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Detection and identification of trypanosomes by polymerase chain reaction in wild tsetse flies in Cameroon

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Abstract

The prevalence of various species and subgroups of trypanosomes in infected flies from three sleeping sickness foci in Cameroon was determined by the use of polymerase chain reaction (PCR). The predominant tsetse species found were *Glossina palpalis palpalis*. Microscopical examination of 943 non-teneral tsetse flies revealed an average infection rate of 10.4%. A total of 90 flies were analyzed for trypanosome identification with primer sets specific for *Trypanosoma* (*Trypanozoon*) *brucei* s.l., *T.* (*Duttonella*) *vivax*, *T.* (*Nannomonas*) *simiae*, and forest type *T.* (*Nannomonas*) congolense. PCR succeeded in identifying 52 of the 90 infected flies. Other primers were also tested on microscope positive/PCR-negative infections, and trypanosome subgroups were detected (Kilifi type and savannah type *T. congolense*). PCR amplification allowed identification of immature infections and revealed mixed-infections. The PCR technique failed to identify 42.2% (38/90) of the parasitologically positive flies and the reasons for this failure are discussed.

Keywords: Trypanosome identification; PCR; Tsetse flies; Cameroon

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1. Introduction

Trypanosoma (*Trypanozoon*) *brucei* is a protozoan parasite that is the etiologic agent of African sleeping sickness, a fatal disease in humans, and nagana, wich limits african livestock production. The parasite is transmitted to mammalian hosts by the bite of an infected tsetse fly. Trypanosomes of the subgenus *Nannomonas* are the most important pathogenic parasites of livestock in Africa. The subgenus includes three species, *T. congolense*, *T. simiae* and *T. godfreyi*, which develop in the midgut and proboscis of tsetse flies. The pathogens occur throughout the area of tropical Africa where tsetse flies are present. In the subgenus *Duttonella*, *T. vivax* causes disease in ruminant livestock. It develops only in the mouthparts of tsetse flies in Africa but the species are also transmitted mechanically by other biting flies (horse flies, stomoxes) in South and Central America. The various trypanosome species infecting the tsetse flies have to be accurately identified for a better understanding of the epidemiology of these diseases.

The classical technique relies on dissection and microscopical examination of the potentially infected tsetse organs (proboscis, midgut and salivary glands) (Lloyd and Johnson, 1924). Whereby identification of trypanosomes is based on location of parasites in the fly organs (Lloyd and Johnson, 1924). The method is laborious and the detection requires large amount of trypanosomes. The visual examination does not allow the identification below the subgenus level and fails to detect mixed infections and to identify trypanosomes found in midgut only (immature infections). The molecular biology method has overcome the limits of sensitivity and specificity of the old methods. The polymerase chain reaction (PCR) has allowed the amplification in vitro of specific DNA sequences (Mullis and Faloona, 1987). This method has been developed to facilitate the detection of many types of parasite (Meredith et al., 1991; Barker et al., 1992; De Bruijn et al., 1993; Laserson et al., 1994), including trypanosomes (Moser et al., 1989; Masiga et al., 1992; Majiwa et al., 1994; McNamara et al., 1995).

PCR was used for detection and identification of trypanosome infections in wild tsetse flies. The study took place in old and active sleeping sickness foci where animal trypanosomiasis can also occur.

2. Materials and methods

Tsetse flies were caught with 'Vavoua' traps (Laveissiere and Grebaut, 1990) in three sleeping sickness foci in Cameroon. In the Mbam focus (4°37′77 N, 11°17′27 E) no case has been diagnosed since 1991, at Fontem (5°40′12 N, 9°55′33 E) and Campo (2°22′52 N, 9°49′93 E) several patients are found each year. The study took place during the rainy season. Several villages of the foci were included in the survey and traps were fitted to presumed man/fly contact areas for 3 days. Traps were harvested twice a day.

Flies were first identified for the different species of *Glossina* and then sorted into males and females, teneral and non-teneral. Non-teneral flies were dissected in a

drop of sterile saline solution and proboscis, midgut and salivary glands successively observed under light microscope. Care was taken to clean clippers after each use during dissection to prevent contamination. Infected fly organs were collected separately in microfuge tubes containing 70% ethanol. Samples were stored at −20°C and taken back to the laboratory until use.

Samples were thawed and ethanol air-dried. After lysis at 55°C for 3 h with proteinase K (200 μg/ml), organs were processed by standard phenol extraction and ethanol precipitation (Maniatis et al., 1982). Pellets were resuspended in 60 μl of sterile water and stored at −20°C. Primers used to amplify target DNA are detailed in Table 1. Specific primers were tested on fly organs where trypanosome species settle during its development. In order to detect dissection contamination, controls were done on flies which midgut was found infected by *T. congolense* by testing their salivary glands with these primers. DNA samples were amplified in a final volume of 25 μl overlaid with paraffin oil. Final concentrations in the reaction mix were 10 mM Tris (pH 9), 50 mM KCl, 3 mM $MgCl_2$, 200 μM of each of the four deoxynucleoside triphosphate (dNTPs), 0.25 unit of Taq (*Thermus aquaticus*) DNA μl polymerase (Appli gene-Oncon., USA) and 1 μM of oligonucle-

Table 1
Primers used for PCR amplification of trypanosome DNA

Specificity	Code	Primer sequence	AP[a]	Reference
T. congolense (forest)	TCF 1	5′-GGACACGCCAGAAGTACTT-3′	350	Masiga et al. (1992)
	TCF 2	5′-GTTCTCGCACCAAATCCAAC-3′		
T. congolense (savannah)	TCN1	5′-TCGAGCGAGAACGGGCACTTT-GCGA-3′	341	Moser et al. (1989)
	TCN2	5′-ATTAGGGACAAACAAATCCCG-CACA-3′		
T. congolense (kilifi)	TCK1	5′-GTGCCCAAATTTGAAGTGAT-3′	294	Masiga et al. (1992)
	TCK2	5′-ACTCAAAATCGTGCACCTCG-3′		
T. simiae	IL 1	5′-CGACTCCGGGCGACCGT-3′	600	Majiwa et al. (1994)
	IL 2	5′-CATGCGGCGGACCGTGG-3′		
T. godfreyi	DDG1	5′-CTGAGGCTGAACAGCGACTC-3’	?	Masiga et al. (1996b)
	DDG2	5’-GGCGTATTGGCATAGCGTAC-3′		
T. brucei	TBR 1	5′-GAATATTAAACAATGCGCAG-3′	164	Masiga et al. (1992)
	TBR 2	5′-CCATTTATTAGCTTTGTTGC-3′		
T. vivax	TVW 1	5′-CTGAGTGCTCCATGTGCCAC-3′	150	Masiga et al. (1992)
	TVW 2	5′-CCACCAGAACACCAACCTGA-3′		

[a] Expected size of the amplification product (Bp).

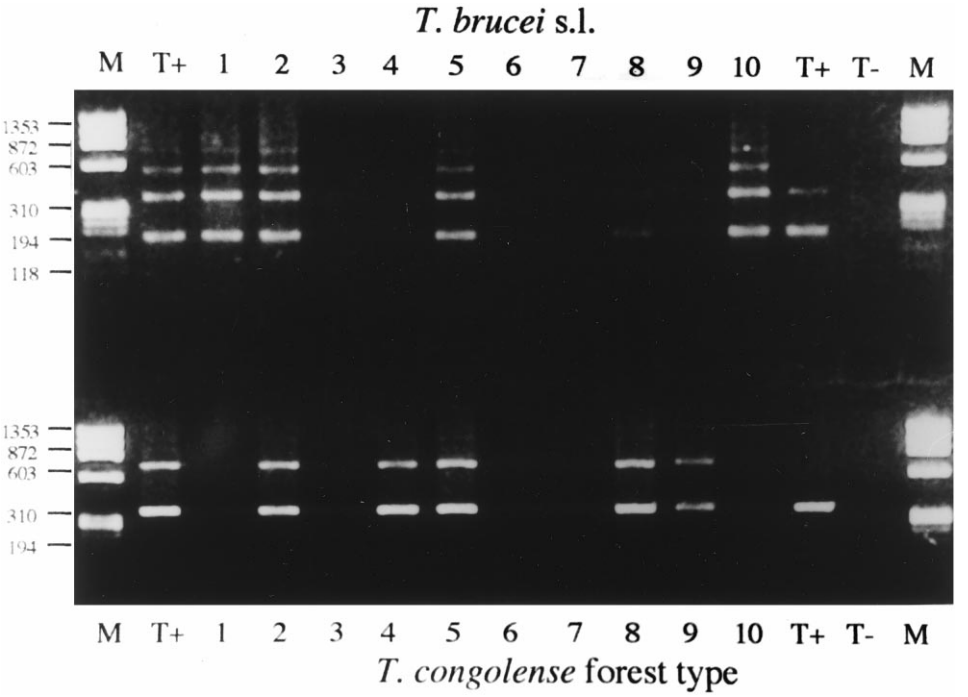

Fig. 1. Ethidium stained gel showing PCR products on parasitological infected midguts (1–10) with TBR1/2 and TCF1/2 primer sets. Lane M contains molecular weight marker (*Hae*III digest of $\phi\chi$174); the size of the fragments are indicated in kb. Lanes marked T+ and T− show positive and negative controls, for each pair of amplification primers. Mixed infections are revealed by the presence of a band on both similar midgut numbers.

otideprimus. Five of the DNA samples were used as template per reaction. Positive (2.5 ng of reference DNA) and negative (without DNA) controls were included in each set of experiments. Tubes were incubated at 94°C for 3 min in an initial denaturation step, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. A final extension was processed at 72°C for 15 min. The PCR reaction was hot started by adding the *Taq* polymerase before the first annealing step at a temperature of 70°C. A 15 μl aliquot of each sample was analysed on an agarose gel, stained with ethidium bromide and photographed under UV illumination (Fig. 1).

3. Results and discussion

A total of 3320 tsetse flies were caught during the rainy season from the 3 foci. Females represented 2295 (69.1%) of these. We have to ascribe this rate to the method of trapping. Indeed, net catches may have caused opposite rates, with a majority of males (Eouzan and Ferrara, 1978). The average apparent density per

Table 2
Comparison of trypanosome location for the two techniques

Fly organ	Positive parasitological results	Positive PCR results proboscis
Proboscis (%)	50 (55.6)	12 (23.1)
Midgut (%)	23 (25.5)	19 (36.5)
At least in salivary glands (%)	8 (8.9)	13 (25.0)
Proboscis and midgut (%)	9 (10.0)	8 (15.4)
Total	90	52

trap (ADP) was 7.9 flies per day with ADPs ranging from 1.5 to 20.7 depending on the locality and the village. *Glossina palpalis palpalis* was the predominant species found in these cameroonese foci and although at Campo three other species were caught (*G. pallicera*, *G. caliginea*, *G. fusca*) they were too few to be considered separately. Of the non-teneral flies, 943 were examined for the visual detection of trypanosomes. Using microscopy, 98 flies were found to be infected, which gave an infection rate of 10.4%. Females had more infections (75/655; 11.5%) than males, but the difference was not statistically significant ($\chi^2 = 2.58$, $P > 0.05$).

Only 90 of these infected flies were analysed in the laboratory, eight being lost during transfer in microfuge tubes or during DNA extraction. Parasitological results for these 90 flies are summarised in Table 2. Just above half (50/90; 55.6%) were found infected only in mouthparts and were supposed to be *T. vivax* infections by parasitological criteria. Immature infections represented 25.5% (23/90) of the infected flies and could not be identified by microscopical examination.

PCR results are shown in Tables 2 and 3. Only one-third (4/12) of proboscis infections were identified as *T. vivax* (Table 3). So, parasitological results would have attributed many *Nannomonas* (*T. congolense*, *T. simiae*) infections to *T.*

Table 3
Distribution by species or subgroups of PCR identified infections in the different tsetse fly organs ($n = 52$)

Infection type	Identified species	P	M	G	PM	PMG	MG	Total
Single infections	*T. brucei*	1	3	2	0	4	0	10
	T. congo F	4	5	0	2	0	0	11
	T. simiae	2	4	0	0	0	0	6
	T. vivax	4	0	0		0	0	4
Mixed infections	*T. brucei*/*T. congo F*	1	5	0	3	2	1	12
	T. brucei/*T. simiae*	0	0	0	0	4	0	4
	T. congo F/*T. simiae*	0	2	0	1	0	0	3
	T. congo F/*T. vivax*	0	0	0	1	0	0	1
	T. simiae/*T. vivax*/ *T. congo F*	0	0	0	1	0	0	1
Total		12	19	2	8	10	1	52

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

vivax, because they were largely found in the proboscis. Forest type *T. congolense* is the most prevalent species, its presence was detected in 53.8% (28/52) of PCR identified infections. This can be explained by the abundance of domestic pigs in the villages in the study area or of wild fauna in the surrounding area, which act as hosts to the tsetse flies. Mixed natural infections were found in 40.4% (21/52) of flies. The majority of which were forest type *T. congolense* (60.7%; 17/28) mixed infections with other trypanosome species. This mixed infection rate is certainly an underestimate, because of the limited range of primers tested. One fly was infected with three species of trypanosomes (*T. vivax*, *T. simiae* and forest type *T. congolense*). But the PCR method does not enable us to determine whether all the species present in one such fly reach an infective stage.

PCR results showed a prevalence of *T. brucei* of 50.0% (26/52). McNamara et al., (1995), Masiga et al., (1996a) had described a similar proportion in natural infected flies from Ivory Coast. Salivary glands infections represented half of *T. brucei* infections (13/26) by PCR, whereas only 8.9% (8/90) were detected by microscopical examination. The detection of small numbers of parasites, which were not seen by microsccopy, is possible by PCR (Masiga et al., 1992), even if all trypanosome species could not be characterized (see next paragraph). This high rate of mature *T. brucei* infections is probably due to presence of old flies in the population. Contamination can be precluded because all controls (negative ones for each batch of experiments and contamination controls) always gave negative results. Most of the *T. brucei* infections (16/26; 61.5%) were mixed with *Nannomonas*, so questions arise if they are *brucei* or *gambiense* subgroup. As the *gambiense* form only infects man and because the *brucei* form and the *Nannomonas* subspecies are zootic pathogens, in a mixed infection with *T. brucei brucei* and *Nannomonas*, the bloodmeal could have been taken on an animal infected by the two trypanosome species (concurrent infection) whereas in the case of a mixed-infection with *T. b. gambiense* and *Nannomonas*, the fly has bitten successively (sequential infection) two infected hosts: a man and an animal. Specific primers to distinguish *T. brucei* subgroups are not yet usable by a single PCR (Schares and Mehlitz, 1996). The possibility to assess the part of each subgroup in these infections will be of great epidemiological importance, since the study took place in sleeping sickness foci.

PCR failed to identify flies which were found to be infected by microscopy (38/90; 42.2%). This is consistent with other surveys (Solano et al., 1995; Woolhouse et al., 1996). The discordant samples were analysed by complementing PCR analyses, with samples amplified for other trypanosome identification: savannah type *T. congolense*, Kilifi type *T. congolense* and *T. godfreyi*. Midguts were also tested for *T. vivax*, although the species cycle is usually confined to the proboscis of the vector. Two midguts gave a positive signal with *T. vivax* primers. So, some trypanosomes ingested with the infected bloodmeal may reach midgut and even if they degenerate some residual traces may persist after digestion. Furthermore, Moloo and Gray (1989) described that *T. vivax* development in tsetse was initiated in the oesophageal region. Kilifi type *T. congolense* was found in three flies and one tsetse carried savannah type *T. congolense*. This confirms that the different types of *T. congolense* are not restricted to limited areas but are widespread throughout the tsetse belt (Woolhouse et al., 1993; McNamara et al., 1995).

For PCR success an essential amplification condition has to be fulfilled: the use of suitable primers. So far, specific primers are not available for all trypanosome species that infect wild tsetse, just those for trypanosomes of medical or veterinary importance exist. Other trypanosome species can exist in the study area, for example *T. uniforme*, parasite of domestic and wild ruminants in Africa for which the infection in the vector is restricted entirely to the proboscis on *T. grayi*, reptile trypanosome and *T. theileri*, ruminant flagellate which develop in the midgut and hindgut of the insect. A part of the unidentified infections are perhaps due to these species, as the analysis of tsetse bloodmeals has revealed that 35.5 and 23.9% of the tsetse preferred hosts were ruminants and reptiles, respectively. Moreover, Bourzat and Gouteux (1990), by morphology and motility criteria, and Gouteux and Gibson (1996), by DNA hybridization, have described high prevalence of *T. grayi* infections in Central Africa. *T. theileri* is usually transmitted by horse flies but tsetse flies could ingest some parasites with an infected bloodmeal, these latter degenerating in the fly midgut. Another reason for the PCR failure might be that divergence between trypanosome sequences can exist according to geographical location, as it has still been observed for *T. vivax* by isoenzyme characterization (Fasogbon et al., 1990) and PCR method (Dirie et al., 1993). Since the non-identified infections concerned 26 proboscis infections (26/38; 68.4%), the preceding assumption can not be preclude. The proteinase K digestion and DNA extraction method with phenol could be involved but infections in midguts and salivary glands, submitted to the same treatment, were characterized. Degradation of target DNA may also account for the failure of some samples to be amplified efficiently.

This is the first report of PCR use for trypanosome identification in Central Africa and results are consistent with studies conducted in East and West Africa (McNamara et al., 1995; Masiga et al., 1996a; Woolhouse et al., 1996). The PCR is a powerful tool for identifying and determining the distribution of trypanosomes in its vectors. Thus, it would be interesting to determine the efficiency of the PCR method in identifying infections non-detected by microscopical examination. The ability to distinguish different trypanosomes in vectors is of interest, particulary for tsetse control in sleeping sickness foci under epidemiological surveillance.

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