The Haemoculture of *Trypanosoma minasense* Chagas, 1908

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Trypanosoma minasense *was isolated for the first time in blood axenic culture from a naturally infected marmoset,* Callithrix penicillata, *from Brazil. The parasite grew profusely in an overlay of Roswell Park Memorial Institute medium plus 20% foetal bovine serum, on Novy, McNeal and Nicolle medium* (*NNN*), *at 27°C, with a peak around 168 hr.*

The morphometry of cultural forms of T. minasense, *estimates of cell population size and comparative growth in four different media overlays always with NNN, were studied. The infectivity of cultural forms to marmosets* (C. penicillata *and* C. jacchus) *and transformation of epimastigotes into metacyclic-like forms in axenic culture in the presence of chitin derivates* (*chitosan*) *were evaluated.*

Key words: *Trypanosoma minasense* - neotropical primates - trypanosomes - marmosets - trypanosomatidae flagellates - culture - Brazil

Trypomastigotes of *Trypanosoma* (*Megatrypanum*) *minasense* were originally described by Chagas (1908) in the blood of a marmoset, *Callithrix penicillata*, from Lassance, State of Minas Gerais (MG), southeastern Brazil. This trypanosome is a widely distributed species detected in 32 species or subspecies of neotropical non-human primates, principally small monkeys (Cebidae), marmosets and tamarins (Callithrichidae), from Panama to southeastern Brazil (Carini 1909, Cerqueira 1924, Dios et al. 1925, Deane & Damasceno 1961, Dunn et al. 1963, Deane et al. 1974, Souza et al. 1974, Souza & Dawson 1976, Deane 1979, Deane et al. 1989, Lourenço-de-Oliveira et al. 1991, Resende et al. 1994, Ziccardi et al. 1994, Deane, pers. commun.).

It is the only *Trypanosoma* in which circadian rhythm in the parasitaemia has been reported (Deane et al. 1974).

Infections with *T. minasense* are usually scanty, and division stages have never been detected in the simian host. It does not infect triatomine bugs and nothing is known about the vectors in nature. Attempts to infect laboratory rodents and dogs have also failed (Hoare 1972).

Rodhain (1937), Deane and Damasceno (1961) and Marinkelle (1966) were unable to cultivate this trypanosome in axenic culture media, such as Novy, McNeal and Nicolle (NNN), and previous attempts to cultivate this parasite from blood samples of marmosets and monkeys from several localities in southeastern and northern Brazil have also been unsuccessful (Lourenço-de-Oliviera, unpublished data).

This paper records the axenic culture of *T. minasense* in a modified "Roswell Park Memorial Institute - Novy, McNeal and Nicolle" (RPMI-NNN) medium.

MATERIALS AND METHODS

A specimen of the marmoset *C. penicillata* (No. 63), caught in Felixlândia (MG) in May 1991, was shown to be naturally infected with *T. minasense* following the examination of thick and thin blood smears. No other blood parasites were detected, and xenodiagnosis made with six nymphs of 3rd and 4th instar *Rhodnius prolixus* was negative. The parasite did not grow in blood-agar culture medium NNN/LIT .

A single further attempt at haemoculture was carried out using the same media (NNN + LIT), 17 months later when the marmoset still showed only *T. minasense* in its blood. The culture was poor on the seventh day and the parasites were subcultured into four different media, over NNN slopes: 1. Roswell Park Memorial Institute (RPMI 1640, Sigma) plus 10mM Hepes, 1.5 mM/1L L-glutamine, 20% of foetal bovine serum (FBS, Microbiológica), penicillin 200 UI/ml and streptomycin 200 µg/ml; 2. Liver Infusion Tryptose (LIT); 3. Schneider's *Drosophila* medium (Schneider 1966); and 4. Dulbecco's Modified Eagle's (DME, Sigma), all supplemented with 20% (FBS) and penicillin 200 UI/ml.

The absence of *T. cruzi* and *T. rangeli* in the cultures was tested by the inoculation of 0.1 ml of the culture (10^6 parasites/ml) into ten 10 days-old albino mice. Their blood were examined (fresh and Giemsa-stained smears) from 7 to 20 days after

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inoculation. Then, all the inoculated mice were sacrificed and haemoculture (NNN + LIT) was made and the cultures microscopically examined during one month.

In addition, we tried to infect 30 nymphs of 3rd and 4th instar *R. prolixus* by feeding them through a membrane (Garcia et al. 1984) on sterile defibrinated sheep blood mixed with the same culture. The search for parasites in the bugs was conducted 5, 13 and 25 days after the blood meal.

A one month old *C. penicillata* marmoset (No. 71), born and reared in the laboratory and negative for trypanosomes, was inoculated intraperitoneally (IP) and subcutaneously (SC) with 0.5 ml of a 13 days-old sample (4th passage) of the original culture of *T. minasense*. The search for parasites in the marmoset was made by thin and thick Giemsa-stained blood smears from day 5 to day 30 post inoculation (p.i).

Two adult *C. jacchus* marmosets (No. 74 and 76) were injected (IP/SC) with 0.5 ml of the 2nd passage of the original culture, previously cryopreserved for one month in liquid nitrogen. The animals were submitted to blood examination (thin and thick smears), xenodiagnosis (six nymphs of 3rd instar of *R. prolixus*) and haemoculture (NNN + LIT) for 30 days.

A third adult *C. jacchus* marmoset (No. 77), was inoculated intravenously (IV) with 0.5 ml of the 2nd subculture of the original isolation of the trypanosome, previously cryopreserved for eleven months, and diluted after quick centrifugation with sterile saline. The animal was examined (thick blood smears) from day 7 to day 120 p.i. Another attempt to infect the same animal was made by injecting it (IV) with material from subcultures, diluted in PBS pH 7.2. The marmoset was then examined from the day 7 to day 15 p.i.

We have tried to stimulate the transformation of *T. minasense* epimastigotes into metacyclic-like trypomastigotes in axenic culture, using chitin derivates (chitosan) according to the method of Wallbanks et al. (1989), using three concentrations: 150, 300, 600 mg of chitosan/5 ml of RPMI. Parasites were sampled at roughly 24 hr periods, when fresh medium (RPMI) was replaced.

The estimates of cell population size were made by a direct count in a haemocytometer (Neubauer).

RESULTS

NNN medium overlaid with RPMI gave the best growth-rate of *T. minasense* (Table I).

The blood of all mice inoculated with *T. minasense* culture, their respective haemocultures and xenodiagnoses (gut contents and faeces) were negative. Based on these experiments we concluded that the culture was free from both *T. rangeli* and *T. cruzi*, and composed purely of *T. minasense.*

The epimastigotes and trypomastigotes found in the culture were quite distinct. Fig. 1 and Table

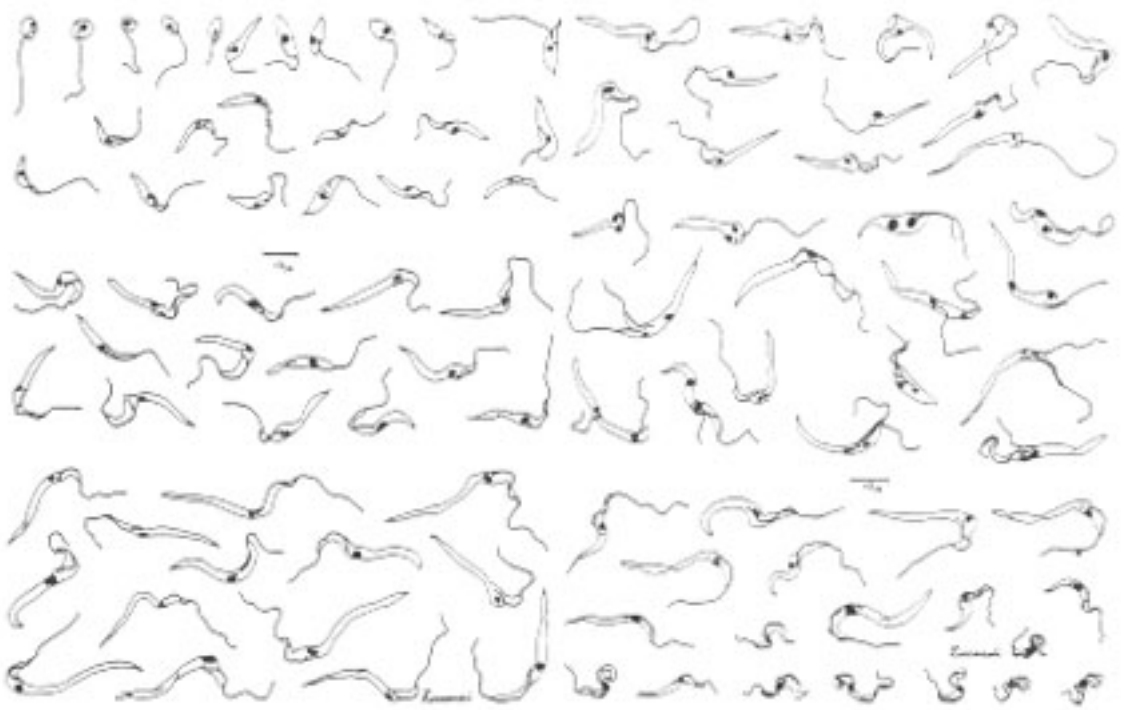

Fig. 1: *Trypanosoma minasense*. Forms found in haemoculture (NNN+RPMI) from a naturally infected *Callithrix penicillata* marmoset (No. 63) caught in Felixlândia (MG), Brazil.

TABLE I

Comparative scores[a] of cultural forms of *Trypanosoma minasense* in different media after 3, 6 and 12 days of culture

Culture media[b]	Days of culture		
	3	6	12
NNN	$1x10^2$	$1.2x10^5$	0
NNN+Dulbecco's	0	0	0
NNN+LIT	$1x10^3$	$7.5x10^4$	0
NNN+RPMI	$1x10^4$	$1.5x10^5$	$1x10^6$
NNN+Schneider's	$1x10^2$	0	0

a: number expressed in parasites/ml; *b*: all media supplemented with 20% (FBS) and incubated at 27°C; media: NNN (Novy, McNeal and Nicolle); LIT (Liver Infusion Tryptose); RPMI (Roswell Park Memorial Institute)

II show, respectively, camera-lucida drawings and the measurements of the most commonly detected cultural forms. We observed a great variety of forms in the culture of *T. minasense*, as follows: round epimastigotes with a long, free flagellum; short, long and slender epimastigotes; and large epimastigotes reaching up to 70.0 µm in length and 4.5 µm in width. Stout epimastigotes, sometimes provided with a free flagellum of up to 42.0 µm long were also seen; epimastigotes in binary fission; and long and short trypomastigotes, some of which appeared to be metacyclic forms.

The injected *C. penicillata* (No. 71) marmoset became infected, presenting the following parasitaemia (parasites/thick blood smear of 5 µl, according to Earle & Perez 1932): 5th day, 2 forms; 7th day, 18; 12th day, 28; 19th day, 18 and 21st day, 7. The blood stream parasites found in the 5th day were little thinner than the typical *T. minasense* trypomastigotes seen later on the 7th day. The peak of parasitaemia was on the 12th day after inoculation. No division stages were seen.

The three inoculated *C. jacchus* (No. 74, 76, 77) failed to become infected.

In NNN + RPMI, the isolate of *T. minasense* presented a maximum growth at 168 hr, with about 8.9×10^6 parasites/ml (Fig. 2).

The percentage of metacyclic-like forms detected in the cultures with added chitosan were periodically evaluated. The highest number of trypomastigotes was recorded after the first change of media, with 300 mg of chitosan/5 ml of RPMI (Table III).

DISCUSSION

Hoare (1972) regarded the species belonging to subgenus *Megatrypanum* as phylogenetically the most primitive representatives of the genus *Trypanosoma* of mammals. At least three species of *Megatrypanum*, have been found in New World monkeys and marmosets: *T.* (*M.*) *minasense*, *T.* (*M.*) *devei* and *T.* (*M.*) *lambretchi*. They are, so far, not known to infect man.

T. lambretchi is easily cultured in Tobie's blood-agar diphasic medium, with either human or rabbit blood, although development is slow (Lambrecht 1965, Marinkelle 1968). Pro-

TABLE II

Morphometric analysis of flagellates found in the axenic culture of *Trypanosoma minasense* (NNN+RPMI) isolated from *Callithrix penicillata*. Ranges given with means in parentheses

Forms	No. measured	L	PK	KN	NA	F	B
Short epimastigotes	23	13.0-43.0 (29.2)	0.9-14.0 (9.4)	0.8-1.3 (1.1)	2.6-12.0 (6.1)	8.0-28.0 (14.1)	2.0-6.0 (3.5)
Intermediate epimastigotes	20	34.0-52.0 (45.0)	9.0-22.0 (16.8)	0.4-2.0 (1.0)	8.0-14.0 (10.5)	7.0-26.0 (14.2)	2.0-4.0 (3.9)
Long epimastigotes	24	41.0-70.0 (54.4)	7.0-35.0 (23.4)	0.4-3.0 (1.4)	5.0-27.0 (11.8)	10.0-21.0 (17.0)	1.9-4.5 (2.8)
Stout epimastigotes	11	39.0-74.0 (48.5)	15.0-39.0 (19.8)	0.4-0.9 (0.7)	1.5-19.0 (9.7)	9.0-42.0 (19.2)	1.9-6.0 (4.1)
Epimastigotes in binary fission	11	30.0-65.0 (48.8)	16.0-31.0 (22.5)	0.2-11.0 (3.6)	3.4-20.0 (11.5)	5.0-30.0 (17.7)	1.8-5.6 (2.8)
Trypomastigotes	18	20.0-53.0 (30.3)	4.0-20.0 (8.8)	1.5-7.0 (4.3)	4.0-17.0 (8.3)	5.0-24.0 (8.2)	1.0-2.8 (2.0)

L: total length (including flagellum), PK: distance from posterior end of body to kinetoplast, KN: distance from kinetoplast to nucleus, NA: distance from nucleus to anterior end of body, F: length of the free flagellum, B: body with (at nucleus level); NNN (Novy, McNeal and Nicolle); RPMI (Roswell Park Memorial Institute)

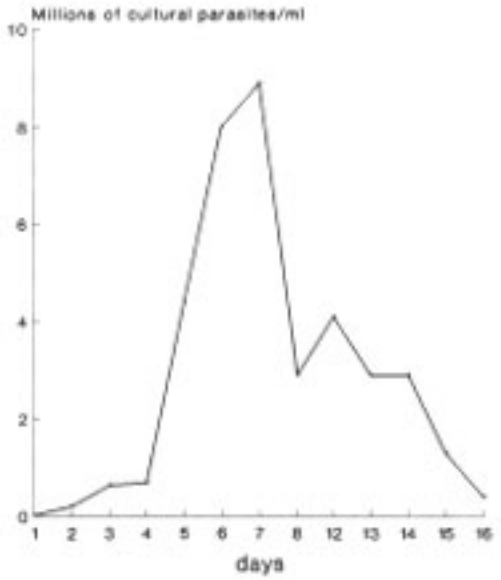

Fig. 2: estimates of cell population size in *Trypanosoma minasense* culture (NNN+RPMI).

TABLE III

Percentage of metacyclic-like trypomastigotes of *Trypanosoma minasense* in culture (NNN+RPMI) in different concentrations of Crab Shell Chitosan (Sigma) were added

Time of culture	Concentration of chitosan[a]		
(hours)	150	300	600
24	---	---	2%
48	---	0	1%
72	14%	---	---
144	14%	0	0
(1st change of medium[b])			
72	5%	4%	6%
144	6%	19.6%	8.4%
240	c	0	0
(2nd change of medium[b])			
56	c	0	0
144	c	0	0
168	c	0	0
(3rd change of medium[b])			
56	c	0	0
104	c	0	0
128	c	0	0

a: mg of chitosan/5ml of RPMI; *b*: RPMI, 20% FBS, 10mM Hepes, 1.5 nM/1 L L-glutamine, penicillin 200 UI/ml and streptomycin 200 µg/ml; c: contamination, scores disregarded; ---: not examined; media: NNN (Novy, McNeal and and Nicolle); RPMI (Roswell Park Memorial Institute)

mastigotes, usually in clusters, were the stages most commonly found, but short, stumpy, slowly-moving epimastigotes and occasional trypomastigotes were also detected in *T. lambretchi* cultures (Marinkelle 1976). According to Marinkelle (1976), there have been no previous reports on the multiplication *in vivo* or *in vitro* of any other trypanosome belonging to the subgenus *Megatrypanum* of neotropical non-human primates.

However, a strain of *T. devei*, from the tamarin *Saguinus midas niger*, was cultivated by Lanham et al. (1984) in blood-agar medium. The trypanosome grew profusely as long slender epimastigotes.

This is the first description of *T. minasense* multiplying in axenic culture, and the description and illustration of the cultural forms presented here now allows a better characterization of this parasite outside the vertebrate host, as well as a comparison with, and differentiation from other *Megatrypanum* species, such as *T. devei* and *T. lambretchi*, as described by Marinkelle (1976) and Lanham et al. (1984), and other New World trypanosomes such as *T. cruzi* and *T. rangeli* (viz. Hoare 1972).

The culture of *T. minasense* also now permits biochemical analysis using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on a discontinuous SDS buffer system, in which the parasite has a quite distinct peptideme from *T. rangeli*, *T. saimrii*-like, *T. cruzi* and *T. conorhini* (Ziccardi 1995).

Recently, we have failed to isolate *T. minasense* from two adult *C. penicillata* marmosets from Minas Gerais, using blood culture in four tubes with NNN + RPMI supplemented with 20% FBS. Its seems that the isolation and maintenance of *T. minasense* in these media is somewhat inconsistent and may depend on the phase of the infection at the time the culture is made. The haemoculture of *T. minasense* from the *C. penicillata* marmoset No. 63, for instance, was positive only in a second attempt made 17 months after the first negative one.

Actually, we have made several previous attempts to cultivate *T. minasense* from blood samples of infected marmosets from Minas Gerais (*C. penicillata*) and Rio de Janeiro (*C. jacchus*), in NNN blood-agar (Miles et al. 1980) and in NNN with overlay of LIT, brain heart infusion (BHI, Difco), RPMI and Schneider's. These either gave negative results or the culture was not successfully maintained. Other attempts with VERO cells with nutrient media such as LIT, RPMI or FBS have also been made. The longest maintenance of *T. minasense* in all these media (76 days) was obtained when the haemoculture was started in NNN + LIT and the flagellates later seeded in BHI, RPMI or Schneider's medium (Lourenço-de-Oliveira, unpublished data).

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