Polymerase chain reaction identification of *Trypanosoma brucei rhodesiense* in wild tsetse flies from Nkhotakota Wildlife Reserve, Malawi

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Abstract

**Background**

*Trypanosoma brucei rhodesiense* is the causative agent of acute human African trypanosomiasis. Identification of *T. b. rhodesiense* in tsetse populations is essential for understanding transmission dynamics, assessing human disease risk, and monitoring spatiotemporal trends and impact of control interventions. Accurate detection and characterisation of trypanosomes in vectors relies on molecular techniques. For the first time in Malawi, a molecular technique has been used to detect trypanosomes in tsetse flies in Nkhotakota Wildlife Reserve.

**Methods**

A polymerase chain reaction (PCR) technique was used to identify the serum resistance associated (SRA) gene of *T. b. rhodesiense* in tsetse flies. Of 257 tsetse flies that were randomly caught, 42 flies were dissected for microscopic examination. The midguts of 206 flies were positive and were individually put in eppendorf tubes containing phosphate-buffered saline (PBS buffer) for DNA extraction. Internal transcribed spacer (ITS)-PCR was first used to isolate all trypanosome species from the flies. TBR PCR was then used to isolate the *Trypanozoon* group. *T. brucei*-positive samples were further evaluated by SRA PCR for the presence of the SRA gene.

**Results**

Of 257 flies caught, 185 (72%) were *Glossina morsitans morsitans* and 72 (28%) were *Glossina pallidipes*. Three were tenellars and 242 were mature live flies. Of the 242 flies dissected, 206 were positive, representing an 85.1% infection rate. From 206 infected flies, 106 (51.5%) were positive using ITS-PCR, 68 (33.0%) being mixed infections, 18 (8.7%) *Trypanosoma simae*, 4 (1.9%) *Trypanosoma vivax*, 3 (1.5%) *Trypanosoma congolense* savanna, 3 (1.5%) *Trypanosoma simiae*, and 1 (0.4%) *Trypanosoma simias*. When subjected to TBR PCR, 107(51.9%) were positive for *T. brucei*. Of the 107 *T. brucei*-positive samples, 5 (4.7%) were found to have the SRA gene.

**Conclusions**

These results suggest that wild tsetse flies in Malawi are infected with human-infective trypanosomes that put communities around wildlife reserves at risk of human African trypanosomiasis outbreaks. Further studies need to be done to identify sources of blood meals for the flies and for surveillance of communities around wildlife reserves.

**Introduction**

Tsetse flies (*Glossina* spp.) are the vectors for the transmission of both human and animal African trypanosomiasis. Animal trypanosomiasis is called nagana, and it affects domestic animals. The reservoir hosts for both human and animal trypanosomiasis are wild animals—particularly antelopes,¹ with domestic animals contributing to a lesser extent.² The human disease (also known as sleeping sickness) is caused by 2 species of *Trypanosoma brucei*. These are *Trypanosoma brucei rhodesiense*, which is confined to East Africa in Malawi and Tanzania, though it is also reported to be present in some parts of Uganda. *T. b. rhodesiense* is commonly associated with acute infection. Chronic disease is mainly caused by *Trypanosoma brucei gambiens*, which is currently confined to West Africa, in particular DR Congo, Angola, Guinea, and Côte d’Ivoire.³

There are several species of tsetse flies (*Glossinidae: Diptera*) that feed on both humans and animals. There are specific foci for these flies. The flies that are good vectors for *T. b. rhodesiense* (namely, *Glossina morsitans* morsitans, *Glossina swynnertii*, *Glossina austeni*, *Glossina longipennis*, *Glossina pallidipes*, *Glossina bravo palpis*, and *Glossina fuscipes fuscipes*) are mainly confined to the southwestern part of Africa, while the flies that are good vectors for *T. b. gambiens* (namely, *Glossins fuscipes fuscipes*, *Glossins taenioides*, and *Glossins morsitans*) are mainly confined to the western part of the continent. However, there is an overlap of these flies, mainly in northwest and southeast Uganda.⁴ It is clear that *Glossins fuscipes* and *Glossins morsitans* are ubiquitous in their locations, making these species of tsetse flies good vectors for both acute and chronic human African trypanosomiasis.

The agents of sleeping sickness belong to the genus *Trypanosoma*, subgenus *Trypanozoon*. There are 3 subspecies of *Trypanosoma brucei*, namely, *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei gambiens*, and *Trypanosoma brucei brucei*. The latter is associated with animal trypanosomiasis and has no effect on human hosts. These species cannot be distinguished morphologically by microscopy. There are molecular techniques that utilise the genetic differences between these parasites that can be used to distinguish them. A commonly used technique involves the serum resistance-associated (SRA) gene, which is lacking in *T. b. gambiens* but is present in *T. b. rhodesiense*. The primers for the SRA gene are used in a polymerase chain reaction (PCR) technique to specifically distinguish the 2 forms.¹¹,¹² Another molecular method uses the internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) of trypanosomes. The ITS region of rDNA is the preferred target for a universal test because of its highly conserved flanking regions and size variability among trypanosomes species and subgroups. Therefore, the ITS1 PCR technique is used to identify almost all trypanosome species.¹³

The current study was designed to identify if the species of tsetse flies that inhabit Nkhotakota Wildlife Reserve harbour *T. b. rhodesiense*.
Methods

Two approaches were used to isolate transmissible *T. brucei* spp. and other trypanosome species: (1) dissection and microscopy, followed by (2) PCR on single midgut samples.

**Trapping of tsetse flies**

After getting clearance from Nkhotakota Wildlife Reserve officials, and in collaboration with the Malawi Ministry of Agriculture, 5 locations in Nkhotakota Wildlife Reserve were purposefully identified as ideal for trapping tsetse flies. Fly traps were deployed at a distance of 100 m apart. A total of 100 traps were deployed, which included both biconical and Epsilon traps (Figure 1) that were baited with phenols and acetone before being deployed. Tsetse flies were harvested every 24 hours for 2 weeks and there was a break of 3 months during the year. All flies were sorted, according to sex and species, using an ordinary microscope. To distinguish between *Glossina morsitans morsitans* and *Glossina pallidipes*, differences in antennal shape and antennal fringe proportions were noted. All tenerals (those that have not yet taken their first blood meal) and dead flies were recorded but were not included in the investigation. Tsetse flies were dissected and each midgut was examined parasitologically (Table 1) using Olympus dissecting and biological microscopes, respectively. Each midgut was then placed in eppendorf tubes containing 200 µL of phosphate-buffered saline (PBS buffer) and were frozen at −20°C for DNA extraction. DNA was extracted from these midguts using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD, USA).

**PCR steps to identify T. b. rhodesiense**

1. **ITS1 rDNA PCR to identify all trypanosome species**

Midguts of all tsetse flies were tested for the presence of trypanosomes using ITS1 PCR, which can even isolate mixed infections in a sample. The ITS1 rDNA primers used were: The forward 5'-CCGGAAGTTCACCGATATTG-3' and reverse 5'-TTGCTGCGTTCTTCAACGAA-3'. PCR was carried out in 25-µL reaction mixtures containing 5X Phusion HF buffer with a 1.5 mM MgCl₂, final reaction concentration (Thermo Fisher Scientific, Waltham, MA, USA), 10 mM of each of the 4 deoxynucleoside triphosphates (dNTPs), primers at 1 µM, 0.25 µl of Taq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), and 3 µl of DNA template. PCR cycles for the primers were: initial step at 98°C for 10 seconds, followed by 35 cycles of 98°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, and final extension at 72°C for 10 minutes using a Techne Genius thermal cycler (Cole-Parmer, Stone, United Kingdom). Amplification products were visualised in 2 % molecular grade agarose gel (Fisher Biotec, Wembley, Australia) stained with ethidium bromide on an ultraviolet (UV) transiluminator (Syngene, Frederick, MD, USA). The PCR product band sizes and species identified are indicated in Table 2 and Figure 2.

2. **TBR PCR to identify all trypanozoons**

Though ITS1 PCR can identify trypanosomes from the Trypanozoon subgenus, TBR PCR is used as a confirmatory test because it exclusively selects Trypanozoon parasites. The TBR1 and TBR2 PCR primers used were 5'-CGAATGAATATTAAACAATGCGCAG-3' and 5'-AGAACCATTTATTAGCTTTGTTGC-3', respectively. PCR was carried out in 25-µL reaction mixtures containing 5X Phusion HF buffer with a 1.5 mM MgCl₂, final reaction concentration (Thermo Fisher Scientific, Waltham, MA, USA), 10 mM of each of the 4 dNTPs, primers at 1 µM,

<table>
<thead>
<tr>
<th>PCR method</th>
<th>Midguts analysed</th>
<th>Positive</th>
<th>Species identified</th>
<th>Expected product band size (base pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1-PCR</td>
<td>206</td>
<td>106</td>
<td>Mixed infection</td>
<td>68 (33%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18 (8.7%)</td>
<td><em>T. brucei</em></td>
<td>480 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 (4.4%)</td>
<td><em>T. vivax</em></td>
<td>250 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 (1.9%)</td>
<td><em>T. godfrey</em></td>
<td>300 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (1.5%)</td>
<td><em>T. simiae</em></td>
<td>400 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (1.5%)</td>
<td><em>T. congolense</em></td>
<td>700 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 (0.5%)</td>
<td><em>T. simersi</em></td>
<td>370 (100%)</td>
<td>Njiru et al.13</td>
</tr>
<tr>
<td>TBR-PCR</td>
<td>206</td>
<td>107</td>
<td><em>T. brucei</em></td>
<td>177 (100%)</td>
<td>Moser et al.14</td>
</tr>
<tr>
<td>SRA-PCR</td>
<td>107</td>
<td>5</td>
<td><em>T. rhodesiense</em></td>
<td>284 (100%)</td>
<td>Radwanska et al.12</td>
</tr>
</tbody>
</table>

Table 2: Results of various polymerase chain reaction (PCR) methods, species identified, and expected PCR product sizes

**Figure 2: Representative gel of ITS1-PCR showing various trypanosome species and mixed infections**

L = molecular ladder; NC = negative control; PC = *T. brucei* positive control; 20, 22, 24 to 30 are positive samples for different species
A total of 257 flies were caught (Table 1). Of these, 185 flies (72%) were *Glossina morsitans morsitans* and 72 (28%) were *Glossina pallidipes*. Among all flies, 140 (54.5%) were females and 117 (45.5%) were males. Three (1.2%) were teneral and 12 (4.7%) were dead on the day of sampling. Only mature live flies (242 total, comprising 133 females and 109 males) were dissected for further analysis. In total 206 flies out of the 242 dissected flies were identified with trypanosomes in their midguts, representing an infection rate of 85.1%.

When the subset of live female flies was studied, 116 (87.2%) of 133 were carrying trypanosomes in their midguts. On the other hand, 90 (82.6%) of the 109 live male flies caught were carrying trypanosomes in their midguts. When the 206 infected samples were subjected to ITS rDNA PCR, 106 (51.4%) were positive (Figure 2), and flies infected with more than 1 trypanosome species were observed. Samples from the 206 infected flies were also subjected to TBR PCR (Figure 3), and 107 samples (51.9%) were positive for *T. brucei*. This observation showed that tsetse flies in Nkhotakota Wildlife Reserve are highly infected with parasites that could be a risk to human disease.

### Discussion

Previous studies have also shown that *Glossina morsitans morsitans* and *Glossina pallidipes* are the most prevalent species of tsetse fly in the wildlife reserves of Malawi. The present study is in agreement with this observation. However, the prevalence of *Glossina morsitans morsitans* was higher than that of *Glossina pallidipes*. It was also noted that the infection rates of both species was not sex dependent, as there was no difference between the proportion of the male (82.6%) and female (87.2%) flies with midgut infections. This shows that the feeding habits of these flies are similar to one another, and that they are not dependent on the egg maturity of female flies. One would have expected a different infection rate if blood meals were directly associated with egg maturity. There would also be a difference if each species had a different preferred host for feeding. The proportion of flies infected was also higher than has been observed in other tsetse flies, both in the region and other areas (unpublished observations). This could partly be explained by a common host animal that is preferred for feeding by the flies in Nkhotakota Wildlife Reserve. It is well established that *Glossina morsitans morsitans* are efficient vectors of trypanosomiasis, as they easily establish infections in their midguts after a blood meal. These flies likely feed on antelopes, which are prevalent in the nature reserve and in their midguts in Nkhotakota Wildlife Reserve carry trypanosomes that are infective to humans.
flies, implying that they are also prevalent in the reservoir hosts. The direct TBR PCR showed that of all infected flies, 107 (51.9%) of 206 were harbouring *Trypanosoma brucei* infection. The proportion of DNA samples from the same infected flies showed that 106 (51.4%) of 206 were positive with ITS rDNA PCR. ITS rDNA PCR does not distinguish one trypanosome species from another. The difference in diagnostic sensitivity between ITS rDNA PCR and TBR PCR is partly explained by mixed infections in some of the tsetse flies. If every fly was infected by a single parasite, the proportions between TBR PCR and ITS rDNA PCR would have been the same. Additionally, ITS1 has approximately 100 to 200 copies, as opposed to species-specific tests that target satellite DNA with over 10,000 copies, making them more sensitive than universal tests like ITS1. As a result of this finding, it became necessary to distinguish the species of *Trypanosoma brucei* in the DNA of 107 positive tsetse flies that were identified after ITS rDNA PCR and TBR PCR. This was done by nested PCR for the SRA gene. From the 107 samples, 5 were found positive for the SRA gene (Figure 4). SRA gene identification confirmed the existence of *Trypanosoma brucei rhodesiense*, the causative agent of the acute human African trypanosomiasis found in Malawi and other countries in the southern African region.

From these molecular studies, it is apparent that animal infections and possibly human trypanosomiasis disease can be transmitted in this wildlife reserve. Indeed, over the years, high levels of human infections of trypanosomiasis have been reported at Nkhotakota District Hospital since records of cases began. The level of human infections has been on the decline, while the data for domestic animal infections is scanty. The present study has also confirmed that both human and animal infections are likely to be caused by the *Trypanosoma brucei* subspecies in and around Nkhotakota Wildlife Reserve. Cases of trypanosomiasis have been reported in tourists and soldiers that have visited some of Malawi's wildlife reserves. Moreover, the cases of trypanosomiasis have also been shown to be subacute in some studies, and showing a genetic susceptibility in some people. Notably, the high infection rates that have been shown in the present study have not translated in a trypanosomiasis epidemic in Malawi. The possible explanation of this is lack of a human–tsetse fly interface.

## Conclusions

The present study has shown that tsetse flies in Nkhotakota Wildlife Reserve have a high infection rate at 85.1%, and more than half of these infections are attributed to *Trypanosoma brucei* species that are causative agents of both human and animal disease. There is a need to sample blood from both animals and humans to establish whether the risk translates to infections in human beings, as well as cattle, pigs, goats, and wildlife in and around the reserves. In addition, there is a strong need to intensify tsetse trapping to reduce the number of flies in the park. Blood meal analysis would assist in identifying the common sources of infection between flies. This would clarify the observation that tsetse flies seem to prefer a common source of host feeding, as suggested by the same infection rate between species and sexes. Detection of trypanosomes in the vector indicates the presence of potentially infective to both humans and animals in the surrounding areas. It is sensible to suggest that resources should be mobilised to prevent and mitigate potential trypanosomiasis epidemics, both in humans and animals.

## Acknowledgements

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## Competing interests

All authors declare that they have no competing interests related to this work.

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