The Development of Species of *Leishmania* Ross,1903 in *Lutzomyia longipalpis* (Lutz & Neiva,1912)

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The development of four isolates of Leishmania from foci of American cutaneous leishmaniasis was studied in Lutzomyia longipalpis. *The suggestion that the differences in the development of the* Leishmania *in the invertebrate host are of great taxonomic significance was confirmed. The pattern of development of three strains was typical of parasites of the subgenus* Leishmania, *the other was similar to* Leishmania *of the subgenus* Viannia. *The identification of the strains using other criteria is in agreement with biological characterization. The results show that the morphological and morphometric study of promastigotes do not clearly define the taxonomic position of the parasites but other studies are needed to confirm this.*

Key words: *Lutzomyia longipalpis* - development - *Leishmania*

It is well established that the various *Leishmania* species behave differently in the lumen of the digestive tract of sandflies. The development of some species is restricted to the anterior region of the gut and others initially establish themselves in the pylorus and ileum and later migrate to anterior parts (Killick-Kendrick 1979).

In 1972, Lainson and Shaw used, amongst other criteria, the development of the parasite in the alimentary tract of the sandfly to separate species of *Leishmania* from the New World into two complexes: the *L. braziliensis* complex and the *L. mexicana* complex. The same authors in 1979 proposed the division of the *Leishmania* genus into three sections, based principally on the behaviour of the parasite within the sandfly, namely Hypopylaria, Peripylaria and Suprapylaria. Later, in 1987, they created the subgenera *Viannia* and *Leishmania* within which were included the species of the sections Peripylaria and Suprapylaria, respectively.

Various studies on the development of flagellates in the alimentary tract of sandflies have been undertaken resulting in substantial contributions to the understanding of the biology of *Leishmania* parasites and their interactions with their vectors (e.g. Lainson & Shaw 1988, Warburg et al. 1989, Killick-Kendrick 1990, Lawyer et al. 1990).

Lutzomyia longipalpis were experimentally infected with four strains isolated from foci of cutaneous leishmaniasis in the southeast of Brazil and their pattern of development within the alimentary tract was observed.

MATERIALS AND METHODS

Leishmania *strains* - Leishmania (Leishmania) amazonensis - (1) *Strain MAKO/BR/78/32R* - Originally isolated from a hamster infected by skin material from a male *Akodon cursor*, captured in the municipality of Caratinga, Minas Gerais, Brazil in July 1978; (2) *Strain MPRO/BR/79/132R* - Originally isolated from a hamster infected by skin material from a male *Proechimys dimidiatus* captured in the municipality of Caratinga in June 1979; (3) *Strain MHOM/BR/79/BH105* - Originally isolated from a hamster infected by skin material from a patient, AWS, a human case with multiple lesions, in the municipality of Viana, Espírito Santo, Brazil in May 1979. The isolation was made from a lesion on the right elbow.

L. (Viannia) braziliensis - Strain MCAN/BR/73/BH348 - Originally isolated from a dog from the region of Barracão in the municipality of Caratinga, in September 1973, by inoculating a strain of the dogs skin into hamsters.

Characterization by isoenzymes and/or monoclonal antibodies indicated that strains 132R, BH105 and 32R are *L. (L.) amazonensis* and strain BH348 is *L. (V.) braziliensis* (Melo & Shaw, personal comunication).

The strains were maintained in *Mesocricetus auratus* and are cryopreserved in liquid nitrogen at the Laboratory of Leishmaniasis of the Department of Parasitology of the Federal University of Minas Gerais.

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Sandflies - Specimens of *Lutzomyia longipalpis* were obtained from a sandfly breeding colony originally from Lapinha Cave in the municipality of Lagoa Santa, Minas Gerais and maintained in the Laboratory of Leishmaniasis of the Centro de Pesquisas René Rachou. When the quantity of insects in the colony were not sufficient further specimens were collected from the Lapinha Cave where *Leishmania* are known not to exist.

Sandfly infection - The sandflies were fed on lesions of hamsters infected with the four above mentioned *Leishmania* strains. Small PVC boxes (3.5cm high with a diameter of 4.0cm) with an inner surface made of fine mesh nylon where the sandflies could feed were used for the infecting meal. The box containing the flies was fixed with tape to the nodule of a hamster previously anaesthetized with Thionembutal at a dose of 500mg in 10ml of saline solution for intraperitonial inoculation using 0.15ml for 100g weight. The box and the hamster were then covered with a black fabric for approximately 30 min. After verifying that the sandflies had fed they were transfered to cages kept in a climatized room with a temperature of 28°C ± 1°C and approximately 90% relative humidity. The sandflies received no form of further food.

Sandfly examination - Sandflies were examined daily from the third to the seventh day of infection. Specimens to be dissected were killed with sulphuric ether and placed in a 0.85g% saline solution. The flies were placed on a slide with a drop of saline and dissection was undertaken using a stereoscope microscope. After dissection a cover slip was placed on top and the gut was examined using a light microscope. The position, number and morphology of the parasites were observed at this stage prior to the rupture of the digestive tract. When wild sand flies were used in this experiment identification was undertaken. Following examination of the freshly dissected material it was fixed with methyl alcohol, and stained with Giemsa's stain, for the study of the morphology and morphometry of the promastigotes.

Promastigote morphology and morphometry - The stained slides were examined by light microscopy with a 100x objective and the flagellates were measured. Thirty randomly selected forms were measured from each strain. The measurements were made directly on the slide using a 10x micrometric eyepiece. The technique used for the morphometric study of the promastigotes was adapted from that described by Hoare (1972) for trypanosomatids. For each strain the following measurements were made in microns: BL - body length, FL - flagellum length, NA - distance of nucleus from the anterior region, KA - distance of the kinetoplast from the anterior region, NP - distance of the nucleus from the posterior region, KP - distance of the kinetoplast from the posterior region. The kinetoplastid index (IK = KP/KA) and the nuclear index (IN = NP/NA) were calculated based on these measurements which indicate the position of the kinetoplast and the nucleus respectively.

Statistical analysis - For the analysis of the localization of the parasites in the digestive tract of the sandflies and in the comparison of the susceptibility of the *Lu. longipalpis* for strains the chi-square test was used (Snedecor & Cochran 1980). Infection in the thoracic midgut and the posterior gut were compared for each strain as well as the indices of infection in each of the three regions of the digestive tract. The comparison between the variables measured in the promastigotes of the *Leishmania* strains were made using Analysis of Variance (Snedecor & Cochran 1980). When necessary the Tukey Test was applied in order to identify differences between the strains.

RESULTS

Susceptibility of Lutzomyia longipalpis to Leishmania *strains* - The result of the susceptibility of *Lu. longipalpis to Leishmania* strains is shown in Table I. *Lu. longipalpis* was susceptible to all the strains used although the percentages of susceptibility varied between 5.2% and 67.3%. By means of the capacity of the strains to infect the sandflies it was possible to separate them into groups which presented statistically significant differences. One group comprising of strains 132R and BH105 presented higher indices of infectivity (67.3% and 66.7%) and the other group, comprising of strains 32R and BH348, had lower indices of infectivity (9.2% and 5.2%).

TABLE I

Susceptibility of *Lutzomyia longipalpis* for *Leishmania* strains

Strain	Flies fed	Flies infected	%
MPRO/BR/79/132R	248	167	63.3
MAKO/BR/78/32R	240	22	9.2
MHOM/BR/79/BH105	360	240	66.7
MCAN/BR/73/BH348	97	5	5.2
Total	945	434	45.9

X^2_3 gl = 313,61; NS = 0.05

Characteristics of the development of infection - L. (L.) amazonensis *(Strain MPRO/BR/79/132R)* - The results of the observation on the localization of the infection with the strain 132R are listed in Table II. In all positive specimens the presence of the parasites in the abdominal midgut was observed. The infection became established and remained in this segment of the di-

gestive tract throughout the course of the infection. The infection in the thoracic midgut underwent a gradual increase reaching 100% on the seventh day. Seven out of 167 flies presented parasites in the hindgut. The parasites were free in the gut lumen and morphologicaly similar to the forms observed in the midgut. Throughout the entire period of observation the infection was abundant in all parts of the digestive tract. The majority of the flagellates were found in the lumen of the tract and were actively moving. In some specimens we observed the formation of rosettes and the presence of dividing forms in the abdominal midgut. From the fourth day of the infection a large proportion of the sandflies presented totally digested blood. In general, the slender, elongate promastigote forms were those most frequently found in the abdominal and thoracic midgut. Nevertheless on the fifth and sixth days of infection short promastigotes were found more frequently in these parts of the alimentary tract. The occurance of broad and round forms with or without a flagellum was very low during the development of the infection. On the seventh day round forms were not observed in any of the specimens.

TABLE II

Localization of infection with *Leishmania amazonensis* (strain 132R) in the digestive tract of *Lutzomyia longipalpis*

Days after infection	Flies infected	Localization of infection (%)		
		TMG	AMG	HG[c]
3	39	51.3	100	0
4	63	85.7	100	7.9
5	45	97.8[a,b]	100	0
6	15	93.3[a]	100	6.7
7	5	100	100	20.0
Total	167	82.9	100	4.2

[a]: in one fly parasites were found in the oesophagus and pharynx
[b]: in one fly parasites were found in the diverticulum
[c]: flagellates free in lumen
TMG: thoracic midgut; AMG: abdominal midgut; HG: hindgut

L. (L.) amazonensis *(Strain MAKO/BR/78/32R)* - Table III summarizes the observations of the distribution of the flagellates of strain 32R in the digestive tract during infection. The great majority of the infections started in the abdominal midgut and remained at this location until the seventh day. The migration to the thoracic midgut was variable and on the last day of observation parasites were not found in this region of the insects digestive tract. The posterior gut was not found to be infected in any of the specimens examined. Soon after the begining of the infection the number of flagellates in the abdominal midgut was low. However, from the fourth day of infection the number of parasites increased significantly and on the fifth day of infection was extremely high and remained so until the end of the period of observation. The parasites were highly active and the majority were moving within the lumen of the intestine. The complete digestion of the ingested blood occured by the fourth day after the infecting meal in 85.7% of the specimens. In the remainder, digestion was complete on the fifth day. Elongate slender forms and short promastigotes were the most frequently encountered forms throughout the midgut of the sandflies. In the abdominal midgut stumpy promastigotes were found with a reasonable frequency in addition to the forms mentioned above. On the sixth and seventh days of infection short promastigotes were the predominant forms found. The presence of the well rounded forms was noted on the fourth day of the infection in the thoracic midgut and on the fifth day in the abdominal midgut.

TABLE III

Localization of infection with *Leishmania amazonensis* (strain 32R) in the digestive tract of *Lutzomyia longipalpis*

Days after infection	Flies infected	Localization of infection (%)		
		TMG	AMG	HG
3	5	40.0	100	0
4	7	42.9	85.7	0
5	7	0	100	0
6	1	100	100	0
7	2	0	100	0
Total	22	27.3	95.5	0

L. (L.) amazonensis (*Strain MHOM/BR/79/BH105*) - The results of the distribution of parasites of strain BH105 are shown in Table IV. In almost all the positive flies parasites were found in the abdominal midgut. The level of infection in the thoracic midgut increased with time and from the sixth day onwards all infected specimens presented parasites in this portion of the digestive tract. The infection observed in the hindgut in some specimens did not show parasites attached to the gut wall. From the start of the dissections the sandflies presented abundant infections but it was impossible to count accurately the number of parasites present. The parasites were highly active with rapid movements of the flagellum and the formation of rosettes was noted in some

specimens as well as the presence of dividing forms. The majority of the flagellates were free within the lumen of the gut but many were found compressed against the gut wall because of the intensity of infection. On the fourth day of infection only one of the positive sandflies had blood in the process of being digested. In all the others the process of digestion was complete. The most characteristic morphological form found in the infections with this strain was a long slender promastigote, which was found throughout the course of infection in various segments of the digestive tract. On the fifth day of infection there was a decrease in the number of the long slender forms which was coincident with an increase in the number of short forms in both the abdominal and thoracic midgut. The short forms were the second most abundant form in these portions of the digestive tract. The short large forms and well rounded forms were seldom found and were restricted to the period between the fourth and sixth days of infection of the midgut.

TABLE IV

Localization of infection with *Leishmania amazonensis* (strain BH105) in the digestive tract of *Lutzomyia longipalpis*

Days after infection	Flies infected	Localization of infection (%)		
		TMG	AMG	HG[b]
3	19	42.1	100	5.3[a]
4	99	91.9	97.0	7.1
5	84	90.5	100	8.3
6	32	100	100	3.1
7	6	100	100	16.7
Total	240	88.8	98.8	7.1

[a]: promastigotes also found in the Malpighian tubes
[b]: flagellates free in lumen

L. (V.) braziliensis (*Sample MCAN/BR/73/BH348*) - A summary of the results obtained with infections of strain BH348 is presented in Table V. Parasites were not observed on any day in the thoracic midgut. In only one specimen, on the fifth day of infection, was the presence of parasites noted in the abdominal midgut. Infection with this strain occurred principally in the hindgut, in the majority of cases restricted to the posterior triangle. Infections were very poor and extremely few parasites were observed in the lumen of the gut with the majority adhering to the intestinal wall by the flagellum. The flagellates exhibited much slower movements than the strains described above. On the fourth day of infection all the flies had completely digested the ingested blood. In the infections with this strain only two morphological forms of promastigote were observed, short promastigotes and rounded forms. No parasites were found in the thoracic midgut during the period of observation. Only in the fifth day of infection short promastigotes forms were seen in the abdominal midgut. The posterior gut was the only segment of the digestive tract where parasites were found on all days of examination. On the fourth and fifth days of infection only short promastigotes were found. By the sixth day both morphological forms were seen - short and rounded promastigotes.

TABLE V

Localization of infection with *Leishmania braziliensis* (strain BH105) in the digestive tract of *Lutzomyia longipalpis*

Days after infection	Flies infected	Localization of infection (%)		
		TMG	AMG	HG[b]
4	1	0	0	100[a]
5	2	0	50.0	100
6	2	0	0	100[a]
7	0	0	0	0
Total	5	0	20.0	100

[a]: infection restrict to the hindgut
[b]: parasites attached to gut wall

Promastigotes morphometry - In Table VI, the means and standard deviations of measurements of various parts of the promastigotes are presented. Using Analysis of Variance it was demonstrated that significant differences existed between the strains studied with respect to all the parameters measured. These differences were localized using Tukey's test. The means of the measurements made with strains 132R and BH105 presented statistically significant differences only in terms of variation in the length of the flagellum. All other variables were not significantly different. Strains 32R and BH348 exhibited measurements of body length, flagellum length and the distance of the nucleus from the anterior region that were not significantly different. The other three parameters measured showed significant differences. The distance of the kinetoplast from the anterior region was the parameter that presented few differences since only strain BH348 showed a difference when compared with the other three strains. The results of the nuclear indices were the following: in strain 132R the nucleus was localized in the anterior half of the promastigote body. In strain 32R, on the other hand, the nucleus was found in the posterior half of the flagellate. The nuclear index of strain BH105 was 1, indicating that the nucleus of the promastigotes of this strain was found exactly in the midlle of the body. Strain BH348 pre-

TABLE VI

Measurements of the *Leishmania* strains (mean and standard deviation)

Studied parameters (μm)	Strain			
	132R	32R	BH105	BH348
BL	12.87 ± 2.34	7.24 ± 1.05	12.48 ± 1.91	8.36 ± 1.14
FL	13.13 ± 2.34	9.79 ± 2.25	17.42 ± 2.70	8.93 ± 1.34
NA	5.85 ± 1.00	4.23 ± 1.25	6.24 ± 1.08	4.07 ± 0.91
KA	2.60 ± 0.52	2.54 ± 1.04	2.50 ± 0.47	1.82 ± 0.70
NP	7.02 ± 2.21	2.89 ± 0.62	6.24 ± 1.66	4.29 ± 1.26
KP	10.14 ± 2.21	4.81 ± 1.09	10.04 ± 2.00	6.54 ± 1.20

BL - body length; FL - flagellum length; NA - distance from nucleus to anterior region; KA - distance from kinetoplast to anterior region; NP - distance from nucleus to posterior region; KP - distance from kinetoplast to posterior region.

sented a nuclear index a little over one, showing that the nucleus was localized in the anterior region very close to the middle of the body. All the kinetoplast indices calculated were greater than one. This indicates that in all the strain studied the kinetoplast was situated in the anterior half of the promastigotes.

DISCUSSION

A series of factors affect the relationship between *Leishmania* and its invertebrate host and consequently the initiation, establishment and evolution of the infection in the sandflies. The relative importance of such factors is extremely difficult to determine since they usually act simultaneously.

Although *Lu. longipalpis* is designated as the only species that transmits visceral leishmaniasis in Brazil it could also be experimentally infected with the other four strains which cause cutaneous leishmaniasis. Coelho et al. (1967a, b) had made this observation when they infected specimens of *Lu. longipalpis* with *Leishmania* species isolated from human cases of cutaneous leishmaniasis. Nevertheless, the susceptibility of the sandfly for the parasite does not necessarily imply that it is capable of transmission. In the view of Killick-Kendrick (1979), the differences in susceptibility of individuals from the same population of a sandfly species are probably genetically determined. Ward et al. (1981), in studies of the genetic polymorphism of sandflies, suggested that intraspecific markers may have importance in susceptibility studies. These observations may explain the fact that some individuals of the group given an infective meal did not develop an infection while others showed great variation in the intensity of infection they developed.

Another fact to be considered in relation to susceptibility is the number of parasites ingested in the infecting meal. When the sandflies are highly susceptible to a given *Leishmania* sample, infections are observed in the stomach of the insect even when few amastigotes are ingested (Shortt 1945, Strangways-Dixon & Lainson 1966). When the susceptibility of the sandfly is low, on the other hand, they do not normally become infected following the ingestion of a small number of amastigotes. The microscopical study of the histiocitomas of the hamsters infected with strains BH105, 132R and 32R showed a high density of amastigotes per field in contrast to the scarcity of parasites observed with BH348. Thus, sandflies feeding on hamsters infected with strain BH348 would have a lower probability of ingesting a large number of parasites with the result that many did not develop infection. Despite the high density of amastigotes found in smears undertaken with strain 32R, the index of infectivity was low. However, those sandflies that were positive presented very intense infections in contrast to observations made with BH348 which resulted in poor infections.

Only a small number of infected sandflies with strains 132 and 105 showed parasites in the hindgut. However the presence of parasites does not characterize the hindgut as a place of establishment of infection as parasites were always free in the gut lumen and morphologically similar to those in the midgut. It is probable that this observation was due to the intensity of the infection in the midgut and was being passively carried to the hindgut. Generally the species of *Leishmania* of subgenus *Viannia* show high indices of infection in the hindgut as observed by Lainson et al. (1979) and Walters et al. (1989).

The results clearly show that the infections caused by parasites of strains 132R, 32R and BH105 established themselves in the abdominal midgut. Parasites were then observed adhering to the stomach wall and some were in the process of division. These and other observed characteristics of the infections permit us to place these strains in the subgenus *Leishmania*. On the other hand, the infections with strain BH348 clearly esta-

blished themselves in the posterior gut and the majority of the parasites encountered were adhering to the pylorus wall. Thus, the observed aspects of the course of the infection show that strain exhibited the typical characteristics of species of the subgenus *Viannia*.

It was confirmed in the present study, as previously suggested by Nicoli (1963), Garnham (1971) as well as Lainson and Shaw (1979), that differences observed in a variety of species of *Leishmania* in terms of their development within sandflies should be considered as being of great taxonomic significance.

The final development step in which the flagellates are to be found in the most anterior regions of the digestive tract was only observed with a very few specimens infected with strain 132R. The effect of temperature on parasite migration to the pharynx and mouth parts may explain this observation. Leaney (1977) working with specimens of *Lu. longipalpis* infected with *L. amazonensis* showed that in specimens maintained at between 22°C and 25°C infections were encountered in the pharynx . In sandflies maintained at 28°C it was found that migration of the parasites to the pharynx did not occur. The specimens of *Lu. longipalpis* used in our experiments were maintained in an acclimatized room with the temperature around 28°C ± 1°C. Thus, it is possible that inhibition of migration occured because of the high ambient temperature.

Until recently, it was accepted that there is only one morphological form of *Leishmania* in the digestive tract of the sandfly in addition to the amastigotes ingested with the blood meal - the promastigote. However, following a series of studies it was concluded that different morphological types of promastigote occur and with an additional developmental stage called the paramastigote (Killick-Kendrick et al. 1974, 1977, Molyneux et al. 1975).

Parasites of strains 132R and BH105 presented very similar morphological and morphometric characteristics. Measurements of body length, flagellum length and the position of the nucleus and kinetoplast agree with the characteristics of nectomonads described by Killick-Kendrick (1979). The measurements were in fact made on the fourth day of parasite infection when the frequency of these forms was considererably higher than any other . The behaviour of the flagellates in the digestive tract of the sandflies was typical of the subgenus *Leishmania* and Lainson and Shaw (1987) have stated that species of this subgenus have large promastigotes. However, comparing the measurements of the promastigotes of strain 32R, which also exhibited behaviour typical of subgenus *Leishmania*, with those of strains 132R and BH105 they showed differences in almost all variables measured. An explanation for this could be that the frequency of nectomonads with strain 32R on the fourth day of infection was not as high in comparison with the other smaller forms. Thus, there occured a greater degree of polymorphism in this strain and consequently the average size of the promastigotes was smaller. Possibly the developmental characteristics of the parasites of strain 32R in sandflies are realy different from those of strains 132R and BH105. This is supported by the observation that in addition to the differences observed in the morphological and morphometrical studies of promastigotes the strain 32R showed a degree of infectivity of much less than 132R and BH105 and indeed similar to that of strain BH348.

The results of the morphological and morphometric studies showed that the body length of flagellates was much smaller than that of promastigotes of strains 132R and BH105. According to Lainson and Shaw (1987), the promastigotes of the *L. braziliensis* complex are notably smaller than those of the *L. mexicana* complex.

The predominant morphological form shown in the hindgut of the sandfly has been described as paramastigote (Killick-Kendrick 1979, Molyneux et al. 1986). However, ultrastructural studies of *Leishmania* in natural and unnatural hosts showed that this form was rare (Walters 1993). Calculation of kinetoplastid indices of the strains studied showed that no paramastigotes were observed on the fourth day of infection. Indeed, these forms were only seen in the hind gut of the sandfly on the sixth day of infection with strain BH348. As paramastigotes only occur in the pylorus and ileum with species of the *L. braziliensis* complex or in the pharynx with species of the two complexes these results were expected. Three flies infected with strain 132R showed the presence of flagellates in the pharynx ever though the probability of finding paramastigotes in this area was very remote.

Our results show that the use of morphological and morphometric studies of promastigotes to distinguish strains of *Leishmania* does not provide a clear definition of the parasites but is only one aspect to be considered within a broader study. Silva et al. (1990) also held this opinion after comparing morphometric data from two strains of *Leishmania* with Suprapylaria type behaviour in the sandfly which presented a series of variables with statisticaly significant differences.

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