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# Molecular xenomonitoring of trypanosomes in tsetse flies

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**Monitoring trypanosomes infections in wild-caught tsetse flies in a given area, is important in prediction of epidemic outbreaks and spread of disease, and could help focus control programs for areas requiring immediate attention in order to limit disease transmission and spread. The main objective of this study is to evaluate the recently developed RIME LAMP and PanTryp LAMP for screening large numbers of tsetse flies for trypanosomes and to assess their sensitivities and specificities for trypanosomes in endemic areas. Wild-caught tsetse flies were dissected and the mid-guts examined by microscopy. The mid-guts were pooled in fives (including one infected gut where applicable), homogenised and DNA extracted by Quiagen kits. TBR- and ITS-PCRs were carried out and examined under ethidium bromide-stained agarose gels while RIMELAMP and PanTryp LAMP were carried out and stained with SYBR green and also observed under ethidium bromide stained agarose gels. A total of 14912 tsetse flies identified as *Glossina fuscipes fuscipes, Glossina. pallidipes, Glossina morsitans, Glossina. swynnertoni*, *Glossina fuscipes quazensis* were trapped from the six different countries. Of these, 8789 were dissected. Both males and female tsetse flies had equal infection rates (12.2%) although overall infection rates varied with country. The highest number of infected tsetse flies was obtained by PanTryp LAMP followed by RIME LAMP, ITS-PCR, TBR-PCR and microscopy respectively. PanTryp LAMP was the most sensitive method followed by ITS-PCR, RIME LAMP and TBR-PCR respectively. However, ITS-PCR was the most specific followed by TBR-PCR, RIME LAMP and PanTryp LAMP respectively. Carrying out LAMP tests in the field provides the simplest and quickest means to estimate trypanosome infection rates in the vector tsetse flies.**

**Key words:** Xenomonitoring, trypanosome, tsetse fly, LAMP.

## INTRODUCTION

Human African trypanosomiasis (HAT) is an important public health problem that affects rural populations of sub-Saharan Africa. The epidemiology of sleeping sickness disease is mediated by the interactions of trypanosomes with the vectors (tsetse flies) which transmit the disease to humans and animal hosts within a particular environment. The disease is usually confined in spatially limited areas referred to as " foci" of the disease found in remote rural areas in Sub Saharan Africa (Simarro et al., 2010). The risk of getting infected with the disease is, therefore, through the bite of a human being by an infected tsetyse fly. Consequently, deploying integrated control methods in areas infested with infected tsetse flies would drastically reduce the prevalence of the disease. With limited resources experienced by endemic countries, methods that would indicate such areas would be very useful to interrupt disease transmission (Franco et al., 2014). After continued control efforts in many of the endemic countries, the number of sleeping sickness cases reported in 2009 dropped below 10,000 for the first time in 50 years. This trend has been maintained in 2010 with 7,139 new cases reported (WHO Fact sheet No 259, January, 2012). In 2010, only the Democratic Republic of the Congo (DRC) reported over 500 new cases per year while Angola, Central African Republic, Chad, Sudan and Uganda reported between 100 and 500 new cases per year. Other countries such as Cameroon, Congo, Cote d'Ivoire, Equatorial Guinea, Gabon Guinea, Malawi, Nigeria, Tanzania, Zambia and Zimbabwe reported fewer than 100 new cases per year. However, current estimates indicated an annual incidence of between 50,000 and 70,000 cases (WHO, Fact sheet No 259, January 2012).

The disease in the DRC and Congo are due to *Trypanosoma brucei gambiense* that causes the chronic form of HAT while Tanzania, Malawi and Uganda are endemic for the acute form called *Trypanosoma brucei rhodesiense,* which may cause disease within weeks (Thomson et al., 2009). Uganda is the only country with both the chronic and the acute form of HAT whose foci are distinct but are feared to overlap hence complicating the diagnosis and treatment regime (Picozzi et al., 2005). HAT is invariably fatal if left untreated and major efforts to control the disease rely on strategic control which involves diagnosis and treatment of infected cases coupled with control of both the vector and reservoir (Simo et al., 2012).

Diagnostic tools appropriate for undertaking interventions to control trypanosome infections are key element to their success. Many diagnostic tests for trypanosome infection in the vector have unsatisfactory performance characteristics and are not well suited for use in the parasite control programs that are being increasingly implemented. It was argued that PCR techniques would simplify analysis of tsetse collected in the field (Moser et al., 1989; Majiwa and Otieno, 1990; Radwanska et al., 2002; Njiru et al, 2005; Adams et al., 2006), but was later noted that such studies tend to exaggerate both mature and immature fly infections rates when compared with microscopy (Farikou et al., 2010 and Simo et al., 2012). The presence of trypanosome DNA in a tsetse fly does not necessarily indicate a mature infection or even an established mid-gut infection as the trypanosome DNA could be from some blood meal taken just prior to analysis (Macleod et al., 2007). Furthermore, Farikou et al. (2010) state that a mid-gut infection does not indicate a mature infection that will be transmitted. However, for control purposes any tsetse fly infection is important for deployment of control strategy instead of waiting until infection is established in humans or animals. Although the application of modern laboratory research techniques to improve diagnostics for trypanosome infection has resulted in some technical advances, uptake has not been uniform. Frequently, pilot or proof of concept studies of promising diagnostic technologies have not been followed by much needed product development, and in many settings diagnosis continues to rely on insensitive and unsatisfactory parasitological (Woo, 1969; Molyneaux, 1975; Nantulya, 1990) or serodiagnostic techniques (Boakye et al., 1999; Njiokou et al., 2004). In contrast, Loop mediated isothermal amplification (LAMP)-based (Notomi et al., 2000; Kuboki et al., 2003; Njiru et al., 2008a) detection of trypanosomes in the tsetse vectors will result in critical advances in the control of both HAT and Animal African Trypanosomiasis (AAT).

Monitoring parasite prevalence in wild-caught vector populations in a given area (known as "xenomonitoring") has often relied on dissection of insect vectors and observation of parasites under a microscope, a process that is time consuming and depends on the skill of the microscopist (Auty et al., 2012). PCR techniques have now gained more use for example: *Plasmodium sp* (Snounou et al., 1993), *Leishmania spp* (Aransay et al., 2000; Dyab et al., 2015), *Oncocerca vulvulus* (Katholi et al., 1995; Rodriguez-Perez et al 1999). Xenomonitoring is important in prediction of epidemic outbreaks of disease and could help focus control programs to areas requiring immediate attention in order to halt disease transmission. This study aimed to evaluate the recently developed RIME LAMP and Pantryp LAMP for screening large numbers of tsetse flies for trypanosomes and to assess

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their sensitivities and specificities for trypanosomes in endemic areas.

## METHODOLOGY

### Tsetse collection and examination

During this study, tsetse flies were collected during field surveys between 2011 and 2012 in different sites for each country (Uganda – Kaberamaido and Dokolo, Tororo and Arua; Tanzania – Serengeti and Urambo; Malawi – Kasungu, Liwonde and Nkhotakota; Republic of Congo, Ngabe and Mboka Lefini, the Democratic Republic of Congo- Itubi and Bandundu). The tsetse flies were tested for *Trypanozoon* infection by dissecting out the mid guts of parasites by light microscopy and then pooled in groups of five (1 infected plus 4 non-infected or five non-infected) and kept in 1× PBS in liquid nitrogen tanks (Malele et al., 2013). The samples were then transported from the field sites to the research laboratories in respective countries. Upon arrival the specimens were stored at -80°C till processed. In the field lab, the tsetse flies were classified as positive if parasites were observed in the mid gut, but were classified as non-infected if no trypanosomes were observed in the mid gut by microscopy.

### Isolation of parasite DNA

The mid guts were homogenized by a pestle in eppendorf tubes. Parasite DNA was extracted from the pooled midguts using the Qiagen kit (Crawley, UK) as per the manufacturer's instructions. The DNA was eluted in 50µL of Tris-EDTA (TE, Sigma Aldrich, Dorset, UK) and stored at -20°C until further analysis.

### Polymerase chain reaction for the detection of parasite DNA

The nucleic acid extracts were analysed with TBR PCR and ITS-PCR, Both PCR assays were performed in a DNA thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT, USA). TBR-PCR assay used two oligonucleotide primers which allowed the amplification of the TBR repeat (284bp) as described by Masiga et al. (1992) that is specific to *Trypanosoma brucei* subspecies. The sequences of these primers are: TBR1F: 5'--CGAATGAATATTAAACAATGCGCAG -3' (25-mer); TBR1R: 5'-AGAACCATTTATTAGCTTTGTTGC -3' (24-mer). Each amplification reaction was made in a final volume of 25µL containing 10 mM Tris–HCl pH 9.2, 1.5 mM $MgCl_2$, 75 mM KCl, 1.25 mM of each dNTP, 12.5pmol of each oligonucleotide primer for TBR-PCR, 1 U of Phusion High-Fidelity DNA polymerase (Thermo scientific, Waltham, USA) and 2µl of DNA template. The temperature program for the TBR-PCR was 1 min at 98°C, followed by 35 cycles of 30 sec at 98°C, 30 sec at 62°C, 2 min at 72°C and a final cycle of 7 min at 72°C. After PCR, 10µL of each sample was run on a 2% agarose gel and stained with ethidium bromide. The remaining volume was stored at -20°C. The ITS-PCR used two oligonucleotide primers with the following sequences: ITSBR 5'-TTG CTG CGT TCT TCA ACG AA-3' (20 mer) and ITSCF 5'-CCG GAA GTT CAC CGA TAT TG-3' (20 mer) for amplification of Internal transcribed spacer (ITS 1) as described by Njiru et al. (2005). Each amplification reaction was made in a final volume of 25µL containing 10 mM Tris–HCl pH 9.2, 1.5 mM $MgCl_2$, 75 mM KCl, 1.25 mM of each dNTP, 100 pmol of each oligonucleotide primer for ITS- PCR 1 U of Phusion High-Fidelity DNA polymerase and 2µl DNA template. The temperature program for the ITS-PCR was 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C and a final cycle of 5 min at 72°C. After PCR, 10 µl of each sample was run on a 2% agarose gel and stained with ethidium bromide. The remaining volume was stored at -20°C.

### Detection of trypanosome DNA using RIME LAMP and PanTryp LAMP

The nucleic acid extracts were analyzed with RIME LAMP (Njiru et al., 2008b) and PanTryp LAMP. The 25 µl LAMP reactions were standardized for optimal reagent concentration. Briefly, the reactions were carried out at 2 µM for FIP and BIP primers, 0.8 µM for loop primer (LF and LB), 0.2 µM for F3 and B3 outer primers, master mix and 8U of Bst DNA polymerase large fragment (New England Biolabs). The reactions were carried out for 1 h at 62°C using the DNA thermal Cycler (Perkin–Elmer Cetus, Norwalk, CT, USA), and terminated by increasing the temperature to 80°C for 5 min. The amplification products are detected by direct visual inspection of the LAMP product after addition of 1 µl of 1/10 dilution of SYBR Green I (Invitrogen), and ethidium bromide stained agarose gels. Nucleic acids extracted from an in-vitro culture of procyclic *T. b. rhodesiense* were used as a positive control. Ultrapure water and gut extract of non-infected laboratory tsetse were used as negative controls. A test was considered positive if a green colour was visible and if the agarose gel showed the characteristic ladder pattern (Njiru et al., 2010).

## RESULTS AND DISCUSSION

### The tsetse flies

In this study, a total of 14,912 tsetse flies were trapped. However, only 8,789 live mature flies were dissected. Tenerals and dead flies were kept frozen and not analyzed any further. The high percentage of dead flies could be due to the high temperatures experienced during fly trapping and exhaustion as flies continually try to escape from the traps. In total, therefore, 8 789 tsetse flies were dissected of which 2,444 (27.8 %) were males and 6,345 (72.2%) were females. Overall, both male and females tsetse flies were equally infected with trypanosomes with a 12.2% infection rate for each sex. Of the total tsetse catches dissected, 1,359 were *G. f. fuscipes* of which 21 (1.5%) were infected; 2,726 were *G. pallidipes*, of which 68 (2.5%) were infected; 992 were *G. morsitans* of which 693 (69.9%) were infected; 622 were *G. swynnertoni* of which 10 (1.6%) were infected; 3090 were *G. f. quazensis* of which 280 (9.1%) were infected with trypanosomes. However, tsetse infection rates with trypanosomes varied from country to country (Table 1). Serengeti II (Tanzania) had the lowest infection rate (1.43%) while Liwonde (Malawi) showed the highest infection rate (94.5%). These data are in agreement with findings reported by Simo et al. (2012) and Auty et al. (2012). Table 2 shows that overall, Pan Tryp LAMP detected more positives (83.1%) followed by RIME-LAMP (67.1%), ITS-PCR (54.3%), TBR-PCR (51.9%) and then microscopy on pools of 5 wild tsetse flies.

### Sensitivity and specificity of TBR- and ITS- PCRs and RIME and PanTryp LAMP tests for the detection of trypanosome DNA

A summary of the sensitivity and specificities of the index tests on the pooled tsetse gut specimens is presented in

**Table 1.** Detection of trypanosomes in tsetse fly midguts by dissection and light microscopy in various countries.

| Country | Tsetse sp | Total catches | Dissected | | | Infected | | Inf rate (%) |
|---|---|---|---|---|---|---|---|---|
| | | | M | F | Total | M | F | |
| **Uganda** | | | | | | | | |
| Ka'maido+ Dokolo | *Glossina fuscipes* | 2391 | 474 | 885 | 1359 | 5 | 16 | 1.55 |
| Tororo | *G. pallidipes* | 1000 | 285 | 363 | 648 | 8 | 12 | 3.09 |
| **Tanzania** | | | | | | | | |
| Serengeti I | *G. pallidipes* | 1141 | 91 | 616 | 707 | 0 | 11 | 1.56 |
| Serengeti II | *G. swynertoni* | 1417 | 2 | 620 | 622 | 2 | 8 | 1.43 |
| Urambo I | *G. pallidipes* | 1329 | 251 | 440 | 691 | 9 | 12 | 3.04 |
| Urambo II | *G. pallidipes* | 1088 | 172 | 508 | 680 | 2 | 14 | 2.78 |
| **Malawi** | | | | | | | | |
| Kasungu | *G. morsitans* | 521 | 121 | 322 | 443 | 51 | 146 | 44.46 |
| Liwonde | *G. morsitans* | 539 | 54 | 253 | 307 | 51 | 239 | 94.46 |
| Nkotakota | *G. morsitans* | 257 | 109 | 133 | 242 | 90 | 116 | 85.12 |
| **DRC** | | | | | | | | |
| Itubi | *G. f. quanzensis* | 2319 | 561 | 1409 | 1970 | 50 | 129 | 9.09 |
| **Congo B** | | | | | | | | |
| Ngabe, Mboka-Lefini | *G. f. quanzensis* | 2910 | 324 | 796 | 1120 | 31 | 70 | 9.02 |
| **Total** | | 14912 | 2444 | 6345 | 8789 | 299 | 773 | - |

Male (M), Female (F), Infected Males (Inf M), Infected Females (Inf F), Infection rate (Inf Rate).

**Table 2.** Performance of TBR-PCR, ITS-PCR, RIME LAMP and Pan Tryp LAMP assays on Pools of 5 wild tsetse midguts collected in 2012.

| Institute/country | Disease Form (HAT) | No. of pools tsetse | No. of pools positive (%) by each test | | | | |
|---|---|---|---|---|---|---|---|
| | | | Microscopy | TBR-PCR | ITS-PCR | RIME-LAMP | Pan Tryp-LAMP |
| Makerere-NEUganda | *T.b.rhod* | 184 | 16 (8.7) | 160 (86.9) | 163 (88.6) | 179 (97.3) | 182 (98.9) |
| Uganda-Kenya border | *T.b.rhod* | 129 | 20 (15.5) | 98 (75.9) | 109 (84.5) | 113 (87.6) | 124 (96.1) |
| TTRI-Tanzania-Serengti | *T.b.rhod* | 102 | 12 (11.8) | 35 (34.5) | 14 (13.7) | 38 (37.2) | 56 (54.9) |
| TTRI-Tanzania-Urambo | *T.b.rhod* | 98 | 19 (19.4) | 33 (33.7) | 16 (16.3) | 40 (40.8) | 70 (71.4) |
| Malawi | *T.b.rhod* | 214 | 197 (92.0) | 137 (64.0) | 129 (60.3) | 199 (92.9) | 212 (99.0) |
| NPHAT-DRC | *T.b.gam* | 130 | 60 (46.2) | 9 (6.9) | 45 (64.6) | 17 (13.1) | 95 (73.1) |
| LSNP-Congo | *T.b.gam* | 78 | 24 (30.8) | 14 (17.9) | 32 (40.0) | 41 (52.6) | 38 (48.7) |
| **Total (%)** | **-** | 935 | 348 (37.2) | 486 (51.9) | 508 (54.3) | 627 (67.1) | 777 (83.1) |

Tsetse midguts were pooled as follows: 1 infected plus 4 non-infected or five non-infected

Table 3. As shown in Table 3, PanTryp LAMP was the most sensitive at 99.9%, followed by ITS-PCR (88.7%), RIME LAMP (87.1%) and TBR-PCR (77.0%). However, ITS-PCR was the most specific at 67.4%, followed by TBR-PCR (65.5%), RIME LAMP (51.4%) and PanTryp LAMP at 33.6% in all countries participating in this study. TBR PCR appeared to be a problem in Malawi, Democratic Republic of Congo (DRC) and the Republic of Congo where it was less sensitive than microscopy. This could be attributed to problems with technical aspects of the methodology as these laboratories were newly set up and staff had limited experienced.

**Table 3:** Sensitivities (Sen), specificities (Spe), positive predictive (PP), and negative predictive (NP) values of the 4 molecular tests against microscopy in detecting trypanosomes in tsetse flies

| Country | TBR-PCR | | | | ITS-PCR | | | | RIME- LAMP | | | | Pan Tryp-LAMP | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sen | Spe | PP | NP | Sen | Spe | PP | NP | Sen | Spe | PP | NP | Sen | Spe | PP | NP |
| Uganda | 100.0 | 14.3 | 100.0 | 100.0 | 100.0 | 12.5 | 9.8 | 100.0 | 100.0 | 2.9 | 8.9 | 100.0 | 100.0 | 1.2 | 8.7 | 100.0 |
| U-K Border | 100.0 | 28.4 | 24.4 | 100.0 | 100.0 | 18.4 | 18.4 | 100.0 | 100.0 | 14.7 | 17.7 | 100.0 | 100.0 | 4.6 | 16.1 | 100.0 |
| Tanzania-S | 100.0 | 74.4 | 34.3 | 100.0 | 100.0 | 97.8 | 85.7 | 100.0 | 100.0 | 71.1 | 31.6 | 100.0 | 100.0 | 51.1 | 21.4 | 100.0 |
| Tanzania-U | 100.0 | 82.3 | 57.6 | 100.0 | 84.2 | 100.0 | 100.0 | 89.6 | 100.0 | 73.4 | 47.5 | 100.0 | 100.0 | 35.4 | 27.1 | 100.0 |
| Malawi | 65.9 | 58.8 | 94.9 | 13.0 | 61.9 | 58.8 | 94.6 | 11.7 | 94.9 | 29.4 | 93.9 | 33.3 | 99.5 | 5.8 | 92.5 | 50.0 |
| DRC | 15.0 | 100.0 | 100.0 | 57.8 | 75.0 | 100.0 | 100.0 | 82.3 | 15.0 | 100.0 | 100.0 | 57.8 | 100.0 | 53.3 | 63.2 | 100.0 |
| Congo | 58.3 | 100.0 | 100.0 | 84.8 | 100.0 | 84.1 | 75.0 | 100.0 | 100.0 | 68.5 | 68.5 | 100.0 | 100.0 | 84.1 | 75.0 | 100.0 |
| Average | 77.0 | 65.5 | 73.0 | 79.3 | 88.7 | 67.4 | 69.1 | 83.4 | 87.1 | 51.4 | 51.2 | 84.4 | 99.9 | 33.6 | 43.4 | 92.8 |

Whenever the prevalence of trypanosomes in tsetse flies is very low (especially in *T. brucei* species), the positive predictive value is never close to 1 even if both the sensitivity and specificity are high. Thus in screening tsetse in the field, it is inevitable that many with positive test results in LAMP will be false positives. Both positive and negative predictive values of a test will depend on the infection rates in tsetse flies.

Mitashi et al. (2012) compared LAMP to PCR in patient diagnosis and reported that the positive predictive value of a test is low in low incidence settings. This study therefore suggest that the variation observed in each country using the same test protocols for each test may be related to variation in trypanosome infection rates in tsetse (Table1, field results). The predictive value indicated the usefulness of each of the test for xenomonitoring of trypanosomes in tsetse flies. Where the infection rates of trypanosomes in tsetse flies were high, both the positive and negative predictive values were comparatively higher. The kappa value of a diagnostic test agreement (not shown in the table) was correspondingly higher than in those with low infection rates.

Unlike other insect vectors such as mosquitoes, black flies and sand flies, both male and female tsetse feed on blood and therefore both are trypanosome vectors. In this study both male and female tsetse were equally infected with trypanosomes at 12.2% infection rates. This is in contrast to earlier reports where male flies (*Glossina morsitans morsitans, G. pallidipes, G. fuscipes fuscipes*) showed higher rates of infection with *T. brucei* than females (Dale et al., 1995; Mauldin et al., 1991; Moloo et al., 1992). In the management of African trypanosomiasis a suite of diagnostics for host-level trypanosomiasis have been developed (Chapuis et al., 2005; Enyaru et al., 2010; Wastling and Welburn, 2011), each with its imperfections (Mitashi et al., 2012). This is coupled to the need to undertake cost-effective trypanomiasis mapping. There is therefore a need to develop novel techniques to overcome the shortcomings of the diagnostics in use.

The major aim of the present study was to evaluate the performance of relatively new rapid tests (RIME LAMP and PanTryp LAMP assays) for the detection of trypanosome DNA in wild-caught tsetse flies. These tests could be used for surveillance to indicate areas with high tsetse infection rates where control measures could be focused. The study data clearly demonstrate that the LAMP DNA detection method is simpler and more sensitive than microscopy and TBR- and ITS- PCRs. This data is also supported by the preliminary laboratory evaluation of the LAMP method using mid guts of laboratory-reared tsetse (Malele et al., 2013). Therefore, LAMP has a superior sensitivity in detecting trypanosome DNA from tsetse midguts in comparison to TBR- and ITS-PCRs respectively. This could be attributed to high copy numbers of RIME, estimated at 500 copies per haploid genome (Bhattacharya et al., 2002) while the ITS region is estimated to have 100 to 200 copies (Desquesnes and Davila, 2002) and TBR about 1000 copies (Masiga et al., 1992). Indeed, the LAMP detection method is more rapid (60 minutes compared to 150 minutes for PCR methods). Like PCR, it is sequence specific, producing sharp and clear bands in trypanosome-positive samples. More importantly, the LAMP detection method does not require expensive laboratory equipment and has the potential to function efficiently in the hands of a moderately trained technician. These factors considerably

overcome some of the many constraints that hamper surveillance and control efforts in trypanosomiasis endemic countries.

## Conclusion

Xenomonitoring of trypanosomes in tsetse using RIME and Pan Tryp LAMP techniques would fast highlight areas of potential disease outbreak and help focus control programs. Therefore, combining field data from the LAMP tests and later confirming with PCR tests in the laboratory would be the simplest and practical means to estimate and map trypanosome infection rates in the tsetse flies.

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## Conflicts of interests

The authors declare that they have no conflicts of interest.

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