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PERFORMANCE ASSESSMENT OF MOLECULAR AND MICROSCOPY TESTS FOR DETECTION OF *TRYPANOSOMA* SPECIES IN *GLOSSINA FUSCIPES FUSCIPES* (*DIPTERA: GLOSSINIDAE*) MIDGUTS IN KAJO-KEJI COUNTY, SOUTH SUDAN

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Abstract: Tsetse flies and Human African Trypanosomiasis (HAT) pose a threat to human health in Kajo-keji County South Sudan. A 6-month study was conducted to assess the performance of molecular and microscopy diagnostic tests to detect trypanosome parasites in tsetse flies, *Glossina fuscipes fuscipes*. Tsetse field surveys were carried out during the wet season. 750 wild tsetse flies dissected and midguts were tested microscopically. Genomic DNA of *Trypanosoma* species was extracted from the guts. Four molecular PCR-and LAMP-based tests were performed. Microcopy method revealed infection rate of 12.0% while the molecular diagnostic tests revealed sensitivity of 15%, 40%, 37.5% and 5% using TBR-PCR, ITS-PCR, RIME-LAMP and Pan Tryp LAMP, respectively. The specificity of TBR-PCR, ITS-PCR, RIME-LAMP and Pan Tryp LAMP tests revealed 100%, 34%, 35% and 31%, respectively. These 4 molecular tests have revealed highly significant (P<0.001) diagnostic results. ITS-PCR (40%) and TBR-PCR (100%) were the most sensitive and specific tests. The PCR and LAMP based assays are yet imperative for rapid and accurate detection of *Trypanosma brucei gambiense* in wild tsetse midguts. Molecular approach is robust and promising and could substantiate microscopy methods in remote rural inaccessible tsetse infested areas. Strategic area wide integrated vector control is needed for the control of HAT in the endemic foci of Kajokeji County South Sudan.

Key words: Assessment, Diagnostics, trypanosomes, tsetse flies, South Sudan

INTRODUCTION

Tsetse flies are the vectors of salivarian section pathogenic mammalian trypanosome parasites which cause Human African Trypanosomiasis (HAT) or sleeping sickness in humans and Nagana disease in cattle (Hu and Askoy, 2006). Seven species and subspecies of tsetse flies identified in the then Sudan including *Glossina fuscipes fuscipes* in South Sudan (Rogers and Robinson, 2004), where *G. f. fuscipes* flies are the sole vectors of the Gambian form of HAT in Central Equatoria State (Mohammed *et al.,* 2008). HAT has resurgent for the last 20 years due to social and economic related activities in Ibba and Maridi Counties, Western Equatoria State, South Sudan (Lukaw and Ochi, 2012).

Detection of trypanosome parasites in tsetse fly infected proboscis, midguts and salivary glands using microscopical methods were laborious, time consuming and sometimes inaccurate (Malele *et al.,* 2013). Moreover, it depends on trypanosomes density in the tsetse fly infected organs or parasitaemia in the human blood, lymph and cerebro-spinal fluid and the test potency. Hence, PCR- and LAMP-based tests are imperative for surveillance and monitoring trypanosomiasis and they could be used to identify infections non-detected by microscopical examination (Morlais *et al.,* 1998) and differentiate trypanosomes which are morphologically indistinguishable but with a very different economic impact (Duvallet *et al.,* 1999).

Recently, PCR-based techniques show that serum resistance associated (SRA) gene is specific for *T. brucei rhodesiense* (Mohammed *et al.,* 2008) or the *T. b. gambiense* surface glycoprotein (TgSGP) that is specific for that subspecies (Berberof *et al.,* 2001 Radwanska *et al.,* 2002). The proficiency to identify different trypanosome parasites in tsetse flies is of a paramount importance in the control of HAT in areas of high tsetse infestations including Kajokeji County. Hence, the recent development and application of Lateral flow test in endemic countries have created valuable analytical diagnostic milestone (Enyaru *et al.,* 2014).

The purpose of this paper was to assess the performance of PCR-based diagnostic tests (ITS-PCR, TBR-PCR, Pan Tryp LAMP and RIME-LAMP) versus microscopy method in detecting *Trypanosoma* species in wild tsetse flies in Kajo-keji County, South Sudan.

MATERIALS AND METHODS

Study Area

The study was conducted in KKC, South Sudan during the wet season which commences in March and ends in November. KKC County lies at geographical latitudes 3.67203 to 4.13238 °N and longitudes 31.1004 to 31.8172 °E. The annual rainfall ranges between 1200 and 2000 mm for over 8 months from March to November. The vegetation covers include different

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grass species and gallery forests which constitute appropriate habitats for the survival of tsetse flies.

Study Design and Sampling Techniques

Tsetse field surveys were conducted and sampling was made once every week from 8:00 a.m to 4:00 p.m hrs for six months during the wet season. The sample size of tsetse was determined by 95% confidence interval at a desired level of 5% (Thrusfield, 1995) and the stratified random sampling method was used for monitoring the prevalence of tsetse and assessing species diversity and distribution. Unbaited biconical traps were deployed (Challier and Larviessier, 1973) in seven sites along the banks of two streams 150 m apart and 10m distant from each. GPS device was used for recording coordinates and elevations of each trap. Total fly catches were stored in cool boxes, identified, sorted into males and females and counted (Brunhes *et al.*, 1998).

Microscopy Method

750 live flies were dissected to check for trypanosomes in the midguts as described in Msangi *et al* (1998). Infection rate (IR%) was determined as 100 (Number of the infected flies / Total number of dissected flies). 80 infected and non-infected midguts with trypanosome species, 40 each were randomly taken and added separately into an Eppendorf tube containing 300µl phosphate buffered saline (PBS) and stored at - 20˚C for genomic DNA extraction.

Genomic DNA extraction

Each midgut was homogenized by a sterile pestle in Eppendorf tube and the trypanosome parasite DNA was extracted using the Qiagen kit as per the manufacturer's instructions. The DNA was eluted in 50µl of Tris-EDTA (TE) and stored at -20˚C for molecular tests.

Molecular tests

TBR-PCR and ITS-PCR: Both PCR tests were performed in a DNA thermal cycler. TBR-PCR test used two oligonucleotide primers specific to *T. brucei subspecies* which allow the amplification of the TBR repeat (284bp) as described by Masiga *et al* (1992). The sequences of these primers are: TBR1F: 5'-CGAATGAATATTAAACAATGCGCAG -3'(25-mer); TBR1R: 5'- AGAACCATT TATTAGCTTTGTTGC -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 mMKCl, 1.25 mM of each dNTP, 12.5pmol of each oligonucleotide primer for TBR-PCR and 1 U of Phusion High-Fidelity DNA polymerase. TBR-PCR was programmed at 98°C for 1 min, 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 operation, 10µl of each sample was run on an ethidium bromide-stained-2% Agarose gel. Similarly, ITS-PCR test used two oligonucleotide primers of the sequences: ITSBR 5'-TTG CTG CGT TCT TCA ACG AA-3' (20 mer) and ITSCF 5'-CCG GAA GTT CAC CGA TAT TG-3' (20mer) for amplification of ITS_1 (Njiru *et al.*, 2005). Each amplification reaction was made in a final volume of 25µl containing 10 m MTris–HCl pH 9.2, 1.5 mM $MgCl_2$, 75 mMKCl, 1.25 mM of each dNTP, 100pmol of each oligonucleotide primer for ITS- PCR and 1U of Phusion High-Fidelity DNA polymerase and 2µl DNA template. ITS-PCR was programmed at 94°C for 5 min, 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 operation, 10µl of each sample was run on an ethidium bromide- stained- 2% Agarose gel.

RIME LAMP and Pan Tryp LAMP: The nucleic acid extracts were analyzed using RIME LAMP and Pan Tryp LAMP. The 25µl LAMP reactions were standardized for optimal reagent concentration. The reactions were carried out at 2µM for FIP and BIP primers, 0.8µM for loop primer (LF and LB), 0.2 µM for F_3 and B_3 outer primers, master mix and 8U of Bst DNA polymerase large fragment (New England Biolabs). The reactions were carried out at 62°C for 1 h using the DNA thermal cycler (Perkin–Elmer Cetus, Norwalk, CT, USA) and terminated at 80°C for 5 min. The amplification products were detected by direct visual inspection of the LAMP product after addition of 1µl 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. gambiense* were used as a positive control. Ultra-pure water and midgut extract of non-infected laboratory tsetse were used as negative controls. Visibility of green colour indicates positive result (Njiru *et al.*, 2010).

Data Management and Statistical Analysis

Data was managed and statistically analyzed by Chi square (χ^2) statistics using Statistical Package for Social Sciences (SPSS) 20 software compatible to windows. All tables were made using Microsoft Excel 2010.

RESULTS

A total catches of 984 *G. f. fuscipes* identified in four Payams of KKC, of which 750 dissected flies showed 12.0% total infection rate, 60% infected male and 40 % infected female flies and 9.15% prevalence rate (table 1). TBR-PCR and ITS-PCR showed infection rate of 15% and 40%, respectively. While, RIME-LAMP and Pan Tryp LAMP showed IR% of 37.5% and 5%, respectively (table 2). TBR-PCR and ITS-PCR in the 40 negative tsetse midgut samples showed IR% of 0.0% and 15%, respectively. RIME-LAMP and Pan Tryp LAMP IR% of 12.5% and 22.5%, respectively (table 3). Hence, the

results of diagnostic Sensitivity, Specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) using PCR- and LAMP based diagnostic tests are shown in table (4). ITS-PCR and TBR-PCR showed the highest sensitivity (40%) and specificity (100%), respectively. Moreover, TBR-PCR showed the highest PPV (100%) and Pan Tryp LAMP had the least NPP of 44.9%.

Table 1: Parasitological results showing the infection rate of Trypanosomes in the wild tsetse midguts in Kajo-keji County, South Sudan.

Location (Payam)	Tsetse flies identified	Fly caches	Dissected flies	Infected flies	Infected		Infection rate (IR%)	Prevalence rate
					Female	Male		
Lire	*G.f.fuscipes*	176	150	00	00	00	00	00
Kangapo I		128	114	00	00	00	00	00
Kangapo II		567	403	65	25	40	16.13	11.46
Liwolo		113	93	25	11	14	26.88	22.12
Total		984	750	90	36 (40%)	54 (60%)	12.00	9.15

Table 2: Percentage from positive wild tsetse midgut samples (40+) from Kajokeji County South Sudan

Disease Form (HAT)	No. of Tsetse midguts	No. of pools positive (%) by each test			
		TBR-PCR	ITS-PCR	RIME-LAMP	Pan Tryp LAMP
T.b.gambiense	40+	6 (15%)	16(40%)	15 (37.5%)	2 (5.0%)

Table 3: Percentage from negative tsetse midgut samples (40-) from KajoKeji County, South Sudan

Disease Form (HAT)	No. of Tsetse midguts	No. of pools negative (%) by each test			
		TBR-PCR	ITS-PCR	RIME-LAMP	Pan Tryp LAMP
T.b.gambiense	40-	0.00+(100.0)	6+(12.5)	5+(12.5)	9+(22.5)

Table 4: Results of the Diagnostic Sensitivity, Specificity, Positive Predictive Value and Negative Predictive Value using PCR-based diagnostic tests

Test	Sensitivity%	Specificity%	PPV%	NPV%	P-value<0.0001
ITS-PCR	40	85	72.7	58.6	P<0.0014
TBR-PCR	15	100	100	54.1	P<0.0054
RIME-LAMP	37.5	90	78.9	59	P<0.0086
Pan Tryp LAMP	5	77.5	18.2	44.9	P<0.000

DISCUSSION

In this study both male and female tsetse were infected with trypanosomes at IR of 12.0%. However, the high tsetse catches of infected male flies might be explained and justified by the fact that male flies have a tendency to respond to stationary objects and traps (Okoh *et al.*, 2011). This is in line with the earlier reports where male flies (*G. m. morsitans, G. pallidipes, G. f. fuscipes*) showed higher rates of infection with *T. brucei* than females (Mauldin *et al.*, 1991 Dale *et al.*, 1995).

Given the imperfections of currently available diagnostics for trypanosomosis, particularly with regard to the invasive and insensitive nature of parasitological diagnosis at patient level, and the often critical need to undertake cost-effective mapping, there is a need to develop new approach to overcome the shortcomings of diagnostics. Detection of trypanosome DNA in wild-caught tsetse flies as surveillance tests to indicate areas with high tsetse infection rates with pathogenic trypanosomes for immediate deployment of control measures is Imperative for key stakeholders. LAMP DNA detection method proved to be simpler and more sensitive than microscopy and TBR- and ITS-PCRs. This has strengthened the preliminary laboratory evaluation of the LAMP method, based on adding known numbers (1 infected + 4 non-infected) midguts of laboratory-reared tsetse. Furthermore, the sensitivity of the test is high to detect trypanosomes DNA in all pools up to 10 midguts per pool which were spiked with procyclic trypanosomes in 10-fold serial dilutions of 10^{6} to 10^{-6}/ml (Malele *et al.*, 2013). Thus, LAMP shows a superior sensitivity of detecting trypanosome DNA from midguts compared to TBR-PCR and ITS-PCR respectively which could be attributed to high copy numbers of RIME, reportedly 500copies/haploid genome (Bhattacharya *et al.*, 2002) while ITS region is estimated at 100-200 copies (Desquesnes and Davila, 2002). Indeed, the LAMP detection method is more rapid taking 60 min compared to 150 min for PCR methods. Similarly, PCR is sequence specific, producing sharp and clear permanent bands in trypanosome-positive samples. More importantly, the LAMP detection method requires no expensive laboratory equipment, which might hamper surveillance and control efforts in trypanosomosis endemic countries. Data combination from the LAMP and PCR tests would be the simplest and practical means to measure trypanosome infection rates in tsetse.

CONCLUSION

PCR- and LAMP-based technology is effective and efficient in identifying the *Trypanosome* species infecting tsetse. Xeno-monitoring for potential disease outbreak and the success of control programme in risky areas of animal and human trypanosomosis using RIME and Pan Tryp LAMP tests is promising. Hence, key stakeholders are urged to strategize area wide integrated pest management approach in the control

of tsetse and trypanosomiasis in Kajo-keji County South Sudan.

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