Occurrence of *Leishmania major* in sandfly urine

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SUMMARY

Promastigotes of *Leishmania major* were frequently detected in the urine droplets discharged by infected *Phlebotomus papatasi* and *P. duboscqi* females during feeding. Parasites were present in the urine of 37·5% *P. papatasi* and 16·1% *P. duboscqi* females, even in those with low intensity gut infections. Free-swimming forms (elongated nectomonads, short slender promastigotes and metacyclic forms) predominated in excreted droplets. Viability of excreted parasites was proved by cultivation on blood agar, and the presence of metacyclic forms in urine droplets was confirmed by specific fluorescence assay with 3F12 antibodies. While the release of promatigotes from the anus of the sandfly was frequent, these were rarely egested from the mouth-parts of sandfly females (1·3% for *P. duboscqi* and 0% for *P. papatasi*) fed on microcapillaries, even if the females were heavily infected. The possible role and significance of the discharge of parasites in sandfly urine are discussed.

Key words: *Phlebotomus* spp., transmission, urine, prediuresis, capillary feeding, metacyclic promastigotes.

INTRODUCTION

In the suprapylarian *Leishmania* spp., including *L. major*, part of the life-cycle takes place both in the midgut and foregut of the sandfly vector. Amastigotes ingested with a bloodmeal transform into promastigotes which attach to the midgut wall and subsequently migrate to the stomodeal valve, oesophagus, pharynx and, eventually, to the mouth-parts (for review see Killick-Kendrick, 1990; Schlein, 1993).

The location of parasites in the sandfly digestive tract is of crucial importance for the transmission to the vertebrate host. In this respect, 2 main hypotheses have been suggested: either parasites emerge from an infected sandfly during feeding only if the proboscis itself is infected (Adler & Theodor, 1935) or they are regurgitated with a backflow of ingested blood. Originally, the regurgitation was supposed to result from a mechanical block of the foregut (Shortt & Swaminath, 1928) or stomodeal valve (Warburg & Schlein, 1986) by the parasites. More recently, the damage to the chitin layer of the stomodeal valve by chitinolytic enzymes produced by the parasites was proposed to be the cause of the regurgitation of parasites from the thoracic midgut (Schlein, Jacobson & Shlomai, 1991; Schlein, Jacobson & Messer, 1992). A third possible way of transmission, the inoculation of parasites into the host skin with sandfly saliva, is supported by the study of Killick-Kendrick *et al.* (1996), who found metacyclic promastigotes of *L. tropica* invading salivary glands of *P. duboscqi*. Contaminative transmission by ingestion of infected sandfly takes place in the genus *Sauroleishmania* (Lainson & Shaw, 1987) and probably occurs in transmission of *Leishmania* to dogs (reviewed by Killick-Kendrick, 1979). In man, contaminative transmission may occur occasionally when an infected biting fly is crushed on the skin (Adler, 1929).

In the present work we studied another possible route of contaminative transmission: the discharge of parasites in sandfly urine. The term 'urine' is used here according to Clements (1992) for the excretory fluid discharged to the exterior during female's feeding on the vertebrate host. In mosquitoes the rapid anal excretion of urine during feeding is called prediuresis (Briegel & Rezzonico, 1985). This phenomenon enables the female to concentrate proteins of the bloodmeal and both physical (pyloric armatures) and metabolic (active transport into Malpighian tubules) filtrations are probably involved (Vaughan, Noden & Beier, 1991). In sandflies, prediuresis was observed in the majority of blood-feeding females studied (Short & Swaminath, 1928; Sádlová, Reishig & Volf, 1999) and previous study of *P. papatasi* and *P. duboscqi* (Sádlová *et al.* 1999) showed that first droplets ejected contained erythrocytes in 25% and 10% of females, respectively.

MATERIALS AND METHODS

Sandfly infections

Phlebotomus papatasi (Cyprus strain) and *P. duboscqi* (Senegal strain) obtained from Professor Killick-

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Kendrick, Imperial College in Ascot, were maintained on 50% sucrose at 25–26 °C and 14/10 LD photo-period.

Leishmania major, LV 561 strain (MHOM/IL/67/LRC-L 137 Jericho-II) was maintained *in vitro* at 23 °C on SNB-9 blood agar (Diamond & Herman, 1954) and, concurrently, in BALB/c mice. Parasites isolated from the mouse were used for sandfly infection after 1–3 *in vitro* subcultures.

P. duboscqi and *P. papatasi* females were fed through a chick-skin membrane with infective food containing either 2×10^6 promastigotes or 1×10^6 amastigotes/ml. Promastigotes from 4-day-old cultures were resuspended in heat-inactivated human blood. Amastigotes derived from mouse lesions were resuspended in human erythrocytes mixed (1:1 v/v) with Schneider's medium supplemented with gentamicin (45 μg/ml).

Examination of urine droplets and capillary contents

Microcapillary feeding (Hertig & McConnell, 1963) was used for testing whether parasites are ejected from mouthparts. Simultaneously, the urine droplets discharged during the feeding were examined. In the first series of experiments, 235 flies were fed on microcapillaries containing SNB-9 (over-lay medium for blood agar) 10 or 14 days after an infective bloodmeal. During capillary feeding, the excreted droplets were caught on a piece of a cover-slip. Immediately after feeding, contents of capillaries were blown onto slides and flies were dissected for detection of *Leishmania* infections. Infected flies (150 females) were the subjects of further analysis.

In the second series of experiments, 100 females were allowed to feed on the capillary for 1·5–2 min only, starting from the first sucking movements of the clypeus. Dissection revealed that 53 females were infected. While during such short feeding time urine excretion usually did not start, only capillary contents were examined.

Amastigote-infected females were fed on capillaries 14 days after infection and excreted droplets were examined for parasite presence as described above.

Parasite morphometry

Capillary contents and urine droplets from infected flies were air dried, fixed with methanol and stained with Giemsa. The body length and width and the flagellum length of parasites were measured under the light microscope with an oil-immersion objective. However, the length of flagellum and the position of the kinetoplast were not visible in some parasites due to the dense background produced by the excreted urine. Therefore, out of the 6 morphological categories defined by Čiháková & Volf (1997) three (short broad promastigotes, rounded promastigotes and paramastigotes) were lumped together into one designated as 'broad forms' (BF). The other 3 categories, i.e. elongated nectomonads (EN), short slender promastigotes (SP1), and metacyclic promastigotes (MP) remained as described previously. Parasites with flagellum which cannot be measured were not classified. Data from gut smears (day 9 after the infective meal; Čiháková & Volf, 1997) were used for comparison of *Leishmania* morphometry in the midgut versus that excreted in droplets.

Leishmania viability test

To examine the viability of discharged parasites, infected *P. duboscqi* females were placed for capillary feeding on the top of a multiwell plate (GAMA n.p. České Budějovice). During capillary feeding, the urine was directed into the wells filled with blood agar and an over-lay medium. The contents of the wells were examined daily for parasites.

Immunofluorescence detection of metacyclic LPG

Excreted droplets of the urine were dried, fixed with methanol and incubated for 30 min with the monoclonal antibody 3F12 specific for the metacyclic stage lipophosphoglycan (Sacks & Da Silva, 1987). Mouse ascitic fluid was diluted 1:20 in 20 mM Tris buffer with 0·1% Tween 20 (Tris–Tw) and 5% fetal calf serum. After washing, slides were incubated for 30 min with FITC-conjugated swine anti-mouse IgG (SEVAC, Prague) diluted 1:20 in Tris–Tw, washed and stained for 2 min with Evans Blue. Positive and negative promastigotes were then detected by fluorescence microscopy using an oil immersion objective. Smears made from 6-day-old cultures of LV561 were used as a positive control. In negative controls, the antibody-incubation step was omitted.

Statistical analysis

All the statistical evaluations were made using the Statgraphics 5.0 programme.

RESULTS

Detection of Leishmania *parasites in urine*

Urine droplets of promastigote-infected sandflies contained promastigotes in 37·5% and 16·1% females of *P. papatasi* and *P. duboscqi*, respectively (Table 1). The difference between these two species is significant after log-linear analysis ($\chi^2 = 4{\cdot}2594$, D.F. = 1, $P = 0{\cdot}0390$) even when the results of individual experiments in one series also differed significantly ($\chi^2 = 34{\cdot}6375$, D.F. = 11, $P = 0{\cdot}0003$). On the other hand, the difference among the actual

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Table 1. Representation of *Leishmania* parasites in urine droplets of *Phlebotomus papatasi* and *P. duboscqi*

Vector species	Time post-infective bloodmeal		
	10th day	14th day	Total
P. papatasi			
Number of infected flies examined	27	13	40
Number (%) of flies with positive droplets	10 (37)	5 (38·5)	15 (37·5)
P. duboscqi			
Number of infected flies examined	31	31	62
Number (%) of flies with positive droplets	8 (25·8)	2 (6·5)	10 (16·1)

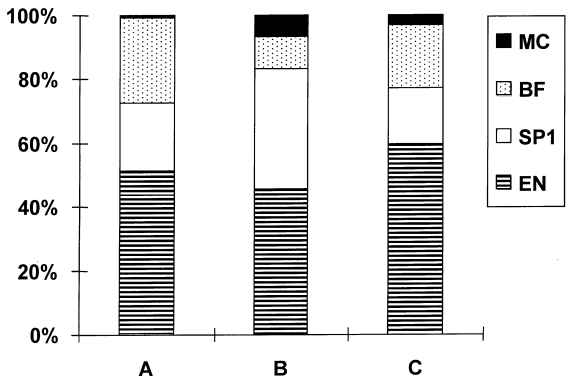

Fig. 1. Relative proportions of morphotypes from smears of gut contents (A) and from excreted droplets 10th (B) and 14th (C) day post-infection in both vector species. EN, elongated nectomonads; SP1, short slender promastigotes; BF, broad forms; MC, metacyclic promastigotes.

numbers of excreted parasites on the 10th and 14th days after infection was not statistically significant ($\chi^2 = 2{\cdot}7924$, D.F. = 1, $P = 0{\cdot}0947$).

In *P. papatasi*, the number of excreted parasites varied from 2 to 125 per female (average 21, median 8 parasites). The range of *P. duboscqi* was 1–260 (average 44, median 11 parasites per female). Parasites were concentrated in the first one or the first few ejected droplets.

Although the majority of females excreting parasites showed heavy (more than 500 parasites per gut) or medium (100–500 parasites per gut) infection, we detected parasites in urine droplets of 2 *P. papatasi* females with as few as 2 or 20 visible parasites in the gut.

Parasites were also detected in the urine of females infected by amastigotes on day 14 after the infective bloodmeal.

Capillary contents

In the first series of experiments parasites were ejected into a capillary by 1 *P. duboscqi* female (1·3 % of infected flies, $n = 79$) and no parasites were detected in capillary contents of infected *P. papatasi* females ($n = 71$). In the second series of experiments, when females were capillary fed for 1·5–2 min only, no parasites were found in 34 infected females of *P. duboscqi* and 19 infected females of *P. papatasi*.

Morphometrical analysis

All parasites found in capillaries ($n = 9$) were metacyclic promastigotes (MP). On the other hand, different morphological forms were detected in urine droplets. The relative proportion of individual forms in urine droplets (Fig. 1) was analysed by log-linear analysis. It differed significantly between the 10th and 14th day of examination ($\chi^2 = 15{\cdot}7854$, D.F. = 3, $P = 0{\cdot}0003$). Differences between the two *Phlebotomus* species were not significant ($\chi^2 = 4{\cdot}0705$, D.F. = 3, $P = 0{\cdot}3814$). The morphometric data of distinct forms are given in Table 2.

Comparison of morphological forms excreted in urine 10 days post-infection versus that in females midgut shows that MP and SP1 were significantly more represented in urine (SP1: $\chi^2 = 7{\cdot}7038$, D.F. = 1, $P = 0{\cdot}0055$; MP: $\chi^2 = 4{\cdot}2458$, D.F. = 1, $P = 0{\cdot}0393$). In contrast, BF were more represented in gut smears ($\chi^2 = 10{\cdot}5090$, D.F. = 1, $P = 0{\cdot}0012$) and proportions of EN differed non-significantly ($\chi^2 = 0{\cdot}7154$, D.F. = 1, $P = 0{\cdot}3976$).

Corresponding morphological differences were obtained when measurements were evaluated for the total material (ignoring morphological categories) using a *t*-test. Excreted promastigotes were shorter ($t = 3{\cdot}357$, $P = 0{\cdot}0008$) and more slender than gut forms: the difference in body width was non-significant in *P. duboscqi* ($t = 1{\cdot}551$, $P = 0{\cdot}1236$) while highly significant in *P. papatasi* ($t = 5{\cdot}309$, $P < 0{\cdot}0001$). Almost no difference was found in flagellar lengths ($t = 0{\cdot}238$, $P = 0{\cdot}8116$).

Cultivation of excreted parasites

The cultivation confirmed that parasites excreted by *P. duboscqi* were viable. The portion of positive

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Table 2. Dimensional morphology of four *Leishmania major* developmental stages excreted in droplets and emerged into the capillary (*)

(EN, elongated nectomonads; SP1, short slender promastigotes; BF, broad forms; MC, metacyclic promastigotes.)

Morphological forms	10 days post-infection					14 days post-infection				
	n	Mean	S.D.	Min	Max	*n*	Mean	S.D.	Min	Max
Body width (μm)										
EN	62	1·8	0·77	0·7	4·3	61	2·3	0·86	1·4	4·3
SP1	51	1·5	0·28	0·7	2·1	18	1·5	0·23	1·4	2·1
BF	14	3·6	1·34	2·9	7·1	20	3·4	0·70	2·9	4·3
MC	9	1·3	0·31	0·7	1·4	3	1·4	0·00	1·4	1·4
MC*	9	0·7	0·00	0·7	0·7	—	—	—	—	—
Body length (μm)										
EN	62	15·7	2·29	14·3	22·8	61	15·6	1·74	14·3	20·0
SP1	51	10·8	1·88	7·1	12·9	18	10·3	2·05	5·7	12·9
BF	14	8·4	2·56	4·3	12·9	20	11·6	3·68	4·3	20·0
MC	9	8·4	1·51	5·7	10·0	3	7·6	1·65	5·7	8·6
MC*	9	9·0	1·01	7·1	10·0	—	—	—	—	—
Flagellar length (μm)										
EN	62	17·6	4·14	7·1	28·6	61	15·2	2·64	10·0	22·8
SP1	51	13·4	3·58	7·1	21·4	18	11·3	2·21	7·1	14·3
BF	14	12·6	4·19	4·3	17·1	20	9·5	2·33	5·2	12·9
MC	9	18·2	4·39	12·9	25·7	3	16·7	1·65	15·7	18·6
MC*	6	20·2	2·1	17·1	22·8	—	—	—	—	—

cultures from sandfly urine (8·3 %, $n = 12$) was lower than the percentage of females with positive urine droplets after Giemsa staining (16·1 %). However, fungal contamination occurred in some cultures and this probably contributed to the underestimation of the presence of viable flagellates.

Detection of metacyclic LPG

The presence of metacyclic promastigotes in the sandfly urine was confirmed by the positive reaction of monoclonal antibodies 3F12 with some parasites. Exact counting of metacyclic forms was, however, hampered in some preparations by non-specific reaction of fluorescein conjugates with excreted liquid. In the control, monoclonal antibodies reacted positively with 17 % of promastigotes from 6-day-old cultures.

DISCUSSION

Recent knowledge implies that not only one, but several modes could be involved in *Leishmania* transmission by sandflies. These may act simultaneously or may be specific for individual vector–parasite species combinations.

From the evolutionary point of view the contaminative route of transmission (passing of infective forms in the excreta) is probably the original route of transmission occurring in monogenetic flagellates. It takes place also in some digenetic trypanosomatids, like in *Trypanosoma lewisi* transmitted by the rat flea or *T. cruzi* transmitted by reduviids. In these cases, the development of parasites in the hindgut enables the contaminative transmission. In 'peripylarian' *Leishmania* species, colonization of the hindgut is maintained prior to migration of promastigotes to the midgut and foregut, which has been interpreted as a recapitulation of the primitive hindgut development of ancestral flagellates (Lainson & Shaw, 1987). In suprapylarian spp., the hindgut development was completely lost. Therefore, the contaminative transmission was thought to be non-functional in the *Leishmania*–sandfly system.

Transmission of *Leishmania* species by sandfly excreta was rejected by Adler (1929) as '*P. papatasi* very seldom defecates during the act of feeding'. However, our recent study showed that 100 % of *Phlebotomus papatasi* and 85 % of *P. duboscqi* females discharged urine during the feeding on human arm (Sádlová *et al*. 1999). Shortt & Swaminath (1928) first described frequent discharge of droplets from the anus of blood-feeding *P. argentipes*. They, however, failed to demonstrate *L. donovani* in droplets and concluded: 'It seems unlikely that the habit of the fly here being discussed can have any bearing on the transmission of this parasite'. On the other hand, living promastigotes were occasionally found in urine droplets of *Lutzomyia youngi* infected by peripylarian species *L. braziliensis* (Y. Tang, personal communication).

In the present study, parasites were found more often in urine than in capillary contents. They were never ejected into the capillary by *P. papatasi*, and only once by *P. duboscqi*. This may correspond with the previously described absence of parasites of LV561 line in the foregut and proboscis of *P. papatasi* and their exceptional occurrence in the

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proboscis of *P. duboscqi* (data shown in Čiháková & Volf, 1997). Similarly, Adler & Theodor (1935) found that *L. infantum* parasites emerge from an infected *P. perniciosus* into the capillary only if the distal part of the proboscis is infected (2 from 52 heavily infected flies in their experiment). An alternative explanation for the low percentage of females ejecting promastigotes into the capillary might be the fact that gorging females only were studied. As described by Warburg & Schlein (1986), *P. papatasi* females which failed to engorge, regurgitated fluid containing *L. major* parasites into capillaries more often than did the feeding ones.

Parasites ejected by *P. duboscqi* into the capillary were morphologically classified as metacyclic forms which corresponds with the data obtained in other parasite–vector combinations. These forms, highly infective for vertebrate host, were selectively egested during the 'forced feeding' also in *L. infantum* – *P. perniciosus* (Adler & Theodor, 1931) and *L. major* – *P. papatasi* (Warburg & Schlein, 1986; Saraiva *et al*. 1995).

Metacyclics were shown to represent an abundant form of promastigotes in the midgut content (Davies *et al*. 1990; Saraiva *et al*. 1995). The present study showed that free-swimming developmental stages including metacyclic promatigotes were even more frequently represented in excreted droplets than in the midgut. On the contrary, broad forms, likely to be attached to the gut by hemidesmosomes were found in urine droplets in lower proportion than in the midgut.

Our findings indicate a certain potential for the contaminative transmission of *Leishmania* and further study is needed to evaluate this possibility. Transmission by bite is certainly the predominant route by which promastigotes enter the host. However, experiments with capillary feeding suggest that it could be successful only in heavily infected flies when the stomodeal valve is heavily colonized by promastigotes and the foregut infection is established. Contaminative transmission, on the other hand, would not be so much conditioned by the number or the localization of parasites. Parasites occurred in urine even in females with low intensity of midgut infections.

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