Characterization of trypanosome infections by polymerase chain reaction (PCR) amplification in wild tsetse flies in Cameroon

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SUMMARY

The polymerase chain reaction (PCR) method was used to characterize trypanosome infections in tsetse flies from 3 sleeping sickness foci in Cameroon. The predominant tsetse species found was *Glossina palpalis palpalis*. An average infection rate of 12·1% was revealed by microscopical examination of 888 non-teneral tsetse flies. PCR amplification analyses for trypanosome identification were carried out on 467 flies, with primer sets specific for *Trypanosoma* (*Trypanozoon*) *brucei* s.l., *T.* (*Duttonella*) *vivax*, *T.* (*Nannomonas*) *simiae* and forest type *T.* (*Nannomonas*) *congolense*. Of 467 flies 93 were positive by microscopical analysis while PCR succeeded in identifying 89 positive flies. Of the PCR-positive flies 34 (38·2%) were negative by microscopical examination. PCR amplification, when compared to the parasitological technique, gave a higher estimate of infection rate of trypanosomes in natural tsetse populations. The PCR technique did, however, fail to identify 40·9% (38/93) of the parasitologically positive flies. The reasons for this failure are discussed. The overall prevalence of mixed infections, assessed by PCR, was 37·1%; the majority (72·7%) involved *T. brucei* and forest type *T. congolense*.

Key words: trypanosome identification, PCR, tsetse flies, Cameroon.

INTRODUCTION

The protozoan parasite *Trypanosoma* (*Trypanozoon*) *brucei* is the aetiological agent of African sleeping sickness in man and nagana in animals. The disease is often fatal for both humans and domestic animals and is an important economic constraint for the concerned African countries. The parasite is transmitted cyclically to its hosts by the bite of an infected tsetse fly. Parasites of the subgenus *Nannomonas* also have an important economical impact in Africa. The subgenus *Nannomonas* includes 3 species, *T. congolense*, *T. simiae* and *T. godfreyi*. In the subgenus *Duttonella*, *T. vivax* causes disease in ruminant livestock. For a better understanding of the epidemiology of these diseases, the different trypanosome species infecting the tsetse flies have to be accurately determined.

Trypanosome detection and species identification in tsetse flies are laborious because they require dissection and microscopical examination of potentially infected insect organs. In fact, the identification is based on the localization of trypanosomes in different vector organs (Lloyd & Johnson, 1924). Trypanosomes found only in the proboscis belong to subgenus *Duttonella* (*T. vivax*). When trypanosomes are located in the midgut and mouthparts, they are classified as *Nannomonas* (*T. congolense*, *T. simiae*, *T. godfreyi*), and trypanosomes in the midgut and salivary glands belong to *Trypanozoon* subgenus (*T. brucei* s.l.). In addition, the sensitivity of trypanosome detection by visual examination of vector organs is probably low, and the presence of parasites is usually only detected in insects with relatively high levels of trypanosomes. Furthermore, microscopical methods fail to identify mixed or immature infections when parasites are only found in the insect midgut. Thus, it is necessary to develop more sensitive techniques for the detection and identification of parasites.

DNA-based methods, such as polymerase chain reaction (PCR) (Saïki *et al.* 1988), have been developed for the detection and identification of parasites to overcome the limitations of microscopical examination (Hide & Tait, 1991; Barker, 1994; Hill & Crampton, 1994). PCR has already been applied to trypanosome identification in experimentally infected tsetse flies and in natural hosts or vectors in West and East Africa (Moser *et al.* 1989; Masiga *et al.* 1992, 1996; Majiwa *et al.* 1994; McNamara, Laveissière & Masiga, 1995; Solano *et al.* 1995; Woolhouse *et al.* 1996).

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Identification of parasites in vectors is necessary to assess vector-control programmes, and to measure risk of infection in epidemic foci. The study presented here describes the trypanosomal fauna in tsetse flies caught in 3 sleeping sickness foci in Cameroon (Central Africa) and prevalence of trypanosome infection is determined both in field and laboratory experiments. We describe how the flies were treated to improve trypanosome detection, and the PCR technique is compared with a microscopical diagnostic method.

MATERIALS AND METHODS

Tsetse fly trap locations

Tsetse flies were caught using 'Vavoua' traps set up in or around villages located in old but active sleeping sickness foci in Cameroon (Laveissière & Grébaut, 1990). The Mbam focus (4° 37′ 77 N, 11° 17′ 27 E), an historical Human African Trypanosomiasis (HAT) focus, is in a savannah–forest mosaic. Fontem (5° 40′ 12 N, 9° 55′ 33 E) is isolated in the Western mountainous area. Campo (2° 22′ 52 N, 9° 49′ 93 E) is located in the primary equatorial forest along the border with Equatorial Guinea.

Several villages were prospected in each of the 3 foci. Traps were placed at each site for 3 days at presumed man/fly contact points. Traps were harvested twice a day.

Dissection

The different species of *Glossina* were first identified, then sorted into females and males, teneral and non-teneral flies. The non-teneral flies were dissected under an ordinary (Leitz) dissection microscope in a drop of sterile 0·9 % saline solution and their midgut, proboscis, and salivary glands were successively examined for the presence of trypanosomes under light microscopy at 400 × magnification. Organs, both infected and uninfected, were then transferred, separately, into microfuge tubes containing 50 μl of 70 % ethanol. During dissection and transfer to microfuge tubes, care was taken to clean the dissecting instruments between each manipulation to prevent contamination. Clippers were cleaned after each use by immersion in detergent (3–5 % sodium hypochlorite) for about 5 min, followed by a brief wash in sterile water and were then dried with a clean absorbent paper. Microfuge tubes were maintained at 10 °C in the field and stored at −20 °C in the laboratory until use.

Sample preparation

Frozen samples were thawed and air-dried. A 1 % lauryl–sarcosine/1 % Triton X-100 solution (100 μl) was added, the tubes were vortexed and incubated at room temperature for 15 min. Then 2 μl of proteinase K (200 μg/ml) were added, and samples were incubated at 55 °C for 3 h. The lysis mixture was further processed by standard phenol extraction (Maniatis, Fritsch & Sambrook, 1982) and nucleic acids were precipitated by the addition of 0·1 vol. of 3 M sodium acetate and 2·5 vol. of absolute ethanol. After centrifugation at 13 000 ***g*** for 15 min, the pellet was rinsed with 70 % (v/v) ethanol, air-dried, and resuspended in 60 μl of distilled water. DNA samples were stored at −20 °C until PCR amplification.

Primers

Primers used in this study are presented in Table 1.

For each fly, 8 PCR amplifications were done. *T. vivax* primers were only tested for proboscis because trypanosomes of these species are known to be located only in this organ. In order to detect dissection contamination, the salivary glands were also tested with *T. congolense* primers when a fly midgut was found to be infected by *T. congolense*. The occurrence of forest type *T. congolense* is restricted to humid regions and it is the only *T. congolense* subgroup found in rain forest (Young & Godfrey, 1983; Schares & Mehlitz, 1996). Since the study areas are located in the rain forest, PCR analysis was limited to forest type *T. congolense*.

PCR amplification

Standard PCR was performed in a 25 μl reaction mixture containing 10 mM Tris–HCl, pH 9, 50 mM KCl, 3 mM $MgCl_2$, 200 μM of each of the 4 deoxynucleoside triphosphate (dNTPs), 1 μM of oligonucleotide primers and 0·25 units of *Taq* (*Thermus aquaticus*) DNA polymerase (Appligene-Oncor, USA). Then 5 μl of the DNA samples were added as template in each reaction mixture. Positive (2·5 ng of reference DNA) and negative (without DNA) controls were included in each set of experiments. The reaction mixtures were overlaid with paraffin oil to prevent evaporation and placed in a programmable heating block (PHC-3 Techne, Cambridge, UK). Samples were first incubated at 94 °C for 3 min for a preliminary DNA denaturation and then processed through 30 cycles consisting of a denaturation step at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. The *Taq* DNA polymerase was added during the first cycle when temperature reached 60 °C ('hot-start PCR'). The last elongation step was extended to 15 min. For each amplified sample 15 μl were resolved on 1·5 % agarose gel electrophoresis, stained with ethidium bromide and photographed under ultraviolet illumination.

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Table 1. Primers used for PCR amplification of trypanosome DNA

Specificity	Code	Primer sequence	AP*	Reference
T. congolense (forest type)	TCF 1	5′-GGACACGCCAGAACTACTT-3′	350	Masiga *et al.* (1992)
	TCF 2	5′-GTTCTCGCACCAAATCCAAC-3′		
T. congolense (savannah type)	TCN1	5′-TCGAGCGAGAACGGGCACTTTGCGA-3′	341	Moser *et al.* (1989)
	TCN2	5′-ATTAGGGACAAACAAATCCCGCACA-3′		
T. brucei	TBR 1	5′-GAATATTAAACAATGCGCAG-3′	164	Masiga *et al.* (1992)
	TBR 2	5′-CCATTTATTAGCTTTGTTGC-3′		
T. simiae	IL 1	5′-CGACTCCGGGCGACCGT-3′	600	Majiwa *et al.* (1990)
	IL2	5′-CATGCGGCGGACCGTGG-3′		
T. vivax	TVW 1	5′-CTGAGTGCTCCATGTCCCAC-3′	150	Masiga *et al.* (1992)
	TVW 2	5′-CCACCAGAACACCAACCTGA-3′		

* Expected size of the Amplification Product (bp).

Table 2. Field data for the prospected areas in Cameroon

Focus	Captured flies	ADT*	Dissected flies	Infected flies (%)
Fontem	900	10·0	240	21 (8·75)
Mbam	697	7·7	206	20 (9·71)
Campo	1365	6·5	442	69 (15·61)
Total	2962	8·1	888	110 (12·39)

* ADT, apparent density/day/trap.

RESULTS

Frequency of tsetse species in the 3 foci and their infection rate

A total of 2962 flies were caught (Table 2). At Campo, where 1365 flies were collected, 4 species of Glossinidae were identified. *G. palpalis palpalis* was predominant (82 % of the captured flies), but *G. caliginea*, *G. pallicera* and *G. fusca* were also present with 9, 7 and 2 % of harvestings respectively. By contrast, in Fontem and Mbam foci only *Glossina p. palpalis* was found. Flies of the *morsitans* group were never caught in any focus. The mean apparent density/trap (ADT) was 8·1 flies/day, and varied from 1·5 to 20·7, depending on the villages. In the field a total of 888 non-teneral flies was examined for the presence of trypanosomes. Among them, 110 had infections in 1 or more of the examined organs, and the average infection rate was 12·1 % (95 % interval of confidence = [8·4; 15·8]). As shown in Table 2, flies are more infected at Campo ($\chi^2 = 8{\cdot}52$, $P < 0{\cdot}05$).

Comparison of microscopical and PCR analysis

In the laboratory, a total of 467 flies with infected or uninfected organs by microscopical examination were treated for PCR analysis. As determined by microscopical examination, 93 of 467 flies had an infection in 1 or more of the dissected organs and PCR was positive in 89 tsetse flies (Tables 3 and 5). Comparison of results revealed that only 59·1 % of parasitologically infected flies (55/93) were positive in PCR experiments. On the other hand, the PCR method allowed the detection of trypanosome infections in 9·1 % of flies not found to be infected by microscopical observation (34/374). We have adjusted these results to entomological data (888 dissected flies) and compared the 2 methods for the whole dissected population. PCR analysis allowed trypanosome identification from parasitologically negative flies in 8·8 % of the fly population; 7·7 % of the flies were positive by the 2 methods. The infection rate is significantly increased by PCR ($\chi^2 = 5{\cdot}85, P < 0{\cdot}05$) (16·5 % by PCR and 12·4 % by microscopy).

PCR analysis of tsetse organ samples

The comparison of infections found in flies positive by the 2 techniques is presented in Table 4. From the supposed *Duttonella* infections, only 4 were identified as *T. vivax*. The majority were due to *Nannomonas* and *Trypanozoon*. Infections in the proboscis were either not characterized by PCR or confirmed except by the concurrent presence of trypanosome DNA in other organs, and particularly in salivary glands. Immature infections were identified for nearly all infected midguts and frequently involved forest type *T. congolense* (Fig. 1).

The PCR identification results are summarized in Table 5. PCR analysis identified trypanosome DNA in 34 parasitologically negative flies. Of these 24 were midgut infections, and 19 (79·2 %) involved *T. brucei* or/and *T. congolense*. Immature infections (in midguts only) were detected for 53·9 % (48/89) of the flies. *T. congolense* was the most abundant species, found in 55·1 % of the flies (49/89), followed by *T. brucei* (42/89, 47·2 %). Sixteen flies (18·0 %) were identified with *T. brucei* at least in their salivary glands. None of the salivary glands tested with forest type *T. congolense* was revealed by PCR.

Prevalence of mixed infections

The distribution of trypanosomes among identified

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Table 3. Trypanosome identification of parasitologically positive tsetse flies, according to Lloyd & Johnson (1924)

Infected fly organ	Trypanosome species	No. of infected flies (%)
Proboscis	*Duttonella*	47 (50·5)
Midgut	*Nannomonas* or *Trypanozoon*	29 (31·2)
At least in salivary glands	*Trypanozoon*	8 (8·6)
Proboscis and midgut	*Nannomonas*	9 (9·7)
Total		93

Table 4. Trypanosome localization and identification of both parasitological and PCR positive flies ($N = 55$)

(P, proboscis; M, midgut; G, at least the salivary glands; *Tbr*, *Trypanosoma brucei*; *Ts*, *T. simiae*; *TcF*, *T. congolense* forest type; *Tv*, *T. vivax*; *Dt*, *Duttonella*; *Nn*, *Nannomonas*; *Tz*, *Trypanozoon*.)

Infected organ	μ* positive	Supposed infection	PCR positive	Single infections				Mixed-infections				
				Tbr	*Ts*	*TcF*	*Tv*	*Tbr/Ts*	*Tbr/TcF*	*Tv/Ts*	*Ts/TcF*	*TcF/Tv*
P	22	*Dt*	10	1	0	4	4	0	1	0	0	0
M	22	*Nn/Tz*	24	3	4	8	0	1	6	0	2	0
G	4	*Tz*	14	6	0	0	0	4	4	0	0	0
PM	7	*Nn*	7	0	0	2	0	0	3	1	0	1
Total	55		55	10	4	14	4	5	14	1	2	1

* Microscopical examination.

Table 5. Distribution by species or subgroups of PCR identified infections in the different tsetse fly organs ($N = 89$)

(P, proboscis; M, midgut; G, salivary glands; *T. congo* F, forest type *T. congolense*.)

Infection type	Identified species	P	M	G	PM	PMG	MG	PG	Total
Single infections	*T. brucei*	1	4	3	0	4	0	1	13
	T. congo F	4	16	0	2	0	0	0	22
	T. simiae	4	9	0	0	0	0	0	13
	T. vivax	8	0	0	0	0	0	0	8
Mixed infections	*T. brucei*/*T. congo* F	1	16	0	3	2	2	0	24
	T. brucei/*T. simiae*	0	1	0	0	4	0	0	5
	T. congo F/*T. simiae*	0	2	0	0	0	0	0	2
	T. congo F/*T. vivax*	0	0	0	1	0	0	0	1
	T. simiae/*T. vivax*	0	0	0	1	0	0	0	1
Total		18	48	3	7	10	2	1	89

species or subgroups is shown in Table 5. Of 89 infected flies, 56 (62·9%) were infected with a single species or subgroup and 33 (37·1%) with 2 species. None of the flies were infected by 3 or more species. Eight flies with mixed infections had a mature infection (in the mouthparts or salivary glands) with 1 species or subgroup, and an immature infection (in the midgut) with another species. *T. vivax* was never found mixed with *T. brucei*.

Distribution of Trypanosoma *species in different foci*

Species or subgroup prevalence and mixed infection rates differed among the 3 prospected foci (Table 6). At Fontem, all flies found with mixed infections (19, 63·3%) were infected with *T. brucei* and forest type *T. congolense*. The PCR analysis did not reveal *T. vivax* infections in this focus. At Campo, only 10 flies (21·7%) were infected with 2 species or subgroups. The number of *G. caliginea* (2), *G. fusca* (2) and *G. pallicera* (8) was too small to investigate tsetse/trypanosome interactions in this particular focus. In the Mbam focus, 30·8% of flies carried mixed infections. Forest type *T. congolense* was the most predominant species.

Infections not identified by the PCR technique

PCR failed to identify flies which were parasitologically positive (38/93). The majority (31 of 38;

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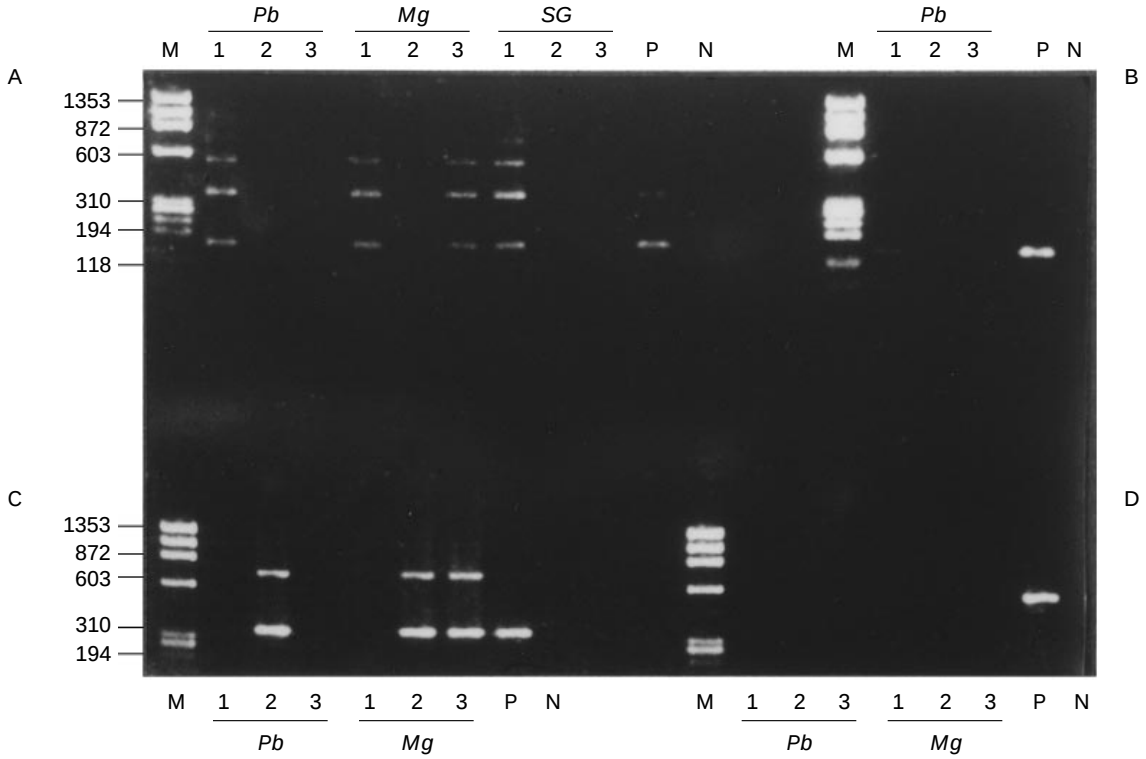

Fig. 1. Agarose gel stained with ethidium bromide showing the PCR amplification products on tsetse flies 1, 2 and 3 using (A) *Trypanosoma brucei* primers TBR-1 and TBR-2, (B) *T. vivax* primers, TVW-1 and TVW-2, (C) forest type *T. congolense* TCF-1 and TCF-2 and (D) *T. simiae* IL-1 and IL-2. *Pb* indicates the results of amplification of tsetse proboscis; *Mg* on tsetse midguts and *SG* on tsetse salivary glands. Lanes marked P and N contain, respectively, positive and negative control DNA samples for each pair of primers. Lane M contains molecular weight markers, consisting of *Hae* III digest of $\phi\chi$174; the size of the fragments are indicated in kb.

Table 6. Trypanosome identification, by PCR, in flies from the different prospected foci ($N = 89$)

(See Table 4 for abbreviations of trypanosome species.)

	Single infections				Mixed-infections					
Focus	*Tbr*	*Ts*	*TcF*	*Tv*	*Tbr/Ts*	*Tbr/TcF*	*Tv/Ts*	*Ts/TcF*	*TcF/Tv*	Total
Fontem	4	4	3	0	0	19	0	0	0	30
Mbam	2	1	6	0	1	2	0	0	1	13
Campo	7	8	13	8	4	3	1	2	0	46
Total	13	13	22	8	5	24	1	2	1	89

81·6%) of these flies were caught at Campo and, generally, trypanosomes were seen in the proboscis by microscopical examination. In order to identify the parasites undetected by PCR, we used savannah type *T. congolense* primers, which may permit determination of whether this *Nannomonas* subgroup exists in Cameroon. But amplification of savannah type *T. congolense* was never observed in our sample.

DISCUSSION

The present study has shown the ability of PCR to improve trypanosome detection and to allow parasite identification in naturally infected flies. We have evaluated the prevalence of trypanosome infections in parasitologically positive and negative wild tsetse flies by means of the PCR method and we have described the trypanosomal fauna in 3 sleeping sickness foci in Cameroon. Because the traditional technique based on microscopical examination is unable to reveal mixed infections or low parasitaemias, PCR is particularly useful in entomological surveys in sleeping sickness foci. However, species-specific primers are not available for DNA amplification of all trypanosome subgroups and the PCR failed to identify some parasitologically positive flies.

The PCR sensitivity and specificity enable better trypanosome detection when compared with micro-

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scopical examination. Indeed trypanosome DNA was identified in parasitologically negative midguts. This result points out the difficulties of parasitological detection. Low parasitaemias often go undetected by microscopical examination while PCR sensitivity is sufficient to detect 1 trypanosome (Masiga *et al*. 1992). It explains why more infections in salivary glands were determined by PCR. We can rule out the problem of contamination because care was taken during dissection and control experiments were negative.

Forest type *T. congolense* was the most prevalent species, and no infection with savannah type *T. congolense* was found. This result confirms that forest type *T. congolense* is restricted to humid areas and is often found in riverine flies of the *palpalis* group (McNamara & Snow, 1991; Masiga *et al*. 1996). Savannah type *T. congolense* is likely to be transmitted by the *morsitans* group (Gibson, Dukes & Gashumba, 1988; Woolhouse *et al*. 1996). *T. brucei* was also frequent in the infected flies; as identification is still complicated (Schares & Mehlitz 1996), we were not able to determine if the vectors were infected with the *gambiense* or *brucei* subgroup. Nevertheless, *T. brucei* infections are often mixed with a *Nannomonas* type, which could be related to bloodmeals on a host infected with several trypanosome species or subgroups. Indeed, the majority of *Trypanozoon* stocks isolated from domestic animals in central Africa have mixed infections with *T. congolense* (Noireau *et al*. 1986). This hypothesis is supported by a recent study at Fontem (Penchenier, personal communication), where domestic pigs were found to be infected with both *T. brucei* and *T. congolense*. Thus, *T. brucei* typanosomes, identified in mixed infections, may belong to the *brucei* subgroup.

No *Duttonella* was found at Fontem, as previously reported by Asonganyi, Suh & Tetuh (1990). In the Mbam and Campo foci, all trypanosome species were found and they showed a similar distribution. This is certainly due to the abundant wild fauna which makes a large host range for different trypanosome species possible. Mixed infection rates differed for the different foci and the most important rate was found at Fontem with 63·3 % of flies, all infected with both *T. congolense* and *T. brucei*. Such a high proportion has been described in midgut infections in Ivory Coast (McNamara *et al*. 1995). Some flies presented both mature and immature infections. They might have sequential infections with a successive development (Gibson & Ferris, 1992). Simultaneous infections could also be involved, with 1 species or subgroup inhibiting the maturation of the other competitive species.

PCR failed to identify 40·9 % (38/93) of the flies found to be infected by microscopical examination, which is close to surveys on wild tsetse populations described by Solano *et al*. (1995) and Woolhouse *et al*. (1996) with 46 and 47 % of unidentified infections respectively. Inhibition of PCR by the organ components is a possible explanation, but in another study phenol extraction and alcohol precipitation was shown to prevent this (Masiga *et al*. 1992). Moreover, in an experimental study with laboratory flies infected by a known *T. congolense* stock, Reifenberg *et al*. (1996) found that PCR on tsetse proboscis confirmed parasitological observations, suggesting that the difference is due to a more fundamental problem with the detection. The majority of non-identified infections were from Campo. Species of trypanosomes such as *T. grayi* (reptile trypanosome), might exist in this area where reptiles are abundant. Bourzat & Gouteux (1990) have previously described high prevalence rate of *T. grayi* infections in *G. p. palpalis* in the Mayombe forest mountains (Congo), and Gouteux & Gibson (1996) found 83 % of *Glossina fuscipes fuscipes* bloodmeals taken on monitor lizard in Ouro-Djafon (Central African Republic). Furthermore, NcNamara & Snow (1991) noted that the presence of these species in the midgut could confound the identification of species. As suitable primers for *T. grayi* are not yet available, we cannot confirm this hypothesis. Some gut infections can also be attributed to *T. vivax*. This species does not usually develop in the fly gut, but we have recently detected *T. vivax* in fly midguts (unpublished observations). Residual traces or *T. vivax* parasites may be present in an infected bloodmeal and may persist after digestion. The presence of *T. vivax* in the midgut could not be due to dissection contamination since PCR controls were always negative.

The limited range of primers used does not allow detection of known *Nannomonas* subgroups, such as *T. godfreyi*, Tsavo type *T. congolense* or Kilifi type *T. congolense*. They are normally located in restricted areas of East and West Africa, but there are no data available in forest areas of Central Africa. Isoenzyme and DNA probe analysis led to a division of the group into several subgroups, but with uncertain taxonomic status (Garside & Gibson, 1995; Gashumba, Baker & Godfrey, 1988; Knowles *et al*. 1988; Young & Godfrey, 1983). More taxonomic groups may yet exist. This could be especially true of *T. vivax*, which is widespread in Africa and different subgroups have been detected by isoenzyme analysis (Fasogbon, Knowles & Gardiner, 1990; Murray, 1982). We used primers designed from sequenced isolates of West Africa, and they may not anneal if different subtypes of *T. vivax* occur in Cameroon. These possibilities could explain why presumed *Duttonella* infections were not confirmed.

Further analyses are required to determine whether or not specific trypanosome strains circulate in Central Africa. Particularly, the presence or absence of *Nannomonas* subgroups has to be confirmed. Fundamental studies are also needed to

distinguish the different *T. brucei* subgroups, to determine the involvement of trypanosomes in these tsetse populations in human sleeping sickness in Cameroon. A larger sample size needs to be examined in order to detect associations between fly species and different typanosomes. Results of tsetse bloodmeal analysis would give important information about the main hosts in the prospected foci. The ability to identify accurately trypanosome species in its vector is crucial for reliable epidemiological data and control programmes.

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