Short Communication

An alternative approach to detect *Trypanosoma* in *Glossina* (Diptera, Glossinidae) without dissection

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Abstract
Background: Determining if a tsetse fly is infected by trypanosomes and thus potentially able to transmit trypanosome-related human and animal diseases is an extremely laborious and time-consuming task to perform, especially under field conditions. In this study we tested a possible alternative approach that uses the entire insect vector for DNA extraction and PCR analysis to detect and identify *Trypanosoma spp*. in field collected tsetse flies.
Methodology: DNA extraction was performed using a method originally developed for tick DNA extraction followed by PCR detection and identification of *Trypanosoma* spp.
Results: Two out of 62 flies captured in Equatorial Guinea carried DNA of *T. brucei* s.l. and *Trypanosoma vivax*. *T. congolense* forest, *T. congolense* savannah and *T. congolense* Kilifi were not detected.
Conclusions: The approach we employed allowed the molecular detection and species identification of trypanosomes using the whole vector body for DNA extraction. Although the approach does not give direct information on tsetse infectivity, it provides valuable information about trypanosome species circulating in a tsetse fly vector population. The method allows an effective processing of a large number of field captured tsetse in a central laboratory.
Key Words: *Glossina*, *Trypanosoma*, PCR, Equatorial Guinea.

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Introduction

African trypanosomes are flagellate protozoa that infect both humans and animals in sub-Saharan countries. They cause human African trypanosomiasis (HAT, sleeping sickness), and several animal diseases (e. g. nagana).

Without adequate treatment, sleeping sickness is a fatal disease. HAT has made a major come-back in the last decades and WHO estimates that the disease is annually killing 66,000 individuals. Two different forms of the disease exist, caused by two different *Trypanosoma brucei* sub-species. *Trypanosoma brucei rhodesiense* is responsible for the acute and severe form of the disease found mainly in East Africa, while *Trypanosoma brucei gambiense* is responsible for the chronic neurological disease found in West and Central Africa [1].

Tsetse flies (*Glossina* spp.) are the vectors responsible for the transmission of trypanosome infection in Africa, in an area approaching 10 million km^2. The trypanosomes they transmit affect humans directly and through the wasting diseases of the livestock and promote a huge economical loss in the African continent [2].

Understanding the complex interaction between tsetse flies and trypanosomes in a given geographical setting is epidemiologically relevant and a key element for the establishment of rational and effective disease control measures [3].

Determining whether a tsetse fly is infected by trypanosomes is extremely laborious and time-consuming. In classical methods the different potentially infected organs (proboscis, salivary glands and midgut) must be carefully removed through dissection of the vector for direct observation of trypanosomes, immediately after capture [4]. More recently, molecular analysis by PCR methods has been used for trypanosome identification in tsetse organs but these methods still need on-site fly dissection by skilled personnel [5-10].

The present study reports an alternative approach to the detection of trypanosomes in the tsetse. Our method consists of PCR-based detection and identification of trypanosomes using the entire vector. Using this approach in remote locations of endemic African countries where difficult field conditions preclude tsetse dissection, tsetse flies can be collected and stored for months until analysis is performed in central laboratories.

Materials and Methods

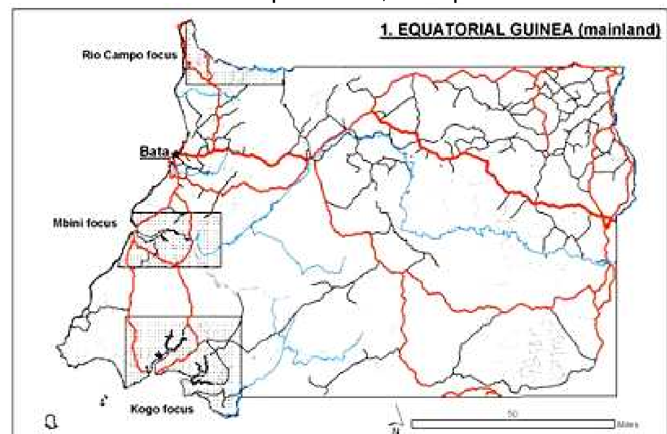
Sampling sites, capture method and processing of tsetse flies

Tsetse flies from Equatorial Guinea (West-Central Africa) were collected during HAT control activities in Equatorial Guinea in July 2005 (the rainy season) in the partially controlled HAT focus of Campo River (Figure 1). This focus is located around the shores of one of the three main rivers of the country, the Ntem River. The main tsetse fly species present on this focus is *Glossina palpalis palpalis*, although *Glossina caliginea* and *Glossina tabaniformis* are also present. Modified pyramidal traps [11] were employed to capture tsetse flies. Traps were hung at sunrise and checked at sunset during three consecutive days. Collected flies were killed by cold (-20ºC), and on-site fly dissection was not performed. Tsetse were dried in silica gel and stored for molecular studies. The tsetse flies were subsequently identified using a modified entomological key described previously [12 -16].

Sample preparation (DNA extraction)

DNA was extracted using a slightly modified protocol developed for DNA extraction in ticks [17]. The tsetse flies that had been conserved at -20ºC were first washed twice in a 70% ethanol solution and twice in sterile distilled water. A sterilized blade was used to longitudinally cut each fly into two equal parts. One half of the fly was collected in a 1.5 ml Eppendorf tube, homogenised with a pipette tip in 200 µl of PBS and boiled at 100ºC for 10 minutes. Tubes were left at room temperature for 5 minutes, followed by a short spin to collect drops from the lid. Subsequently, 20 µl of 10% SDS were added. The tubes were vortexed and spun for 5 minutes at 14,000 rpm (or full speed). Two hundred microlitres phenol: chloroform: isoamylalcohol (P:C:AI = 25:24:1 pH7,8) were added to each tube followed by gentle mixing and spin for 5 minutes at 14,000 rpm. The upper phase was transferred to a clean tube, previously filled with 200µl P:C:AI and gently mixed. Subsequently, tubes were spun for 5 minutes at 14,000 rpm and the upper phase recovered to a clean tube. For DNA precipitation 17.5 µl of 3M Sodium Acetate and 400 µl of absolute ethanol were added. The tubes were incubated at -20ºC overnight. After the overnight incubation a spinning at 14,000 rpm for 15 minutes was performed. The supernatant was discharged and the pellet washed with 70% ethanol, dried at 37ºC in an incubator, and re-suspended in 50 µl of TE (TRIS + EDTA).

Figure 1. Map of mainland Equatorial Guinea showing the tsetse capture site, Campo River.



Polymerase Chain Reaction

To confirm that no PCR inhibition was observed when using the flies' DNA, a PCR reaction targeting the tubulin gene of *Glossina* was performed as previously described [18].

For trypanosome detection, DNA extracted from the flies was organized in pools of four samples, composed of 1 µl of each fly DNA to a final volume of 4 µl, and then analysed by PCR (ITS and species-specific primers). Whenever a pool yielded a positive amplification signal for the

primers tested, each sample in that pool was individually tested to validate the result and to identify the positive sample(s) in the pool. In this case, 1 µl of fly template DNA was used in PCR. All PCR assays were performed with a final volume of 25 µl of reaction mixture.

PCR with ITS1 primers for detection of *Trypanosoma* spp. was performed as described by Njiru and co-workers [19]. PCR assays with species-specific primers (Table 1) were based on previously described protocols for *T. congolense* savannah [7], *T. congolense* Kilifi [5] and *T. congolense* forest [5], with the following modified amplification conditions: initial step of denaturation at 94ºC for 5 minutes, followed by 35 cycles at 94ºC for 1 minute, 60ºC for 1 minute, 72ºC for 1 minute, and final extension at 72ºC for 5 minutes. For *T. brucei* the original amplification conditions [20], were also modified: initial step at 95ºC for 1 minute, followed by 40 cycles of 95ºC for 1 minute, 55ºC for 1 minute, 72ºC for 1 minute, and final extension at 72ºC for 10 minutes. *T. vivax* PCR was performed using the primers described by Masake and co-workers [21] and the amplification conditions according to Morlais and co-workers [22]. Positive controls (DNA from identified parasites) and negative controls (no template) were used in all PCR assays.

All PCR products obtained were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Table 1. Primers used for trypanosome detection.

Specificity	Code	Primer sequence	Product size(bp)	Reference
Trypanosoma spp.	ITS1 CF ITS1 BR	5’- CCGGAAGTTCACCGATATTG -3’ 5’- TTGCTGCGTTCTTCAACGAA -3’	Species-specific sizes	19
T. congolense forest	TCF1 TCF2	5’- GGACACGCCAGAAGGTACTT -3’ 5’- GTTCTCGCACCAAATCCAAC -3’	350	5
T. congolense savannah	TCS1 TCS2	5’- CGAGCGAGAACGGGCAC -3’ 5’- GGGACAAACAAATCCCGC -3’	316	7
T. congolense Kilifi	TCK1 TCK2	5’- GTGCCCAAATTTGAAGTGAT -3’ 5’- ACTCAAAATCGTGCACCTCG -3’	294	5
T. brucei	TBR1 TBR2	5’- CGAATGAATATTAAACAATGCGCAGT -3’ 5’- AGAACCATTTATTAGCTTTGTTGC -3’	177	20
T. vivax	TVM F TVM R	5’- TCGCTACCACAGTCGCAATCGTCGTCTCAAGG -3’ 5’- CAGCTCGGCGAAGGCCACTTGGCTGGGGTG -3’	400	21

Results

DNA extracted from all tsetse flies (all identified as *Glossina palpalis palpalis*) from Rio Campo (Equatorial Guinea) was successfully amplified with the *Glossina* spp. tubulin primers, confirming that no PCR inhibitors were present in DNA extract.Among the 62 flies, only one tsetse had an amplification signal with ITS primers, corresponding to *T. vivax*, as shown in Table 2.

Samples were subsequently tested with species-specific primers to confirm the sensitivity and specificity of the ITS primers. None of the flies were carrying *T. congolense* forest, savannah or Kilifi DNA. The sample positive for *T. vivax* with ITS primers was also positive with the specific primers for *T. vivax*. An additional sample was positive for *T. brucei* s.l. An overall carrier status of 3.23% of the tsetse was therefore obtained (Table 2). The PCR results did not reveal any multiple trypanosomal infections.

Discussion

In this study we present a new approach to detect and identify trypanosome parasites in tsetse flies, based on DNA extraction from tsetse entire bodies followed by PCR. This approach has been successfully used in other vector borne diseases,

such as tick-transmitted or malaria parasites [17, 23].

Our aim was to determine whether the "whole insect body" approach was also feasible in field collected tsetse flies.

Table 2. Identification of trypanosome species by PCR, using ITS1 and species-specific primers, in *Glossina palpalis palpalis* from Equatorial Guinea (N = 62).

Trypanosome	ITS1 primers (%)	Species-specific primers (%)
T. brucei s.l.	0	1 (1.61%)
T. congolense forest	0	0
T. congolense savannah	0	0
T. congolense Kilifi	0	0
T. vivax	1 (1.61%)	1 (1.61%)
Total	1 (1.61%)	2 (3.22%)

The tsetse flies used in the present study were obtained during HAT control activities in Equatorial Guinea, aimed at increasing the efficacy of trap-based tsetse control measures. On-site fly dissection was not performed. The only possibility of acquiring data on the trypanosomes circulating in the tsetse population was to use the conserved flies. For this reason we could not directly compare the sensitivity of our approach with data reported when using microscopy and PCR detection of trypanosomes in dissected tsetse [5 - 10]. Further studies to determine the comparative sensitivity of our approach are needed.

Between the infecting blood meal and maturation into infective stages, trypanosomes have a complex life cycle in tsetse flies. Determining whether a trypanosome present in a tsetse is infective for the vertebrate host requires internal organ localization which then permits the putative identification of the protozoon subgenus. Although this alternative technique only determines the trypanosome carrier status of tsetse flies and the number of positive specimens in our study was very limited, the overall proportion of tsetse flies carrying trypanosomes we found in Rio Campo (3.23%, 2 out of 62) is in the range of the infection rates obtained in studies using PCR techniques in dissected flies from geographically related regions [3]. Furthermore, this alternative technique overcomes the difficulties associated with fly dissection.

The use of ITS1 primers allows a two- to five-fold reduction in sample processing costs when compared to PCR performed with the species-specific primers, since the number of reactions required per sample is reduced to a single one [24]. Although this characteristic could render the use of ITS primers more cost-effective, the sensitivity of these primers is known to be lower than the species–specific primers [19]. The very limited number of positive samples (2) we found precludes effectively comparing the performance of the two sets of primers (Table 2).

We believe that one of the main advantages of our approach relates to the field work. In the majority of entomological tsetse surveys, a team of trained technicians goes to the transmission foci and performs tsetse dissection *in loco*. This usually means at least one week of field work, with related costs (team transportation, *per diem*, housing, fuel, adequate material and conditions for fly dissection). For many HAT control programs the investment in entomological control activities of this type is too high. With our technique, tsetse flies can be simply collected by unskilled personnel, stored at room temperature and shipped in dry tubes to a central lab where our method can be implemented. We believe that entomological and parasitological surveillance can thus be more cost-effectively achieved.

To our knowledge this is the first report about trypanosome detection and identification in tsetse flies without dissection. Other studies are currently being performed in our laboratory. This method may be integrated as an additional and useful tool in HAT and animal trypanosomiasis control activities.

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