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Haementeria lutzi Pinto, 1920 (Hirudinea: Glossiphoniidae) as a putative Vector of *Trypanosoma evansi* (Kinetoplastida: Trypanosomatidae) in the Pantanal Matogrossense (MS, Brazil)

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Abstract

In the present study, it was shown under experimental conditions that *Trypanosoma evansi* could be mechanically transmitted to *Rattus norvegicus* by leeches (*Haementeria lutzi*). Additionally, we also described some aspects related to the behavior of the *Trypanosoma evansi in* the leeches after an infective blood feeding, as follows: a) 10 minutes after the parasites were ingested; they promptly progressed to the coelomic cavity. b) Approximately, from 10 to 30 minutes inside the gut, rounded and dividing forms together with stumpy and slender trypomastigotes showed a random dispersion. c) 24 hours after, the trypanosomes also invaded both, the salivary glands as well as the proboscis cells. Our results suggest that leeches of the species *Haementeria lutzi* could have some role as a probable alternative vector of Trypanosoma evansi at wetlands in Brazil.

Keywords: *Trypanosoma evansi*; *Haementeria lutzi*; Mechanical transmission; Experimental infection; Leeches

Introduction

Trypanosoma evansi is the aethiological agent of an equid disease called "Mal de Caderas" or "Surra". It is mechanically transmitted by biting flies (Tabanidae and Stomoxidae) and can infect in addition to horses, other domesticated livestock including dogs, bovines, buffaloes and camels as well as several species of sylvatic mammals, such as: deer, ocelot, vampire bats, coatis and capybaras [1-3].

The *Trypanosoma evansi* presents the widest geographical range of all the pathogenic trypanosome species occurring in many countries of South America, Africa and Asia where foci of the infection were related to flooded areas [4-6].

Correlation between the wet habitats and transmission of *Trypanosoma evansi* has been suggested because such conditions are considered suitable for the development of the insect vectors [7,8]. Coincidentally those habitats also offer ideal environments for the development of leeches.

Leeches live mainly in aquatic environments and among the 700 species actually recognized, only 100 are marine and 90 terrestrial while the remnants live in freshwater habitats [9].

Most leeches are hematophagous, feeding on blood from vertebrate and invertebrate animals. They comprise a group of highly specialized annelids distributed worldwide on all continents except Antarctica, achieving the highest diversity in the Holarctic region with one-half of all mainland species [9-11].

The leeches have an ancient origin [12-14] and a very important coevolutive history with the trypanosomes being already described as vectors of trypanosomes of fish, amphibians and reptiles [15,16].

Vassal [17] presented the first record suggesting a probable involvement of leeches on the transmission of *T. evansi*. He tested the blood ingested by the annelids (species not mentioned) from infected animals by injecting into rats and found it was infective immediately after blood meal but not four hours later. Basewitz [18] in Brazil reported to have used leeches (*Haementeria officinalis*) in treating a horse, which developed "Mal de Caderas" as a result of the treatment.

Tubangui [19] in Manila has used two species of leeches in experimental infections; namely the water's leech, *Hirudinaria manillensis*, and land's leech, *Haemadipsa zeylanica*. In the experiments carried out by injecting blood of mice obtained from leeches that had previously sucked infected animals, it was determined that in the aquatic species, *Trypanosoma evansi* was infective up to one hour and twenty minutes after feeding. On the other hand, in the land-living species, the trypanosomes remained viable up to four hours and fifteen minutes.

In interrupted-feeding experiments, carried out by the same author with animals previously infected, it was determined that the water's leech was unable to transmit the *Trypanosoma evansi*, differently the land dwelling species was capable transmit the parasite mechanically to the new hosts, because they survived in the proboscis for as long as thirty minutes.

Taking into account, the previous reports suggest a possible involvement of leeches on the transmission of *Trypanosoma evansi*. In the present paper, we investigated the potentiality of experimental transmission of *Trypanosoma evansi* by *Haementeria lutzi* as well as some aspects of its development in the leeches.

Materials and Methods

The strain of *Trypanosoma evansi* (TECAP-S01) was originally

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obtained from one capybara and the leeches were of the species *Haementeria lutzi* [20]; obtained from a colony initiated in our laboratory since 2005. Both the trypanosomes and the first specimens of leeches that originated the colony were collected in Pantanal of Mato Grosso do Sul, Brazil and kindly supplied by the Dr. Heitor M. Herrera.

The trypanosomes were maintained cryo-preserved and when necessary sub passaged in albino Wistar rats (*Rattus norvegicus*) for parasite multiplication.

Parasite multiplication was achieved from a rat presenting a parasitemia of 6×10^9 parasites/ml of blood that was infected four days before. The purification was accomplished through an anion exchanger column (DEAE cellulose) [21].

After counting in a Neubauer chamber the inocula was adjusted to yield, 10^3 parasites/g of body weight, that were injected intraperitoneally into a young male rat weighting 150 g. Blood samples for parasitological follow-up were taken every day.

In the transmission experiment, initially the leeches were fed on infected rats for approximately 5 minutes, then they were artificially removed with a tweezer and 30 minutes after the infective feeding was interrupted, they were exposed to non-infected animals.

The assay was achieved in Petri dishes with distillated water by exposing the tails of five laboratory-raised rats (*Rattus norvegicus*) to fifty leeches (ten leeches for each rat). Blood samples for parasitological follow-up were taken every day for 70 days when the animals were killed.

In the experiments related to development of *Trypanosoma evansi* in the leeches, twenty five young leeches previously fed on two highly infected rats (3×10^{10} parasites/ml of blood) were processed as follows:

For direct observations, five alive leeches were placed with a drop of water individually on a glass slide covered with cover slips and submitted to a microscopic examination (Carl Zeiss model: Axio lab. A1). After fifteen minutes of direct observation, the leeches still alive, were removed for a clean tupperware® container with 200 ml of water.

For the freeze dry material, the samples were made fifteen minutes, thirty minutes, twenty-four hours and forty eight hours after infective feeding, five leeches were used for each point and ten as negative controls. The leeches were quick-freezed in liquid nitrogen, separately embedded it in Tissue Tek® and transversal serial sections made on a cryostat microtome (Leica Cryocut CM 1800) from the entire specimens that were fixed with methanol and stained by Giemsa.

For the smears, five leeches were macerated individually and the samples collected were dried at room temperature, fixed in methanol and also stained by Giemsa stain.

All the experiments were carried out in accordance with guidelines defined by the Committee of Ethics in Animal Experimentation of the Oswaldo Cruz Foundation Rio de Janeiro, Brazil. Number: P0183-03.

Results

In the transmission experiments, one of the five rats was positive after infective feeding; this animal presented a long pre patent period of 45 days and the parasitemia reached 10^9 parasites/ml. All the five rats were followed up until 70 days when the animals were killed. No trypanosome was observed in any of the four negative rats.

The observation of *Trypanosoma evansi* in alive leeches during the first fifteen minutes after the infective feeding showed the parasites dispersed randomly in the gut and presenting intense movements. The morphology varied between stumpy and slender trypomastigotes as well as rounded and dividing forms. From ten to fifteen minutes, a high quantity of parasites had crossed the gut wall reaching the coelomic cavity. Once having reached the coelomatic cavity, they were promptly detected close to proboscis sheath and salivary glands. The trypanosomes could easily progress toward those structures because when leeches were engorged, the gut diverticula enlarged up until very close to them (Figure 1).

After fifteen minutes, most of the parasites have reduced their movements.

In the freeze-dried sections, after fifteen minutes, the parasites presented the same morphology as the observed in the inoculum. Thirty minutes after feeding, in the gut's blood, several rounded forms were noted randomly dispersed together a high quantity of trypomastigotes. In the coelomic cavity the parasites formed clusters composed mainly by rounded forms, but it was possible to observe some unbound trypomastigotes too (Figure 2).

On the smears one hour after feeding, the clusters of rounded forms were very frequent when compared to isolated slender and stumpy trypomastigotes as well as rounded and dividing forms (Figures 3 and 4).

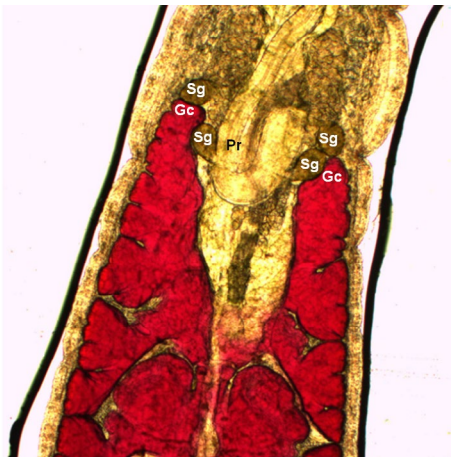

Figure 1: General view of the anterior body part of an alive *Haementeria*, ten minutes after blood feeding. Note the proximity of both the salivary glands (Sg) and the proboscis (Pr) with gut cecum (Gc) (100 X).

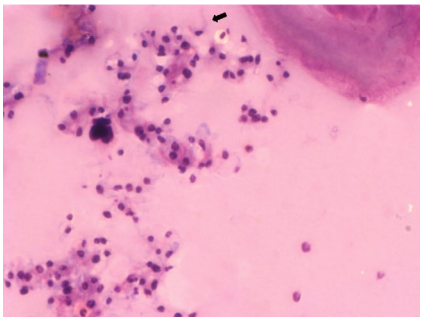

Figure 2: Freeze dry section of a leech, thirty minutes after the infective blood feeding with *Trypanosoma evansi*. In the coelomic cavity are seen several clusters of trypanosomes localized in the anterior region of the leech close to the proboscis. Note several rounded forms together to trypomastigotes (arrow) (2.300 X).

Twenty-four hours after feeding, in the gut, the distribution of the parasites and their morphological heterogeneity remained the same as already described. In the coelomic cavity, the trypanosomes tended to converge to the anterior region of the leeches, where they could be seen attached on the wall of the proboscis sheath as well as inside of it. At the same time, the parasites were already making contact with the outer surface of basal membrane of proboscis cells (Figure 5).

In the proboscis they were located in the thick middle layer composed of muscle cells, where it was possible to observe some parasites per cell, most of them as rounded forms (Figures 5 and 6).

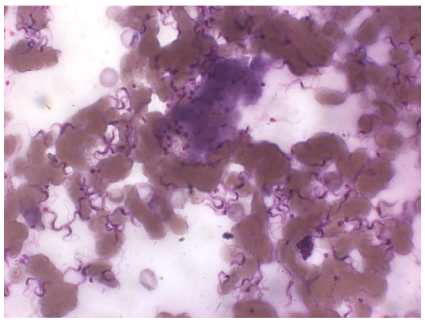

Figure 3: Smear from a macerated leech one hour after feeding on a rat infected with *Trypanosoma evansi*: clusters of rounded forms can be observed together with isolated slender and stumpy trypomastigotes (1000 X).

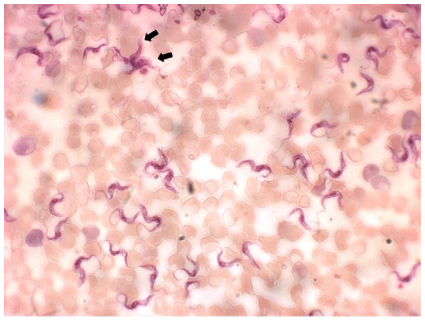

Figure 4: Smear from a leech macerated one hour after feeding on a rat infected with *Trypanosoma evansi*: note the presence of several isolated slender and stumpy trypomastigotes in addition to some dividing forms (arrows) (2.500 X).

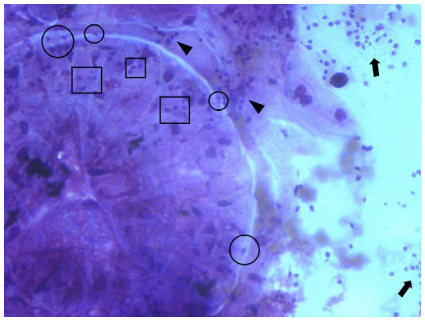

Figure 5: Freeze dry section of a leech, twenty-four hours after infective blood feeding with *Trypanosoma evansi*. In the coelomic cavity, several trypanosomes can be seen assembled in the anterior region of the leech, close to the proboscis (arrows). Some parasites were attached to the proboscis sheath wall (arrowheads) and inside of the proboscis sheath (circles). In the proboscis, they were located in the thick middle layer composed of muscle cells, most of them as rounded forms (squares) (1000 X).

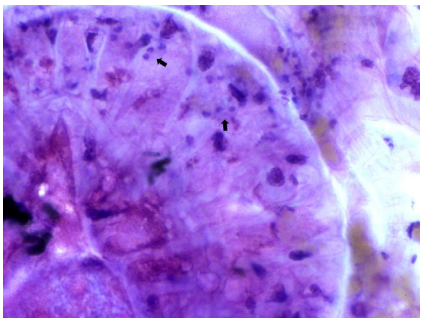

Figure 6: A detail from the anterior figure is showing the parasites inside the proboscis cells (arrows) (3.000 X).

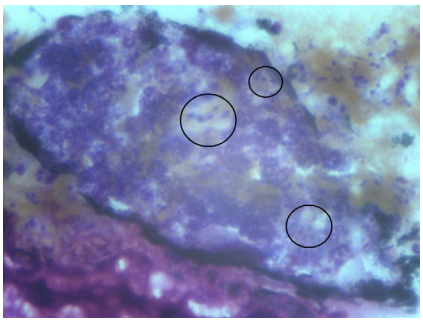

Figure 7 Freeze dry section of a leech, twenty-four hours after infective blood feeding with *Trypanosoma evansi*. Several trypanosomes in the salivary glands are observed with extracellular location in both the central and peripheral regions (circles) (1.500 X).

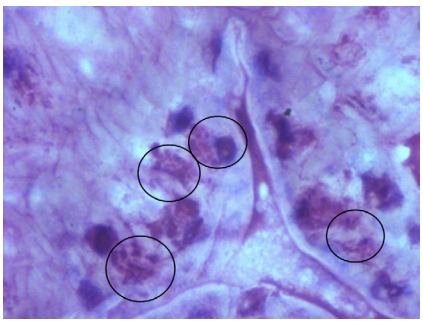

Figure 8: Freeze dry section of a leech, forty-eight hours after infective blood feeding with *Trypanosoma evansi*. In the proboscis, the parasites are observed inside the cells, closely associated with the tri-radiate proboscis lumen (circles) (1500X).

In salivary glands, the trypanosomes were observed with extracellular location in both the central and peripheral regions (Figure 7).

Forty-eight hours after feeding, in the gut, the morphology of the parasites and dispersion patterns remained the same. In the proboscis, they were observed closely associated with the trirradiate proboscis lumen (Figure 8).

Leeches that were used as controls presented no parasites or similar structures like those observed in infected ones.

Discussion

We showed that *Trypanosoma evansi* could be mechanically

transmitted by *Haementeria lutzi* under experimental conditions. Although it was not possible to be determined if leeches could have remained infective after a longer period than 30 minutes. It is probable that 30 minutes are sufficiently extended to allow these hirudinids, in natural conditions, to feed in a non-infected animal if they had dislodged from an infected one.

Blood-sucking flies are naturally subject to frequent interruptions during the blood meal due to the efforts of their victims to repel them, but even leeches with their persistence to keep attached on their victims could be shaken or brushed from an animal by branches of trees and other vegetation through which the animal may be passing.

The pleomorphism of *Trypanosoma evansi* observed in the leech was probably related to the parasite characteristics, because the Pantanal strains of *Trypanosoma evansi* were already described as presenting a high morphological variation, including those isolated from capybaras [22].

Trypanosoma evansi have already been considered a monomorphic trypanosome by some authors [23-25], nevertheless, the description of morphometric variations among different strains have been contested this idea [22,26-28].

In relation to rounded forms, the first study that described those forms in *Trypanozoon,* was carried out by Holmes [29]. In a posterior study, Salvin-Moore and Breinl [30] regarded the spherical forms as "latent bodies", capable of renewing the infection either by relapse or by passage into another host in the absence of trypomastigote in the blood. Later, Ormerod and Venkatesan [31] observed amastigotes forming large masses in the choroid plexus of rats.

Our observations of dividing forms in the smears one hour after feeding, suggest that the parasite probably could be able to multiply in the leeches' gut at least while the blood digestion was not achieved yet. Then, the *Trypanosoma evansi* could probably survive for several hours or may be days under those conditions; differently what occurs in the gut of tabanids and stable fly where the parasites could survive just for 8 hour in the maximum [32,33].

The blood digestion in the tabanids can keep four days in the maximum [34], on the other hand, in leeches it can remain for weeks or months depending of certain conditions, such as the age of the leech and temperature of the environment. Therefore, the stored blood suffers a very slow modification, presenting a gradual haemolysis, which in some cases, the red blood cells persisting for up to 18 months [35].

It is likely that the slower blood digestion process of leeches in comparison to the insect vectors could have afforded the greatest lifetime of trypanosomes in the leeches.

In respect to the probable invasion of salivary glands and cells of proboscis, we think it could represent some degree of adaptation of *Trypanosoma evansi* to persist in the infection and probably be correlated with the behavior observed among others Salivaria in their biological vectors.

In tabanids that are considered the major vectors of "Mal de caderas", all attempts to demonstrate the cyclic development of *Trypanosoma evansi* have failed [24,36] and the effect of the interruptions times between feeds on donors and recipients during *Trypanosoma evansi* transmission, showed that the probability of transmission dropped precipitously between 15 min and 24h after interruption [37].

There are several reports about the development of mammals' trypanosomes in leeches [38-43] and it was proposed that trypanosomes that develop in the anterior-station of arthropods evolved quite recently from a leech-aquatic-vertebrate stock [44].

In our view, it is quite probable that *Haementeria lutzi* could have some role of as an alternative vector for *T. evansi*, in wetlands from Brazil, in function of the following informations: 1) *Haementeria lutzi* can be commonly found in that area. 2) The strain of *Trypanosoma evansi* we have used was isolated from the same place, from a capybara that is a semi-aquatic rodent, which lives in large groups along the riverbanks grazing on the lush grasses and aquatic vegetation an important natural host of both the *Trypanosoma evansi* and *Haementeria* in Brazil [1]. 3) It was already observed that *Haementeria lutzi* were able to feed on a great number of other hosts that participate in the life cycle of *Trypanosoma evansi* in the Pantanal Matogrossense, such as: horses, cattle, buffalos, dogs and feral pigs (data not shown).

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