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"IN VITRO" DEVELOPMENT OF TRYPANOSOMA BUTANTANENSE ARANTES AND FONSECA, 1931

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SUMMARY

The Authors studied the "in vitro" maintenance of the flagellate **Trypanosoma butantanense** isolated from a specimen of **Waglerophis merremii** (Serpentes, Colubridae). For the passages, Eagle and L.I.T. media were enriched with (i) whole blood, (ii) plasma, or (iii) snake red blood cells. Best results were obtained with media supplemented with whole blood, red blood cells proving better than plasma. For the **in vivo** transmission, inocula of infected blood diluted in Eagle proved the most efficacious. **W. merremii**, as well as young **Crotalus durissus terrificus** showed high susceptibility to **T. butantanense**, while young **Bothrops alternatus** were less susceptible. The Authors suspect a possible biological resistance in adult **C. d. terrificus.** Transmission of **T. butantanense** both by oral route or through the leech **Haementeria gracilis** (Hirudinae) was negative.

Key words: **Trypanosoma butantanense.** Trypanosoma transmission. Trypanosome culture.

INTRODUCTION

ARANTES & FONSECA [1] had the opportunity to detect a specimen of **Waglerophis merremii** (Wagler, 1824) **(Xenodon merremii)** [13], commonly known as "boipeva", strongly parasitized by flagellates described and classified as **Trypanosoma butantanense.** The high parasitemia shown both by the naturally and experimentally infected snakes, called the attention of those authors, who observed trypanosomes outnumbering the erythrocytes even in the absence of evident pathogenic effects for the host. As stated earlier [3], snakes are seldom parasitized by trypanosomes; if present, parasitemia is generally low, only a few cases exhibiting higher counts of flagellates in the peripheral blood.

Among the various species of trypanosomes described in Brazilian snakes, **T. butantanense** shows not only distinct morphological characteristics but, as a biological peculiarity, intense replication in the peripheral blood. For other species of snake trypanosome, there is no reference as to the organism's multiplication in the vertebrate host.

Searching flagellates in snakes for many years, PESSOA et al. [12] have not come across **T. butantanense** again. Recently, however, the detection of the parasites in a specimen of **W. merremii** permitted the elaboration of this paper.

MATERIALS AND METHODS

Original infected snake

A specimen of **W. merremii** ("boipeva") infected by trypanosomes was captured by J. NAVAS in December, 1977, in Araçoiaba da Serra, São Paulo State, Brazil. The parasite was identified as **T. butantanense** Arantes and Fonseca, 1931, and is being maintained **in vivo** since then, through inoculation into adult and young snakes.

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All snakes used in the transmission experiments were previously checked as to the presence of trypanosomes, by direct blood examination. The transmission routes were: (i) subcutaneous: drops of **T. butantanense** infected blood diluted in saline solution or tissue culture Eagle's medium [8], in volumes varying from 0.5 to 2.0 ml, were injected laterally into the median region of the snake's body; this experiment included the inoculation of adult snakes (19 **W. merremii)** (Wagler, 1824), one **B. pradoi** (Hage, 1948), and two **B. neuwiedi pauloensis** (Amaral, 1925), one adult and 47 young specimens of **C. durissus terrificus;** (ii) **per os:** the capacity of **T. butantanense** to penetrate through the digestive tract mucosa was investigated by dripping infected blood into the oral cavity of adult snakes: one **B. pradoi** and one **W. merremii;** (iii) — by leeches: one **W. merremii** highly parasitized by **T. butantanense** was placed in a container with water along with specimens of **Haementeria gracilis,** at room temperature (± 24°C), for 24 hours.

"In vitro" maintenance

Blood samples of four adult infected **W. merremii** were used as inocula for the **in vitro** maintenance of **T. butantanense,** the samples being collected aseptically after dissection and with a heparinized syringe, from the left aortic arch [11]. For each ml of harvested blood, 0.04 ml (50 U/ml) heparin were employed. Approximately 0.15 ml of infected blood harbouring 4-4.5 million flagellates, were dispensed in culture bottles containing 15 ml of L.I.T. medium.

Harvesting of blood from infected snakes

In the first transmission experiments, blood harvest was carried out through sectioning of the snake's tail. Both for the **in vitro** maintenance of the parasite and for suplementing culture media, blood was harvested through puncture of the aortic arch. For the separation of red blood cells, an International centrifugue type PR-2, was used at 200 g for 10 min (+4°C).

Culture media

Aliquots of 15 ml each of the following media, supplemented with 0.2 ml whole blood, 1.0 ml plasma, or 0.1 ml packed blood cells, were distributed in 80 ml tissue culture bottles: (i) Eagle's [8] supplemented with 10% (v/v) inactivated calf serum, plus 50 μg streptomycin and 200 U0 penicillin/ml; (ii) L.I.T.[5]: this medium consisted of the following components (g/ml): liver infusion (0.003g); tryptose phosphate broth (0.005); NaCl (0.004); KCl (0.0004); $Na_2HPO_4 . 7H_2O$ (0.015); dextrose (0.001), plus 2% lysed sheep erytrocytes, 10% inactivated calf serum, 100 μg streptomycin, and 50 U0 penicillin/ml.

Trypanosome countings

Countings of the trypanosomes present in the "in vitro" cultures were made at the 2nd, 4th, 7th and 10th incubation day, in an improved Neubauer haemocytometer.

Subculturing of the trypanosome

Cultures were maintained at room temperature (± 24°C) for 7 to 10 days. For the subsequent passages, 5 ml of the original passage were transferred to 10 ml of similar fresh medium.

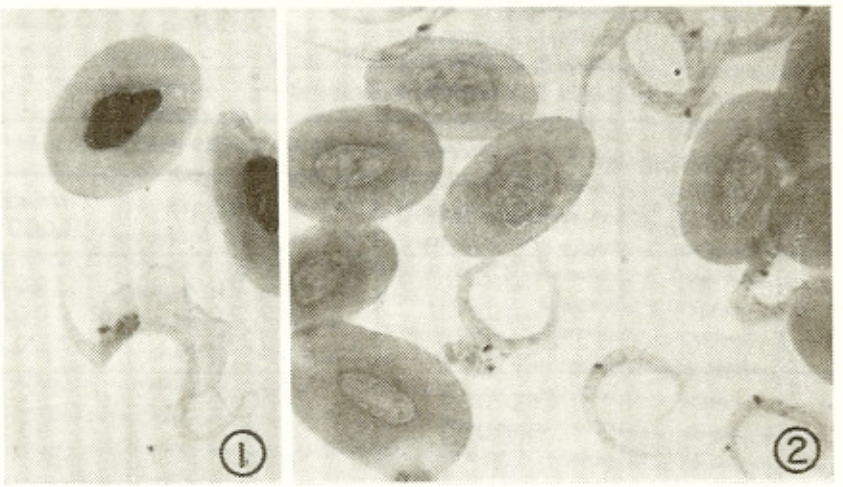
Examination of snake blood and "in vitro" cultures

Whole fresh blood samples and Giemsa stained smears were examined and when positive, photomicrographed.

RESULTS

From the **W. merremii** positive for **T. butantanense** (Figs. 1, 2), the original inoculum was passed into a snake of the same species. This snake (NH-514), found positive on the 3rd day, was the initial reservoir for the trypanosome studies in the present experiments. Subsequent passage into **W. merremii** NH-511 and NH-512, led to a higher parasitemia especially in the latter, within eleven days. Number NH-514 was sacrificed five months later and samples of its blood inoculated on L.I.T. and Eagle's media. After 24 h, intense growth was observed: in Eagle, the trypanosomes formed groups, and in L.I.T., individual parasite thrived, various evolutive and dividing forms being identified. Similar procedure was adapted for "boipevas" NH-511 and NH-512 (Figs. 3, 4).

Samples of infected blood from NH-511, diluted in: a) L.I.T. — were injected into 8 young



Figs. 1 and 2 — **T. butantanense** in Giemsa stained blood smears from **W. merremii.** 1) Trypomastigote form with perceptibe undulant membrane, and blepharoplast. 2) Several trypanosomes among erythrocytes. Magnification: 1.600 ×

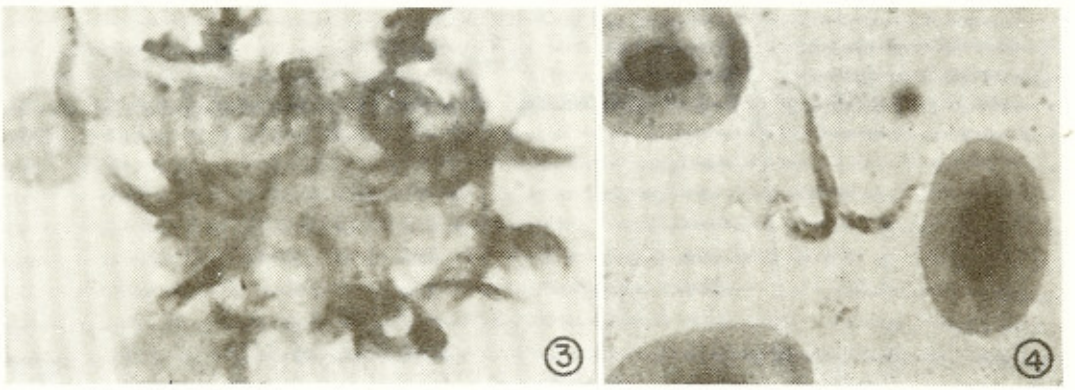


Fig. 3 — **T. butantanense** in Eagle's medium. Parasites grouping. Giemsa staining. Magnification: 800 ×. Fig. 4 — **T. butantanense** in L.I.T. medium. Non-organized multiplicative staining forms. Magnification: 2.000 ×

C. d. terrificus, and 4 **B. neuwiedi pauloensis**, of which only one young "cascavel" (**C.d. terrificus**) survived, exhibiting high parasitemia 23 days later. This specimen, although positive for **T. butantanense**, survived in captivity for five months; b) Eagle — were injected into two especimens of **W. merremii** (NH-516, NH-517) and one **B. pradoi** (NH-518). Snakes NH-516 and NH-517 proved positive 20 days later but NH-518 was negative up to its death, on the 31st post inoculation day.

Blood from inoculated **W. merremii** NH-531 showing high parasitemia on the 15th post-inoculation day, was distributed into 80 ml bottles containing 15 ml L.I.T. medium enriched with whole blood, plasma or packed red blood cells. These cultures were replicated on the 7th day to homologous media. Parasite countings in the inoculum and cultures are shown in **Table I** and **Graph I**, countings in each culture type being performed on the 2nd, 3rd and 7th day. At the 1st passage, a peak on the growth rate was observed on the 2nd day, whereas countings carried out on the 7th day revealed a lower number of organisms in the there types of cultures. In the 2nd passage a steady decrease of the parasite numbers was recorded.

TABLE I

Multiplication of *T. butantanense* in vitro in L.I.T. medium

(*W. merremii* NH-531)

PASSAGE	COUNTING DAY	L.I.T. medium supplemented with normal snake blood — Number of parasites / ml		
		WHOLE BLOOD	PLASMA	PACKED RED BLOOD CELLS
1 st	0	290.000	290.000	290.000
	2	4.740.000	3.045.000	2.800.000
	7	1.682.000	180.000	270.000
2 nd	0	560.000	60.000	86.666
	3	39.000	0	93.000
	7	0	0	37.500

GRAPH I

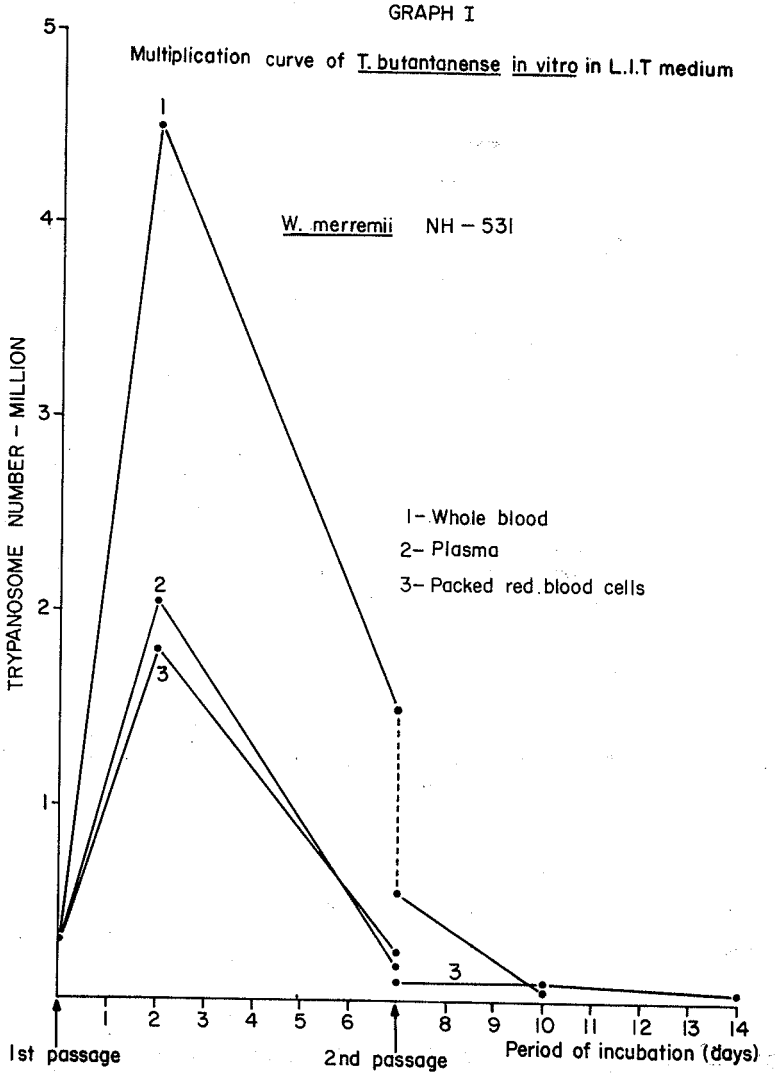


Table II and Graph II show the results obtained in a similar test with experimentally infected **W. merremii** NH-534. Intense multiplication was observed between the 4th and 7th days, in the 1st passage. Henceforth, and in the 2nd and 3rd passages, a decrease in the number of inoculated organisms persisted.

Of the two young **B. alternatus**, inoculated in 04/10/78, NH-64 died positive after 199 days; NH-65 was negative up to its death in 10/30/78 (203 days).

TABLE II

Multiplication of T. butantanense in vitro in L.I.T. medium

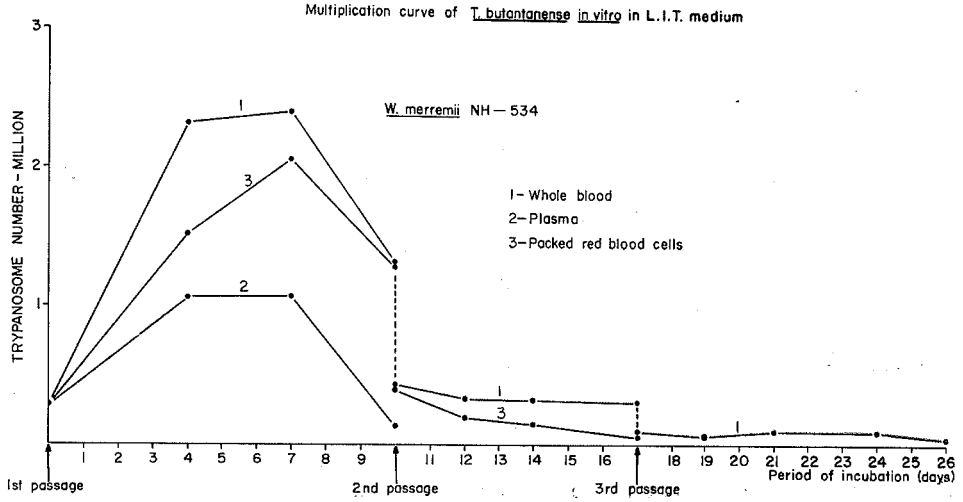
(W. merremii NH-534)

PASSAGE	COUNTING DAY	L.I.T. medium supplemented with normal snake blood — Number of parasites / ml		
		WHOLE BLOOD	PLASMA	PACKED RED BLOOD CELLS
1 st	0	267.666	267.666	267.666
	4	2.326.000	1.050.000	1.510.000
	7	2.409.000	1.068.000	2.058.000
	10	1.310.000	127.500	1.281.000
2 nd	0	436.666	n. c. o.	406.216
	2	330.000	n. c. o.	240.000
	4	330.000	n. c. o.	202.000
	7	315.000	n. c. o.	57.000
3 rd	0	105.000	n. c. o.	n. c. o.
	2	60.000	n. c. o.	n. c. o.
	4	96.500	n. c. o.	n. c. o.
	7	96.000	n. c. o.	n. c. o.

n. c. o. = not carried out.

GRAPH II

Multiplication curve of T. butantanense in vitro in L.I.T. medium



The inoculated young **C. d. terrificus** developed a higher degree of parasitemia.

The tentative transmission of trypanosomes by oral route, to the snakes **B. pradoi** (NH-519), and **W. merremii** (NH-535) failed.

Leeches (**Haementeria gracilis**), placed together with **W. merremii** (NH-530), positive to **T. butantanense** by subcutaneous inoculation, died during or after the suction.

DISCUSSION

The experimental inoculations of **T. butantanense** in its natural host, as well as in other species of snakes were carried out to investigate the susceptibility of the species and of each specimen to this flagellate. All specimens of **W. merremii** and young **C. d. terrificus** exhibited high susceptibility to the parasite; less susceptible were the two young **B. alternatus**, of which only one developed a low parasitemia. In the conditions of our experiments, the adult and young **B. neuwiedi pauloensis** and the adults **B. pradoi** and **C. d. terrificus** were negative; these results, however, do not exclude a probable susceptibility of these snakes to **T. butantanense.** The different results obtained with adult and young specimens of "cascavel" **(C. d. terrificus)**, might suggest a biological resistance of adults of this species to **T. butantanense.**

The **in vivo** maintenance of **T. butantanense** through successive subcutaneous inoculation in snakes was initially performed with saline-diluted infected blood. As dilution in Eagle's proved by far more effective, this diluent was adapted throughout the experiments.

Transmission of **T. butantanense** by oral route was shown to be non-productive, leading to the conclusion that **T. butantanense** may not easily penetrate the mucosae, and that the parasite must have direct access to the host's blood in order to cause infection.

Difficulties in the identification of snake trypanosome vectors and transmission mechanisms have already been stressed [3]. Several Authors suggest Phlebotominae [6], Glossina [10], Culicidae [7,3], and Hirudinae (leeches) [2,4] as vectors or possible vectors, in the case of reptiles or frogs.

Since **W. merremii** inhabits mostly humid places, where Hirudinae can be found, tentative transmissions of **T. butantanense** were tried with **Haementeria gracilis**; in our experimental conditions, however, results have not proved this invertebrate to be probable vectors of this trypanosome species.

The cultivation of reptile trypanosomes, for a better knowledge of their biology regarding their various evolutive forms "in vitro" and experimental transmission, led several Authors to test different culture media. FROMENTIN [9] studied **T. therezieni**, a parasite of **Chamaeleo brevicornis**, using different types of culture media such as N.N.N., Noller, Almeida's, Parker, 199, and Hanks' variously supplemented with either human blood, rabbit blood, guinea-pig red blood cells or Hobler's medium; CHRISTENSEN et al. [6] used Senekji's agar slants, and Noguchi's semisolid agar for **T. tchecadactyli**; DE BIASI et al. [3] studied in N.N.N. medium evolutive forms of **T. salamantae** and **T. phylodriasi**; ARANTES & FONSECA [1] investigated the development of **T. butantanense** in N.N.N. medium.

In the present experiments with **T. butantanense**, the trypanosomes were inoculated in L. I.T. and Eagle, intense multiplication occurring in both media. However, in Eagle, the organisms grouped in clusters (Fig. 3), and in L.I.T., they were rather individualized; it can be concluded therefore, that for our experimental purposes L.I.T. proved more adequate for the **in vitro** cultivation of **T. butantanense.** In two experiments with L.I.T., it was observed that the ideal moment to passage the cultures in order to keep its growth rate in the logarithmic phase would be around the 3rd to the 5th days, when a maximal multiplication occurred (Figs. 1 and 2).

The culture medium granted a more copious growth when supplemented with whole blood, the next best results being obtained with packed red blood cells supplemented medium.

In a subsequent series of experiments, the critical moment for subculturing the flagellates in order to assure the establishment of **in vitro** cultivable strains of **T. butantanense**, will be investigated, as well as the **in vitro** development of the different morphological phases of the parasite.

RESUMO

Desenvolvimento "in vitro" do Trypanosoma butantanense **Arantes e Fonseca, 1931**

Os Autores estudaram a manutenção "in vitro" do flagelado **Trypanosoma butantanense**, isolado de espécime de **Waglerophis merremii** (serpente, Colubridae). Para a realização das passagens, os meios L.I.T. e Eagle foram suplementados com (i) sangue total, (ii) plasma, (iii) hemácias de serpentes. Os melhores resultados foram obtidos com meios enriquecidos com sangue total, sendo as hemácias mais eficientes do que o plasma.

Para a transmissão "in vitro" o sangue infectado, diluido em meio Eagle, foi o mais efetivo; **W. merremii**, assim como filhotes de **Crotalus durissus terrificus**, apresentaram maior suscetibilidade ao **T. butantanense**, ao passo que os filhotes de **Bothrops alternatus** foram menos suscetíveis.

Os Autores suspeitam de uma possível resistência biológica dos **C. d. terrificus** adultos ao flagelado. Não foi possível transmitir o **T. butantanense** por via oral ou através de sanguessuga **Haementeria gracilis** (Hirudinea).

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