

Monitoring the susceptibility of *Glossina palpalis gambiensis* and *G. morsitans morsitans* to experimental infection with savannah-type *Trypanosoma congolense*, using the polymerase chain reaction

S. RAVEL*, P. GRÉBAUT*, C. MARIANI*, V. JAMONNEAU*, D. CUISANCE†, R. H. GOODING‡ and G. CUNY*

*IRD, UR035, Laboratoire de Recherche et de Coordination sur les Trypanosomoses IRD–CIRAD, TA 207/G, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

†CIRAD, Programme Econap, TA 30/F, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

‡Department of Biological Sciences, The University of Alberta, Edmonton, Alberta, T6G 2E9, Canada

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General *Glossina palpalis gambiensis* and *G. morsitans morsitans* (Diptera: Glossinidae) were fed on mice infected with savannah-type *Trypanosoma (Nannomonas) congolense*. The infection was monitored by checking the post-feeding diuresis fluid (midgut infection) and saliva (mature infection) of individual flies for parasites, at different times post-infection, using microscopical examination and a PCR-based assay. The results indicated that both tsetse species supported established midgut infections by 10 days post-infection and that maturation occurred after 24 days in *G. m. morsitans*. Although, for both diuresis fluid and saliva, the results of the microscopy showed good concordance with those of the PCR, the PCR identified more positive samples. Monitoring allowed determination of the status of the infection in individual flies, which was confirmed, 48 days post-infection, by the microscopical examination of the midguts and probosces dissected out of the flies and by the PCR-based amplification of any trypanosome DNA in these organs. Again, in terms of the detection of trypanosomes in the dissected organs, there was good concordance between the results of the PCR and those of the microscopy, although PCR revealed many more mature infections than did microscopical examination, particularly in the *G. p. gambiensis* investigated.

There was a higher prevalence of immature infection in *G. p. gambiensis* than in *G. m. morsitans* ($P < 0.05$) but the inter-specific differences seen in the prevalences of any infection and of mature infection were not statistically significant. The intrinsic vectorial capacity for *T. congolense* of both tsetse species therefore appeared quite similar, although the true vectorial competence of *G. p. gambiensis* remains to be determined.

Trypanosoma (Nannomonas) congolense is one of the most economically important trypanosomes. The animal trypanosomiasis it causes in sub-Saharan Africa seriously limits use of large areas for livestock production. The savannah type of *T. congolense* is the most

prevalent in cattle (Nyeko *et al.*, 1990; Reifenberg *et al.*, 1997b; Solano *et al.*, 1999) and mainly transmitted, cyclically, by tsetse flies.

Specific associations between particular species of trypanosomes and various tsetse species and subspecies have been suspected for many years. For example, field and laboratory observations indicate that tsetse flies in the *morsitans* group are the major

Reprint requests to: S. Ravel.
E-mail: ravel@mpl.ird.fr; fax: +33 4 67 59 39 20.

vectors for trypanosomes in the subgenus *Nannomonas* (Harley and Wilson, 1968; Roberts and Gray, 1972; Moloo and Kutuza, 1988; Van den Bossche, 2000), and that members of the *palpalis* group are poor vectors of *T. congolense* (Willett *et al.*, 1964; Reifenberg *et al.*, 1997a; Kazadi, 2000). Such tsetse–trypanosome associations influence the vectorial capacity of the tsetse, which is one component that determines transmission risk. Vectorial capacity depends on several factors (Molyneux, 1980; Welburn and Maudlin, 1999), which are generally classified (Reisen, 1989) as intrinsic (i.e. vectorial competence) or extrinsic (i.e. ecological). The vectoring ability of tsetse flies is linked to the life-cycle of *Trypanosoma*, which is divided into two phases within the fly: establishment in the midgut and maturation. In *T. congolense*, maturation occurs in the mouthparts. Progression from establishment to maturation is not automatic and only a portion of flies with midgut infections develops mature infections (Maudlin and Welburn, 1994). The kinetics of the establishment of the midgut infection and, more importantly, the kinetics of the maturation of trypanosome infections in tsetse should play an important role in transmission. It seems reasonable to assume, because of the short life-span of tsetse, that the longer the time taken for maturation of midgut infections in a particular species, the less important that species will be for transmission of the trypanosome in the field.

The present, experimental study had three main objectives. Firstly, to compare the vectorial competence of *Glossina palpalis gambiensis* with that of *G. morsitans morsitans*, with respect to *T. congolense* of the savannah type. Secondly, to characterise the infection in terms of the establishment and maturation times of *T. congolense* in each tsetse species, by monitoring the course of the infection in individual flies. Lastly, to compare a PCR-based assay with microscopical examination, as methods for determining the course of the infection in individual flies and the prevalences of infection in both tsetse species.

MATERIALS AND METHODS

Tsetse Flies

The *G. p. gambiensis* and *G. m. morsitans* used were from colonies, maintained at the CIRAD–EMVT (*Centre de Coopération Internationale en Recherche Agronomique pour le Développement: Département Élevage et Médecine Vétérinaire*) in Montpellier, France, derived from flies collected in Burkina Faso and Zimbabwe, respectively.

Trypanosome

The *T. congolense* clone E325 (savannah type) used was isolated from wild *G. pallidipes* caught in Uganda (Uilenberg *et al.*, 1973).

Infection of Mice and Tsetse Flies

The stabilate of *T. congolense* E325 was thawed and injected intraperitoneally, at 0.5 ml/mouse, into eight BALB/c mice. The murine infections were monitored by examining samples of tail blood by phase-contrast microscopy at $\times 400$. When the parasitaemias reached 3×10^7 – 10^8 trypanosomes/ml, the teneral flies were fed on the bellies of the mice (*G. p. gambiensis* on four mice and *G. m. morsitans* on the other four). The engorged flies were placed in individual cages and maintained, at 23°C, by feeding on uninfected rabbits (one for each tsetse species) until the end of the experiment. Both rabbits were monitored for parasites twice a week, by examining — again by phase-contrast microscopy at $\times 400$ — buffy coats (Murray *et al.*, 1977) prepared from blood samples taken from the ear. The feeding of the experimental flies was supervised by a veterinarian, in accordance with the French rules for the use of animals in experiments. Flies of the same age that had not fed on an infected mouse were maintained on other rabbits, as negative controls.

Monitoring the Tsetse Infections

To assess the development of the trypanosome infections in the tsetse, samples were

collected from the infected and negative-control flies by taking advantage of two aspects of tsetse physiology: the tendency of hungry tsetse flies to probe and salivate onto warm objects (Burt, 1946) and the diuresis that occurs immediately after feeding (Lester and Lloyd, 1928). Parasites in the fluid produced during post-feeding diuresis ('diuresis fluid') indicate midgut infections whereas parasites in the saliva indicate mature infections. On days 10, 14, 17, 24, 30 and 43 post-infection, each fly was first offered a drop of phosphate-buffered-saline-glucose (Burt, 1946; Gidudu *et al.*, 1995) on a warm slide (37°C), to salivate into, and then a live rabbit on which it could take a bloodmeal. The diuresis fluid each fly expelled while taking a bloodmeal was collected on another slide. The samples of saliva and diuresis fluid were examined by phase-contrast microscopy ($\times 400$) and then checked for trypanosome DNA in a PCR-based assay (see below). Infection was also assessed by dissecting each fly 48 days after the infective feed, and subjecting the midgut and proboscis to both phase-contrast microscopy and the PCR-based analysis. Flies that died after day 10 of the experiment were also dissected (as soon as they had been found dead) and examined for trypanosome infection.

PCR

Each tsetse sample (saliva, diuresis fluid, midgut or proboscis) was suspended in 30 μ l sterile water and then incubated, for 1 h at 56°C and then 30 min at 95°C, with 30 μ l 5% Chelex 100 resin (Bio-Rad, Hercules, CA). The suspension was then centrifuged (10,000 $\times g$, 5 min) and 10 μ l of the supernatant solution so produced were used for the subsequent DNA amplification. PCR was carried out using a DNA thermocycler (MJ Research, Cambridge, U.K.) in a 50- μ l reaction mixture containing 10 pmol of each primer (TCS1 and TCS2; Masiga *et al.*, 1992), 0.2 mM of each deoxyribonucleotide, 1 \times incubation buffer with 1.5 mM MgCl₂ and 0.5 U Taq polymerase (QBIogene, Ilkirch,

France). Samples were initially denatured at 94°C for 3 min then processed through 45 cycles, each having a denaturation step at 94°C for 30 s, an annealing step at 55°C for 30 s and an extension step at 72°C for 1 min. The final elongation step was lengthened to 5 min. The amplification products were checked by electrophoresis in 2%-agarose gels and visualised by ethidium-bromide staining under ultra-violet light.

RESULTS

Of the 87 *G. p. gambiensis* (45 males and 42 females) and 78 *G. m. morsitans* (37 males and 41 females) that fed on the infected mice, 22 *G. p. gambiensis* (13 males and nine females) and 11 *G. m. morsitans* (six males and five females) died within a few days and before they had chance to establish a midgut infection. The present data relate only to the 132 flies that survived for at least 10 days after the infective feed.

Monitoring

The samples of saliva and diuresis fluid collected from the negative-control flies were never found positive by microscopy or PCR. At the time the tsetse flies were first examined for *T. congolense* (i.e. 10 days post-infection), trypanosomes were detected, mainly by PCR, in the diuresis fluid of six flies (three of each species) that had fed on infected mice. Trypanosomes were found in the diuresis fluid of 20 other flies on days 14 or 17, and consistently thereafter until the final dissection. All the parasites observed by microscopy appeared immotile. Parasites were detected, by PCR only, in the saliva of two *G. m. morsitans* from day 24 until the final dissection of the flies. Trypanosomes were found in the saliva of six additional *G. m. morsitans* from day 30 onwards (two positive by both PCR and microscopy and four by PCR only). No trypanosomes were found in the saliva of any *G. p. gambiensis*, either by microscopy or PCR.

There was a good level of concordance between the results of the PCR and those of the microscopy with regard to detection of *T. congolense* in the saliva and diuresis fluid (Table 1). No sample was found both positive by microscopy and PCR-negative but the PCR-based assay revealed more trypanosome-positive samples of diuresis fluid and saliva than the microscopy.

Prevalence of Infection

The prevalences of any, immature and mature infection were determined after dissection and study of the midguts and probosces, both by microscopical examination and by PCR. Infections were scored as immature if they were found in the midgut only and as mature if they were found both in the midgut and proboscis (Table 2).

Most (101) of the 132 tsetse flies that survived more than 9 days post-infection were negative for trypanosomes, both by the PCR-based and microscopical examination

of the midguts and the probosces. Of the 31 PCR-positive midguts, only 27 were positive for trypanosomes by microscopy. Of the 31 corresponding probosces, 13 were negative and nine positive using both techniques, and nine others were PCR-positive but negative by microscopy. Eight of the latter nine probosces were from *G. p. gambiensis*. The use of PCR therefore revealed many more mature infections than did microscopical examination, especially in the *G. p. gambiensis*.

The organs found positive by microscopy were never found PCR-negative. Nothing in the organs from the negative-control flies was amplified by the PCR (data not shown).

Although the PCR results indicated that a higher proportion of *G. p. gambiensis* (29.2%) than of the *G. m. morsitans* (17.9%) were infected with *T. congolense*, the difference was not statistically significant ($\chi^2 = 2.3$; $P > 0.05$). The prevalence of immature infection was, however, significantly higher in the *G. p. gambiensis* than in the *G. m. morsitans*

TABLE 1. The results of the microscopical and PCR-based examinations, for *Trypanosoma congolense*, of samples of diuresis fluid and saliva from the two species of tsetse flies

	No. of samples:				
	Positive by microscopy and:		Negative by microscopy and:		Investigated
	Positive by PCR	Negative by PCR	Positive by PCR	Negative by PCR	
Diuresis fluid	21	0	47	198	266
Saliva	2	0	14	132	148

TABLE 2. The prevalences of immature and mature infection detected, by microscopy or PCR, in the tsetse fed on mice infected with *Trypanosoma congolense* E325

Tsetse species	Sex	Dissected	No. of tsetse:			
			Found with immature infections		Found with mature infections	
			By microscopy	By PCR	By microscopy	By PCR
<i>Glossina p. gambiensis</i>	Male	32	8	3	1	7
	Female	33	8	7	0	2
	Both	65	16	10	1	9
<i>Glossina m. morsitans</i>	Male	31	0	0	3	4
	Female	36	2	3	5	5
	Both	67	2	3	8	9

(15.4% *v.* 4.4%; $\chi^2 = 5.8$; $P < 0.05$). There was no significant difference between the two species with respect to the prevalence of mature infections. Consequently, the *G. p. gambiensis* and *G. m. morsitans* expressed similar intrinsic vectorial capacities (Le Ray, 1989; Table 3). Neither tsetse species displayed a significant sex-related difference in the prevalence of immature or mature infection.

On day 48 after their infective feeds, seven of the tsetse flies (one *G. p. gambiensis* and six *G. m. morsitans*) appeared (both by microscopy and PCR) to have no trypanosomes in their midguts although they had PCR-positive probosces. Because of the definitions of 'mature' and 'immature' infections, these tsetse flies were not included in Table 2.

Determination of the Parasitic Status of the Tsetse Flies

The 31 tsetse flies with PCR-positive midguts on day 48 included 26 flies that displayed PCR-positive diuresis fluid throughout the experiment. Only two flies appeared to have negative diuresis fluid throughout the experiment but positive midguts on day 48. As for the remainder, collection of diuresis fluid failed in one fly and the other two died just after day 10 (the first day samples were collected) and their diuresis fluid was therefore only sampled once (and found negative). The saliva samples of eight of the nine *G. m. morsitans* that had positive probosces on day 48 contained parasites whereas, as previously indicated, no parasites could be found in any of the saliva samples from any of the *G. p. gambiensis*.

Infection of Rabbits

The rabbit used to feed the *G. m. morsitans* was positive for *T. congolense* on day 36. It was treated with a trypanocidal drug (3.5 mg diminazene aceturate/kg body weight) and immediately replaced with another rabbit. This replacement rabbit was itself found infected 15 days later and received the same trypanocidal treatment. The rabbit used to feed the *G. p. gambiensis*, however, was never found parasitaemic during the experiment.

DISCUSSION

The present results demonstrate the value of monitoring trypanosome infections in tsetse by detecting parasites, in the diuresis fluid (midgut infections) and saliva (mature infections) of individual flies, using PCR on samples collected at different times post-infection. This procedure indicated that midgut infections of savannah-type *T. congolense* established within 10 days of the infective feed in both tsetse species, and that maturation took more than 17 days and perhaps as long as 24 days in *G. m. morsitans*. The results of previous research indicated shorter times for establishment and maturation of *T. congolense* in *G. m. morsitans*. Gidudu *et al.* (1996), for example, using the same stock of *T. congolense*, observed parasites in the saliva on day 14, and Welburn and Maudlin (1989) found midgut infections as early as 3 days post-infection and mouthpart infections by day 6. The relatively low temperature at which the present study was conducted (23°C, instead of the usual 25°C) may have made the

TABLE 3. Estimates of the intrinsic vectorial capacity (*i.e.* procyclic index \times metacyclic index) in two species of tsetse experimentally infected with *Trypanosoma congolense* E325

Tsetse species	Procyclic index*	Metacyclic index†	Intrinsic vectorial capacity
<i>Glossina p. gambiensis</i>	0.292	0.473	0.138
<i>Glossina m. morsitans</i>	0.179	0.75	0.134

*No. of tsetse with midgut infection/no. dissected.

†No. of tsetse with proboscis infection/no. with midgut infection.

infections develop relatively slowly; it is generally assumed that the time taken for a trypanosome infection to mature in tsetse flies depends only on temperature and the species of trypanosome (Dale *et al.*, 1995).

Although the prevalence of immature infection was higher in the *G. p. gambiensis* than in the *G. m. morsitans*, the intrinsic vectorial capacities (IVC) of the two species were, according to the PCR results, the same. In general, the relevant data collected in the past have indicated that flies of the *morsitans* group are more susceptible to infection with *T. congolense* than those of the *palpalis* group (Moloo and Kutuza, 1988; Reifenberg *et al.*, 1997a). These older data come from studies based exclusively on microscopy, however. If the present analysis is confined to the results of the microscopy, the IVC of *G. m. morsitans* does indeed appear higher than that of *G. p. gambiensis* (0.119 *v.* 0.015). It is only when the results of the present PCR-based assay, with its greater sensitivity, are analysed that the IVC of the two species appear the same. The type of host used to infect the tsetse flies may also affect estimates of IVC; Moloo and Kutuza (1988) used goats, Reifenberg *et al.* (1997a) used rabbits, and mice were employed in the present study.

A strong association was observed between the presence of trypanosomes in the diuresis fluid and their presence in the midguts of tsetse flies (of either species). At least in the *G. m. morsitans*, there was also a strong association between the presence of trypanosomes in the saliva and their presence in the probosces. Analyses of saliva and diuresis fluid should therefore make it possible to determine the parasitic status of the flies (mature *v.* immature) without having to follow the classical strategy of dissection and microscopical examination (Welburn *et al.*, 1994; Reifenberg *et al.*, 1997a; Kazadi *et al.*, 1998) and without having to kill the flies. Flies of known parasitic status can therefore be used for subsequent studies of co-infection and/or infection facilitation by different *Trypanosoma* species.

The PCR technique was clearly more sensitive than microscopy in detecting trypanosomes in saliva and diuresis fluid. PCR also revealed many more mature infections, probably because the small numbers of trypanosomes that are often present in the proboscis escaped detection by microscopy. PCR was no more sensitive than microscopy in detecting infections in midguts that had been dissected out of the flies, probably because infected midguts generally harbour such large numbers of parasites that they are rapidly detected under the microscope.

PCR revealed the presence of trypanosomes in the probosces of some tsetse flies that had PCR-negative midguts. The inhibition of the PCR-based amplifications by the midgut contents is an unlikely cause of this apparent discrepancy because several attempts to overcome any such inhibition, by diluting the midgut samples, still gave no amplification of trypanosome DNA (unpubl. obs.). Some tsetse infected with *T. congolense* do seem to lose their midgut infections whilst retaining parasites in the proboscis (Jordan, 1964).

Curiously, no parasites were detected in the saliva samples of the nine *G. p. gambiensis* found to have mature infections whereas virtually all of the *G. m. morsitans* with mature infections ejected trypanosomes during successive salivation assays. The results of the salivation assays in a previous study (Kazadi, 2000), undertaken with a *T. congolense* clone, were 100% negative in *G. p. gambiensis* ($N = 194$) but 100% positive in *G. m. morsitans* ($N = 168$). In the present study, moreover, the two rabbits used to feed the *G. m. morsitans* flies (including the nine with mature infections) became positive for *T. congolense*, thereby demonstrating the infectiousness of these flies. Despite the evidence of nine *G. p. gambiensis* with mature infections, however, the blood of the rabbit used to maintain these and the other *G. p. gambiensis* remained negative for *T. congolense* throughout the experiment. It seems that, notwithstanding the presence of parasites in their probosces, the infected *G. p. gambiensis*

were unable to expel, and therefore to transmit, *T. congolense*. This phenomenon seems to depend on the species of trypanosome, since mobile, mature, metacyclic forms of *T. brucei gambiense* were observed in the saliva of the same strain of *G. p. gambiense* during a previous study (Ravel *et al.*, 2003). It is also likely that, as suggested by Reifenberg *et al.* (1996), the subspecies of *Glossina* is also important. In the study by Reifenberg *et al.* (1996) on *G. tachinoides* (another member of the *palpalis* group), 25% of the flies subsequently found to have positive probosces (when dissected) produced parasite-free saliva. The present results, obtained under laboratory conditions, may help to explain, but not predict, what is seen in the field.

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