LEISHMANIA (VIANNIA) UTINGENSIS N. SP., A PARASITE FROM THE SANDFLY LUTZOMYIA (VIANNAMYIA) TUBERCULATA IN AMAZONIAN BRAZIL

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Summary:

A leishmanial parasite isolated in 1977 from a specimen of the sandfly *Lutzomyia tuberculata* from Pará State, Amazonian Brazil, has been characterized following its comparison with other species of *Leishmania* from the same region, using isoenzyme profiles, monoclonal antibodies and characterization of the mini-exon gene repeat, using the polymerase chain reaction technique (PCR). It is described here under the name of *Leishmania (Viannia) utingensis* n. sp.

KEY WORDS : *Leishmania (Viannia) utingensis* n. sp., *Lutzomyia tuberculata*, sandflies, Brazil.

Résumé : *LEISHMANIA (VIANNIA) UTINGENSIS* N. SP., PARASITE DU PHLÉBOTOME *LUTZOMYIA (VIANNAMYIA) TUBERCULATA* EN AMAZONIE BRÉSILIENNE

Une *Leishmania* isolée en 1977 d'un spécimen de *Lutzomyia tuberculata* de l'état de Pará, Amazonie brésilienne, est différenciée des autres espèces de *Leishmania* de la même région par les profils enzymatiques, les anti-corps monoclonaux et la caractérisation, par PCR, des mini-exons répétés dans les gènes. Elle est nommée *Leishmania (Viannia) utingensis* n. sp.

MOTS CLÉS : *Leishmania (Viannia) utingensis* n. sp., *Lutzomyia tuberculata*, phlébotome, Brésil.

INTRODUCTION

In 1977 a leishmanial parasite was isolated from a single specimen of the phlebotomine sandfly, *Lutzomyia (Viannamyia) tuberculata* (Mangabeira) (Fig. 1), taken from the trunk of a large tree in Utinga forest on the outskirts of Belém, Pará State, Amazonian Brazil. Initially thought to possibly be *L. (Viannia) braziliensis* (Lainson & Shaw, 1979), it was later listed among the "unnamed parasites of the subgenus *Viannia*" by virtue of its peripylarian development in the sandfly host, morphology, and behaviour in hamsters and *in vitro* culture (Lainson & Shaw, 1987). In the present paper, we give a more complete description of the organism and show it to differ from all those species of *Leishmania* previously recorded in the Amazon Region of Brazil, following characterization of the mini-exon gene repeat, using the polymerase chain reaction technique (PCR), isoenzyme electrophoresis profiles, and monoclonal antibodies (McAbs).

MATERIALS AND METHODS

MORPHOLOGY OF AMASTIGOTES AND PROMASTIGOTES

Following retrieval of the parasite (Register Number M4964) from our cryobank, it was maintained in Diffco B45 blood-agar culture medium (Walton *et al.*, 1977) and the skin of hamsters inoculated intradermally with promastigotes into the dorsal surface of the hind feet.

For amastigotes, animals were sacrificed eight days post inoculation (d.p.i.) and impression smears made from the skin excised from the point of inoculation. Preparations were air-dried, fixed in aqueous Bouin's fluid for 20 minutes, and washed repeatedly in 70 % ethyl alcohol until colourless. The smears were then stained for one hour by Giemsa's method, differentiated in a graded series of acetone-xylol mixtures and mounted under a cover-slip in "Permout"®.

Promastigotes of 7-day-old cultures were washed twice by centrifugation in pH 7.2 phosphate buffered saline (PBS) and the sediment used to prepare thin smears. These were air-dried, fixed in absolute methyl alcohol and stained by Giemsa's method. Measurements of 50 amastigotes and 40 promastigotes were made using the oil immersion lens, × 10 oculars and an eyepiece micrometer. All measurements are in µm and given as means, followed by the standard deviation and the range in parentheses. Photomicrographs were prepared using a Zeiss "Photomicroscope III" and Kodak TMX 100 film.

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Fig. 1. – *Lutzomyia* (*Viannamyia*) *tuberculata*. Freshly dissected female genitalia (spermathecae). Bar = 0.05 mm.

POLYMERASE CHAIN REACTION

The oligonucleotides used in the PCR were with the following sequences (Fernandes *et al.*, 1994): S 1629:5'-GGG-AAT-TCA-ATA-TAG-TAC-AGA-AAC-TG-3' and S 1630:5'-GGG-AAG-CTT-CTG-TAC-TTT-ATT-GGT-A-3'. Agarose gel (1 %) electrophoresis of mini-exon PCR amplification products and a molecular weight marker (Φ X 174 DNA/Hae III) were visualized following staining with ethidium bromide. A negative control, with no added DNA, was included.

ENZYME ELECTROPHORESIS

We used the enzymes ASAT, ALAT, PGM, GPI, MPI, G6PD, MDH, ACON, PEP and 6PGDH (for methods and abbreviations, see Miles *et al.*, 1980), and the profiles of isolate M4964 were compared with those of *L. (V.) braziliensis* (MHOM/BR/1975/M2903) from Serra dos Carajás, Pará; *L. (V.) braziliensis* sensu lato (MHOM/BR/1975/M2904) also from Serra dos Carajás; *L. (V.) guyanensis* (MHOM/BR/1975/M4147) from Monte Dourado, Pará; *L. (V.) lainsoni* (MHOM/BR/1981/M6426) from Benevides, Pará; *L. (V.) naiffi* (MDAS/BR/1979/M5533) from Monte Dourado, Pará; *L. (V.) shawi* (MCEB/BR/1984/M8408) from Parauapebas, Pará; *L. (Leishmania) amazonensis* (IFLA/BR/1967/PH8) from Utinga Forest, Belém, Pará; and *L. infantum chagasi* (MCER/BR/1981/M6445) from Salvaterra, Marajó island, Pará.

MONOCLONAL ANTIBODIES

The isolate was tested with 23 McAbs prepared against the above-listed *Leishmania* (*Viannia*) species responsible for cutaneous leishmaniasis in the Amazon Region of Brazil and neighbouring parts of South America

(McMahon Pratt *et al.*, 1986; Hanham *et al.*, 1991). They were used together with the indirect immunofluorescence/fluorescein-labelled avidin technique (Shaw *et al.*, 1989).

EXPERIMENTAL INFECTIONS IN LABORATORY-BRED SANDFLIES

Using the membrane-feeding technique of Ward *et al.* (1978), culture forms of both M4964 and *L. (V.) braziliensis* (M 2903) were separately fed to laboratory-bred *Lutzomyia* (*Lutzomyia*) *longipalpis* and *Lutzomyia* (*Viannamyia*) *furcata*. The sandflies were dissected five days later, when all the ingested blood had been digested.

RESULTS

BEHAVIOUR IN CULTURE MEDIUM

Growth of the parasite is rapid, with the production of abundant, large rosettes of dividing forms. The separate flagellates are small and extremely active – a characteristic of most of the species within the subgenus *Viannia* (Lainson & Shaw, 1987).

BEHAVIOUR IN THE SKIN OF HAMSTERS

Following the intradermal inoculation of enormous numbers of log-phase promastigotes, only a small number of free and intracellular amastigotes could be detected in the stained smears of skin excised from the site of inoculation eight days p.i. No visible skin lesion could be detected up to two months p.i., when parasites were encountered with great difficulty: in this respect the parasite resembles *L. (V.) naiffi* (Lainson &

Fig. 2. – *Leishmania (Viannia) utingensis* n. sp. Intracellular and free amastigotes in the skin of a hamster inoculated eight days previously with promastigotes. a-f. Division stages: one parasite (f), shows the conspicuous flagellar vacuoles of the two daughter amastigotes which are on the point of separation. g-k. Smaller products of division. Bouin fixation, Giemsa staining. Bar = 10.0 μ m.

Fig. 3. – *Leishmania (Viannia) utingensis* n. sp. Log-phase promastigotes from an 8-day culture in Diffco B45 blood-agar medium. a-b. Dividing forms. c-f. Small non-dividing flagellates. Methyl alcohol fixation, Giemsa staining. Bar = 10.0 μ m.

Shaw, 1989; Lainson *et al.*, 1990) and the newly described *L. (V.) lindenbergi* (Silveira *et al.*, 2002).

MORPHOLOGY OF AMASTIGOTES AND PROMASTIGOTES (Figs 2 and 3)

Mean measurement of amastigotes was $3.0 \pm 0.7 \times 2.1 \pm 0.3$ ($2.2\text{--}4.0 \times 1.5\text{--}2.2$), $n = 50$. The parasite shows no morphological features by which to differentiate it from most of the species in the subgenus *Viannia*, with which it shares the characteristic of very small, compact amastigotes in which the kinetoplast is typically large and positioned between the anterior end and the middle of the nucleus (Shaw & Lainson, 1976). The mean body measurement of the promastigotes was $8.1 \pm 2.0 \times 2.3 \pm 0.6$ ($5.2\text{--}11.1 \times 0.7\text{--}3.0$). The mean length of the free flagellum was 9.3 ± 2.7 , $n = 30$, but the wide range of 4.0–21 suggests that many flagella were broken during the washing process.

POLYMERASE CHAIN REACTION (Fig. 4)

Using the oligonucleotides S1629: 5' and S1630: 5' the PCR clearly shows that isolate M4964 belongs to the

subgenus *Viannia*, and differentiates it from the two representatives of the subgenus *Leishmania* examined, *Leishmania infantum chagasi* and *L. (L.) amazonensis*. Among species of the subgenus *Viannia*, the parasite could be separated from *L. (V.) lainsoni*, but not from *L. (V.) braziliensis*, *L. (V.) guyanensis*, *L. (V.) naiffi*, and *L. (V.) shawi*.

ISOENZYME PROFILES (Fig. 5)

Shaw *et al.* (1991) showed that failure of the enzyme ACON to migrate from its origin towards the negative pole was a means of separating parasites of the subgenus *Viannia* from those of the subgenus *Leishmania*, and the present result for this enzyme again supports the previous allocation of M4964 to the subgenus *Viannia*.

All the enzymes used separated M4964 from *L. infantum chagasi* and *L. (L.) amazonensis*, and the combined use of the seven enzymes ASAT, ALAT, PGM, G6PD, MPI, 6PGDH and MDH enabled separation of the parasite from all the known reference strains of species within the subgenus *Viannia*.

Fig. 4. – Agarose gel (1 %) electrophoresis of mini-exon PCR amplification products from neotropical *Leishmania* spp., visualized by ethidium bromide staining. Lane PM: molecular weight marker (Φ X 174 DNA/Hae III). Lane 1: *Leishmania (V.) braziliensis* sensu lato (M2904). Lane 2: *L. (V.) utingensis* n. sp. Lane 3: *L. (V.) shawi* (M8408). Lane 4: *L. (V.) guyanensis* M4147). Lane 5: *L. (V.) naiffi* (M5533). Lane 6: *L. (V.) lainsoni* (M6426). Lane 7: *L. (V.) braziliensis* (M2903). Lane 8: *L. (L.) amazonensis* (PH8). Lane 9: *L. infantum chagasi* (M6445). Lane CN: negative control (no DNA added). Lane PM: molecular weight marker.

Fig. 5. – Electrophoresis of the enzymes ASAT, ALAT, PGM, GPI, G6PD and ACON of *Leishmania* species from Amazonian Brazil. The parasites under comparison are:

1. *L. (L.) amazonensis* (PH8); 2. *L. (V.) braziliensis* (M2903); 3. *L. (V.) guyanensis* (M4147); 4. *L. (V.) shawi* (M8408); 5. *L. (V.) lainsoni* (M6426); 6. *L. (V.) naiffi* (M5533); 7. *L. (V.) braziliensis* sensu lato (M2904); 8. *L. (V.) utingensis* n. sp., (M4964); 9. *L. (L.) infantum chagasi* (M6445) and 10. *L. (L.) amazonensis* (PH8).

Species of <i>Leishmania</i>	Monoclonal antibodies										
	B2	B12	B5	B18	B19	N2	N3	M2	W1	WA2	LA2
<i>L. (V.) braziliensis</i>	+	+	+	+	−	+	−	−	−	−	−
<i>L. (V.) guyanensis</i>	+	+	−	−	+	−	−	−	−	−	−
<i>L. (V.) shawi</i>	+	+	−	−	−	−	−	−	−	−	−
<i>L. (V.) naiffi</i>	+	+	−	−	−	+	+	−	−	−	−
<i>L. (V.) lainsoni</i>	−	−	−	−	−	−	−	−	−	−	+
<i>L. (V.) utingensis</i> n. sp	+	−	−	−	−	+	−	−	−	−	−
<i>L. (V.) lindenbergi</i>	+	+	−	−	−	+	−	−	−	−	−

Table I. – Comparison of the serodeme profiles of *Leishmania (Viannia) utingensis* n. sp. with other species of the subgenus *Viannia* from the Amazon Region of Brazil.

MONOCLONAL ANTIBODIES (Table I)

The subgeneric position of the parasite was once more confirmed, by the McAb B2 which is specific for the taxon *Viannia* (McMahon-Pratt *et al.*, 1982, 1985). Among the known species within the subgenus, it was distinguished from *L. (V.) braziliensis* and *L. (V.) guyanensis* by its failure to react with McAbs B18 and B19, respectively; from *L. (V.) naiffi* by its non-reaction with McAb N3, which recognizes that parasite; from *L. (V.) shawi* which, unlike M4964, does not react against McAb N2; from *L. (V.) lainsoni* by its failure to react with McAb LA2, specific for that parasite, and its positive reaction to McAb B2 to which *L. (V.) lainsoni* does not react; and from *L. (V.) lindenbergi* (Silveira *et al.*, 2002) which reacts with McAb B12, whereas M4964 does not.

In conclusion, M4964 can be distinguished from other member of the subgenus *Viannia* by its unique serodeme, as determined by monoclonal antibodies, and a combination of the profiles for seven different enzymes. Of particular note is the fact that mobility of the parasite's ASAT is similar to that of *L. (V.) braziliensis* and faster than that of *L. (V.) lindenbergi*, while mobility of its G6PD is intermediate between that of *L. (V.) braziliensis* and *L. (V.) naiffi* and *L. (V.) lindenbergi*. The G6PD of the latter parasite migrates less than any other named species of the subgenus *Viannia*. As a result of the present study we propose the name of *Leishmania (Viannia) utingensis* n. sp. for the isolate M4964, which has for so long remained incognito.

DEVELOPMENT IN EXPERIMENTALLY INFECTED SANDFLIES

In *Lutzomyia (Lutzomyia.) longipalpis*, both *L. (V.) braziliensis* and *L. (V.) utingensis* n. sp. showed typical peripylarian development in both the pylorus and ileum. In *Lutzomyia (Viannamyia) furcata*, *L. (V.) utingensis* n. sp. underwent a similar development, whereas *L. (V.) braziliensis* showed attached flagellates only in the ileum.

SPECIFIC DIAGNOSIS

LEISHMANIA (VIANNIA) UTINGENSIS N. SP

Type host: the phlebotomine sandfly *Lutzomyia (Viannamyia) tuberculata* (Diptera: Psychodidae: Phlebotominae).

Type locality: Utinga forest, on the outskirts of Belém, Pará State, North Brazil.

Strain designation: ITUB/BR/1977/M4964.

Amastigotes: non-dividing parasites small and compact, $3.0 \pm 0.7 \times 2.1 \pm 0.3$ ($2.2-4.0 \times 1.5-2.2$), $n = 50$. Promastigotes: small. Body of non-dividing parasites $8.1 \pm 2.0 \times 2.3 \pm 0.6$ ($5.2-11.1 \times 0.7-3.0$), $n = 40$. Mean length of free flagellum 9.3 ± 2.7 , ranging from 4.0-21.0, $n = 30$.

Behaviour in sandfly host: prolific peripylarian development, characteristic of the subgenus *Viannia*, with promastigotes and paramastigotes predominantly attached to the wall of the pylorus.

Behaviour in hamster: no visible lesion produced at the site of intradermal inoculation two months p.i. Amastigotes rare or undetectable in the skin, but their presence can be confirmed by culture in blood-agar medium.

Behaviour in *in vitro* culture: exuberant growth with abundant, large rosettes.

Isoenzyme profiles: the combined use of enzymes ASAT, ALAT, PGM, G6PD, MPI, 6PGDH and MDH distinguishes the parasite from other members of the subgenus *Viannia*.

Monoclonal antibodies: major epitopes in distinguishing *L. (V.) utingensis* n. sp. from other members of the subgenus *Viannia* are B12, B18, B19, LA2, N2 and N3. Type material: hapantotype slides of amastigotes and promastigotes in RL's collection, in the Instituto Evandro Chagas. Promastigotes and amastigotes maintained in the Institute's cryobank.

Etymology: the specific name is derived from that of the forest (Utinga) in which the infected sandfly was captured.

DISCUSSION

In addition to the above discussed characteristics separating *L. (V.) utingensis* from other species of the subgenus *Viannia*, it is of interest that in *Lutzomyia furcata* the parasite developed abundant attached forms in the pylorus and ileum, whereas *L. (V.) braziliensis* produced attached forms only in the ileum. This suggests differences in the lectin receptors of the two species.

Lutzomyia (Viannamyia) tuberculata was first described from the forest of Aurá, on the outskirts of Belém, Pará (Mangabeira, 1941), and the sandfly has subsequently been recorded in many other areas of north Brazil, French Guyana, Surinam, Venezuela, Colombia and Panamá (Young & Duncan, 1994). It is predominantly a forest species and is commonly taken from the trunks of the larger trees. It is likely, therefore, that the mammalian host of *L. (V.) utingensis* is an arboreal animal.

To date, the parasite has not been found in man, and as *Lutzomyia tuberculata* shows no anthropophilic tendencies it is unlikely that this sandfly plays any part in the epidemiology of American leishmaniasis. It nevertheless remains possible that other sandflies are hosts of *L. (V.) utingensis* n. sp., and if these include an anthropophilic species it may well be that this parasite may find its way to man. In this respect, it is noteworthy that the parasite developed abundant attached forms in the pylorus and ileum of experimentally infected *Lutzomyia (Lutzomyia) longipalpis*, and that we have on several occasions encountered heavy peripylarian promastigote infections in wild-caught specimens of another member of the same subgenus, *Lutzomyia (Lutzomyia) gomezi*, captured in the forests of Pará (Lainson & Shaw, 1979 and unpublished observations). Unfortunately, we have consistently failed to isolate this parasite which, like *L. (V.) utingensis* n. sp., did not produce a visible lesion in the skin of inoculated hamsters. Unidentified promastigotes were recorded in a single specimen of *Lutzomyia gomezi* taken from a tree trunk in Colombia (Young *et al.*, 1987) and, as this sandfly feeds avidly on man, it remains a suspected vector of *Leishmania* throughout its wide geographic range in Latin America. It might equally well be argued, however, that the development of abundant attached promastigotes of *L. (V.) utingensis* in the pylorus of two members of the subgenus, *Viannamyia* (*Lutzomyia tuberculata* and *Lutzomyia furcata*) indicates a specificity of *L. (V.) utingensis* for sandfly species of this group of sandflies. Only continued attempts to identify the parasite of *Lutzomyia gomezi* and the examination of further specimens of *Lutzomyia tuberculata* and other members of the subgenus *Viannamyia* will answer this question.

ACKNOWLEDGEMENTS

To Constância M. Franco, Yara L.L. Jennings, Iorlando R. Barata, Raimundo N. Barbosa Pires and Antonio J. de Oliveira Monteiro for technical assistance.

This work formed part of an MSc thesis (RRB), Centro de Ciências Biológicas da Universidade Federal do Pará, Brasil and received the support of Grant 049426 from the Wellcome Trust, London (RL).

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Reçu le 18 février 2003

Accepté le 4 mars 2003