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Seasonal Monitoring of *Glossina* Species Occurrence, Infection Rates, and *Trypanosoma* Species Infections in Pigs in West Nile Region, Uganda

Sadiya Maxamhud,¹ Johanna F. Lindahl,^{1–3} Albert Mugenyi,⁴ Richard Echodu,⁵
Charles Waiswa,^{4,6} and Kristina Roesel^{2,7}

Abstract

Introduction: Trypanosomiasis is a parasitic infection caused by the protozoa *Trypanosoma*. It is exclusively associated with *Glossina* species habitats and, therefore, restricted to specific geographical settings. It affects a wide range of hosts, including humans. Animals may carry different *Trypanosoma* spp. while being asymptomatic. They are, therefore, potentially important in unpremeditated disease transmission.

Aim: The aim of this study was to study the potential impact of the government tsetse fly control program, and to elucidate the role of pigs in the *Trypanosoma* epidemiology in the West Nile region in Uganda.

Methods: A historically important human African trypanosomiasis (HAT) hotspot was selected, with sampling in sites with and without a government tsetse fly control program. Pigs were screened for infection with *Trypanosoma* and tsetse traps were deployed to monitor vector occurrence, followed by tsetse fly dissection and microscopy to establish infection rates with *Trypanosoma*. Pig blood samples were further analyzed to identify possible *Trypanosoma* infections using internal transcribed spacer (ITS)-PCR.

Results: Using microscopy, *Trypanosoma* was detected in 0.56% (7/1262) of the sampled pigs. Using ITS-PCR, 114 of 341 (33.4%) pig samples were shown to be *Trypanosoma vivax* positive. Of the 360 dissected tsetse flies, 13 (3.8%) were positive for *Trypanosoma* under the microscope. The difference in captured tsetse flies in the government intervention sites in comparison with the control sites was significant ($p < 0.05$). Seasonality did not play a substantial role in the tsetse fly density ($p > 0.05$).

Conclusion: This study illustrated the impact of a government control program with low vector abundance in a historical HAT hotspot in Uganda. The study could not verify that pigs in the area were carriers for the causative agent for HAT, but showed a high prevalence of the animal infectious agent *T. vivax*.

Keywords: *Trypanosoma brucei*, animal African trypanosomiasis, nagana, tsetse flies, latent infection, swine

¹Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden.

²Department of Biosciences, International Livestock Research Institute, Nairobi, Kenya.

³Department of Clinical Sciences, Swedish University of Agricultural Science, Uppsala, Sweden.

⁴Coordinating Office for Control of Trypanosomiasis in Uganda, Ministry of Agriculture, Animal Industry and Fisheries, Makerere, Uganda.

⁵Department of Biology, Faculty of Science, Gulu University, Gulu, Uganda.

⁶Department of Pharmacy, Clinical and Comparative Studies, School of Veterinary Medicine and Animal Resources, Makerere University, Kampala, Uganda.

⁷Department of Veterinary Medicine, Institute for Parasitology and Tropical Veterinary Medicine, Freie Universität Berlin, Berlin, Germany.

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In the February 2022 issue of Vector-Borne and Zoonotic Diseases (vol. 22, no. 2; 101–107), the article entitled Seasonal Monitoring of *Glossina* Species Occurrence, Infection Rates, and *Trypanosoma* Species Infections in Pigs in West Nile Region, Uganda has been updated on March 3, 2022 after first online publication of February 15, 2022 to reflect Open Access, with copyright transferring to the author(s), and a Creative Commons License (CC-BY) added (<http://creativecommons.org/licenses/by/4.0>).

Introduction

AFRICAN TRYPANOSOMIASIS ARE vector-borne infections in animals or humans caused by different species of the parasite *Trypanosoma*. African trypanosomiasis is a disease that is restricted to sub-Saharan Africa (SSA) as it is exclusively associated with *Glossina* species (tsetse fly) habitats (Franco et al. 2013). Infectious human African trypanosomiasis (HAT) is caused by two *Trypanosoma brucei* subspecies, *T. b. gambiense* and *T. b. rhodesiense*.

Gambiense human African trypanosomiasis (g-HAT) results in a more chronic form of the illness with the infection not progressing as rapidly as Rhodesiense human African trypanosomiasis (r-HAT). r-HAT results in acute symptoms apparent within days or weeks after infection. Clinical signs for HAT include recurring episodes of fever, headache, and weakness, with progressing symptoms of meningoencephalitis (CDC 1976, Wenk et al. 2003, Cattand et al. 2006).

Animals, including pigs, have been shown to be carriers of the human pathogenic *T. brucei* ssp. and are, therefore, potentially important in maintaining disease transmission. Efforts to control the disease have, therefore, focused on vector control as well as active and passive surveillance in humans and animals (Büscher 2002, Wenk et al. 2003, Njiokou 2010).

HAT is still endemic in 36 SSA countries. However, cases have reduced significantly the past decade, with <10,000 HAT cases being reported for the first time in 50 years in 2009. The number of cases has continued to reduce throughout the years, with <1000 cases being reported in 2019 (WHO 2020). A very effective tool to reduce disease transmission and the tsetse fly apparent density (AD) has been the deployment of tsetse fly targets. Tiny targets are pieces of netting fabric, with the colors blue and black, saturated in insecticides (pyrethroids).

These have been applied since 2011 (Tirados et al. 2015, WHO 2015, Stanton et al. 2018) and have been deployed in large-scale programs to control sleeping sickness in countries such as Chad, Guinea, Côte d'Ivoire, the Democratic Republic of Congo (DRC), and Uganda (Tirados et al. 2015).

With fewer than 2200 HAT cases reported globally in 2016, the World Health Organization (WHO) has set the goal to achieve zero transmission of g-HAT to humans by 2030 (Büscher et al. 2018). This may, however, be challenged by factors that can contribute to continuous transmission, including latent human infections and animal reservoirs (Büscher et al. 2018).

Animal African trypanosomiasis (AAT), also known as nagana, is an important limitation in livestock production due to its high mortality (FAO 2009). It can affect a wide range of animals and cause production losses for farmers, which can subsequently lead to substantial economic losses. Furthermore, AAT can be caused by a wide range of *Trypanosoma* species, such as *Trypanosoma vivax*, *Trypanosoma congolense*, and *T. brucei*.

T. vivax accounts for up to half of total *Trypanosoma* infections in West Africa, and is involved in high number of cattle lost annually (Chamond et al. 2010, Holt et al. 2016, FAO 2019, Autheman et al. 2021). However, many animals can be asymptomatic when infected with certain *Trypanosoma* species, but are able to play a role as disease reservoirs (N'Djetchi et al. 2017). The detection of potential animal

reservoir hosts is, therefore, of high relevance to avoid re-emergence of a disease that has been brought under good control (Waiswa et al. 2003, Ng'ayo et al. 2005, Waiswa 2010).

Pig production is becoming increasingly important as a livelihood activity in Uganda, including in the northwestern part of the country. When infected with *Trypanosoma*, pigs are usually asymptomatic, excluding infections caused by *Trypanosoma simiae* and *Trypanosoma suis* (Holt et al. 2016). The northwestern part of Uganda is an important region for cross-border trade and nongovernmental organizations, resulting in frequent movement of people between Uganda, DRC, and South Sudan. This is a crucial and important aspect as the frequent movement can play an addition role in disease transmission (Migchelsen et al. 2011, Büscher et al. 2017, Wamboga et al. 2017). The predominant *Glossina* species in the West Nile region is *Glossina fuscipes fuscipes* of the Palpalis group (Echodu et al. 2011, Opiro et al. 2017). The West Nile region is characterized by riverine forest along the tributaries of the White Nile.

The objectives of this study were to investigate control and intervention sites: (1) to determine the prevalence of *Glossina* species, (2) to determine *Trypanosoma* infection rates in tsetse flies, and (3) to determine the prevalence of *Trypanosoma* spp. in pigs.

Materials and Methods

Study design and study sites

The repeated cross-sectional study took place between February and June 2019 during both dry season (February to March) and rainy season (April to June), in an attempt to compare the density of tsetse flies and infection rate between the two seasons. The study took place in the northwestern part of Uganda that is close to the borders with DRC and South Sudan (Fig. 1).

Data show that, between 1905 and 1920, there were on average 31 HAT-related deaths per 10,000 recorded annually in the West Nile region (Berrang-Ford et al. 2006). HAT cases in northwest Uganda have been reported between 2004 and 2010 by Balyeidhusa et al. (2012) and Tirados et al. (2015), illustrating that this area was still an active HAT foci at that time. In 2010, a total of 97 cases was reported in the region (Balyeidhusa et al. 2012). However, more recent data from 2018 found no *T. b. gambiense* infection in humans in the same region (Selby et al. 2019).

The two selected districts, Arua and Maracha, were considered suitable surveillance sentinel sites due to its known history of trypanosomiasis and a relatively high density of pigs (Balyeidhusa et al. 2012, Tirados et al. 2015). Sampling, as well as the deployment of tsetse fly traps, took place in six government intervention sites and six control villages. Control sites included villages with no known ongoing vector control. Intervention sites included villages where continuous vector control through the deployment of tiny targets and tsetse fly monitoring was taking place since 2011. The interventions in the area were conducted by the coordinating office for control of trypanosomiasis in Uganda (COCTU).

Sampling and dissection of *Glossina* species

There was a total of 48 pyramidal tsetse traps set up monthly, with 4 traps deployed in each village. The traps were arranged along rivers and valleys (Fig. 2) and emptied

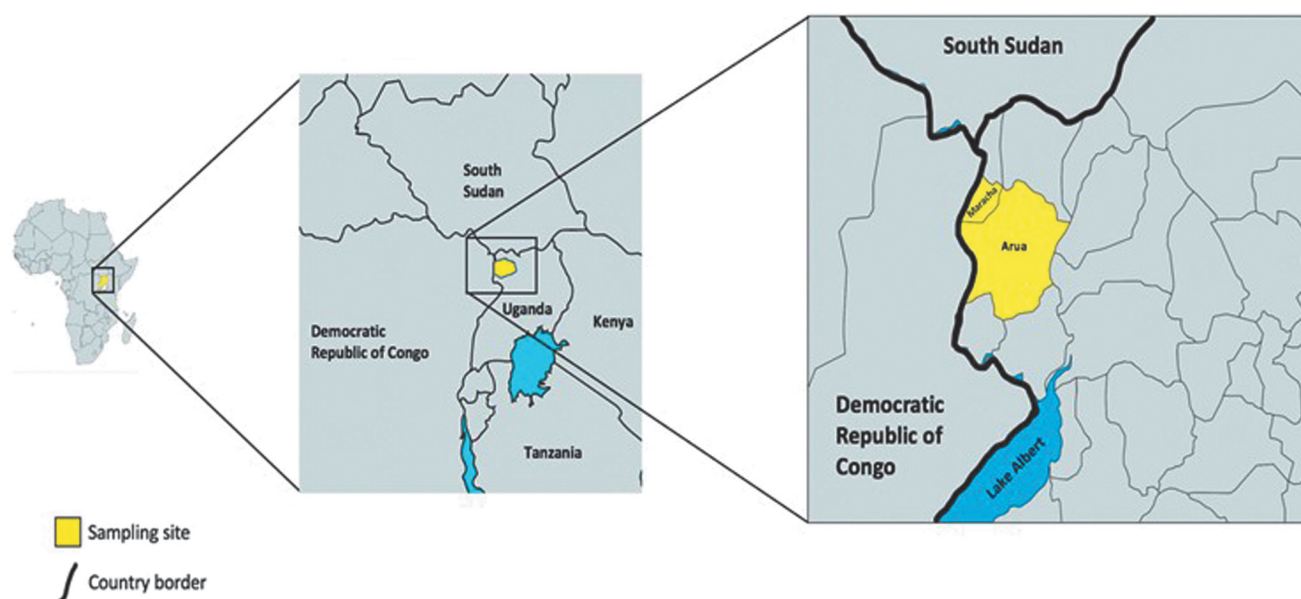


FIG. 1. Geographical illustration of the sites where sampling and deployment of tsetse traps took place in Uganda. Color images are available online.

every 24 h for 72 h, each month, throughout a period of 5 months. The tsetse flies that were collected from the traps were subsequently stored and transported in an ice box to the local laboratory in Arua town for dissection.

Dissection was performed on living tsetse flies under a stereomicroscope (Stemi 2000, Zeiss, 1.8–20 $\times$). Isotonic saline solution (Ringer's solution; Sigma Life Science) was initially positioned on the slide to maintain the organs in a hydrated environment during dissection. To prevent cross-contamination, the dissected organs, salivary glands, proboscis, and midgut, were positioned in separate ends of the slide. If a bloodmeal was present, it was noted down. The forceps that were used throughout the dissection was cleaned between each dissection with 97% ethanol and wipes.

Pig sampling

Pig sampling took place in the same villages where traps were being deployed in Arua and Maracha. For each village, a minimum of 20 pigs were sampled every 4 weeks during the entire period of 5 months. The sample size was based on a two-proportion power calculation in STATA 14.2 (StataCorp Ltd, College Station, TX), assuming a power of 80%, and a proportion of 5% positive animals in intervention villages,

and 50% in control villages. Recruitment of the pigs in the villages was based on availability and willingness of farmers to participate.

Microscopic detection

The pig sampling involved capillary blood being drawn from the ear vein using a lancet for perforation. The hematocrit centrifugation test (HCT) technique was used to extract and examine the white blood cells of the blood sample, known as the buffy coat, to uncover potential trypanomastigote movements under a microscope (Woo 1969). The capillary tubes were subjected to a centrifugal force of 12,000 rpm for 3 min. To analyze possible trypanosome movement microscopically, the buffy coat was extracted by cutting the capillary tube ~1 mm below the buffy coat with a diamond tipped pencil. This was subsequently extruded onto a microscopic slide and covered with a cover slip. The sample was then observed and examined under a microscope (Leica DM500).

During the last 2 months of the study, blood samples were also subsequently smeared onto labeled Whatman Fitzco/-Flinder Technology Agreement (FTA) cards (GE Healthcare Life Sciences), air dried at room temperature, stored, and transported to Gulu University for molecular analysis.



FIG. 2. Deployment of tsetse trap in one of the selected villages in Arua district. Color images are available online.

Even though microscopy is the most common technique for detection of an active infection, for higher sensitivity and identification of genetic diversity, molecular techniques are recommended (Abdi et al. 2017). Hence, a subset of 341 blood samples were analyzed through both microscopic and molecular means during the last two sampling periods (May and June 2019).

Molecular analyses

The molecular analyses were conducted at the Bioscience Research Laboratory located at Gulu University for detecting the presence of *Trypanosoma* species.

Before DNA extraction, the filter paper was cut into smaller pieces, using a razor blade or a scissor, and dissolved in 200 μ L of premade 1 $\times$ phosphate buffered saline (PBS) (AmericanBIO). For DNA extraction, a commercial DNA extraction kit was used (DNeasy Blood & Tissue Kits; Qia-gen). Samples were stored at -20°C , until analyses for the internal transcribed spacer (ITS), a valuable marker for *Trypanosoma* species differentiation, using the nested ITS PCR protocol as described by Cox et al. (2005), with minor modifications. This method was selected as it is an economical method that can be performed by most laboratories.

For the initial PCR, a total volume of 25 μ L was prepared consisting of the following components: 12.5 μ L Go-Taq Green master mix (Promega, Madison, WI), 0.1 μ L of both the external primers ITS-1 (5'-GATTACGTCCTGCCA TTTG-3') and ITS-2 (5'-TTGTTGCGCTATCGGTCTTCC-3'), 0.5 μ L MgCl_2 , 6.8 μ L PCR water, and 5 μ L template of DNA.

The reaction conditions were 1 cycle of 95°C for 7 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with final extension of 72°C for 7 min using Simpli Amp Thermocycler machine. The second PCR included using 2 μ L of PCR product from the first reaction together with a total reaction volume of 23 μ L that consisted of the following components: 12.5 μ L of Go-Taq Green master mix, 0.1 μ L of each internal primers ITS-3 (5'-GGAAGCAAA AGTCGTAACAAGG-3') and ITS-4 (5'-TGTTTCTTTTCTCCGCTG-3'), 0.5 μ L of MgCl_2 , and 9.8 μ L of PCR water. The same reaction conditions as described for the first reaction were used. Amplification products were lastly visualized in 2% molecular grade agarose gel.

Statistical calculations

The AD of fly population was calculated by dividing the number of flies caught during a period of 72 h with the product of deployed traps and days of deployment and is expressed as flies per trap per day (FTD) (FAO 2009). *t*-Test was in addition also applied to observe the differences in the results and to appreciate if it was statistically significant. The statistical analysis was performed using the software STATA 14.2 (StataCorp Ltd.).

Ethical considerations

Ethical approval to conduct this study was obtained from the College of Veterinary Medicine, Animal Resources and Biosecurity at Makerere University, Kampala, Uganda (Ref.: SBLS/SM/2019); the institutional research ethics committee at the International Livestock Research Institute, Nairobi,

Kenya (Ref.: ILRI-IREC2019-13); and the institutional animal care and use committee at the International Livestock Research Institute, Nairobi, Kenya (Ref.: IACUC2019-14). In addition, we obtained permission from the Uganda National Council of Science and Technology to conduct research in the selected sites in Uganda (Ref.: NS 669). All participants gave informed consent to participate.

Results

Tsetse fly density

A total of 587 tsetse flies were collected throughout the entire sampling period, with 236 tsetse flies collected during the dry season (January to March 2019) and 351 tsetse flies collected during the rainy season (April to June 2019). There was no statistically significant difference in the tsetse fly density between the two seasons ($p > 0.05$). Of the collected tsetse flies, 360 (61%) were alive and dissected for further microscopic analysis. The difference in the tsetse fly AD from the intervention sites (0.60 FTD) in comparison with the control sites (1.05 FTD) indicated there was a statistically significant difference ($p < 0.05$).

Fly infection rates

Out of the 360 dissected flies, 13 (3.6%) were microscopically positive for *Trypanosoma* with 3 of them were having bloodmeals present. Approximately 85% (11/13) of the positive flies had *Trypanosoma* present in the proboscis. About 15% (2/13) of the microscope positive tsetse flies had *Trypanosoma* detected in the midgut. In addition to this, 59 of the dissected flies (16.4%) had bloodmeals present.

Pig infection rates

A total of 1,262 pigs were sampled during a period of 5 months, and 0.56% (7/1262) of the sampled pigs were microscope positive for *Trypanosoma*. Owing to the low prevalence of microscope positive samples, a subset of 341 samples were furthermore analyzed using the ITS1-PCR method for detection and differentiation of *Trypanosome* species in blood.

The results revealed that 114 of 341 (33.4%) were *Trypanosoma* positive, including the seven microscope positive samples. All (100%) of the PCR-positive samples were shown to be positive for *T. vivax* by the ITS PCR that targets the gene encoding the small ribosomal subunit, giving the PCR products a unique size (Fig. 3). *T. brucei* was not detected.

Discussion

A total of 587 tsetse flies were collected throughout the entire sampling period in both Maracha and Arua districts. No morphological analysis was performed on the collected tsetse flies. Out of the 360 flies that were dissected, 13 (3.6%) were *Trypanosoma* positive under the microscope.

This study found a significant difference in tsetse fly catches between government intervention and control sites. There was a significant higher concentration of tsetse flies being captured in the control sites, explained by the effectiveness of the ongoing vector control with deployment of tiny targets and regular monitoring occurring in the selected

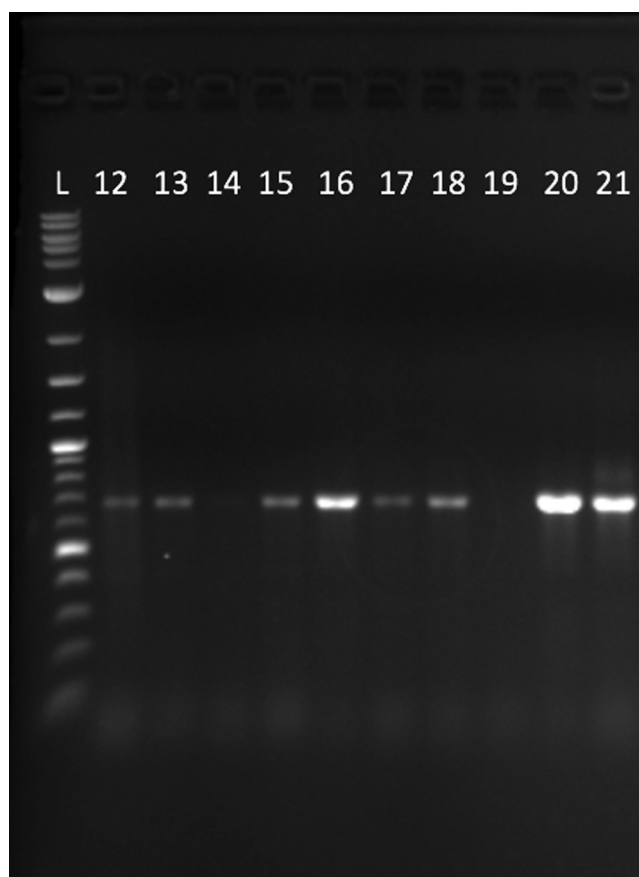


FIG. 3. Representative gel visualization of *Trypanosoma vivax* with a 100 bp ladder (L) used as a size marker. All the samples, excluding sample 19, showing ~620 bp band size of the PCR product corresponding to *T. vivax*.

intervention sites (Tirados et al. 2015). Because of tiny target deployment, there has been a significant reduction in tsetse flies.

According to Tirados et al. (2015), an overall 90% tsetse fly decline was illustrated within a 12-month period in West Nile region after the mass deployment of the tiny targets, with the AD showing a reduction from 2.4 tsetse FTD in Arua and Maracha district to 0.5 tsetse FTD within a 3 years period (2010–2013) (Tirados et al. 2015, Lehané et al. 2016).

Out of the 13 *Trypanosoma* infected tsetse flies, 85% had *Trypanosoma* present in the proboscis that could suggest an infection with *T. vivax* (Bouyer et al. 2015), *T. brucei* s.l., or *Nanomonas*. *T. vivax* is a causative agent of nagana in cattle, and this is consistent with the finding of *T. vivax* in the infected pigs. The molecular data suggested a *T. vivax* infection being present in 33.4% (114/341) of the blood samples collected from pigs. This furthermore shows that the majority of the sampled pigs could act as a reservoir for the causative agent for the wasting disease nagana in cattle.

Less than 1% (7/1262) of the sampled pigs were microscopically positive, which is very low. This can be explained by the lower sensitivity of microscopy, as using the HCT method for detection of *Trypanosoma* under microscopy has shown to have a sensitivity of ~56% (39–80%) when used for detection of *Trypanosoma* (Bonnet et al. 2015). In this

study, however, the sensitivity of the HCT methods was much lower, with only 7 out of 114 (6%) PCR-positive samples being correctly identified. Interobserver variability in microscope reading, as well as the likelihood of low parasitemia, also plays a significant role in the sensitivity of microscopy, especially under field conditions (Ng'ayo et al. 2005).

The ITS PCR method uses primers that target conserved regions of the ITSs that are known to vary in size between different *Trypanosoma* species. It has been shown to be a reliable method that can be used to screen biological samples accurately for the prevalence of different *Trypanosoma* species causing African trypanosomiasis.

Existing published data show that *T. vivax* has been detected in pigs, but it is not very commonly reported as pigs have been described resistant to infection with *T. vivax*. This *Trypanosoma* species is most commonly found in cattle.

No *T. brucei* was detected in our study. Data from 2018 showed that, after screening 10,000 people for trypanosomiasis across four districts in the northwestern part of Uganda, no current *T. b. gambiense* infection was detected (Selby et al. 2019). This indicates a low prevalence of g-HAT in the study area. Furthermore, another study conducted in the West Nile region by Cunningham (2017) reported, similarly to our study, that pigs in the region were negative for both *T. b. gambiense* and *T. b. rhodesiense*. Our results also support earlier results by Balyeidhusa et al. (2012), who concluded that there is an apparent lack of domestic animal reservoir of g-HAT in northwest Uganda.

However, this study, as well as the studies concluded by Ng'ayo et al. (2005), Biryomumaisho et al. (2009), and Hamill et al. (2013), illustrated the important role pigs play as domestic host for *T. vivax*.

Conclusion

The tsetse fly control programs are a very important component in the fight against African trypanosomiasis, and this study indicates the success of the ongoing vector control. The low prevalence of tsetse fly density and low incidence of *Trypanosoma* infection in the vector were most likely due to the ongoing vector control activities, which seem to be efficient in reducing vector populations and subsequently *Trypanosoma* infections. This study further supports earlier reports of the importance of pigs as a reservoir for *T. vivax* in Uganda and indicates that the pigs may be a less important reservoir for HAT in the region.

Limitation

A significant limitation is that not all pig samples were analyzed through ITS-PCR. Another limitation is that, due to limited resources, the *Glossina* spp. bloodmeals stored on FTA paper could not be further analyzed for species identification, mammalian host preference, and infections with *Trypanosoma* spp.

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Authors' Contributions

S.M., J.F.L., K.R., A.M., and C.W. contributed to idea formulation, data collection, data interpretation, and writing of the report. S.M., R.E., J.F.L., and K.R. contributed to quantitative data analysis and interpretation.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Johanna F. Lindahl

Department of Medical Biochemistry and Microbiology

Uppsala University

Po Box 582

Uppsala SE-751 23

Sweden

E-mail: johanna.lindahl@slu.se