, isoenzymes, PCR products

INTRODUCTION

Trypanosoma rangeli Tejera, 1920 is a parasite of human beings, domestic and sylvatic mammals, widely distributed in the Latin America, being trans-

mitted by triatomine bugs mainly of the genus *Rhodnius*. This species is harmless to their vertebrate hosts, but can be pathogenic for its insect vectors (Hoare 1972, D'Alessandro-Bacigalupo and Saravia 1992). *Trypanosoma lewisi* (Kent, 1880) is a non-pathogenic parasite of rats (mainly *Rattus* spp.) transmitted by fleas, being found throughout the world where these mammals occur. *T. lewisi* is the type-species of the subgenus *Herpetosoma*, which includes several species with a high degree of host-restriction, differently from *T. rangeli*

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(Hoare 1972, Molyneux 1976). The most common hosts of the *Herpetosoma* species are rodents and some lagomorphs, but *T. lewisi* (or *T. lewisi*-like) has been also reported in primates, including human beings, sometimes as opportunist infections (Johnson 1933, Maia-da-Silva *et al.* 2010, Sousa 2014). There is close resemblance between the bloodstream trypomastigotes of *T. rangeli* and the “adult” forms of *T. lewisi*, besides between their stages in axenic cultures (Hoare 1972; D’Alessandro 1976; Sousa 1999, 2014). Some phylogenetic studies validated only the inclusions of *T. lewisi* in the *Herpetosoma* subgenus (Maia-da-Silva *et al.* 2010) and placed *T. rangeli* within the *T. cruzi* clade (e.g., Hamilton *et al.* 2007) despite the lack of biological and morphological affinities between these species (Sousa 1999).

In 1991, Steindel *et al.* isolated a very peculiar *T. rangeli* strain (SC-58) from the wild rodent *Phyllomys dasythrix* (Santa Catarina Island, southern Brazil). It presented morphology and biological behavior typical of this species, but had uncommon isoenzyme patterns. Subsequently, Macedo *et al.* (1993) compared this strain together with another of the same origin (SC-61) with seven *T. rangeli* isolates from the Central and north part of South Americas by their DNA fingerprints and molecular hybridization patterns with specific nDNA and kDNA probes. A phenetic analysis of these results, as well as from isoenzyme and RAPD analyses (Steindel *et al.* 1994), evidenced two main distinct groups in *T. rangeli*, one of them including only the isolates SC-58 and SC-61. Macedo *et al.* (1993) also discovered the absence of KP1 minicircles in the SC-58 strain, a finding subsequently confirmed by Vallejo *et al.* (2002) in other strains from Santa Catarina, besides from other countries (Colombia and Panama). The stocks of *T. rangeli* were subsequently denoted as KP1(+) and KP1(−) according to the presence or absence of this minicircle class (Vallejo *et al.* 2002). Studies on the PCR products from the spliced-leader intergenic region (SL-IR) confirmed the dichotomy in *T. rangeli* and expanded the knowledge on their geographic distribution in the Latin America (Grisard *et al.* 1999a; Vallejo *et al.* 2003, 2015; Urrea *et al.* 2005, 2011; Salazar-Antón *et al.* 2009). Several authors reported associations between the two main *T. rangeli* groups and their regional vectors, besides their development pattern within these insects (Machado *et al.* 2001; Vallejo *et al.* 2003, 2015; Urrea *et al.* 2005, 2011; Salazar-Antón *et al.* 2009). Other authors, proposed the subdivision of *T. rangeli* into five lineages (A–E), and the SC-58 was classified in the lineage D (Maia-da-Silva *et al.* 2007, 2009) based on SL-IR and ribosomal gene

sequencing. Some studies have associate the KP1(+) strains with the lineage A, and KP1(−) strains with C and D (Ocaña-Mayorga *et al.* 2015, Vallejo *et al.* 2015), but correlations between these genotypes are still lacking for several *T. rangeli* isolates.

In the recent decades, the literature on biochemical and molecular characterization of *T. rangeli* has greatly increased, this being accompanied by a drastic reduction of morphological and biological studies. Consequently, there are still gaps in our knowledge on *T. rangeli*, as some aspects of its life-cycle in vectors and vertebrate hosts, as well as in axenic cultures (Hoare 1972, Añez 1983, D’Alessandro-Bacigalupo and Saravia 1992, Grisard *et al.* 1999b, Sousa *et al.* 2008). However, nowadays it is possible to obtain new and consistent conclusions on living organisms by combining the above-mentioned approaches. In the present work, we compared some morphological and biological features of both KP1(−) strains isolated from *P. dasythrix* with those from some KP1(+) reference stocks of *T. rangeli* from several Latin American countries, besides *T. lewisi*. Classical biochemical and molecular analyses were also performed to confirm their genetic differences.

MATERIALS AND METHODS

Parasite strains

All trypanosome stocks analyzed in the present work proceeded from the Trypanosomatid Collection at Oswaldo Cruz Institute, and are identified by their code-numbers (in parentheses). The KP1(−) strains of *T. rangeli* were: SC-58 (CT-IOC 270) and SC-61 (CT-IOC 272/349). The KP1(+) strains were: Macias (CT-IOC 273), Choachi (CT-IOC 271), H14 (CT-IOC 038), San Agustín (CT-IOC 282) and R1625 (CT-IOC 002). Most of these strains had been also classified in different lineages based on SL-IR and ribosomal gene sequencing (Maia-da-Silva *et al.* 2007), as follows: A (Macias, Choachi, San Agustín), C (R1625) and D (SC-58). Data on the origin of these isolates were described by Steindel *et al.* (1994) and on their identification as KP1(+) and KP1(−) are in Sincero *et al.* (2015).

The stock of *T. lewisi* used throughout this study was isolated by M. P. Deane (Deane strain) (Sousa 2014), and deposited in the Trypanosomatid Collection with two code-numbers: CT-IOC 009 (blood forms, experimentally infected rats) and CT-IOC 095 (axenic cultures).

Morphological and biometrical studies

These studies were performed on Giemsa-stained smears of bloodstream trypomastigotes of four *T. rangeli* strains (SC-58, SC-61, Choachi and Macias) and *T. lewisi* (only adult forms), as seen under optical microscopy (×1,000). Firstly, these stages were drawn with the aid of a camera lucida coupled to a microscope. Measurements (in centimeters) were taken directly on these drawings, and

these values transformed into micrometers by a multiplication factor deduced from the size of a scale bar (cm) corresponding to 10 μm , which was included in all drawing plates (Sousa *et al.* 2008). The blood smears with trypomastigotes of *T. rangeli* were provided by M. Steindel (Federal University of Santa Catarina), proceeding from experimentally infected mice. Those of *T. lewisi* were donated by N. Thomas (IOC, Fiocruz), proceeding from experimentally infected rats.

In the present study, the biometrical parameters chosen for comparisons were: total length (TL) of the parasite body (free flagellum included) and the nuclear index (NI) (Dias and Freitas 1943, Hoare 1972). Measurements of trypomastigotes were taken from the parasite posterior end (P) to the middle of the nucleus (PN), from the middle of nucleus to the anterior end (NA), and the free flagellum length (F) (see Fig. 1, SC-61). The parasite total length (TL) was determined by $\Sigma = \text{PN} + \text{NA} + \text{F}$. The nuclear index ($\text{IN} = \text{PN}/\text{NA}$) indicates the position of the nucleus in the trypanosome body, as follows: $\text{NI} = 1$, central nucleus; $\text{NI} < 1$, nucleus nearest the parasite posterior end; $\text{NI} > 1$, nucleus nearest the anterior end.

Parasite growth in axenic cultures

All comparative studies on the growth of *T. rangeli* and *T. lewisi* strains in axenic cultures were carried out using liver infusion-tryptose both (LIT) supplemented with 20% fetal calf serum (LIT-20) from the same bath. Cultures were begun by seeding 2.0×10^6 cells of each strain into 16 \times 150 mm screw-cap tubes containing 4 mL of medium and kept at 27.3°C ($\pm 0.4^\circ\text{C}$). Before seeding, each stock had been twice passed (weekly intervals) in a biphasic medium (N.N.N. overlaid with LIT-20), and once in LIT-20. The cultures under study were examined on the 4th, 7th, 10th, 13th, 17th and 20th days for determining the number of cells/mL using Neubauer hemocytometers. The arithmetic mean and maximum growth from each trypanosome stock were used to compare the isolates under study.

Biochemical and molecular analyses

For these analyzes, parasite cells were grown in LIT-20, harvest by centrifugation (1,500 g, 15 min, 4°C), washed twice in saline-EDTA (0.1 M, pH 8.0), and the pellets stored in nitrogen liquid until use.

The trypanosomes under study were compared by multilocus enzyme electrophoresis (MLEE) on agarose gels according to standard procedures, as described elsewhere. The stocks were tested for activity of five enzymatic loci: malate dehydrogenase (E.C.1.1.1.37; MDH), isocitrate dehydrogenase (E.C.1.1.1.42; IDH), phosphoglucosmutase (E.C.2.7.5.1; PGM), glucose phosphate isomerase (E.C.5.3.1.9; GPI) and malic enzyme (E.C.1.1.1.40; ME).

For polymerase chain reaction (PCR), the DNA from each species was extracted by the DNAzol method, as described elsewhere. The amplifications were performed using the primers 121 (5'-AAA TAA TGT ACG GG(T/G) GAG ATG CAT GA-3') and 122 (5'-GGT TCG ATT GGG GTT GGT GTA ATA TA-3'), which were described as *T. cruzi*-specific, but can also amplify kDNA minicircle sequences from other trypanosome species (Sousa *et al.* 2008, 2014). The PCR products from each isolate (15–18 μl) were loaded onto the slots of 1.6% agarose gels, and electrophoresed at 70–73 V, for 3 h. Molecular size marker (100 bp DNA ladder) was included into the gels. After ethidium bromide staining, the gels were examined and photographed under ultraviolet light.

RESULTS AND DISCUSSION

Morphological and biometrical studies

The results of the biometry of the bloodstream trypomastigotes from KP1(-) and KP1(+) *T. rangeli* stocks, besides the “adult” forms *T. lewisi*, are presented in Table 1. All *T. rangeli* isolates studied herein presented TL (mean and ranges) within the variation reported by several authors for this species and the so-called *T. saimiri* (Pifano 1949, Groot *et al.* 1951, Deane and Damasceno 1961, Hoare 1972, D’Alessandro 1976, Miles *et al.* 1983, Ziccardi and Oliveira 1998). Likewise, the TL values of *T. lewisi* were also within the variation reported by Hoare (1972). Table 2 presents these findings. However, the TL values reported by Suárez *et al.* (2008) for some KP1(-) strains of *T. rangeli* strains from *Rhodnius pallescens* and *R. colombiensis* were not in accordance with our results and those from different authors (Table 2), suggesting either problems in the scale calibration used in the measurements, or that these stocks could belong to another *T. rangeli*-like species, as summarized by Hoare (1972).

The close resemblance between the bloodstream trypomastigotes of *T. rangeli* and the adult forms of *T. lewisi* has been largely confirmed in several publications, and among their peculiarities, the most remarkable one is the nucleus position at the anterior portion of the parasite body ($\text{NI} \geq 1.1$) (Pifano 1949, Groot *et al.* 1951, Hoare 1972, D’Alessandro 1976, Molyneux 1976, Miles *et al.* 1983, Guhl and Vallejo 2003, Maia-da-Silva *et al.* 2010, Sousa 2014). In the present study, this feature was confirmed in the blood trypomastigotes of the KP1(+) strains of *T. rangeli* and in *T. lewisi*. However, most bloodstream forms of the KP1(-) strains (SC-58 and SC-61) presented $\text{NI} \leq 1.0$, since their nucleus was mainly central or placed in the posterior portion of the body (Table 1; Fig. 1). Interestingly, the finding of *T. rangeli* and *T. rangeli*-like stocks displaying bloodstream trypomastigotes with $\text{NI} \leq 1.0$ had been only reported in isolates of wild vertebrates and triatomines from the Brazilian Amazon, including *T. saimiri*, which is considered a junior-synonym of *T. rangeli* (Ziccardi and Oliveira 1998) (Table 2). At present, it is unknown if *T. saimiri* and other *T. rangeli*-like stocks from this region present KP1 minicircles, but several isolates from there were classified in the lineage B according to SL-IR and RNA gene sequencing (Maia-da-Silva *et al.* 2007, Vallejo *et al.* 2015). Recently, Sincero *et al.* (2015) proposed that they belong

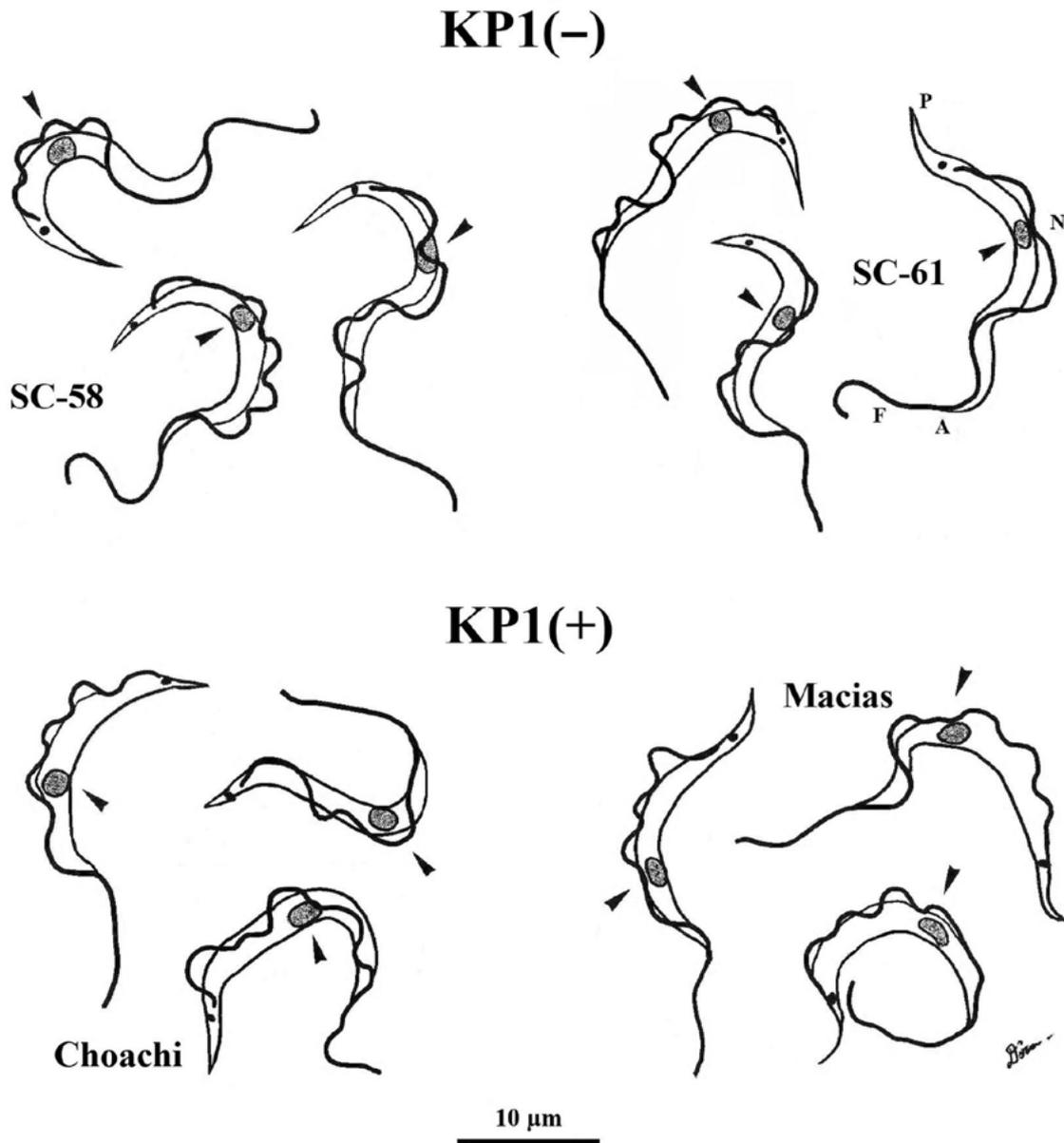


Fig. 1. Camera lucida drawings of representative bloodstream trypomastigotes of *Trypanosoma rangeli* KP1(-) and KP1(+) strains from experimentally infected mice by metacyclic trypomastigotes grown in DMEM medium. The position of the nucleus in each trypomastigote is indicated by arrowheads. Parasites were from Giemsa-stained smears of each strain, as seen under optical microscopy ($\times 1,000$). In a trypomastigote of the strain SC-61 are indicated the reference points for taking measurements: anterior end (A), posterior end (P), nucleus (N) and free flagellum (F).

to an ancestral group of *T. rangeli*, apart from KP1(+) and KP1(-), then named Amazonian group.

Parasite growth in axenic cultures

Fig. 2 presents the results of the comparative study on the growth in axenic culture of KP1(-) and KP1(+) *T. rangeli* strains, besides *T. lewisi*. It is noteworthy, that the KP1(-) strains from *P. dasythrix* (SC-58 and

SC-61) were more fastidious than all the KP1(+) stocks examined, and to a lesser extent than *T. lewisi*, this indicating marked differences in their nutritional requirements. This finding suggests that the KP1(-) strains could be easily eliminated in mixed cultures with KP1(+) *T. rangeli* stocks. We did not know another study that comparatively analyzed the growth of isolates of *T. rangeli* (with or without *T. lewisi*) in axenic cultures

Table 1. Biometrical study of bloodstream trypomastigotes from Giemsa-stained smears of KP1(-) and KP1(+) *Trypanosoma rangeli* strains, and *T. lewisi* (adult forms). Biometrical parameters: TL (total length, flagellum included), and NI (nuclear index) according to Dias and Freitas (1943). Means, standard deviations and ranges (in parenthesis) are in μm ($n = 30$, unless indicated). Percent of forms with $\text{NI} \leq 1.0$ are also presented. The trypomastigotes of *T. rangeli* and *T. lewisi* were from experimentally infected mice and rats, respectively.

Parameters	<i>Trypanosoma rangeli</i>				<i>T. lewisi</i> *
	KP1(-)		KP1(+)		
	SC-58	SC-61	Choachi	Macias	
TL (μm)	33.0 \pm 4.2 (24.8–38.8)	31.3 \pm 4.1 (24.0–38.3)	31.8 \pm 2.3 (27.0–35.2)	33.1 \pm 2.1 (26.5–36.8)	28.2 \pm 4.2 (19.8–35.0)
PN (μm)	12.1 \pm 1.5 (8.7–14.6)	11.7 \pm 1.7 (8.3–16.1)	14.1 \pm 1.3 (11.7–17.4)	15.1 \pm 1.6 (12.6–18.7)	13.2 \pm 2.4 (9.1–18.1)
NA (μm)	12.1 \pm 2.6 (6.1–17.0)	10.9 \pm 2.3 (6.5–15.2)	8.5 \pm 1.2 (7.0–10.4)	7.9 \pm 1.5 (3.5–10.2)	6.4 \pm 2.0 (1.7–8.9)
NI (PN/NA)	1.0 \pm 0.3 (0.6–1.9)	1.1 \pm 0.3 (0.7–2.0)	1.7 \pm 0.3 (1.2–2.2)	2.0 \pm 0.6 (1.4–4.7)	2.5 \pm 1.8 (1.3–8.5)
NI \leq 1.0 (%)	60.0	56.6	0.0	0.0	0.0

NI indicates the position of the nucleus in the trypanosome body, as follows. NI = 1, central nucleus. NI < 1, nucleus nearest the posterior end. NI > 1, nucleus nearest the anterior end. The anterior end is that where the flagellum emerges. PN: distance from the parasite posterior end to the middle of the nucleus). NA: distance from the middle of nucleus to the parasite anterior end.
(*) n = 15.

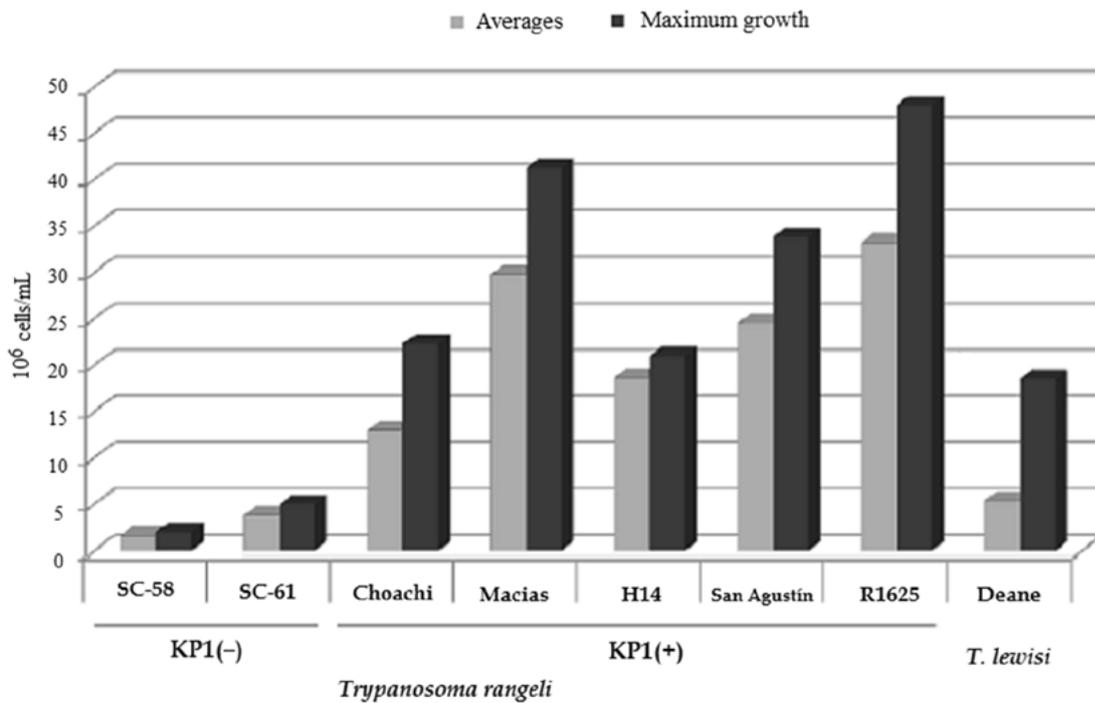


Fig. 2. Comparative growth in axenic cultures of *Trypanosoma rangeli* strains [KP1(-) and KP1(+)] and *T. lewisi*. Data (10^6 cells/ μL) are the averages and the maximum growth from the values taken at the 4th, 7th, 10th, 13th, 17th and 20th days of cultivation in liver infusion-tryptose broth supplemented with 20% fetal calf serum (LIT-20) at $27.3 \pm 0.4^\circ\text{C}$.

Table 2. Total length (TL, flagellum included) and nuclear index (NI) of bloodstream trypomastigotes of *Trypanosoma rangeli*, *T. saimiri**, and *T. lewisi* according to data from several authors. The averages and the ranges (in parentheses) are in μm , excepting NI.

Ref.	<i>Trypanosoma rangeli</i>				<i>T. saimiri</i> *		<i>T. lewisi</i>		
	Piñano 1949	Groot <i>et al.</i> 1951	Hoare 1972	D'Alessandro 1976	Miles <i>et al.</i> 1983	Ziccardi and Oliveira 1998	Deane and Damasceno 1961	Ziccardi and Oliveira 1998	Hoare 1972
TL	-(a) (30.5-38.0)	30.0-31.2 (b) (25.0-37.0)	27.0-32.2 (§) (25.0-37.0)	26.4-33.8 (§) (25.0-37.0)	32.1 (c) (28.0-36.0)	30.9 (f) (29.0-36.0)	30.9** (g) (25.2-36.4)	31.4 (h) (24.0-40.0)	30.6 (§) (21.0-36.5)
NI	-	1.6-1.7 (1.1-2.8)	1.6-2.0 (1.1-2.8)	1.6-2.0 (1.1-2.2)	1.6 (1.1-2.2)	1.4 (0.8-2.0)	1.4 (0.8-1.8)	1.5 (0.7-2.2)	1.5 (0.7-2.2)

NI indicates the nucleus position in the trypomastigote body, as follows. NI = 1, central nucleus. NI < 1, nucleus nearest the posterior end. NI > 1, nucleus nearest the anterior end. The anterior end is that where the flagellum emerges.

(*) *T. saimiri* is considered a junior synonym of *T. rangeli* (Ziccardi and Oliveira (1998)). (**) data are the sum of the averages of the body length and the free flagellum. (***) nucleus at the anterior portion of the parasite body; NI values were not specified. (§) values summarized from data provided by several authors.

Origin of the blood trypomastigotes: (a) dog natural infection. (b) human natural infection and mouse experimental infection. (c) mouse experimental infection; human original host, reference strain R1625. (d) mouse experimental infection; originally from *Didelphis marsupialis*. (e) mouse experimental infections; originally from *Rhodnius robustus* (data from 3 isolates). (f) *Callithrix penicillata* and *Saimiri* sp. natural infections. (g) *Saimiri sciureus sciureus* natural infection. (h) *Saimiri* sp. natural infection.

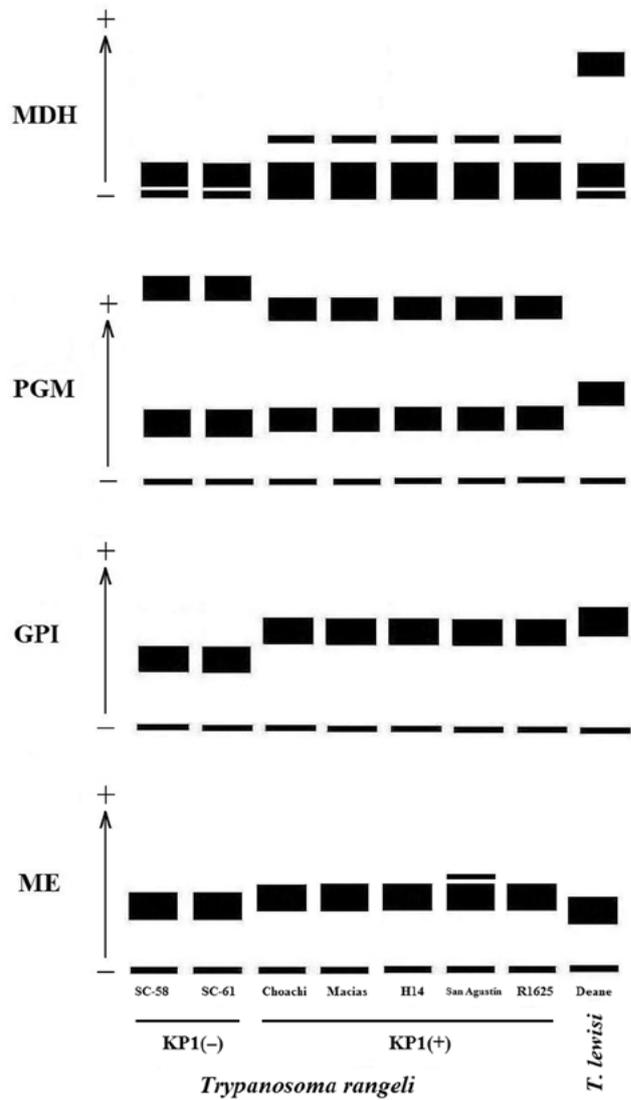


Fig. 3. Diagrammatic representation of the electrophoretic patterns of malate dehydrogenase (MDH), phosphoglucosmutase (PGM), glucose phosphate isomerase (GPI) and malic enzyme (ME) displayed by KP1(-) and KP1(+) *Trypanosoma rangeli* strains, and *T. lewisi*.

under standardized conditions, as used herein. However, it would be of interest to study other *T. rangeli* lineages to better understand the correlations between their phenotypes and genotypes.

Biochemical and molecular analyses

The KP1(+) and KP1(-) strains of *T. rangeli* analyzed herein were easily distinguished by the electrophoretic mobility of their MDH, PGM and GPI isoenzymes. The present study also showed that both KP1(+) and KP1(-) stocks can be distinguished from

played a single large band (~330 bp), while two distinct bands (~350 bp, and another with 300 bp) were found the KP1(-) stocks (SC-58 and SC-61). These two amplicons were also seen in KP1(-) samples from *R. ecuadoriensis* analyzed by Ocaña-Mayorga *et al.* (2015; Fig. 1A). It is interesting to note that *T. rangeli* stocks from *Rhodnius* spp. (Pallescens lineage) were identified in the KP1(-) group and lineage C, an association that has been proposed (Vallejo *et al.* 2015). However, the reference strain R1625 (human origin) was classified in the lineage C (Maia-da-Silva *et al.* 2007), although being KP1(+), as reported by Sincero *et al.* (2015) and confirmed by our results. Accordingly, it is necessary to identify (or review) the KP1 type of several *T. rangeli* strains, mainly those previously classified in the lineages B, C and E (Maia-da-Silva *et al.* 2007, 2009), in order to better establish the correlations between the groups obtained with distinct genotypic markers. Otherwise, as previously reported by Sousa (2014), the primers 121/122 generated two different amplicons for *T. lewisi* (400 and 700 bp), enabling its clear distinction from *T. rangeli*, besides *T. cruzi*.

CONCLUSIONS

As far we know, KP1(+) and KP1(-) *T. rangeli* strains have not yet been compared by their morphological and biological features, and the present paper evidenced other peculiarities of the SC-58 and SC-61 isolates from *P. dasythrix* from Santa Catarina Island. Recent molecular studies (Sincero *et al.* 2015) point to a subdivision within the KP1(-) group, which corroborate the uniqueness of these isolates, besides others from wild *Panstrongylus megistus* from the same region (Vallejo *et al.* 2002, 2015). This may reflect a process of speciation within *T. rangeli*, as advanced by some authors (Macedo *et al.* 1993, Guhl and Vallejo 2003, Vallejo *et al.* 2003), closely associated to the vector lineages (Machado *et al.* 2001; Vallejo *et al.* 2002, 2015; Urrea *et al.* 2005, 2011; Maia-da-Silva *et al.* 2007; Salazar-Antón *et al.* 2009; Barreto-Santana *et al.* 2015; Sincero *et al.* 2015). At present, with so much available knowledge on *T. rangeli* genetic diversity, we question whether the KP1(-) group from southern Brazil could be a subspecies. Otherwise, we emphasize the importance of better knowing other wild stocks, as those from the lineages B and E (Maia-da-Silva *et al.* 2007, 2009), by their morphological and biological features as well,

either to verify whether they actually can be identified as *T. rangeli* or belong to another similar species, as those cited by Hoare (1972) and D'Alessandro-Bacigalupo and Saravia (1992). However, accounting the extensive variability of *T. rangeli* throughout the Latin America, only after more comparative studies, using different approaches and reference stocks from distinct origins, it will be possible to conclude whether there are one or more subspecies/species included in this taxon.

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