Molecular identification of *T. brucei* s.l. in tsetse flies after long-term capture in field traps

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Abstract

Background: Tsetse flies (*Glossina* spp.) are responsible for the transmission of trypanosomes, agents of animal and Human African Trypanosomiasis (HAT). These diseases are associated with considerable animal and human economical loss, morbidity and mortality. The correct identification of trypanosomes species infecting tsetse flies is crucial for adequate control measures. Identification presently requires technically difficult, cumbersome, and expensive on-site fly dissection. To obviate this difficulty we explored the possibility of correctly identifying trypanosomes in tsetse collected, under field conditions, only for number determination.
Methodology: Tsetse flies, that remained exposed for weeks in field traps in the Vista Alegre HAT focus in Angola, were obtained. The flies were not dissected on site and were stored at room temperature for months. DNA extraction using the whole tsetse bodies and PCR analysis were performed in 73 randomly chosen flies.
Results: Despite the extensive degradation of the tsetse, DNA extraction was conducted successfully in 62 out of the 73 flies. PCR analysis detected the presence of *Trypanosoma brucei* s.l DNA in 3.2 % of the tsetse.
Conclusions: This approach could be cost-effective and suitable for vector-related HAT control activities in the context of countries where entomological trained personnel is missing and financial resources are limited.

Key words: *Glossina, Trypanosoma*, PCR

J Infect Dev Ctries 2009; 3(9):735-738.

Received July 28, 2009 – Accepted August 01, 2009

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Introduction

In tropical regions of Africa, *Glossina* (Diptera, Glossinidae) or tsetse flies can carry and transmit several species of trypanosomes (*Trypanosoma vivax*, *Trypanosoma congolense*, *Trypanosoma simiae*, *Trypanosoma brucei* s.l.), which can infect a large number of vertebrates, including man (*Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense)* [1,2]. The associated animal (nagana, surra) and human (sleeping sickness or HAT) diseases, often fatal without adequate drug treatment, pose very important health and economic constraints affecting the sustainable economic development in vast areas of African land [3].

Effective HAT and animal disease control measures rely on the determination of both tsetse and vertebrate reservoir infection rates [4]. To be able to provide important epidemiological information it is therefore fundamental to have access to sensitive and effective techniques for trypanosome detection and identification [5].

The presently used standard method for trypanosome detection in tsetse flies is based on the direct microscopic observation of the parasite after organ dissection of freshly collected flies. This method, often performed under field conditions, is laborious and dependent on skilled personnel and does not allow trypanosome identification below the subgenus level [6].

In recent years, several molecular methodologies for trypanosome identification have been developed based on the polymerase chain reaction (PCR) technique [4]. PCR-based diagnostic procedures largely overcome the problems associated with the

lack of sensitivity and specificity of standard methods [5].

In the present study, we show the results of the molecular detection of trypanosomes in heavily deteriorated, field-trapped tsetse, without previous dissection of the flies.

Material and Methods

Study site

Tsetse flies used in this study were collected in 2005, in the sleeping sickness focus located near Vista Alegre village (8º 17' 00" S and 14º 48' 19" L), in Uíge Province, Angola.

Tsetse capture, conservation and identification

After capture, the flies remained for weeks in the field pyramidal traps and were subsequently stored at room temperature for a maximum of six months. When observed in our lab in January 2006, the flies exhibited a very dry and deteriorated appearance.

Tsetse flies were identified by an experienced entomologist using a modified entomological key [7-11]. The specimens were numbered and preserved at 4ºC in dry tubes with silica gel until molecular analysis in November 2007.

DNA extraction

DNA extraction was performed with the General Electric kit (illustra™ tissue and cells genomic Prep Mini Spin Kit) using the whole tsetse body in 73 randomly chosen tsetse from Vista Alegre focus as described in the manufacturer’s instructions, with one exception: final elution was performed with 25 µl of DNA elution solution.

DNA amplification

The success of the tsetse DNA extraction was confirmed by amplifying part of the *Glossina* tubulin gene, using the primers GmTub F (5´-ACG TAT TCA TTT CCC TTT GG-3´) and GmTub R (5´-AAT GGC TGT GGT GTT GGA CAA C-3´) [12], with the following PCR modifications: annealing temperature of 50ºC, 40 cycles of amplification, and 10 pmol of primers.

DNA samples that displayed a positive amplification signal for the tsetse tubulin sequence were further tested to detect *T. brucei* s.l. DNA using TBR 1 (5´-CGA ATG AAT ATT AAA CAA TGC GCA G-3´) and TBR 2 (5´-AGA ACC ATT TAT TAG CTT TGT TGC-3´) primers [13] with 1 µM of primers and the following modified amplification conditions: initial denaturation at 95ºC for one minute, followed by 40 cycles at 95ºC for one minute, 55ºC for one minute, 72ºC for one minute, and final extension at 72ºC for 10 minutes.

To confirm the correct identification of *T. brucei* s.l., the TBR positive samples were further tested using ORPHON 5J-u (5´-GAT CCC TCT CCA CCA ATC GAC CG-3´) and ORPHON 5J-l (5´-AAC TGC CCC GAC CTC CGC AGT-3´) primers [14], with 40 cycles of amplification. Samples were scored as positive for *T. brucei* s.l. DNA detection whenever they displayed a PCR amplification signal for both TBR and ORPHON 5J primers.

All PCR reactions were performed using Ready-to-go PCR Beads (General Electric Healthcare) with 2 µl of DNA, in a final volume of 25 µl. Positive and negative (no template) controls were included in each PCR reaction. All PCR products obtained were separated by electrophoresis in a 2% agarose gel stained with ethidium bromide and photographed under UV illumination.

Results

Sixty-five flies used in our experiment were identified as *Glossina palpalis palpalis*; the remaining eight flies could only be identified as belonging to the *palpalis* group, due to their extensive deterioration.

DNA was successfully extracted in 62 of the 73 tsetse, as revealed by PCR amplification of the tubulin gene in these flies. It should be noted that 10 out of the 62 flies presented a very weak PCR signal for tubulin, probably reflecting the extensive deterioration of the tsetse flies when they arrived at our lab.

The 62 *Glossina* sp. were further tested for the detection of *T. brucei* s.l. DNA. The PCR with TBR primers showed that two of these tsetse carried *T. brucei* s.l. DNA. Results were further confirmed by the amplification of *T. brucei* s.l. with the ORPHON J primers in these samples.

Discussion

The possibility of detecting trypanosomes in tsetse organs after dissection using molecular methods such as PCR has been in use for some years [15]. Recently our group proposed an alternative approach that allowed the detection of trypanosome DNA in well-preserved tsetse flies collected from Equatorial Guinea, using the insect whole body [16]. This approach has already been used in other vector-borne diseases, such as malaria [17] or tick-transmitted parasites [18].

With the present work our goal was to test the validity of the same approach in extensively deteriorated flies.

Vector control activities in HAT endemic countries almost collapsed after the independence of most of these countries but remain a key point in the fight against this neglected disease [19]. The method here proposed opens new perspectives in vector-related HAT control activities and offers a convenient tool to bypass the present depletion of entomologically skilled personnel. In fact, in this study we used tsetse captured in the Vista Alegre HAT focus that were kept for weeks in the traps and stored at room temperature for a maximum period of six months during 2005 until they arrived at our lab in January 2006. They were then stored at 4ºC until DNA extraction was conducted in November 2007. These conditions precluded the efficient DNA extraction in some of the flies, but it was still possible, two years after capture, to determine that 3.2 % (2/62) of them were carrying *T. brucei* s.l. DNA. This figure is within the range of *T. brucei* detection in tsetse either using the dissected organs [5] or the whole body approach [16,20].

Our data suggests that storage of the tsetse for six months at room temperature is probably the limit for a reasonable DNA integrity in order to allow good extraction efficiency with the technique here described. This is further confirmed by the complete impossibility to detect tubulin in *Glossina* sp. that were stored at room temperature for an undetermined number of years at the Angolan HAT Research Reference Centre (CRIV) of the Angolan National HAT Control Program in Viana, Angola, which we tested in parallel (data not shown).

The ideal situation for obtaining information on trypanosomes infecting tsetse implicates skilled personal performing onsite dissection and the use of both microscopic observation of the trypanosomes and molecular identification techniques. Unfortunately, most HAT endemic African countries presently do not have such resources. For this reason, even if our method does not allow the determination of tsetse infectivity, its value is substantial since it delivers important information on the infection rate of tsetse carrying trypanosome DNA in a given area and allows the identification of the trypanosome species involved.

This method presents the possibility for inhabitants of HAT foci of countries with limited resources for HAT control activities to be assigned to collect flies from their endemic areas and send them to a central laboratory where the flies can be identified and processed for molecular detection of trypanosomes.

Knowing the infection rates of tsetse in each endemic focus allows the identification of region(s) that should be prioritized for vector-control activities. Data on the trypanosomes circulating in a tsetse population has to be updated and made available at least annually in order to be useful for control activities.

Our approach offers a low-cost, field-applicable solution, as maps of spatial distribution of tsetse and the trypanosomes in the tsetse population could be obtained more easily, providing updated information about the areas of increased risk of transmission of human and animal trypanosomiasis.

Acknowledgements

We would like to thank Prof. António Grácio, Head of the Unidade de Entomologia Médica/UPMM, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, and Dr. Nuno Rolão from the Unidade de Patologia Tropical/CMDT, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, for the critical review of this article and helpful suggestions.

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Conflict of Interest: No conflict of interest is declared