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Microbiome frequency and their association with trypanosome infection in male *Glossina morsitans centralis* of Western Zambia

Njelembo J. Mbewe[a,*], Cornelius Mweempwa[a], Samuel Guya[b], Florence N. Wamwiri[b]

[a] Department of Veterinary Services, Tsetse and Trypanosomiasis Control Section, Chilanga, 350001, Zambia
[b] Kenya Agriculture and Livestock Research Organisation,Biotechnology Research Institute, Kikuyu, 362 00902, Kenya

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ABSTRACT

Tsetse flies (Diptera: Glossinidae) are considered primary cyclical vectors that transmit pathogenic trypanosomes in Africa. They harbour a variety of microbes including *Wolbachia*, *Sodalis* and the salivary gland hypertrophy virus (SGHV) which are all vertically transmitted. Knowledge on tsetse microbiome and their interactions may identify novel strategies for tsetse fly and trypanosomiasis control. Area-wide application of such strategies requires an understanding of the natural microbiome frequency in the different species and subspecies of *Glossina* in their geographical populations. Consequently, this study determined the prevalence of *Sodalis, Wolbachia,* SGHV and trypanosome infections in *Glossina morsitans centralis* from two sites of Western Zambia. We also explored possible associations of the microbes with trypanosome infections. Male *G. morsitans centralis* samples were collected from two sites (Lyoni and Lusinina) in Western Zambia. The age structure of the flies at each site was determined using the wing fray method. DNA was extracted from the samples and analyzed for *Wolbachia, Sodalis,* SGHV and trypanosome presence using PCR. Associations and measures of associations between trypanosome infection and microbes in the fly were determined. The flies from the two locations (Lusinina, $n = 45$ and Lyoni, $n = 24$) had a similar age structure with their median fray category not being significantly different ($p = 0.698$). The overall prevalence of *Wolbachia* was 72.5% (95% CI: 61.6–83.3%), *Sodalis* was 15.9% (95% CI: 7.1–24.8%), SGHV was 31.9% (95% CI: 20.6–43.2%) and *Trypanosoma* species was 23.2% (95% CI: 13–33.4%). The prevalence of *Wolbachia* was significantly higher in Lusinina than Lyoni ($p = 0.000$). However this was not the case for *Sodalis,* SGHV and *Trypanosoma* species. Despite the low number of flies that were positive for both trypanosome and *Sodalis* (6; 8.7%), a statistically significant association ($p = 0.013$; AOR 6.2; 95% CI: 1.5–25.8) was observed in *G. morsitans centralis.* The study showed that the prevalence of microbiota may vary within the same species of the tsetse depending on the geographical location as was the case of *Wolbachia.* Further it showed that infection with *Sodalis* could affect vector competence. The study concludes that *Sodalis* could be an ideal candidate for symbiont-mediated trypanosomiasis control interventions in *G. morsitans centralis.*

1. Introduction

Tsetse flies (Diptera: Glossinidae) are the major vectors of African trypanosomes, the causative agent of human African trypanosomiasis (HAT or sleeping sickness) and African animal trypanosomosis (AAT or nagana). Trypanosomiasis is a debilitating disease that mainly affects the rural poor. Therefore, interventions that are designed for disease and vector control should be affordable and sustainable. The tsetse fly microbiome consists of diverse organisms including symbiotic and non-symbiotic microbes. These symbionts live in close proximity to developing trypanosomes in the midgut and therefore, may potentially influence infection development by creating parasite-refractory tsetse.

Two microbial endosymbionts have been described in tsetse flies (Aksoy, 2000). The obligate *Wigglesworthia glossinidia,* which is transmitted through the maternal milk gland secretions, is essential for maintaining fecundity and proper maturation of tsetse immunity (Dale and Maudlin, 1999; Pais et al., 2008; Weiss and Aksoy, 2011). *Sodalis glossinidius*, a facultative intra- and extra-cellular endosymbiont, is acquired by the intra-uterine larvae through maternal milk gland secretions (Aksoy et al., 1997). The density of *Sodalis* infection varies between species, and flies that harbour greater densities of *Sodalis* have been suggested to be more susceptible to trypanosome infection (Geiger et al., 2007). Some tsetse

* Corresponding author. Tel.: +260 977 433470; fax: +260 211278537.
E-mail address: njelembombewe@yahoo.com (N.J. Mbewe).

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individuals are also infected by the parasitic *Wolbachia pipientis* (Cheng et al., 2000). *Wolbachia* infection may result in a variety of reproductive abnormalities, the most important phenotype being cytoplasmic incompatibility (CI). *Wolbachia*-mediated CI has been used to control mosquitoes in pilot projects (Brelsfoard and Dobson, 2009; Laven, 1967). The recent demonstration of CI expression in *G. morsitans* morsitans infers that this phenomenon could presumably be applied to control tsetse flies as well. In addition to the three microorganisms described, some tsetse flies may also harbour the salivary gland hypertrophy virus (SGHV) (Abd-Alla et al., 2011). SGHV infection is acquired vertically either through infected milk gland secretions or trans-ovum (Abd-Alla et al., 2010). It is present in several tsetse species including *Glossina fuscipes* fuscipes, *Glossina pallidipes, Glossina swynnertoni* and *G. morsitans* morsitans (Alam et al., 2012; Kariithi et al., 2013; Malele et al., 2013). Often, SGHV is found to co-infect the host together with maternally-transmitted endosymbionts (Alam et al., 2012). Symptomatic SGHV infections, which are characterised by enlarged salivary glands, are observed at high virus titres (Abd-Alla et al., 2007). However, the majority of infected flies are asymptomatic. The pathological outcomes of SGHV infection are especially marked in colonised populations of *G. pallidipes*, where these effects are characterised by interplay between the virus and the endosymbionts (Boucias et al., 2013). *Wolbachia*, *Sodalis* and SGHV are all vertically transmitted i.e., mother to offspring. After emergence from the pupa, the tsetse fly may acquire trypanosome infection upon feeding on an infected host. The presence of all or any combination of symbiont/parasite infections may affect the host physiology due to inter–community interactions (Wang et al., 2013).

In several insect species, the midgut microbiota has been shown to influence insect host vector competence (Dong et al., 2009; Gonzalez-Ceron et al., 2003; Weiss and Aksoy, 2011). The increase in knowledge on tsetse symbiosis may reveal rational prospects of using these microbes in strategies to control tsetse flies (Aksoy et al., 2008; Rio et al., 2004). However, understanding of the natural infection frequency in the different species and subspecies of *Glossina* in its geographical populations will be required for area-wide application of such strategies.

Historically, *G. morsitans centralis* has been an important vector of AAT in the common fly belt of Zambia, Botswana, Namibia and Angola. Despite its eradication in the three latter regions (Chilongo, 2013), this species remains an important vector for AAT in some parts of Zambia (Willemse, 1991). Most of the literature concerning tsetse research in Zambia is based on the eastern province, specifically on *G. pallidipes* and *G. morsitans* morsitans in the Luangwa valley. Subsequently, there is limited information available with regard to *G. morsitans* centralis, which is confined to the western part of the country. In this paper, we determined the prevalence of *Sodalis, Wolbachia,* SGHV and trypanosome infections in *G. morsitans* centralis from two sites in Western Zambia. We also explored possible associations of the microbes with trypanosome infections.

2. Materials and methods

2.1. Study Area

Flies were captured from Lusinina in Sesheke district ([latitude – 17.1705, longitude 24.5119]; Western province) and Lyoni in Kazungula district ([latitude – 16.5485, longitude 25.2159]; Southern province) which are about 99 km apart. Mixed farming is the major economic activity, within the main vegetation type of miombo woodlands. The weather comprises of three seasons, warm and wet (November–April), cool and dry (May–August) and hot–dry (September–October). Lusinina is located within the 2014 sequential aerosol technique (SAT) block where as Lyoni lies outside the block (TTCS, 2014). Although both areas are outside the National game parks, wild animals are seen occasionally. *G. morsitans centralis* is the only tsetse species found in this area.

2.2. Sample collection and wing fray analysis

Tsetse flies were captured in May 2014 on screen fly rounds (Ford et al., 1959; Potts, 1930). Black screens with a blue band at the centre were used. They were baited with butanone released at 5 g/h from an open bottle and octenol dispensed at 0.5 mg/h from a 5×5 cm polythene sachet. The attendants stopped at 200 m intervals to catch flies from the screen and surroundings using hand nets. Six fly rounds of about 5 km each were conducted along path/road networks that passed through suitable tsetse habitats in each area. At the end of the fly round, flies collected were sorted according to species and sex. All male flies collected were put aside for this study whereas females were used for other examinations to assess the effectiveness of SAT in the study area.

Male flies were used to estimate population age using the wing fray method (Jackson, 1946). The wings from randomly selected flies were carefully snipped off the body and examined under a dissection microscope. The degree of fraying as indicated by the tearing in the trailing edge of the wings was scored on a scale of 1–6. The mean wing fray value (MWFV) was calculated for samples from each site and the mean age obtained from a reference table (Buxton, 1955). The remaining proportion of flies was preserved in 95% ethanol until DNA extraction.

2.3. DNA extraction and detection of infecting microbes

Upon removal from ethanol storage, individual flies were blotted dry on a paper towel and given unique identifiers. They were then air-dried overnight at room temperature. Total genomic DNA was extracted from individual whole fly samples (minus wings) using the DNeasy® Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. To determine the prevalence of the different microbes, a PCR reaction was carried out in 20 μl volumes using 1 μl of template DNA in a buffer containing 5X PCR reaction buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 500 nM of each primer and 0.3 μl of GoTaq® Flexi DNA polymerase 5 units/μl (Promega, Madison, WI, USA). The presence of *Wolbachia* was determined by amplification of the 16 S rRNA gene (Doudoumis et al., 2012). Assays for *Sodalis* and SGHV were performed using previously published primers (Abd-Alla et al., 2007; O'Neill et al., 1993). The internal transcribed spacer 1 region was amplified for detection of *Trypanosoma* species using the ITS1CF/BR primers (Njiru et al., 2005). For each PCR run, a negative control using molecular grade water (HyClone Laboratories Inc., UT, USA) and a corresponding positive control were included. The quality of template DNA was verified by amplification of insect-specific 12 S rRNA gene (Simon et al., 1994). Samples that were identified as *Trypanozoon* species using the ITS1assay were further tested for the presence of the serum- resistance associated (SRA) gene (Gibson et al., 2002). After completion of the PCR run, 10 μl of the amplification products were analyzed by electrophoresis in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) on a 2% agarose gel together with a 100 bp DNA ladder size standard (Invitrogen, Carlsbad, CA, USA) and visualized using ethidium bromide (EtBr) staining.

2.4. Statistical analysis

Mood's median test was used to examine inter-group differences in wing fray category median. Microbiome prevalence data was exported to STATA IC version 11 for management and analysis. A multiple logistic regression was used to determine associations