African Journal of Biotechnology Vol. 9(38), pp. 6408-6412, 20 September, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

*Full Length Research Paper*

# Detection of *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in *Glossina fuscipes fuscipes* (*Diptera: Glossinidae*) and *Stomoxys* flies using the polymerase chain reaction (PCR) technique in southern Sudan

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Accepted 5 May, 2010

**Ethanol-fixed entire bodies of the tsetse fly, *Glossina fuscipes fuscipes*, and unidentified stable flies, *Stomoxys* spp., collected from near Juba town, southern Sudan, were tested for *Trypanozoon* trypanosomes infections using polymerase chain reaction (PCR) technique for the first time in Sudan. The crude target DNA sequences were extracted by incubation of entire flies in Nonindet PCR template buffer containing proteinase-K. The DNA amplification sets of conditions were adjusted for each pair of primers employed. The oligonucleotide primers used included $TBR_{1-2}$, $SRA_{A-E}$, $SRA_{B537-B538}$ and $TgsGP_{FOR-REV}$. The results showed that 74.4% of *G. f. fuscipes* and 39.36% of *Stomoxys* spp. were infected with *Trypanozoon* trypanosomes. Out of the 117 examined *G. f. fuscipes*, 46.2, 24.8, 35.04, 17.09 and 10.26% were due to *T. b. gambiense* ($TgsGP_{FOR-REV}$), *T. b. rhodesiense* ($SRA_{A-E}$), *T. b. rhodesiense* ($SRA_{3537-3538}$), mixed infection with *T. b. gambiense* and *T. b. rhodesiense* and *T. b. brucei*, respectively. However, infections in *Stomoxys* spp. of 2.13 and 37.2% were due to *T. b. rhodesiense* and *T. b. brucei*, respectively.**

**Key words:** *Glossina fuscipes fuscipes*, *T. b. gambiense*, *T. b. Rhodesiense*, vectoral capacity, infection rate, PCR technology.

## INTRODUCTION

At the end of the 19th century, sleeping sickness or human african trypanosomosis (HAT) decimated about a quarter million people in central Africa including the whole of Uganda, Kenya shores and islands of Lake Victoria, Rwanda/Burundi, the Congo and northward into Equatoria Province of southern Sudan. This devastating epidemic was attributed to *Trypanosoma brucei gambiense* or the Gambian form of the disease (Bloss, 1960; Ford and Katondo 1971). However, at the time of the epidemic there were no alternative effective diagnostic tools, which might have led to the suspicion that the causative agent was anything other than *T. b. gambiense*. Nevertheless an outbreak of epidemic proportions attributed to *T. b. rhodesiense* was reported in sudanese/Ethiopian border in the early 1970s (Baker *et al*., 1974). Considering the current instability of people and livestock due to the latest war, there is a high probability of the spread and overlap of the two types of sleeping sickness in the rest of southern Sudan.

Although seven species of *Glossina* were recognized in Sudan (Lewis, 1949), *Glossina f. fuscipes* has been incriminated as the only vector of *T. b. gambiense*, the causative agent of HAT in southern Sudan (Snow, 1983). Empirical data have also shown that some biting flies can transmit pathogenic trypanosomes, but only few species in nature are considered epidemiologically important (Leak and Rowlands, 1997). Obtaining accurate knowledge of the dynamics of natural trypanosome infections in vector

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**Abbreviations: PCR,** Polymerase chain reaction; **HAT,** human African trypanosomosis.

**Table 1.** The PCR reaction buffer compositions.

| Materials (1rxn) | $TBR_{1-2}$ (µl) | SRA (µl) | $TgsGP_{FOR-REV}$ (µl) |
|---|---|---|---|
| 10x buffer | 2 | 2 | 2 |
| 200µM for each dNTPs | 2 | 2 | 0.5 |
| 1 µM specific primer -1 | 0.5 | 0.5 | 0.5 |
| 1 µM specific primer - 2 | 0.5 | 0.5 | 0.5 |
| 2.5 unit *Taq* DNA polymerase | 0.3 | 0.2 | 0.5 |
| Crude template DNA | 2 | 2 | 2 |
| Sub-total volume | 7.3 | 7.2 | 6 |
| De- ionized water | 12.7 | 12.8 | 14 |

Insects and the adventitious host will significantly contribute to a better understanding of the epidemiology of trypanoso-moses (Woolhouse et al., 1993).

The classical techniques used to detect trypanosome infection in flies (Lloyd and Johnson, 1924) are of limited sensitivity and specificity (Jordan, 1974; Woolhouse et al., 1994). Currently, trypanosome-detection techniques have significantly improved by using very high specific molecular tools such as the Polymerase chain reaction (PCR) (Majiwa and Otieno, 1990; Masiga et al., 1992; McNamara et al., 1995). In this paper we present results of experiments performed to identify and estimate the natural *Trypanozoon* trypanosomes infection rate of *Glossina fuscipes fuscipes* and *Stomoxys* spp in Juba area, southern Sudan using PCR technique (Masiga et al., 1992). The area had an active focus of sleeping sickness during the time of the study.

## MATERIALS AND METHODS

### Study area

The flies were collected from locations, near Juba town (4°40` - 5°N; 30°30'- 3°45`E), Central Equatoria, Southern Sudan. The area has an equatorial or semi-equatorial climate. The most dominant feature in the area is River Bahr El Jebel, which originates from Lake Victoria. Patches of evergreen thickets and double storey gallery forests grow along the banks of the river, and are infested with *Glossina fuscipes fuscipes*. The main trees of the gallery forest are *Ficus religiose, Tamarindus indica, Azadirachta indica, Anogeissus leiocarpus* together with *Combretum* spp. and various climbers and grasses. This riverine vegetation is however interrupted by several villages, hamlets and small numerous plots for subsistence farming. In most cases these plots have thick green hedges, which are deemed suitable habitats for *G. f. fuscipes* together with other biting flies including *Stomoxys* spp.

### Capture of flies

*G.f. fuscipes* and *Stomoxys spp.* were caught in five unbaited biconical traps (Challier et al., 1977). Traps were placed 200 m apart and catches were collected at 24 h intervals for three consecutive days. Captured flies were killed, counted and examined for tenerality. Only non-teneral male and female tsetse flies and 10% of engorged male and female *Stomoxys spp.* were used. These were preserved separately in ethanol in an eppendorf tube until examined for trypanosome infection using PCR technique (EANETT, 2005).

### Preparation of crude DNA templates

The ethanol was suck using a sterilized pipette. The fixed flies were left to dry by air. Then each fly was crushed under liquate nitrogen using a sterilized mortar and incubated in 100 µl template preparation buffer (10 mM Tris-Cl pH 8.3; 50 mM KCl; 1.5 mM MgCl; 0.9% Nonindet P40). 25 µl Proteinase - K was added to each tube at concentration of 60 mM. Thereafter, the mixture was incubated at 55°C for one hour in a water bath. The proteinase was denatured by increasing the temperature to 95°C for 10 min. Such preparations were either used immediately or stored at -20°C.

### Primers

The oligonucleotide primers used (Table 1) to amplify the target DNA sequences includes $TBR_{1-2}$ (Moser et al., 1989), $SRA_{A-E}$ (Gibson et al., 2002), $SRA_{B537-B538}$ (Welbum et al., 2001) and $TgsGP_{FOR-REV}$ (Radwanska et al., 2002).

### PCR cycling

PCR amplifications were carried out in 20 µl reaction mixture. The reaction buffer compositions were adjusted for each pair of primers used as shown in Table 1. The reaction mixtures were cycled in a programmed PCR machine for each pair of primer used as follows: for TBR-primers; initial denaturation 94°C/3 min (1 cycle), denaturation 92°C/30 s, annealing 60°C/45 s, extension 72°C/45 s (30 cycle), final extension 72°C/5 min (1 cycle). For SRA-primers; initial denaturation 95°C/3 min (1 cycle), denaturation 95°C/30 s, annealing 60°C/30 s, extension 72°C/60 s (35 cycle), final extension 72°C/5 min (1 cycle). For TgsGP-primers; initial denaturation 95°C/5 min (1 cycle), denaturation 94°C/45 s, annealing 63°C/45s, extension 72°C/120 s (45 cycle), final extension 63°C/10 min (1 cycle). Then 20 µl of each sample was electrophoresed through 1.5 - 2% agarose containing 0.5 µg $ml^{-1}$ Ethidium-bromide and the voltage was set at 60 V for the electrophoretic mobility to visualize the amplified DNA and compared to a standard DNA consisting of a known amount of bp.

## RESULTS

Overall the biconical traps caught 249 and 938 *G. f. fuscipes* and *Stomoxys* spp., respectively. A number of

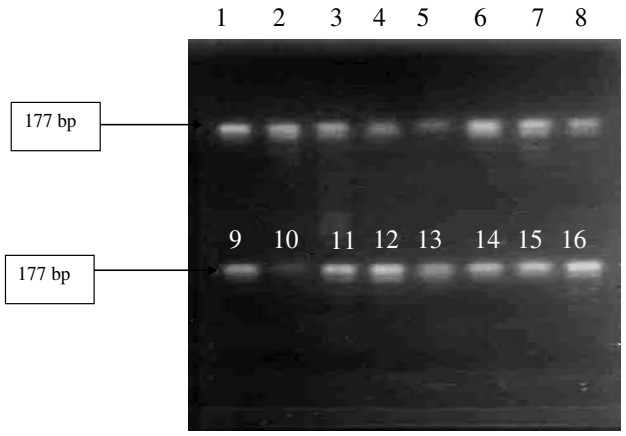


**Figure 1.** Ethidium-bromide stained 1.5% agarose gel showing PCR amplification of crude template of whole *Glossina fuscipes fuscipes* and *Stomoxys* flies using $TBR_{1-2}$ primer sets: 1-16 *Trypanosoma brucei* lanes show 177 bp using ΦX174 Hae III Digest 100-bp marker.

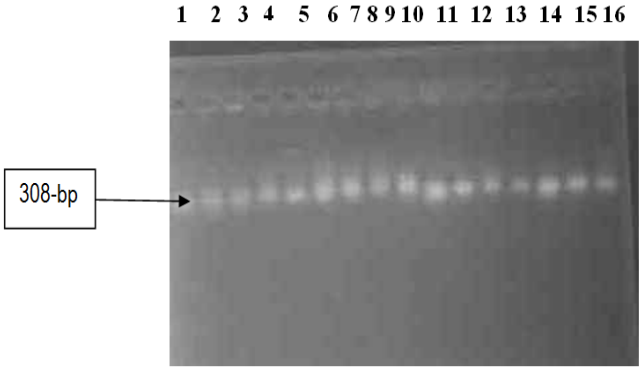


**Figure 2.** Ethidium-bromide stained 1.5% agarose gel showing PCR amplification of crude template of *Glossina fuscipes fuscipes* females using $TgsGP_{FOR-REV}$. 1-16 *T.b.gambiense* lanes show 308-bp using ΦX174 Hae III Digest 100-bp marker.

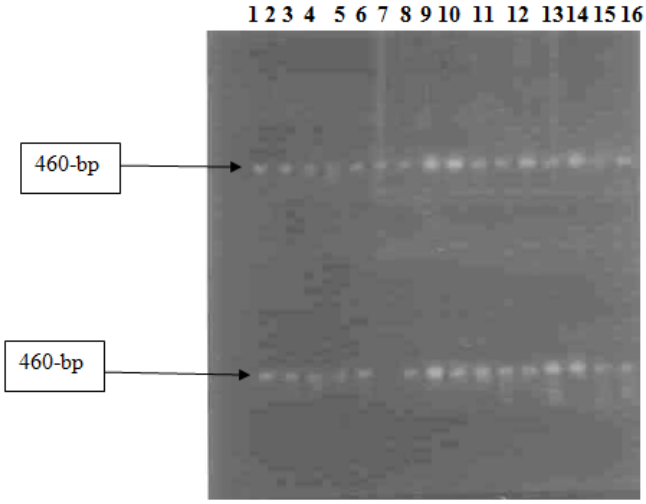


**Figure 3.** Ethidium-bromide- stained 1.5% agarose gel showing PCR amplification of crude template of *Glossina fuscipes fuscipes* males using $SRA_{A-E}$ and $SRA_{3537-3538}$ pair primer sets: 1-16 first row *T. b. rhodesiense* $SRA_{A-E}$ and1-16 second row *T. b. rhodesiense* $SRA_{3537-3538}$ lanes show 460-bp using ΦX174 Hae III Digest 100-bp marker.

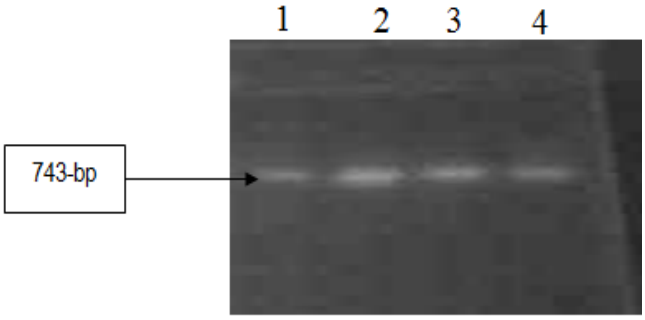


**Figure 4.** Ethidium-bromide - stained 1.5% agarose gel showing PCR amplification of crude template of *Stomoxys* using $SRA_{B537-B538}$ primer sets. 1,2,3,4 replicates *T. b. rhodesiense* lanes show 743-bp using ΦX174 Hae III Digest 100-bp marker

117 non-teneral male and female *G. f. fuscipes* and 94 engorged male and female *Stomoxys* spp were examined using $TBR_{1-2,}$ $SRA_{A-E}$, $SRA_{B537-B538}$ and $TgsGP_{FOR-REV}$ $SRA_{B537-B538}$ primers for infection with *Trypanozoon* trypanosomes. Among the 117 *G. f. fuscipes* examined, 87 reacted positively with $TBR_{1-2}$ primers: 54, 29 and 41 reacted positively with $TgsGP_{FOR-REV}$, $SRA_{A-E}$ and $SRA_{B537-B538}$ pair primers, respectively, while 20 of the samples reacted positively with the three set of primers used. In contrast 37 and 2 out of 94 *Stomoxys* flies reacted positively with $TBR_{1-2,}$ and primers, respectively. Amplification products of expected band size for *Trypansoma brucei* group, *T. b. gambiense*, and *T. b. rhodesiense* are clearly visible in Figures 1, 2, 3, and 4.

The analysis of the results obtained (Tables 2 and 3) indicated that the overall infection rate of *Trypanozoon* was 74.4% based on the number of the non-teneral *G. f. fuscipes* flies. The burden of flies harboring *T. b. gambiense* trypanosome was 46.2% (54/117). Of the 54 infected flies, 34 were infected with *T. b. gambiense* and 20 with *T. b.* $SRA_{B537-B538}$ *gambiense* and *T. b. rhodesiense*. The rate of flies infected with *T. b. rhodesiense* using $SRA_{A-E}$ and primers was 24.8% (29/117) and 35.04% (41/117), respectively: Of the 29 flies that reacted with $SRA_{A-E}$, 20 flies harboring *T. b. gambiense* and *T. b. rhodesiense* mixed infection; Of the 41 flies that reacted with $SRA_{B537-B538}$, 21 flies reacted as well with $SRA_{A-E}$ and 20 flies harboring *T. b. gambiense* and *T. b. rhodesiense* mixed infection.

The overall mixed infection of *T. b. gambiense* and *T. b. rhodesiense* was 17.09% (20/117). The remaining

**Table 2.** *Trypanozoon* infection rates detected by PCR technique in wild *G. f. fuscipes* and *Stomoxys* flies.

| Fly spp. | $TBR_{1-2}$ | $TgsGP_{FOR-REV}$ | $SRA_{A-E}$ | $SRA_{B537-B538}$ | Multiple |
|---|---|---|---|---|---|
| *G.f.fuscipes* | 74.4%(87/117) | 46.2%(54/117) | 24.8%(29/117) | 35.0%(41/117) | 17.0%(20/117) |
| *stomoxys* | 39.4%(37/94) | 0 | 0 | 2.13%(2/94) | 0 |

**Table 3.** The number of wild *G. f. fuscipes* and *Stomoxys* flies infected with *Trypanozoon* trypanosomes detected by PCR technique.

| | $TBR_{1-2}$ | $TgsGP_{FOR-REV}$ | $SRA_{A-E}$ | $SRA_{B537-B538}$ |
|---|---|---|---|---|
| $TBR_{1-2}$ | 12 | 54 | 29 | 41 |
| $TgsGP_{FOR-REV}$ | 54 | 34 | 20 | 20 |
| $SRA_{A-E}$ | 29 | 20 | 9 | 29 |
| $SRA_{B537-B538}$ | 41 | 20 | 29 | 12 |

infection was attributed to *T. b. brucei* of 10.26% (12/117). None of the *Stomoxys* flies were found infected with *T. b. gambiense*, nevertheless, 2.13% (2/94) and 37.2% (35/94) of these *Stomoxyinae* were infected with *T. b. rhodesiense* and *T. b. brucei*, respectively.

## DISCUSSION

The present work shows that *G. f. fuscipes* is the potential vector of sleeping sickness ascribed to *T. b. gambiense* or *T. b. rhodesiense* in the study area and *Stomoxys* species may play a role in transmission of *T. b. rhodesiense* because they usually feed on animals as well as humans. The *G. f. fuscipes* is probably the only vector of sleeping sickness; this is because no other *Glossina* spp. had been found in the area. According to Ford (1963) and Ford and Katondo (1977), the distribution of *G. f. fuscipes* overlaps with that of *G. morsitans submorsitans* in the study area in southern Sudan. However, recently only *G. f. fuscipes* was detected in the area (Mohammed, 2004). Although the lack of capture of *G. m. submorsitans* could as well be due to the absence of this species in the area, the use of the bi-conical trap as the only catching device and without odour bait might have also contributed to the dearth in catches *G. m. submorsitans* (Mohamed-Ahmed et al., 1993).

The species-specific oligonucleotide primers for PCR amplification becomes obtainable for different trypanosomes. Consequently, correct identification and classification of trypanosomes in archived samples, tsetse and other biting flies is possible (Stijn et al., 2008). In view of the fact that the amplification of the crude preparations of templates from a whole fly produced clearly visibly an expected band size for *Trypansoma brucei* group, *T. b. gambiense*, and *T. b. rhodesiense* is an indication of infection. Thus, this indicates that tsetse flies of *G. f. fuscipes* are the only vectors transmitting human african Trypanosomosis in the study area. To date no evidence has been reported elsewhere in Africa of biological transmission of trypanosomes responsible for HAT in vectors other than *Glossina* (Leak, 1999). Conversely, there was ample evidence that biting flies *Stomoxys* are responsible for the maintenance and spread of Nagana diseases causative trypanosomes including *Trypanozoon* (Karib, 1961; Abdulla et al., 2005).

The PCR technique (Masiga *et al*., 1992; EANETT, 2005) was used in the present study to identify and detect trypanosomes infections in tsetse and stable flies. This was done in order to estimate the role of *G. f. fuscipes* and *Stomoxys* spp. in the epidemiology of HAT in southern Sudan as the traditional trypanosome detection techniques (Lloyd and Johnson, 1924; Mohamed-Ahmed *et al*., 1989) which rely on microscopic examination could identify parasites to the subgenus level only and would not differentiate between *brucei rhodesiense, brucei gambiense* or *brucei brucei* trypanosomes.

Some authorities base infection rates on the number infected out of the total non-teneral male and female flies whereas others base infection rates on total flies examined including tenerals (Jordan, 1974; Mihok et al., 1992). In the present work infection rates was based on non-teneral *G. f. fuscipes* flies examined for infection using PCR. This was done because non-teneral tsetse flies are more likely to get infected than teneral ones which have yet to obtain their first blood meal (Welburn and Maudlin, 1992).

Recent and previous devastating sleeping sickness epidemics in sudan were attributed to the Gambian form of the disease only (Bloss, 1960) although at the time of those epidemics, effective diagnostic techniques such as the PCR were not developed to verify the exact cause. Moreover, Sudan lies in the interface of the geographical belt of both types of diseases and that *T. b. rhodesiense* had caused an epidemic outbreak in Sudan eastern border (Baker et al., 1974).

The present results indicate that *G. f. fuscipes* and *Stomoxys* spp found in Bahr El Jebel State were infected

with *T. b. rhodesiense*. This suggests that the *rhodesiense* form of the disease might have been present in the area but had been overlooked due to inefficient diagnostic techniques in both human and vectors.

## ACKNOWLEDGMENTS

Tropical Medicine Research Institute (TMRI), National Centre of Research, Animal Resources Research Corporation and the East African Network for Trypanosomosis (EANETT) financial and technical support throughout this study are appreciated.

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