UNIVERSITY OF NAIROBI

EPIDEMIOLOGY OF *TRYPANOSOMA* INFECTIONS IN CATTLE AND *GLOSSINA* FLIES AT THE HUMAN-WILDLIFE-LIVESTOCK INTERFACE OF AKAGERA NATIONAL PARK, RWANDA

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A thesis submitted in fulfilment of the requirements for the award of degree of Doctor of Philosophy in Applied Veterinary Parasitology of the University of Nairobi

Department of Veterinary Pathology, Microbiology and Parasitology

Faculty of Veterinary Medicine

University of Nairobi

2022

DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other university

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Title of the work : **Epidemiology of *Trypanosoma* infections in cattle and *Glossina* flies at the human-wildlife-livestock interface of Akagera National Park, Rwanda**

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DEDICATION

This thesis is dedicated to:

My late Mum, for your motivation, support, and prayers up to this far

Rest in eternal peace.

My spouse Francine, my daughter Tya Lesly and my son Rick Dylan. This is a fruit of your patience with my long time absence and late nights' work.

Be encouraged, success has no limit

ACKNOWLEDGEMENTS

My sincere gratitude goes to my supervisors Prof Ndichu Maingi, Prof Samuel M.Githigia, Dr. Daniel Masiga, and Dr. James Gashumba for their invaluable advice and guidance throughout this work. The Borlaug Higher Education for Agricultural Research and Development (BHEARD) program offered this Ph.D. scholarship; under the Michigan State University. The program was financially supported by the United States Agency for International Development (USAID), as part of the Feed the Future initiative, under the CGIAR Fund, award number BFS G-11-00002, and the predecessor fund the Food Security and Crisis Mitigation II grant, award number EEM-G-00-04-00013. I am grateful to them.

I wish to acknowledge the additional financial support from Rwanda Dairy Development Project (RDDP), funded by the International Fund for Agricultural Development (IFAD) - project ID: 2000001195 through Rwanda Agriculture and Animal Resources Board. I would like to express my sincere gratitude to the International Centre of Insect Physiology and Ecology (ICIPE) for offering a Dissertation Research Internship Programme (DRIP) Fellowship. The fellowship received financial support from the German Ministry for Economic Cooperation and Development (BMZ) through the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) ICTDL Project Contract Number 81235250 and Project Number 18.7860.2-001.00.

I thank the administration of Kayonza, Gatsibo, Nyagatare and Kirehe districts in Rwanda, for the permission to conduct the fieldwork. I thank African Parks through Akagera National Park management and Rwanda Development Board for the research permission. African Parks manages Akagera National Park; a non-profit conservation organisation that manages protected areas on behalf of governments across Africa, through a public-private partnership with the Rwanda Development Board;

The Rwanda Agriculture and Animal Resources Development Board (RAB) and the School of Veterinary Medicine, the University of Rwanda are acknowledged for providing the laboratory facilities. I thank the Animal Production and Health Division of the Food and Agriculture Organization of the United Nations (FAO), in the framework of the Programme Against African Trypanosomosis (PAAT), for the collaboration and guidance throughout the study time.

I gratefully acknowledge the management, staff and interns of the Molecular Biology and Bioinformatics Unit (MBBU) of ICIPE, for access to research facilities, collaboration, enthusiasm, and good network. The Animal Resources Officers at the study districts and sector level, private veterinarians, students and farmers who facilitated the fieldwork, data collection and analysis are acknowledged. I acknowledge my beloved family especially my spouse Francine Gasengayire and children Tya Lesly Ineza and Rick Dylan Simba, for their endurance, prayers and emotional support. I thank my extended family and friends for their continued support as well.

God bless you

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND ACRONYMS

AAT	: African Animal Trypanosomiasis
AD	: Apparent Density
ANOVA	: Analysis of Variance
CATT	: Card Agglutination Test for Trypanosomiasis
CGIAR	: Consultative Group on International Agricultural Research
DNA	: Deoxyribonucleic acid
EDTA	: Ethylene Diamine Tetra Acetic acid
Exo1-rSAP	: Exonuclease I and recombinant Shrimp Alkaline Phosphatase
FTD	: Fly per Trap per Day
G.	: *Glossina*
GALVmed	: Global Alliance for Veterinary Medicines
GIS	: geographical information system
HAT	: Human African Trypanosomiasis
ICIPE	: International Centre for Insect Physiology and Ecology
IFAD	: International Fund for Agricultural Development
LST	: Lysis, Storage and Transportation
MINAGRI	: Ministry of agriculture and animal resources
NISR	: National Institute of Statistics of Rwanda
NP	: National Park
OR	: Odds Ratio
OIE	: Office International des Epizooties
PAAT	: Programme Against African Trypanosomiasis
PATTEC	: Pan-African Tsetse and Trypanosomiasis Eradication Campaign
PCP	: Progressive Control Pathway
PCR / HRM	: Polymerase Chain Reaction / High Resolution Melting
PCV	: Packed cell volume
RAB	: Rwanda Agriculture and Animal Resources Development Board;
RDT	: Rapid diagnostic test
REMA	: Rwanda Environment Management Authority;
SPSS	: Statistical Product and Service Solutions;
SRA	: Serum Resistance-Associated gene
UNFAO	: Food and Agriculture Organization of the United Nations;
WHO	: World Health Organisation.

ABSTRACT

African Trypanosomosis is a major neglected tropical disease of animals and humans in low resource settings in Africa. The disease is cyclically transmitted by *Glossina* (tsetse flies) spp. and mechanically by biting flies such as *Tabanus* spp. and *Stomoxys* spp. and has enormous negative effects on the health and life of both humans and animals. The socio-economic and health impact of the disease is often felt at the edge of protected, tsetse-infested wildlife areas. In Rwanda, tsetse flies and trypanosomosis are reported in areas around the Akagera National Park but the situation has previously not been well documented. This study aimed at determining (i) the distribution of species of *Glossina* (ii) the *Trypanosoma* species circulating in tsetse flies, their infection rate, and the endosymbionts, (iii) the hosts' preference for the tsetse flies, and (iv) the *Trypanosoma* species circulating in cattle, at the wildlife-livestock interface of Akagera National Park in Rwanda.

To determine the distribution of *Glossina*, a longitudinal stratified sampling entomological survey was carried out inside the park and its surroundings. Biconical traps were deployed in 55 sites for six consecutive days of each study month from May 2018 to June 2019 and emptied every 48hours. Flies caught in the traps were identified using FAO keys for entomological taxonomy. The number of flies per trap per day (FTD) was used to determine the apparent density (AD) of the flies. Pearson chi-square ($\chi 2$) and parametrical tests (t-test and ANOVA) were used to determine the variability between the variables. Logistic regression was used to determine the association between predictors of tsetse flies occurrence. A selected sample of 1101 tsetse flies, recovered from the traps, was analysed for trypanosome species and endosymbionts using PCR, and 2-gene High Resolution Melting analysis for blood meal source. A total of 1037 blood samples collected between March and July 2019 from randomly selected cattle (local and local x Friesian breeds) in four districts neighboring the National Park, and were examined for species of *Trypanosoma*. Four districts viz. Kayonza, Gatsibo,

Nyagatare and Kirehe were selected for their proximity to the park and for being adjacent to protected game reserves in Tanzania. The presence of trypanosomes in the blood samples was determined by microscopy, immunological rapid test, and PCR coupled with High-Resolution Melt (HRM) analysis. Sanger sequencing was done on the amplicons to complement the analysis. The Cohen Kappa test was used to compare the level of agreement between the diagnostic methods.

Thirty-nine thousands and five hundreds sixteen (39,516) tsetse flies were collected using the traps, of which 73.4% (29,019) and 26.6% (10,497) were from inside the park and the interface area, respectively. Female flies accounted for 61.3% (24,223) while 38.7% (15,293) were males. Two species of *Glossina*, i.e. *G. pallidipes* [n=29,121, 7.4 flies/trap/day (FTD)] and *G. morsitans centralis* (n=10,395; 2.6 FTD) were identified. The statistical difference was significant between the two species (p=0.000). The flies were more abundant during the wet season (15.8 FTD) than the dry season (4.2 FTD) [p=0.000]. Large numbers of flies were trapped around the swamp areas (69.1 FTD) inside the park and in Nyagatare District (11.2 FTD) at the interface [p=0.000]. One thousand and one hundred and one (1101) tsetse flies (771 *Glossina pallidipes* and 330 *Glossina morsitans centrali*s) were analysed for trypanosome infections. The overall infection rate was 13.9% (153/1101) in the head and proboscis (HP) and 24.3% (268/1101) in thorax and abdomen (TA) of the flies. Eight species of trypanosomes were identified. For each species, head +proboscis and thorax+ abdomen were analyzed in parallel and are presented as HP/TA. Of these species, *Trypanosoma (T.) brucei brucei* accounted for 4.1/7.1%, *T.congolense* Kilifi (2.2/2.1%), *T.congolense* savannah (1.6/1.2%), *T. evansi* (0/0.9%), *T.godefreyi* (1.2/3.1%), *T. grayi* (0/1.08%), *T.simiae* (2.08/3.7%), *T.theileri* (0/2.08%) and *T.vivax* (5.2/3.7%). Mixed infections were 2.2/0.8% (25/9). No *T.brucei rhodesiense* was found in tsetse flies analyzed.

The endosymbionts found in tsetse flies were the bacteria *Sodalis* (2.8%; 31/1101) and *Wolbachia* (4.8%; 53/1101). No *Spiroplasma* and SGH Virus were found in all samples analyzed. The preferred hosts for blood meal by the tsetse flies were buffalo (36.5%), warthog (14.1%), cattle (10.6%), savannah elephant (8.7%), bushbuck (7.3%), and human (5.7%).

The overall prevalence of trypanosome infections in cattle was 5.6%, 7.1%, and 18.7% by thin blood smear, Buffy coat technique, and PCR/HRM, respectively. Microscopy showed a low sensitivity (28.9%) while the rapid test (VerY Diag) showed a low specificity (32.5%). Trypanosomes were detected in cattle blood, including species that are pathogenic to cattle (i.e. *T. brucei brucei, T. congolense* savannah, *T. evansi* and *T. vivax*) and *T. theileri* which is non-pathogenic.

T. congolense was the most prevalent (10.7%), followed by *T. vivax* 5.2%, *T. brucei brucei* 2%, and *T. evansi* 0.7% by PCR/HRM analysis. Lower trypanosome infections were observed in Ankole Friesian cross-breeds than indigenous Ankole. No human-infective *T. brucei rhodesiense* was detected. There was no significant difference between the mean PCV of infected and non-infected animals ($p>0.162$).

The study results on tsetse distribution, endosymbionts, hosts preference, and trypanosomes infections, corroborate other similar regional findings. *Glossina pallidipes* were found in higher numbers and therefore conceivably the most important vectors of trypanosomes. This study confirms that the cattle reared around the Akagera NP are infected by *Trypanosoma* species causing African Animal Trypanosomiasis (AAT), and the area should, therefore, be targeted in control activities. Future studies should focus on the AAT impact assessment on cattle production and information on the use of trypanocides to help policymakers prioritize target areas and optimize intervention strategies.

CHAPTER ONE: GENERAL INTRODUCTION

1.1. Background information

African Trypanosomes constitute a group of vector-borne parasites causing African Animal Trypanosomiasis (AAT) or ‘’nagana’’ (Giordani *et al*., 2016) and Human African Trypanosomiasis (HAT) or “sleeping sickness” (Büscher *et al*., 2017). The disease is caused by protozoan parasites in the Genus *Trypanosoma*, which are transmitted cyclically by tsetse flies (Jordan, 1976; Maudlin *et al*., 2004) and, for some species, mechanically by biting flies such as Tabanids and *Stomoxys* (Desquesnes, 2004; Ahmed *et al.*, 2016). Animal African Trypanosomosis (AAT) and Human African Trypanosomosis (HAT) are conferred little priority since they are essentially rural challenges. Furthermore, AAT occurs in poor and vulnerable settings of Africa, where it is still overlooked by funders and even governments of the endemic countries themselves (PATTEC, 2001).

In comparison to other diseases, AAT is often neglected by veterinary authorities because it mainly affects poor livestock keepers and frequently shows a chronic presentation (Diall *et al*., 2017a). Trypanosomosis affects many mammalian species (Ruiz *et al*., 2015); including some wildlife animals such as buffaloes and warthogs, which are the reservoirs of the infection (Anderson *et al*., 2011; Auty *et al*., 2012). Many wild animals carry trypanosomes with no clinical signs (Auty *et al*., 2012). In areas of human-livestock-wildlife interface, the wildlife can act as a constant source of infections (Van den Bossche *et al*., 2010). Sleeping sickness threatens more than 50 million people in Africa and is characterised as a neglected tropical disease (Simarro *et al*., 2012; WHO, 2013; Franco *et al.*, 2020).

A sub-Saharan area of roughly 10 million km^2 is infested by tsetse flies and more than 60 million cattle are exposed to AAT (Giuliano and Mattioli, 2009). This area is suitable for increased agricultural production on the African continent (Swallow, 2000). It has been shown that trypanosomosis has harmed African populations' ability to generate agricultural surpluses

in the past, and that tsetse flies continue to have a significant impact on the continent's current economic performance (Alsan *et al*., 2015).

Approximately, losses of over 3 million cattle and other livestock species linked to trypanosomosis occur in Africa (Weining and Giuliano, 2020). The annual financial losses attributed to trypanosomosis, i.e. reduction in livestock productions like meat and milk, the cost of treatment, and/or vector control, have been estimated at US $4.75 billion (PATTEC, 2001; Weining and Giuliano, 2020). In East Africa alone, the economic losses from cattle trypanosomosis were estimated to be approximately US $2.5 billion (Shaw *et al.,* 2014).

Agriculture and animal resources play a key role in the economy of Rwanda, with livestock contributing about 16% (NISR, 2020). Among other livestock, cattle are the main source of income for farmers in Eastern Province, a region dedicated to cattle production (MINAGRI, 2015). A survey conducted in 2007 showed that roughly, 40% of the national cattle population are raised in Nyagatare, Gatsibo and Kayonza districts of Eastern Province (Chatikobo *et al*., 2009). The same survey revealed that vector-borne diseases are the major animal health challenges accounting for 35.8%, of which trypanosomosis alone accounted for 8.2%. The intense cattle rearing is found at the interface of Akagera National Park because two-thirds of the park area was occupied by farmers who returned home in 1994 from exile and did not have land for their cattle (Hajabakiga, 2004).

1.2. Problem statement

In Rwanda, the Akagera National Park (NP) and its surrounding areas are a refuge for tsetse flies (Gashururu *et al*., 2021) and a source of *Trypanosoma* infections to livestock (Chatikobo *et al*., 2009; Mazimpaka *et al.,* 2017). Akagera NP is adjacent to other game reserves in Tanzania, with Ibanda Game Reserve in the North and Kimisi Game Reserve in the South.

This area is infested by the savannah species of tsetse flies, believed to be efficient vectors of human and animal infective trypanosomes (Mihok *et al*., 1992; Gashururu *et al*., 2021). The park has suitable habitats for tsetse survival and hosts a high concentration of preferred wild animals such as warthogs and buffaloes on which the flies constantly feed (Macpherson, 2013; Macpherson, 2019). These animals are the natural reservoirs of trypanosome infections (Auty *et al*., 2012). Around the park, there are many cattle farms and the settled farmers ' homesteads surround the entire length of the park (Udahemuka *et al*., 2020). The interface between the livestock and the wildlife reservoirs plays a significant role in the AAT epidemiology in infested areas (Van den Bossche *et al*., 2010). There is a risk of diseases occurrence, originating from wild animals and/ or shared between humans, livestock and wild animals (Ntivuguruzwa *et al*., 2020). In terms of epidemiology, this is an area in which the tsetse fly challenge to livestock is mainly found at the edge of a tsetse-infested park and AAT impact on livestock is highest along the wildlife-livestock interface (Van den Bossche *et al*., 2010).

Tsetse–transmitted trypanosomes have been prevalent and reported around Akagera NP. Farmers rearing livestock in this area are aware of AAT (Chatikobo *et al*., 2009; Mazimpaka *et al*., 2017), and trypanosomes were detected in cattle and tsetse flies vectors (Mihok *et al*., 1992). The latter study reported an overall infection rate of 5.5% in cattle and *T. congolense* accounted for 79% of the infections, followed by *T. vivax* accounting for most of the remaining 21%. In tsetse flies, the *T. congolense* and *T. vivax* infections in proboscis each accounted for 3.8% (Mihok *et al*., 1992). The situation of resistance to trypanocides has been reported in many African countries (Delespaux *et al*., 2008). Few sporadic cases of *T.b. rhodesiense* sleeping sickness were reported in Rwanda around the year 1990 (Clerinx *et al*., 1998). However, no recent positive cases have been reported in the area (Simarro *et al*., 2010; Franco *et al*., 2020; WHO, 2020). The transmission of trypanosomes in an area involves many other factors including the tsetse fly endosymbionts (Wang *et al*., 2013) and the feeding preferences

(Auty *et al*., 2016). This information is missing in tsetse flies of the Akagera region of Rwanda. To contribute to the understanding of interactions and relationships between humans, wildlife- and livestock, there is need to investigate the endosymbionts community and the feeding preferences for tsetse flies that are important in the transmission dynamics of African Trypanosomiasis in this setting. The drugs being used to treat animals in the area for so long are Diminazene aceturate and Isometamidium chloride. The presence of considerable drug resistance to Diminazene aceturate in Bukora Ranch, which is not far from the study area was reported by Mihok and others (1992). However, no extended research has been carried out in all risky areas of the country to determine the situation on the group, with regards to trypanocidal drug resistance. There is need to know whether the trypanocidal treatment failure being reported by farmers (personal communication from the veterinary services) is due to drug resistance development or other factors such as poor drug quality on the local markets and/or under dosing.

1.3. Study objectives

1.3.1. Overall Objective

To contribute to a better understanding of the epidemiological factors of African Trypanosomes at the human-wildlife-livestock interface of Akagera National Park in Rwanda.

1.3.2. Specific objectives

1. To determine the spatial distribution, abundance, species composition and seasonal variations of *Glossina* (tsetse flies) in the study area;
2. To characterize the trypanosome species in tsetse flies, determine their infection rates and endosymbionts community in the study area;
3. To determine the blood meal sources (hosts preference) for the tsetse flies in the area;

4. To determine the occurrence, diversity and distribution of *Trypanosoma* species circulating in cattle in the study area.

1.4. Working hypothesis

1. Tsetse flies are abundant in the study area and *Trypanosoma* infections are prevalent in cattle and the tsetse flies around the park.
2. Tsetse flies harbor human infective trypanosomes, but cattle do not.
3. Cattle are the most preferred hosts for blood meals of the tsetse flies.

1.5. Knowledge gaps and justification

The key requirement to eradicate and or eliminate African Trypanosomosis is to understand the transmission dynamics in the target area. Despite the basic information above, there is no reliable data on the disease and little is known about the tsetse flies and trypanosomes in Rwanda. The parameters determining the epidemiology of tsetse-transmitted trypanosomes are not well understood to date. These parameters are, but are not limited to, the structure and distribution of the vectors; infection rates in the vectors and their host preferences; diversity of *Trypanosoma* species in the vectors and susceptible hosts and the efficacy status of available trypanocidal drugs.

This information is crucial to the design and put in place evidence-based strategies for the control of the vector and tsetse-transmitted trypanosomosis (Diall *et al.*, 2017b; Stanton *et al*., 2018). This study contributes valuable data (entomological and occurrence of infections) to set adequate and focused control measures in the country. In the same vein, these findings help the country to comply with FAO Progressive Control Pathway (PCP) to reduce and eliminate AAT. On the human side, the generated data is valuable to validate the elimination of Human African Trypanosomosis (HAT) (Franco *et al.*, 2020).

CHAPTER TWO: LITERATURE REVIEW

2.1. African trypanosomes

2.1.1. General considerations

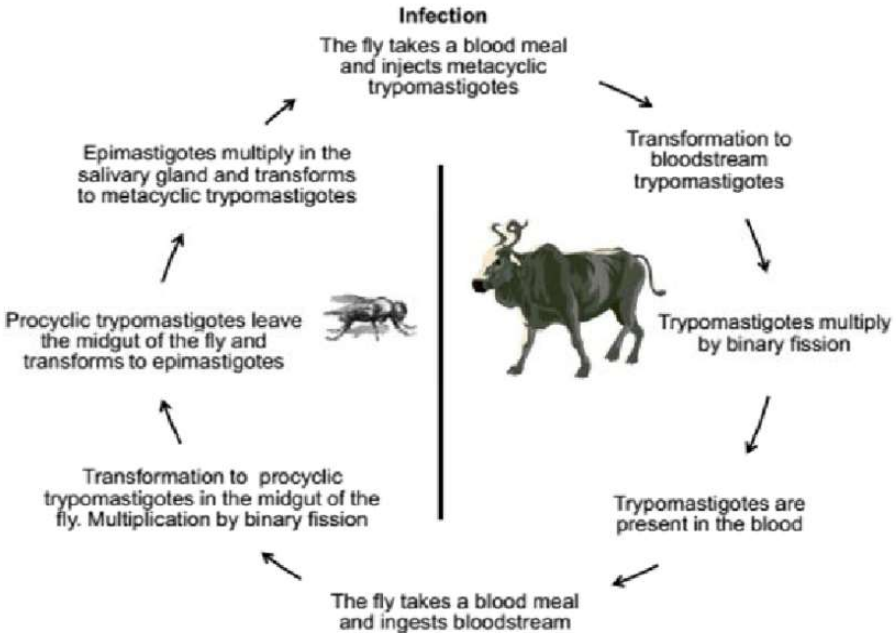
Trypanosomes, literally meaning "the body into a tailspin", are protozoan parasites that are flagellated, unicellular, elongated and typically a little curved (Mulligan, 1970). Trypanosomes fall under the Phylum of Protozoa, Order *Kinetoplastida;* Family *Trypanosomatidae* and Genus *Trypanosoma* (Maudlin *et al.*, 2004). Trypanosomes are divided into two distinct sections: the Stercoraria (subgenera *Schizotrypanum, Megatrypanum* and *Herpetosoma*) transmitted by vector faecal contamination (posterior station development) and the Salivaria (subgenera *Duttonella, Nannomonas, Trypanozoon*), transmitted by inoculation through the tsetse fly bite (anterior station development) (Radostis *et al*., 2000; Maudlin *et al.*, 2004). Within the salivarian section, the parasites are classified according to their morphology and site of development in the vector (OIE, 2013). Except for *Trypanosoma cruzi*, the stercorarian species are normally non-pathogenic but may confuse the parasitological diagnosis of trypanosomosis (Maudlin *et al*., 2004). Typically, the salivarian group is the only one that exhibits antigenic variability because of having the genes of the variant surface glycoprotein (VSG) (Matthews *et al*., 2015).

The *Nannomonas* subgenus has two main clades, one for *T.congolense* subgroups and the one for *T. simiae, T. godfreyi* and *T. simiae* Tsavo (Gibson, 2003). There are three distinct genotypes of *T.congolense* viz. savannah type (Tropical Africa), riverine/forest type (West & Central Africa) and Kilifi type (East Africa) (Gibson, 2003). *T.congolense* savannah strains are experimentally more virulent than the forest and Kilifi strains (Bengaly *et al*., 2002**;** Masumu *et al*., 2006; Van Den Bossche *et al*., 2011**;** Motloang *et al*., 2014)**.** Among the *Trypanozoon, T.brucei* species has three subspecies: *T.brucei brucei* affecting animals, *T.brucei rhodesiense* and *T.brucei gambiense* affecting humans (Maudlin *et al*., 2004).

2.1.2. Multiplication and transmission of trypanosomes to the susceptible hosts

The multiplication of trypanosomes occurs in the blood, lymph vessels and tissues, as well as cardiac muscle and central nervous system (OIE, 2013). In the mammalian host, the trypanosomes are extracellular and are frequently found in blood circulation (Maudlin *et al*., 2004). The multiplication is primarily done asexually by binary fission but sexual reproduction is also possible (Peacock *et al.*, 2006), through the exchange of genetic material during development in the tsetse fly vector (Morrison *et al*., 2009, Holzmuller *et al.*, 2010).

The procyclics are immature forms while metacyclics are mature forms of the parasite and both occur in the tsetse fly. In the fly, the parasite undergoes two stages of differentiation for successful development and transmission. Depending on the species, the parasite establishes in the midgut and then matures in the mouthparts (*Nannomonas*) or salivary glands (*Trypanozoons*) (Maudlin *et al*., 2004). The lifecycle of *T.vivax (Duttonella)* takes place exclusively in mouthparts (Dagnachew & Bezie, 2015). The fly is infected when it takes the blood meal from a host that has bloodstream trypomastigotes. After ingestion by a fly, the bloodstream trypomastigotes are located in the fly gut and are transformed into procyclic trypomastigotes. After their multiplication, the procyclic trypomastigotes leave the fly gut to form the procyclic epimastigotes in the mouthparts (Figure 2.1). The latter multiply and form the metacyclic trypomastigotes, which are infective to mammalian hosts when the fly is taking its blood meal. Epimastigotes forms have long or truncated posterior ends, while the infective metacyclics are very small and do not divide. In the mammalian host, the metacyclic form multiplies and migrates through the lymph to the bloodstream (Vickerman *et al*., 1988a). The bloodstream form is infective to other hosts. Transmission occurs when an infected fly inoculates the metacyclic form of the parasites in its saliva as it takes a blood meal from another mammalian host (Leak, 1999). Once the fly is infected, it remains so for life (Radostis *et al*., 2000).



The main phases in the life cycle of a trypanosome
Source: (Dagnachew & Bezie, 2015)

Figure 2.1: The life cycle of trypanosomes in the tsetse fly vector and mammalian hosts

All trypanosome species have a transmitting vector, which is usually an arthropod, except *T. equiperdum*, which is transmitted through a venereal way (Matthews *et al.*, 2015). *Glossina* cyclically transmits trypanosomes and it is the main mode of transmission for African Trypanosomiasis. In this mode of transmission, *Glossina* are infected by the bloodstream form of the parasite when they are feeding on an infected host (OIE, 2013). The non-cyclical transmission is possible in the absence of *Glossina* vectors when the trypanosome infection is established into a herd (Maudlin *et al.*, 2004). The biting flies such as tabanids, stomoxys and hippoboscidae can transmit mechanically the infection to susceptible hosts (Leak, 1999). Vampire bats transmit *T.evansi* in America, the parasite multiplies in the blood of bats and can survive but there is no other morphological transformation. It is, therefore, called mechanical transmission as well. Similarly, *T.vivax* can be mechanically transmitted by biting flies and seems to have reached America through the importation of cattle (Bowman, 2014).

Vertical transmission can occur intra-utero and during partum, as well as through iatrogenic means and carnivores that feed on infected cadavers (OIE, 2013).

2.2. African trypanosomes and the disease

Both domestic and wild animals are affected by trypanosomes at varying levels (Giordani *et al*., 2016; Auty *et al*., 2012). The host preference depends on the trypanosome species. Furthermore, the pathogenicity of the parasite depends on the host species affected, species and strains of trypanosomes as well as the nutritional status of the animals (Desquesnes *et al*., 2013; Maudlin *et al*., 2004). Several trypanosomes species are pathogens that cause serious disease in livestock; for example, *T. congolense, T. vivax* and *T. b. brucei, T. simiae, T. godfreyi, T. suis, T. equiperdum* and *T. evansi*. Every species of trypanosome has a diverse host range depending on the region (Maudlin *et al*., 2004). The disease in cattle is called *Nagana*, a Zulu word meaning "to be depressed" (WHO, 2013). However, other names are depending on the host species affected and trypanosome species. Infection with *T. evansi* in equines is called ‘’Surra’’, while the one with *T.equiperdum* in equids is called ‘’Dourine’’ (Maudlin *et al*., 2004). Animal African Trypanosomiasis (AAT) is found throughout Sub-Saharan Africa's tsetse-infested belt (Simarro *et al*., 2010). The human form of trypanosomosis, also known as sleeping sickness, is caused by two subspecies of *T. brucei*: *T. brucei rhodesiense* and *T. brucei gambiense*, whereas *T. brucei bruce*i is not pathogenic to humans (Büscher *et al*., 2017). Human African trypanosomosis (HAT), is found in more restricted endemic areas (Simarro *et al*., 2010). In cattle, the clinical signs are numerous, but not specific. In general, anaemia is the most important physiological characteristic and pathological consequence (Maudlin *et al*., 2004). However, other symptoms exist such as weakness, weight loss, loss of appetite, conjunctival petechiae, pyrexia, swelling of lymph nodes, cachexia, oedema, immunosuppression or abortion are observed (Giordani *et al*., 2016). Clinical signs are the first suspicions of trypanosomal infection in cattle when tsetse flies are present, although none is

specific (OIE, 2021). In endemic areas, the same clinical signs can be seen in other parasitic diseases such as babesiosis, hemonchosis, theileriosis and anaplasmosis (Desquesnes and Dávila, 2002).

2.3. Detection of trypanosomes from their vectors and cattle hosts

Several diagnostic tests are used to detect trypanosomes, i.e. parasitological (Murray, 1977), Immunological (OIE, 2013; Boulangé *et al*., 2017) and molecular techniques (Simwango *et al*., 2017). Techniques differ in sensitivity and each presents its advantages and drawbacks (Boulangé *et al*., 2017; OIE, 2013; Ng'ayo *et al*., 2005; Kivali *et al*., 2020).

2.3.1. Parasitological techniques

The first-line method is microscopy consisting of visualizing the parasite in biological samples. It is based on the detection of the parasite itself (parasitology), its constituents (proteins), and the host's immune response (serology). Direct examination of motile trypanosomes in fresh blood obtained from peripheral veins (ear vein, jugular vein, or the tail) between slide and cover-slip by wet, Giemsa-stained thick or thin films (OIE, 2021).

The parasite concentration methods include:

2.3.1.1. Haematocrit centrifuge technique (HCT) - Woo method

Separation of blood components by centrifugation in a heparinised capillary tube, coupled with haematocrit. The method requires rapid implementation (Woo, 1970).

2.3.1.2. Buffy coat technique (BCT) - Murray method

The method derives from the Woo method and consists of the centrifugation of blood in heparinized capillary tube. The capillary tube is cut with a diamond-tipped pencil to get the buffy coat which is then examined between slide-coverslip with a dark-ground or a phase-contrast microscope (Murray, 1977).

2.3.1.3. Mini Anion-exchange chromatography technique (mAECT)

It is widely used for the diagnosis of *T.gambiense* sleeping sickness (OIE, 2021). Anion exchange chromatography on diethylamino-ethyl resin (DEAE) is also used to concentrate and separate trypanosomes from other blood elements in larger volume samples. Blood is passed through a DEAE-cellulose column equilibrated with a phosphate buffered saline (PBS) solution of an ionic strength matched to the blood of the animal species being examined (Camara *et al*., 2010). The red blood cells, negatively charged than trypanosomes, remain in the column while trypanosomes pass through. The pellet is then examined under the microscope (Uilenberg, 1998).

2.3.1.4. Inoculation

In case of low parasitaemia, multiplication of the parasite by an intraperitoneal injection of fresh infected blood into an immunocompromised rodent. A drop of blood collected from the tail tip is examined by a wet film under a microscope (Schoening, 1924;OIE, 2021)

2.3.2. Immunological/serological techniques

Antibodies persist long in blood circulation after recovery or treatment. Antibodies indicate a present or past contact with the parasite but do not indicate an active infection. Serodiagnosis is, therefore, primarily used in epidemiological studies (OIE, 2021; Boulangé *et al*., 2017).

2.3.2.1. Indirect Fluorescent Antibody Test (IFAT)

Detection of anti-trypanosome (lgG) antibodies by immunofluorescence. The IFAT is preferred for small-scale surveys (Katende *et al*., 1987) and requires a fluorescence microscope, a material that is expensive to purchase. The test is sensitive, slow and cross-reactions between species are possible (Uilenberg, 1998; OIE, 2021).

2.3.2.2. Card Agglutination Test for Trypanosomiasis - CATT / T. evansi

Based on the direct agglutination of the parasites by specific type M immunoglobulins (lgM), produced by *T.evansi*. A pure preparation of *T. evansi*, expressing a predominantly variable antigen (VSG RoTat 1.2) is coated on a card and stained. Agglutinate, resulting from the binding of IgM present in the serum or infected animal blood to the parasitic antigens fixed on the card, is visible with a naked eye (OIE, 2021). The test is quick and easy to perform in the field and requires little expertise compared to other serological tests. The CATT / *T. evansi* is suitable for confirming an active infection (Uilenberg, 1998).

2.3.2.3. Complement Fixation Test (CFT)

The test is highly specific for *T.equiperdum*, and detects anti-trypanosome antibodies by complement fixation coupled with a haemolytic system. It is complex, expensive, and slow and requires expertise (Schoening, 1924; OIE, 2021).

2.3.2.4. Antibody detection Enzyme-Linked Immunosorbent Assay (Ab-ELISA)

ELISA is the "gold standard" for AAT serological diagnosis. It is based on the detection of anti-trypanosome antibodies (lgG) by enzymatic immuno-absorption. Antibodies are detected indirectly, most often using total soluble *Trypanosoma* antigens derived from trypanosomes in the blood of infected rodents (Luckins, 1977; Desquesnes, 1977) , or a recombinant protein (Boulangé *et al.*, 2002; Pillay *et al.*, 2013). More recently, an Ab-ELISA test for *T. vivax* and *T. congolense* (VerYDiag ® by CEVA) was introduced in the form of lateral flow rapid field test (Pillay *et al*., 2013; Boulangé *et al*., 2017). VerY Diag eliminates the problem of standardization and reinforces specificity, but lacks sensitivity in very early infections or on the contrary at a very late stage (Boulangé *et al*., 2017).

2.3.2.5. Trypanolysis

Detection of anti-VSG antibodies by binding of complement to immune complexes lysing living trypanosomes. The trypanolysis test is based on cell lysis by the complement system. It makes it possible to detect the antibodies recognizing a single multicopy epitope of the VSG RoTat 1.2 variant of *T. evansi* type A. The lysis of the trypanosomes, in the presence of complement, is observed under a microscope. The specificity is limited to the genus level (Tehseen *et al*., 2015).

2.3.3. Molecular techniques

The methods identify the species and subspecies of trypanosomes five days after infection in both the insect and the mammalian host, through the detection of the parasite's nucleic acid (DNA) (Matovu *et al*., 2010). The methods that are often used are conventional PCR and quantitative PCR. The use of molecular tools is more reliable and has been used to identify species, subspecies, strains, and populations of trypanosomes in the epidemiological investigation. The Polymerase chain reaction (PCR) can detect even a single trypanosome in a sample (Adams and Hamilton, 2008). However, the effects of genetic exchange and recombination between organisms can influence these techniques significantly (François *et al*., 2016). Molecular based tools for the identification and diagnosis of trypanosomes have undergone considerable developments. Those are the species-specific identification techniques (DNA probes, species-specific PCR, Loop mediated isothermal Amplification - LAMP), generic approaches (Ribosomal length-based – Internal Transcribed Spacer, Restricted Fragment Length Polymorphism- RFLP, Fluorescent Fragment Length Barcoding – FFLB and sequence analysis. The species-specific PCR is highly specific and sensitive, nevertheless, there is no recognition of unknown species using the primers. it is used when the trypanosome diversity is known and is time consuming especially when there is an increase in trypanosome species identified (Adams and Hamilton, 2008). The development of the Loop-mediated

isothermal Amplification (LAMP) to detect trypanosomes brought advancement. LAMP is fast, specific, sensitive and relatively simple, requires little laboratory equipment and results are easily readable. However, false positive results are frequently observed, as the technique is more prone to contamination. The kit for the *Trypanozoon* species was developed from the perspective of sleeping sickness (Mitashi *et al*., 2013). The technique can detect an active infection since it targets the parasite DNA (lifespan of the parasitic DNA estimated at 48 h), thus making the tool more sensitive and more specific (Jarra and Snounou, 1998).

2.3.4. Perspective on the future towards the field tests

The challenge in molecular diagnosis of trypanosomes is still the development of a cheap, effective and fast test, which can be used in field conditions with limited facilities and infrastructure. Methods such as LAMP and FFLB should be an answer in that regard but they are only adapted to the medically important species (Adams and Hamilton, 2008). Currently, serological tests seem to be suitable field tests because of fulfilling the ASSURED criteria: « *Affordable by those at risk of infection, Sensitive, Specific, User-friendly, Rapid, Equipment-free, Delivered to those who need* it» (Mabey *et al*., 2004). Molecular tools, though more sensitive and specific, do not fulfill the above criteria because they require an equipped laboratory, high technical expertise and are therefore difficult to use in the field (Mabe *et al*., 2004; Magamba, 2021).

2.4. Tsetse fly (*Glossina*) – the vector

2.4.1. Taxonomy, description and ecology

The tsetse flies (*Glossina*) belong to the Kingdom: *Animalia*, Phylum: *Arthropoda*, Class: *Insecta,* Order: *Diptera*, Sub-Order: *Cyclorrapha*, Family: *Glossinidae, and* Genus: *Glossina.* There are 33 species and subspecies within the genus *Glossina* (Krafsur, 2009). Tsetse flies are blood-sucking insects, distinguished from other haematophagous insects by their long

proboscis pointing forward and a particular wing vein pattern, which forms a cell called hatchet cell in a form of Butcher's cleaver. Their size ranges between 6 - 13 mm (Bowman, 2014). According to their preferred habitats, *Glossina* are divided into three groups namely the savannah, riverine and the forest group: they are respectively the *morsitans* group (subgenus *Glossina)*, *palpalis* group (subgenus *Nemorhina*) and the *Fusca* group (subgenus *Austenina*)(Leak, 1999). The *Morsitans* group is primarily found in a variety of savannah habitats, woodlands, and evergreen thickets. The *Palpalis* group is mainly found in humid/moist areas: the forests and extends into riverine and lakeside habitats (Radostis *et al*., 2000; Leak, 1999). This habitat includes lakeshores, riverbanks, swamps and forests. The *Fusca* group is restricted to areas of thick forests (forest dwelling species). *Morsitans* and *Palpalis* are diurnal while *Fusca* is nocturnal (Leak, 1999, Maudlin *et al*., 2004). Further characterisation of *Glossina* species and subspecies can be done according to their morphology, physiology, genetics, biochemistry and their host range (Maudlin *et al*., 2004). Recent genetic and evolutionary discoveries within the *Glossina* genus provide useful information which could help in novel tsetse and AT control (Attardo *et al*., 2019).

2.4.2. Distribution of Tsetse flies

Tsetse flies are restricted to the continent of Africa, between the deserts of Sahara and Kalahari. Extreme hot temperatures and lack of moisture limit the further extension of the fly towards the north, while low temperatures limit their extension to the south (Leak, 1999); (Maudlin *et al*., 2004). The tsetse fly is populating more than 10 million square miles in Africa, roughly a third of the continent, of potential agricultural and grazing land (Figure 2.2), making it inhospitable for agricultural activities and a large human population vulnerable to infection (Giuliano and Mattioli, 2009). Thirty seven countries of Africa are found within the tsetse fly belt (Swallow, 2000).

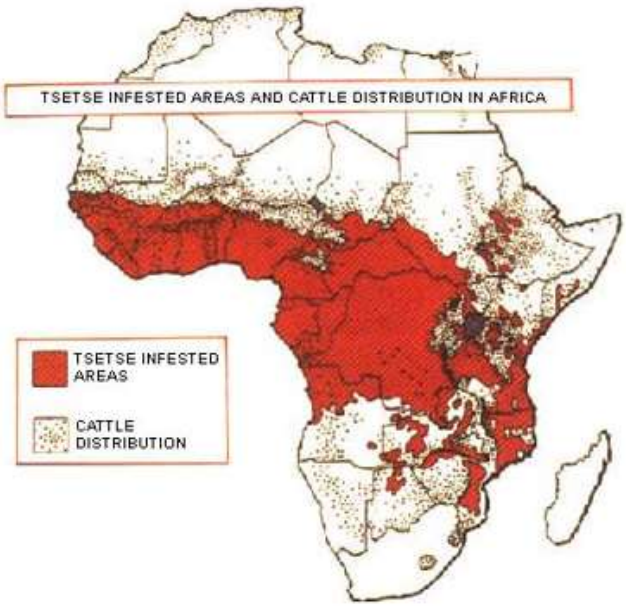


Figure 2.2: Tsetse distribution in the continent of Africa

Source: http://www.fao.org/docrep/u8480e/U8480E8f.jpg

2.4.3. History of tsetse flies in Rwanda

Information on the distribution of *Glossina* and trypanosomes in Rwanda was much documented during the colonial period (Henrard, 1951; Evens, 1953; Benoit, 1955; Berghe and Lambrecht, 1956; Evens and Meyus, 1957). However, little has been done to update this information afterward (Mihok *et al*., 1992). Three species of tsetse flies, i.e. *G. pallidipes* (Subgenus *Glossina* (*morsitans*) group - Austen 1903*), G. morsitans centralis* (Subgenus *Glossina* (*morsitans*) group - Machado 1970), and *G. brevipalpis* (Subgenus Austenina (*Fusca*) group - Newstead 1910) were reported in the lowland eastern savannah region of Rwanda (Evens and Meyus, 1957; Moloo, 1985; Katondo, 1984). The fourth species *G.fuscipes martinii* (Subgenus Nemorhina *(palpalis)* group - Zumpt, 1935) was once reported in the southwest region of Rwanda bordering Burundi (Ford, 1977). However, the species has not been reported again since then (Moloo, 1993). Population growth has over time reduced the suitable habitats for *Glossina* due to land acquisition (NISR, 2020). The formerly known hotspots comprised of the former Mutara region and the affiliated protected areas including Akagera NP.

The de-gazettement of roughly two-thirds of the protected land comprising the entire Mutara hunting area (300 km^2) and part of Akagera National Park for resettlements and farming activities after the year 1997 greatly reduced the protected areas (Hajabakiga, 2004; Sun *et al*., 2018). This reduced the Akagera NP to about 1,120 square kilometres out of the 2500 km^2 initially gazetted (Apio *et al*.,2015; Sun *et al*., 2018).

2.4.4. Susceptibility to *Trypanosoma* and vector competence among *Glossina* species

All species of *Glossina* are susceptible to *Trypanosoma* infections and therefore can transmit the disease (Roditi and Lehane, 1998). However, susceptibility varies among species of *Glossina*. The savannah (morsitans group) are the most effective vectors of trypanosomes to livestock (Aksoy *et al*., 2003). The savannah species cause the greatest threat to livestock since they colonise the places where animals are usually reared and can adapt easily to other ecological niches (Peacock *et al*., 2012). Moreover, the vector competence can vary between the groups, species, sub-species and even between the colonies of the analogous species in the laboratory (Kubi *et al*., 2006). The teneral flies, newly emerged unfed flies, are highly susceptible to trypanosome infection compared to other blood fed flies (Walshe *et al*.,2011).

2.4.5. Development and multiplication of trypanosomes in the vector and the hosts

Glossina get trypanosome infection when taking a blood meal from the mammalian hosts. In the course of cyclical transmission within a tsetse fly, the trypanosome goes through a developmental cycle (Leak, 1999). Within a tsetse fly, the developmental cycle depends on the trypanosome species and lasts from a few days to a few weeks. To survive in tsetse mid-gut, trypanosomes change their metabolism and morphology within the fly (Vickerman *et al*.,1988). Trypanosomes undergo two distinct stages: procylic and metacyclic forms. The parasite experiences significant physiological, biochemical and morphological changes before reaching the final metacylic stage (Leak, 1999).

Trypanosomes are first established in the tsetse midgut and mature in the mouthparts for *Nannomonas* species or in salivary glands for *Trypanozoon* species. When the trypanosomes reach the midgut, they quickly differentiate into procyclic forms and replicate. They migrate towards the proventriculus and then to the mouthparts, in which they differentiate into epimastigotes, colonizing the proboscis or salivary glands, depending on the species of the parasite (Abbeele *et al*., 2000). There, trypanosomes differentiate into metacyclic forms. The metacyclic are infective forms to susceptible hosts through tsetse blood meal (Vickerman *et al.*, 1988b). The development of *Duttonella* species takes place exclusively in the mouthparts. When the tsetse fly is taking the blood meal, the metacyclic forms are inoculated in the mammalian host (Maudlin *et al*., 2004). These metacyclic forms develop and multiply at the site of infection, producing a swelling (chancre) and, subsequently, trypomastigotes are released into the bloodstream via the lymphatic system (Vickerman et al., 1988b). Throughout the developmental life in the mammalian host, the trypanosomes can change their surface antigenic coat with the help of the variable surface glycoprotein (VSG) to escape the immune system of their host (Maudlin *et al*., 2004). The physiology of a host infected by trypanosomes is affected, such as the changes in hormones, nutrient concentration and blood pH (Seed, 2001). It is believed that the infected host gets more frequent tsetse bites (Baylis and Mbwabi, 1995), probably because of the excretion of trypanosome catabolites in the urine, acting like chemical attractants to tsetse (Sawalhy *et al*.,1995).

2.5. Factors affecting the transmission of trypanosomes by *Glossina* vectors

Several factors influence the transmission of trypanosomes (Leak, 1999; Wang *et al*., 2009). Some factors are related to the tsetse fly genome, others to the parasite itself and those resulting from the interactions between the host and the parasite (Figure 2.3) (Roditi and Lehane, 1998; Nayduch and Aksoy, 2007).

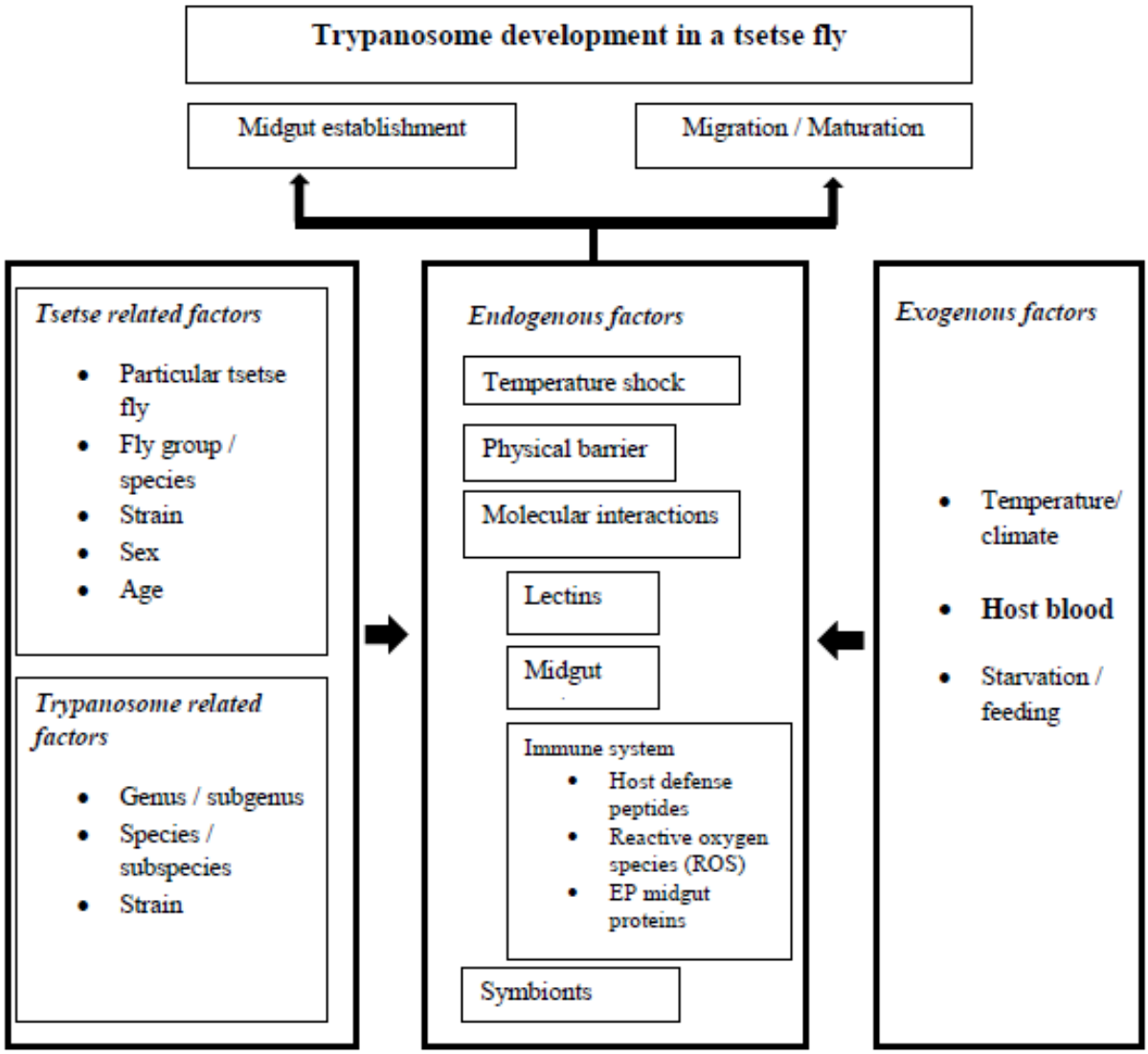


Figure 2.3: Summary of the factors influencing the transmission of trypanosomes

Source: Adapted and reshuffled from Akoda (2009)

2.5.1. Tsetse fly endosymbionts

Endosymbionts are organisms which make a symbiotic association with organisms (Weiss *et al*.,2013). Tsetse flies can carry up to four organisms, which are vertically transmitted, including the obligate *Wigglesworthia*, commensal *Sodalis*, parasitic *Wolbachia*, and the Salivary Gland Hypertrophy Virus (SGHV). The symbionts have an impact on tsetse physiology, including fecundity, vector competence and nutrition (Wang *et al*., 2013). The symbionts-trypanosomes relationship modulates the vector competence of tsetse flies and has

therefore the potential for vector and /or disease control (Weiss *et al*.,2013; Geiger *et al*., 2018; Herren *et al*., 2020). *Sodalis glossinidius* , *Wigglesworthia glossinidia* (Enterobacteriaceae) and *Wolbachia* (Rickettsiaceae) are the bacteria species that reside in the tsetse fly gut (Figures 2.4 and 2.5) as the facultative symbionts (Geiger *et al*., 2018). *Sodalis* can also be found in salivary glands and hemolymph either inside or outside the cells (Balmand *et al.*, 2013). *Sodalis* and *Wigglesworthia* favour trypanosome infection in tsetse fly (Farikou *et al*., 2010; Hamidou *et al*., 2013; Wamwiri *et al*., 2014; Rio *et al*., 2019); and could potentially be used as a target for vector control (Geiger *et al*.,2015; Demirbas-uzel *et al*., 2018; De Vooght *et al*., 2018; Roma *et al*., 2019). *Sodalis* has a possibility of being used for transgenesis in tsetse flies (De Vooght *et al*., 2018) and paratransgenesis (Demirbas-uzel *et al*., 2018). However, the mechanism underlying this vector competence-symbionts interaction is unknown especially for *Sodalis* (Griffith *et al*., 2018). There is a genetic heterogeneity between *Sodalis* populations and differences in genetic structure between *Glossina* species (Geiger *et al*., 2005), disease foci (Farikou *et al*., 2011) and in their relationship with a particular trypanosome species in a fly (Geiger *et al*., 2006). Factors such as geographic location, *Glossina* species, age and sex may however affect the ability of *Sodalis* to act on tsetse competence (Channumsin *et al*., 2018). On another hand in infected arthropods, *Wolbachia* causes disorders in reproduction such as parthenogenesis and especially the cytoplasmic incompatibility (CI) (Alam *et al*., 2011), thus impairing host fertility, lifespan, immunity and development (Kambris *et al.*, 2010; Glaser and Meola, 2010;). *Wolbachia* prevalence in tsetse flies of the morsitans group usually ranges between 9.5 and 100% (Doudoumis *et al*., 2012a; Doudoumis *et al*., 2013). *Wolbachia* strains are genetically diverse within tsetse populations but are closely interrelated (Cheng *et al*., 2000; Doudoumis *et al*., 2012b).

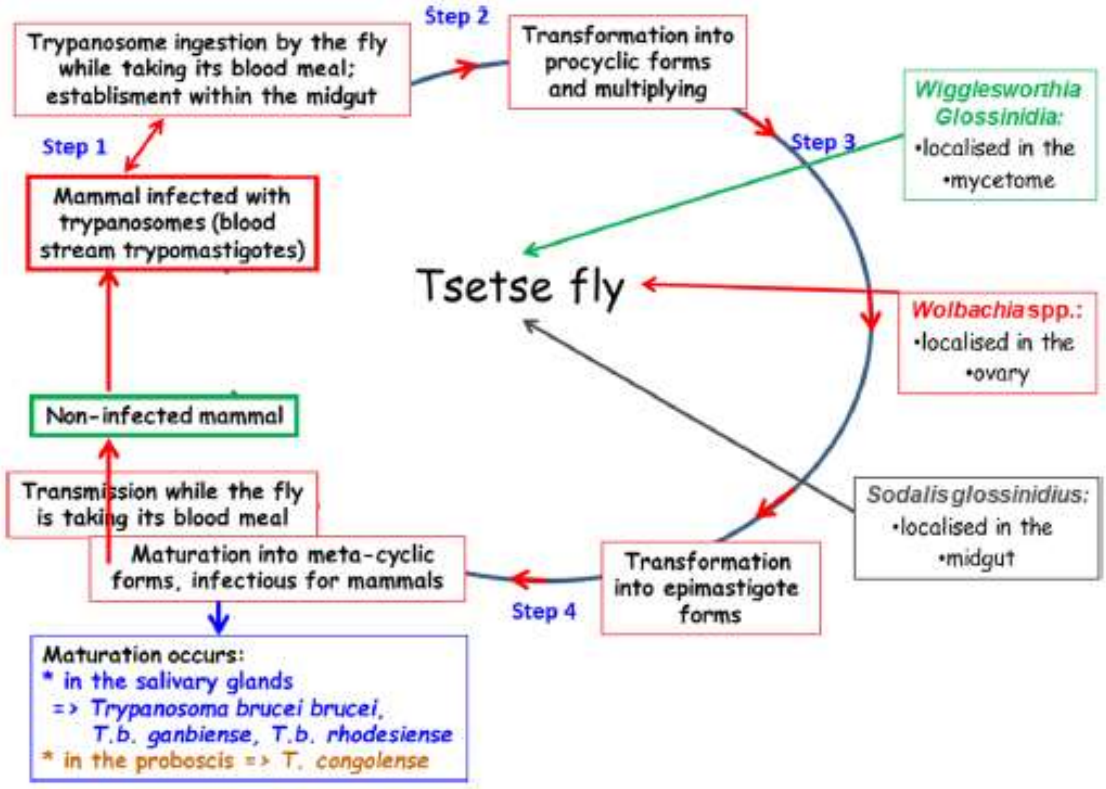


Figure 2.4: Trypanosome life cycle and three symbionts into the Tsetse fly

Adapted from Geiger *et al*., (2018).

Spiroplasma, another symbiont, has been shown to decrease vector competence in tsetse flies (Schneider *et al*., 2019), and It has been found in different tsetse fly species from various African countries (Doudoumis *et al*., 2017). The Salivary Gland Hypertrophy Virus (SGHV, Hytrosaviridae family) is an insect virus colonising the salivary glands (Fig 2.4) (Lietze *et al*., 2011). Although some other tsetse species are susceptible, the virus is common in *Glossina pallidipes* thus the name of *Glossina pallidipes* Salivary Gland Hypertrophy Virus (GpSGHV) (Kariithi *et a*l., 2017). It is important in that it decimates tsetse colonies, hence hampering the production of sterile male tsetse, which are used in the sterile male technique for control of tsetse-transmitted trypanosomes. Its use as a biological control agent for tsetse is not yet clear (Demirbas-uzel *et al*., 2018). The SGHV replicates in the salivary gland, inducing the hypertrophy of the later gland and thus causing the observed reproductive malfunction (Kariithi *et al*., 2016; Kariithi *et al*., 2017). In *Glossina pallidipes*, the establishment of trypanosomes in

salivary glands was linked to the symptoms of the salivary gland hypertrophy (Peacock *et al*., 2012). The prevalence of SGHV in field populations is usually low (0.2%–5%) and SGHV infected flies present reduced fertility and fecundity (Geiger *et al*., 2018). The infection with the virus in salivary glands increases the vulnerability of the organ to trypanosome infection due to the decreased immunity (Kariithi *et al*., 2016; Orlov *et al*., 2018). SGHV prevalence in field tsetse populations differs according to locations and tsetse fly species (Alam *et al*., 2012; Malele *et al*., 2013; Wang *et al*., 2013).

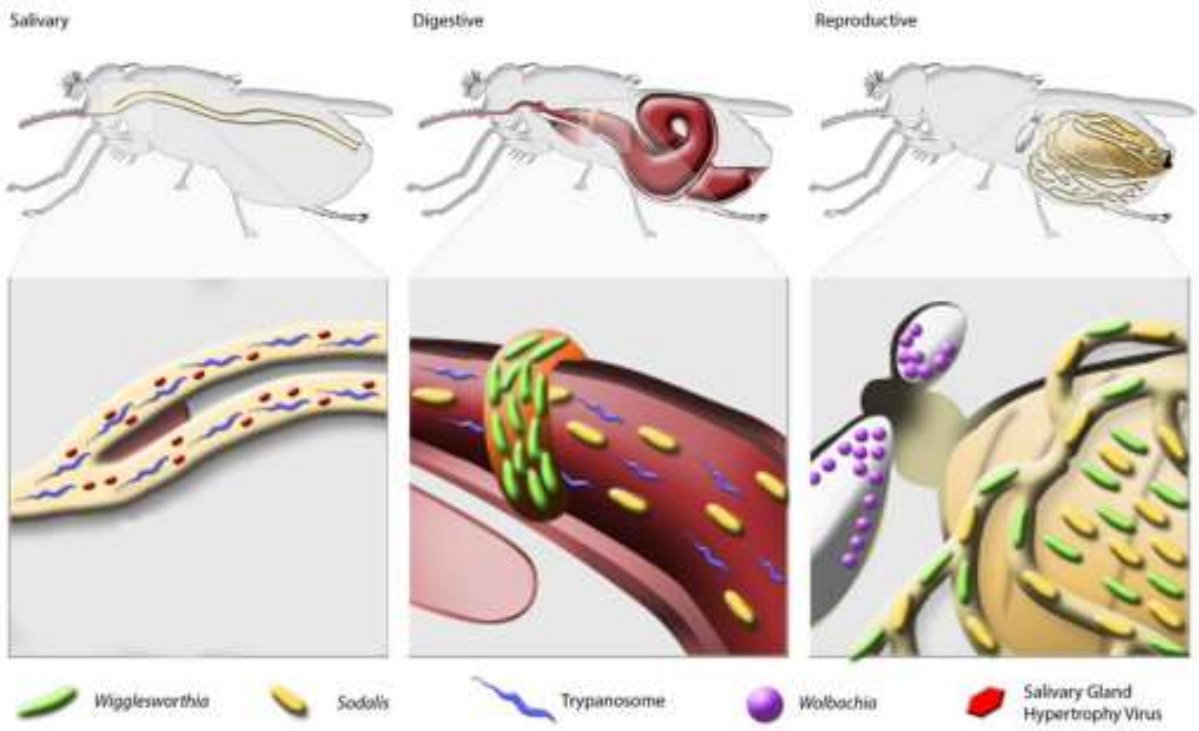


Figure 2.5: Localization of symbionts and SGHV in tsetse

Adapted from Wang *et al*., (2013).

2.5.2. Tsetse blood meal sources and evolution of their identification

The transmission of trypanosomes involves multiple host species and the knowledge of the individual contribution of each host species is required to better understand the transmission mechanisms (Auty *et al*., 2016). Tsetse flies are pure hematophagous flies (Leak, 1999). Their distribution is driven by the availability of suitable hosts in an area, which determines their

dispersal (Chikowore *et al*., 2017), especially the savannah group (Van den Bossche, 2001), (Munang'andu *et al*., 2012). The host choice and tsetse fly preference depend on the available fauna. However, some hosts are preferred (such as buffalo, warthog and bushbuck) while others are rarely fed on (such as zebra), regardless of their abundance in an area (Muzari *et al*., 2010; Auty *et al*., 2016; Rodrigues *et al*., 2019). The ability of tsetse flies to maintain and transmit the trypanosome infection and their feeding patterns play a substantial role in the epidemiology of AT in wilderness areas (Bauer *et al*., 1995). Tracking the feeding patterns of tsetse flies and their host choice is a key element in understanding the vector-host interactions, and subsequently predicting the reservoirs of trypanosomes in an area. The reservoirs are potentially involved in AT transmission (Ngonyoka *et al*., 2017; Rodrigues *et al*., 2019)**.** Detecting and identifying the blood meal sources in tsetse flies is essential to understanding the transmission dynamics and /or the epidemiology of tsetse–transmitted trypanosomosis (Isaac *et al*., 2016). Therefore, Tsetse fly blood meal identification is a valuable approach to estimate the risk of parasite transmission between domestic and wild hosts. This provides a baseline tool for improving and /or designing effective control strategies (Bauer *et al*., 1995).

The host DNA in a blood meal is stable within the insect for several days and allows reliable host identification by PCR (Boakye *et al*., 1999). Several molecular techniques are used to determine the blood meal sources in tsetse flies, using the DNA barcoding and the mitochondrial gene sequence (Hebert *et al*., 2003). Both generic and specific markers are designed, and primers were designed to identify the cytochrome oxidase I (COI) gene (Ivanova *et al*., 2006). PCR reactions targeting the vertebrate mitochondrial cytochrome b (Cytb) sequences were thereafter used to identify the blood meal sources (Muturi *et al*., 2011; Mej *et al*. 2012; Omondi *et al*., 2015) and Vertebrate 16S (Omondi *et al*., 2015). New technologies are evolving to ease the identification of blood meal sources. The real-time high-resolution melting (HRM) analysis-based approach was established to identify and differentiate rapidly

the domestic from wild vertebrate species in East Africa Bitanyi *et al*., (2011). A rapid on-site DNA meta-barcoding of environmental samples such as MinION sequencing was developed to identify and differentiate the sequence source from different hosts (Ji *et al*., 2013). Recent technologies include the DNA meta-barcoding approach (Gaithuma *et al*., 2020) and a rapid, low-cost approach that uses three genes: COI, cytochrome b, and 16S ribosomal RNA, based on their distinctive high-resolution melting profiles of the PCR products (Ouso *et al*., 2020).

2.6. Wildlife – livestock- human interface and trypanosome transmission

The insufficiency of basic data from the field on the interactions at the livestock/wildlife/environment interface relative to the diseases is still a problem in many settings (Wambwa, 2002). Notwithstanding recent interest in animals as sources of emerging diseases in humans, the importance and role of livestock – wildlife interfaces in disease ecology has been largely overlooked *(*Wiethoelter *et al*., 2015). The pathogens'ability to infect the wildlife hosts is an important risk factor for the emergence of human and livestock diseases (Cleaveland *et al*., 2001). The transmission of animal diseases may be bidirectional at these interfaces, with indigenous wildlife infections crossing over into livestock or diseases traditionally seen in livestock entering wildlife populations (Osofsky, 2005). The most commonly reported ungulate species in wildlife/livestock disease outbreaks are primarily from the family Bovidae, with the buffalo and bovine being the most prominent (Cleaveland *et al*., 2001). Given their close phylogenetic connection to the ancestral (wild) cattle, this is perhaps not surprising (Osofsky, 2005). Additionally, the emergence of the disease in humans has been linked to pathogen maintenance in wildlife populations and spill over to livestock (Morse *et al*., 2012). As a result, there is a growing interest in using knowledge synthesis methods to track down and quantify the zoonotic origins of diseases (Jones *et al*., 2013). List A of the Office International des Épizooties (OIE) classifies the most important diseases at the interface.

The wildlife-livestock interface is a source of many significant diseases between wild and domestic animals (Wambwa, 2002). African trypanosomosis is an example of the most significant diseases at the interface. Trypanosomiasis, a natural disease of wildlife, has crossed over to domestic animals due to the infringement of people into areas usually inhabited by game animals (Van den Bossche *et al*., 2010). Trypanosomes are maintained in a variety of wildlife species including elephants, rhinos, buffalo, warthog and hippo (Auty *et al*., 2012). Tolerance has evolved in game animals over millions of years of co-evolution with the parasite. Because of their susceptibility to trypanosomosis, the vast majority of cattle, sheep, and goats succumb to acute, subacute, or chronic infections, resulting in significant production losses (Wiethoelter *et al*., 2015).

CHAPTER THREE: SPATIAL DISTRIBUTION, ABUNDANCE, SPECIES COMPOSITION AND SEASONAL VARIATIONS OF *GLOSSIN*A AT THE HUMAN-WILDLIFE-LIVESTOCK INTERFACE OF AKAGERA NATIONAL PARK , RWANDA

3.1. Introduction

Glossina (tsetse flies) are biological vectors of trypanosomes, which cause trypanosomiasis in both humans and animals (Jordan, 1976; Maudlin *et al*., 2004; Büscher *et al*., 2017; Cayla *et al*., 2019). The distribution patterns of tsetse species in an area are a key element to better understand the transmission dynamics of trypanosomosis (Dicko *et al*., 2015; Ngonyoka *et al*., 2017). This information is a requirement for strategic, risk-based control of the vectors and the disease (Diall *et al*., 2017b; Shaw *et al*., 2017). The presence and abundance of *Glossina* are associated with the environment (Chikowore *et al*., 2017). The habitat, land use, and ecological settings are determinants of tsetse fly distribution and therefore the disease transmission (Cecchi *et al*., 2008; Munang'andu *et al*., 2012). On top of this, the availability of hosts and their distribution in an area determine the dispersal of tsetse flies, especially the savannah group (Chikowore *et al*., 2017).

Environmental changes play a role in changing disease transmission (Van den Bossche *et al*., 2010). Habitat fragmentation and human activities reduce considerably the distribution and abundance of savannah species, leading them to be confined to protected areas where they find a conducive environment and hosts to feed on (Ducheyne *et al*., 2009; Lord *et al*., 2018; de Gier *et al*., 2020). However, this situation results in an increased challenge of tsetse bites on livestock and humans around the protected infested area (Van den Bossche *et al*., 2010). This chapter determined the current spatial distribution, abundance, diversity, and seasonal variations of *Glossina* species in Akagera region of Rwanda.

3.2. General study area

The study was carried out in the Eastern Province of Rwanda (coloured pink in Figure 3.1). The province is the largest in the country (9,813Km2) with more than one-third of the total surface (26,338 km2) of the country (MINAGRI, 2015). The province is located in the savannah lowlands characterized by a depressed relief, and shares borders with Tanzania in the East and Uganda in the North, with altitudes ranging between 1 200 m and 1 700 m. The province is home to the famous Akagera National Park at its border with Tanzania, where *Glossina* are still reported. The province has seven districts, however, three districts, i.e. Kayonza (1,937Km2), Gatsibo (1,582 Km2) and Nyagatare (1,741 Km2), were selected because of their proximity to the park and being adjacent to protected game reserves in Tanzania. Adjacent areas of the fourth district (Kirehe 1,192 Km2) were included for data collection. The interface area outside the Akagera NP is dominated almost entirely by cattle farms. The rains follow a bimodal pattern with an average rainfall of about 1000 mm per annum, even though it is less regular compared to other regions of the country, leading to frequent dry spells. At the interface, the vegetation cover consists of grassland, woodland, and often bushland closer to the park where the study was conducted. The temperature varies between 19°C and 29°C. The long dry season spans the months of June, July up to August while the long wet season comprises the month of March, April, and May (REMA, 2011). Because of the availability of grazing land (Beyi & Dahl, 2016), the area is dedicated to livestock production. Cattle are the dominant domestic animals kept in the area, the indigenous Ankole breed is predominant in the districts of Kayonza and Gatsibo, while crossbreed Ankole x Friesians is the main breed kept in Nyagatare. Genetic improvement is increasing through cross-breeding with bulls and artificial insemination. Other small livestock species such as goats are also found. The husbandry system is open grazing on individual farms often fenced by *Euphorbia tirucalli* or on the open lands along the park boundary.

Cattle farms are concentrated along the interface area with Akagera NP and are consequently exposed to tsetse fly challenge and trypanosomiasis.

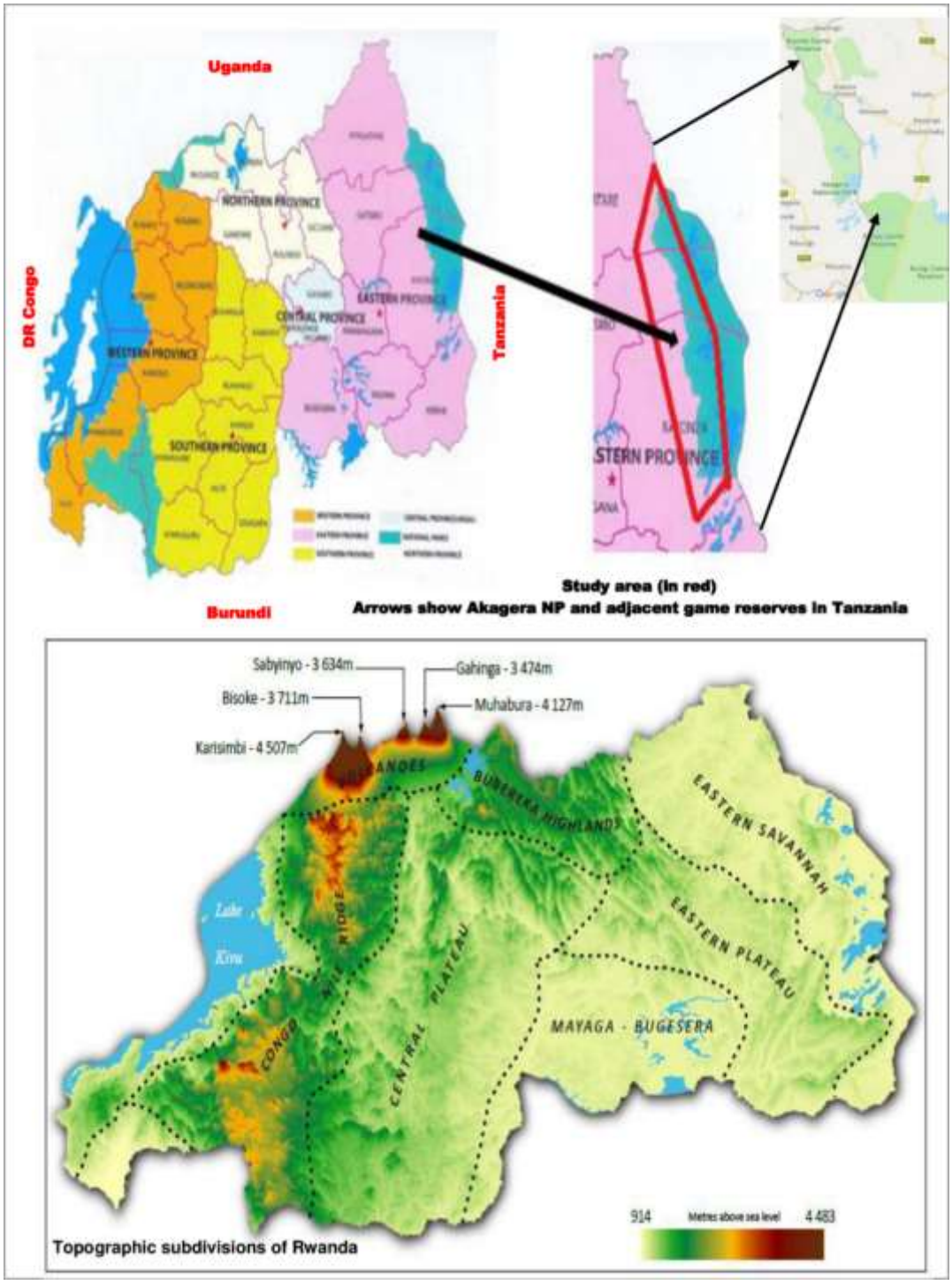


Figure 3. 1: Overview of the study area

Adapted and reshuffled from REMA (2011) and https://hongwkim.files.wordpress.com/2016/09/map.jpg (Accessed on 10th/10/2021)

Established as a national park in 1934, the park draws its name from the Akagera River that runs along the international border with the United Republic of Tanzania (Macpherson, 2019). Akagera NP is the only protected savannah region in Rwanda and therefore the only shelter for savannah-adapted species of tsetse. Akagera NP has a sub-arid savannah habitat, subdivided into three ecosystems: Swamp (wetland and wetland fringes), mountain, and savannah. There are different vegetation types within the above ecosystems mainly grasslands, bushed grasslands, wooded grasslands, woodland (mainly Acacia), dry and humid forests (Viljoen, 2010; Macpherson, 2019). The wetlands are permanently flooded areas with marshlands and a complex system of lakes fed by the Akagera River. The wetlands make 30% of the park surface and the terrestrial part makes the remaining 70%. The altitude is characteristic of the eastern region; however, it has a mountainous north to the south-central ridge along the western boundary that can reach nearly 1750m (Macpherson, 2013).

This specific entomological study focused on Akagera NP and extended to its surrounding areas located in three neighbouring districts (i.e. Kayonza, Gatsibo, and Nyagatare) as shown in Figure 3.2.below.

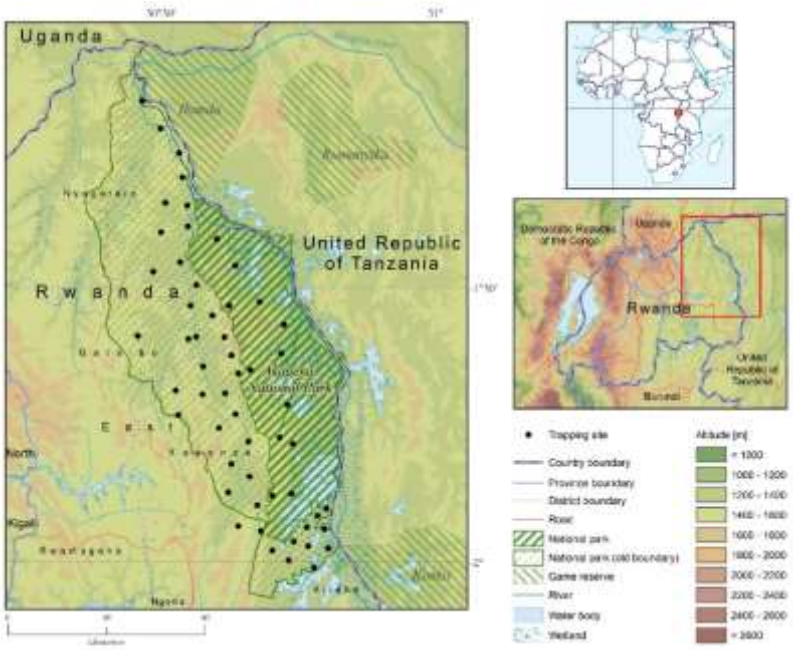


Figure 3.2: Map showing sampling sites (indicated by black dots) for tsetse flies

3.3. Ethical consideration

This research followed the protocols for care and use of animals. The ethical permission was approved by the ethics committees of the Faculty of Veterinary Medicine - University of Nairobi (REF: FVM BAUEC/2019/246) and the College of Agriculture and Veterinary Medicine – University of Rwanda (REF: 030/19/DRI).

3.4. Materials and methods

3.4.1. Entomological survey

A longitudinal stratified sampling was carried out from May 2018 to June 2019 to determine seasonal variations in tsetse fly populations as described by Leak and Vreysen (2008). Inside the park, ecosystems were used for the stratification of sampling, whereas the districts were used at the interface area outside the park. In each of three park ecosystems (i.e. swamps, savannah and mountain), locations were randomly selected, nonetheless, the most suitable sites for tsetse were purposefully chosen. At the interface, in addition to stratification using the districts, the same approach was used to select the sites. The information on the potential *Glossina*–habitat suitability such as near human residence, the density, and distribution of cattle population, communal watering points, grazing areas, overnight cattle collection, and the farmers' knowledge on the existence of flies was taken into account. Fifty-five (55) sites (12 inside the park and 43 along the interface) were therefore selected for the study. To determine the effect of the distance to the park at the interface, sites located less than 3 km from the park border were considered as close, while those located more than 3km were considered as distant. Biconical traps (Challier, 1973) supplied by Vestergaard Frandsen (Lausanne, Switzerland), were used in the study. These traps are widely used and are efficient in sampling and population monitoring (Challier and Eyraud,1977). Traps were deployed for six consecutive days of each month (3months in the rainy season and 3 months in the dry season) and emptied every 48

hours (Nnko *et al*., 2017). Two traps were deployed in the same site at a distance of 200m (Ngonyoka *et al*., 2017), and the fly catches of the two traps were later combined to represent a site. The number of traps deployed in an area was determined by the area size (Mulandane *et al*., 2020). The trap effectiveness was improved by using a 3 weeks-old cow urine and acetone as baits, kept in plastic bottles with an opening dispensing the odour. Grease was applied at the bottom of the trap support to prevent ants from climbing to the trap. Each trapping site was georeferenced by a global positioning system device (Garmin Ltd, Kansas, USA) to generate a map later.

Figure 3.3: A biconical trap deployed with attractants in the study area

Flies were morphologically identified using a stereomicroscope (Opta Tech SK392, Poland) as described in the FAO training manuals (Pollock, 1982b); (Leak et al., 2008). For each site, records of number, species, sex, and other biting flies were taken. Flies with damaged or lost body parts were excluded.

3.4.2. Data analysis

Data was entered into the SPSS software (SPSS Inc., IL, USA). The average number of flies caught per trap per day (FTD) referred to as the apparent density (AD), was obtained after dividing the total number of flies caught in a trap by the number of days the trap has been in place. The term 'abundance' refers to the apparent density to express the average number of flies available in a specific site (Ngonyoka et al., 2017). Pearson chi-square ($\chi 2$) was used to determine the variations between the variables such as the area, season, district, species, sex and localities. The P-value of less than 0.05, significant at 95% of confidence interval was taken into account. Parametrical tests (t-test and ANOVA) were used to compare whether the apparent density differed between factors/predictors such as area, season, ecosystem, month, district and locality. Logistic regression was used to determine the association between the occurrence of tsetse flies and the associated predictors (cited above and explained in the design), followed by the determination of odds ratios (OR) for each predictor.

3.5. Results

A total of thirty-nine thousands and five hundreds sixteen (39,516) tsetse flies were collected, of which 29,019 (73.4%) and 10,497 (26.6%) were from Akagera NP and the interface area, respectively (Figure 4.1.). The difference in the density of tsetse flies between the two areas was statistically significant (p=0.000).Two species of *Glossina*, namely *G. pallidipes* (n=29,121; 7.4 overall FTD) and *G. morsitans centralis* (n=10.395; 2.6 overall FTD) were identified, of which the females accounted for 61.3% (n=24,225), while 38.7% (n=15,291) were males (Table 4.1). The difference in the density of the two species was statistically significant (p=0.000). Both of the identified species belong to the savannah (*morsitans*) group.

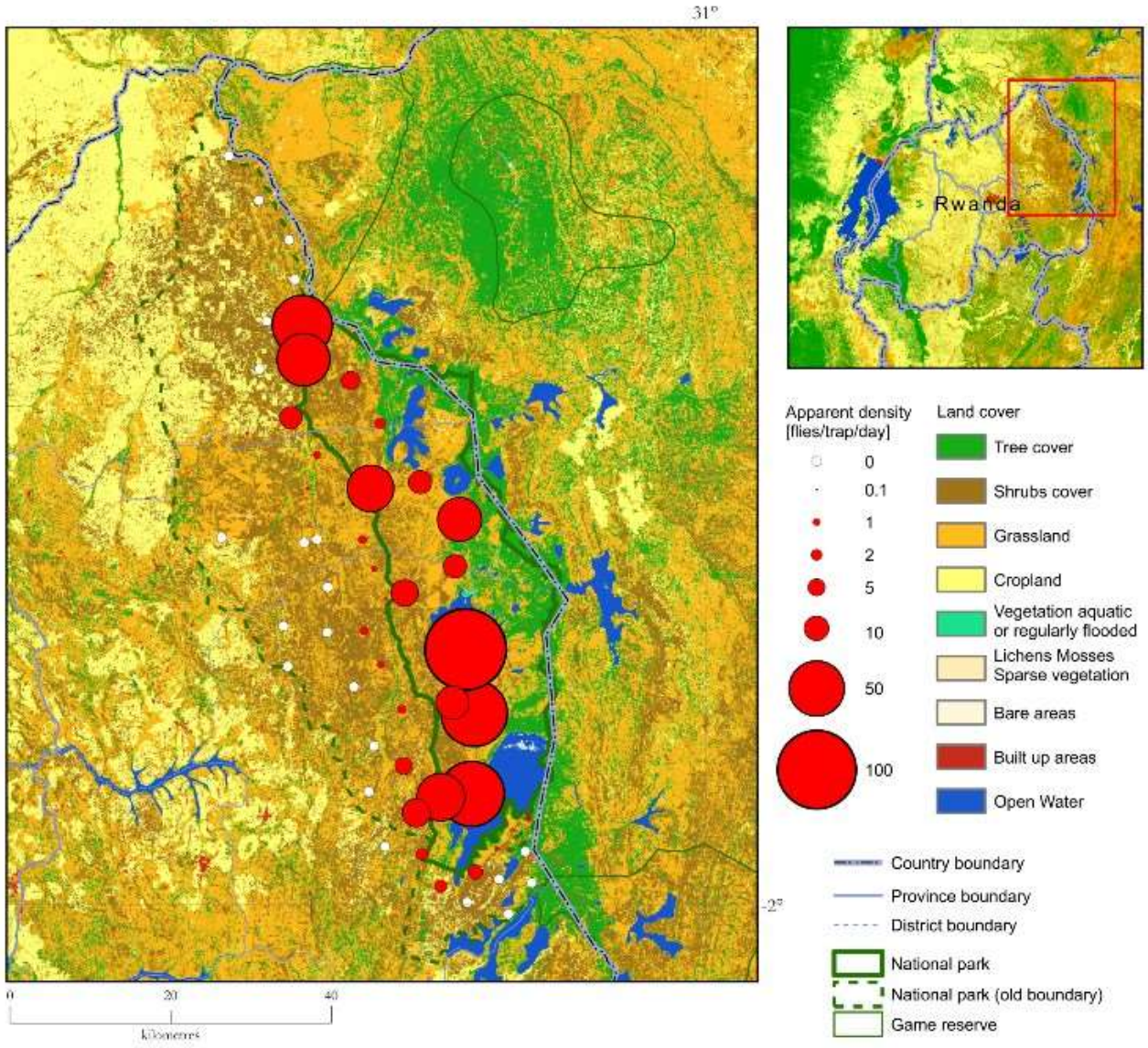


Figure 3 4: Apparent density with land cover (Seasons and species combined)

As the map shows, high catches were mainly found inside the park and a narrow band of Nyagatare District. High tsetse densities were associated with swampy vegetation.

3.5.1. Seasonal variation in distribution of tsetse flies

The flies were more abundant during the wet season (n=31,295; 15.8 FTD) compared to the dry season (n=8,221; 4.2 FTD). Figures 4.1 and 4.2 show the seasonal distribution in densities and species with details of the respective months.

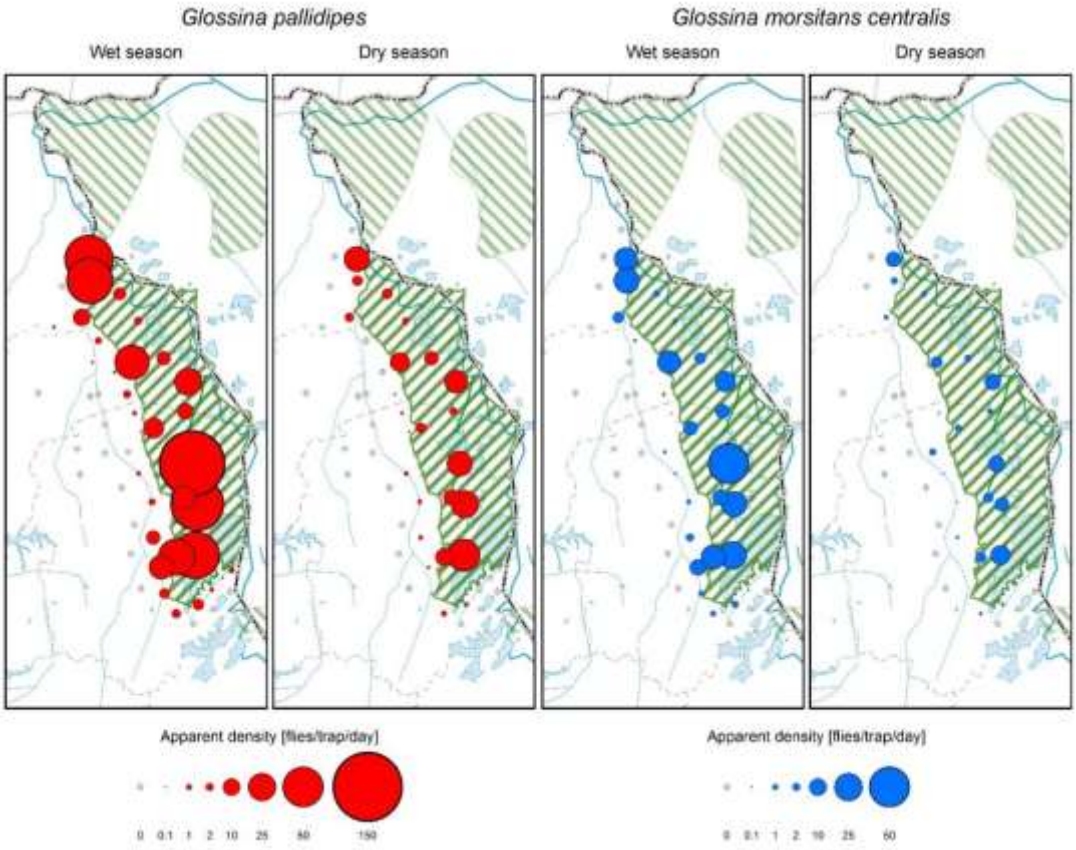


Figure 3.5: Seasonal occurrence of *G. pallidipes* and *G. morsitans centralis* in and around Akagera National Park. The area of the circles is directly proportional to the apparent density.

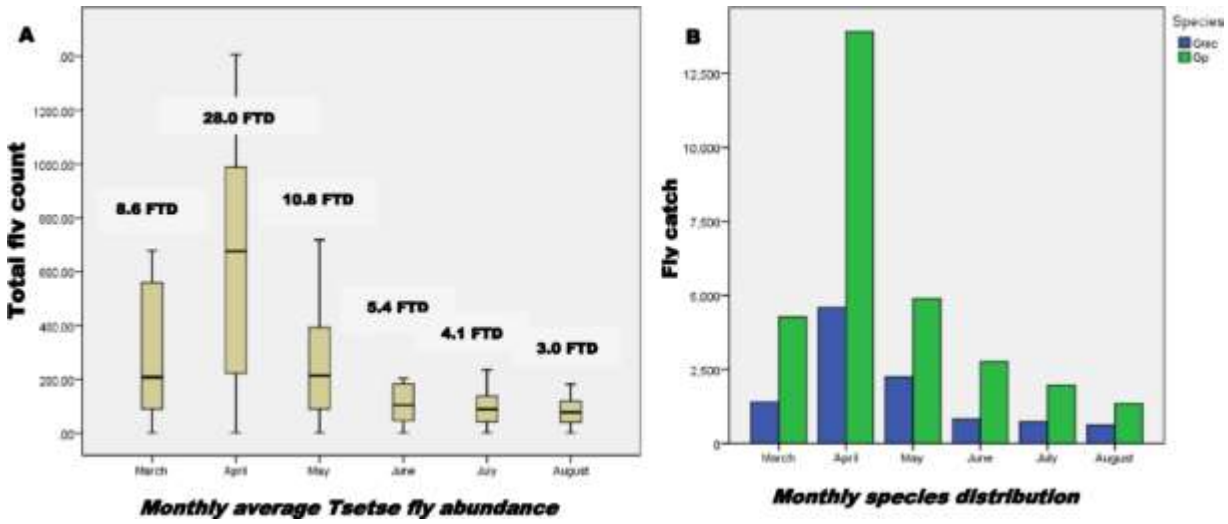
Variations of flies in season and areas by species and sex are detailed in Table 3.1.

Table 3. 1: Distribution of *Glossina* by species and sex across study areas and seasons

Variable	*Glossina morsitans centralis*			*Glossina pallidipes*			Total	Overall FTD	p-value
	M	F	Σ	M	F	Σ			
Dry season	786	1380	2166	2288	3767	6055	8221	4.2	
Wet season	3250	4979	8229	8967	14099	23066	31295	15.8	< 0.000
Interface	894	1485	2379	3114	5004	8118	10497	3.4	
Akagera NP	3142	4874	8016	8141	12862	21003	29019	33.6	< 0.000

M= Male F=Female Σ= Total FTD= Flies/Trap/Day

Glossina were more abundant in Akagera NP than it was at the interface and the wet season was associated with high fly catches compared to the dry season. There was a variation in sex composition across seasons and study areas (Figure 3.6). The female flies were more abundant than the males. The variations in density were observed within collection months (Figure 3.6)



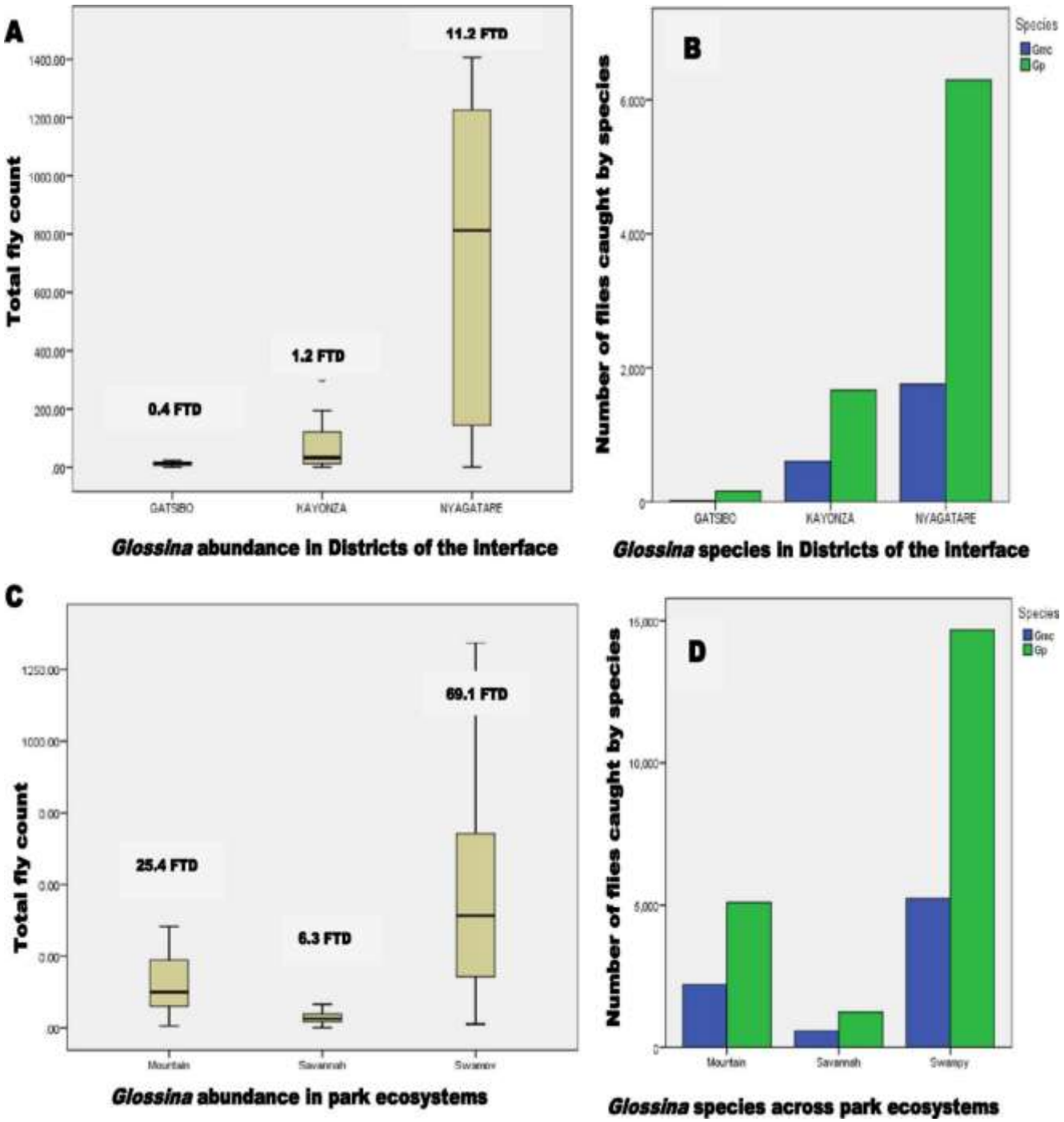
Gmc = Glossina morsitans centralis; *Gp= Glossina pallidipes;* FTD= Fly per Trap per Day

Figure 3 6: Monthly fly abundance (A) and species composition (B)

The difference is highly significant in April (p=0.00, 95% CI) compared to other months.

3.5.2. Distribution of tsetse flies across districts and park ecosystems

Inside the park, the fly distribution differed between ecosystems, where a higher fly catch was recorded from the swamps area (n=19,904; 69.1 FTD) compared to the mountains (n=7,304; 25.4 FTD) and the savannah (n=1,811; 6.3 FTD). The difference in fly catch between ecosystems was statistically significant (p=0.000). Variations in the fly catch were observed outside the park, within the three districts surrounding the park. Tsetse catch was n=8,053 (11.2 FTD); n=2,266 (1.2 FTD); n=178 (0.4 FTD) respectively for Nyagatare, Kayonza and Gatsibo (Figure 3.7.). The difference in catch was statistically significant p=0.000 when Kayonza and Gatsibo were compared to Nyagatare, but not significant between Gatsibo and Kayonza (p=0.107). The effect of the distance from the park to tsetse density is shown in Figure 3.8.



Gmc = *Glossina morsitans centralis*; Gp= *Glossina pallidipes;* FTD=Fly per Trap per Day.
(A, B) are Districts of the interface and (C, D) are ecosystems of Akagera NP.

Figure 3.7: The abundance and distribution of Glossina within the study strata.

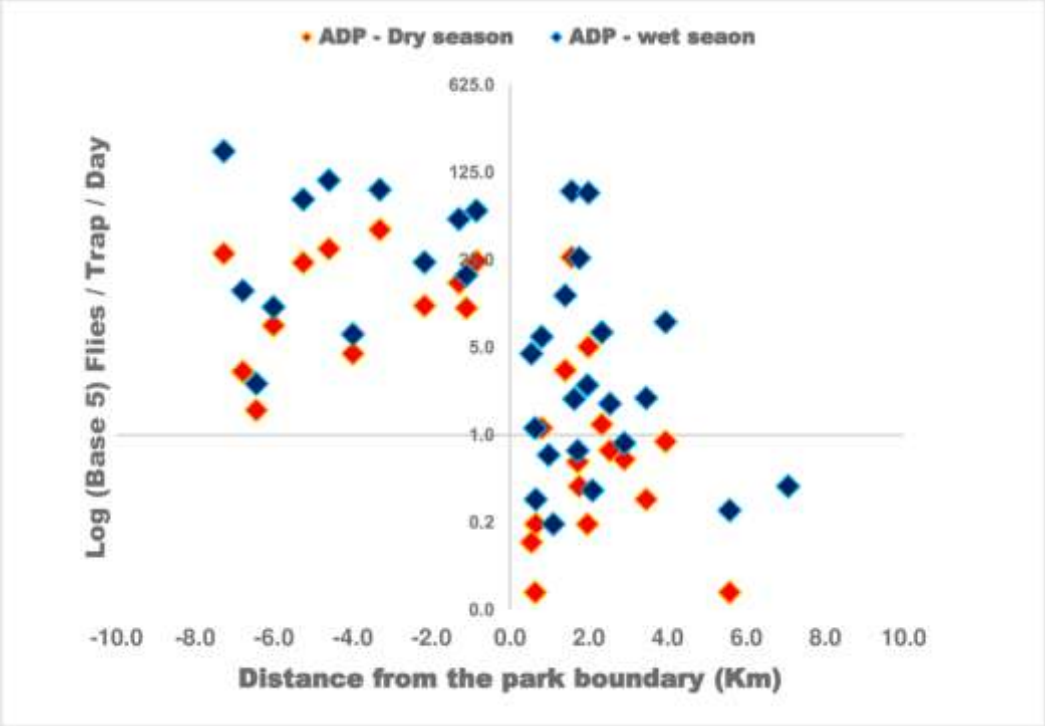


Figure 3.8: Tsetse density with reference to the distance from the park boundary

In this figure, the negative distance values indicate the sites inside Akagera park, whereas positive values show the sites at the interface area. Outside the park, the furthest tsetse fly was captured 7 Km from the park boundary and it was during the wet season.

3.5.3. Maximum likelihood analysis for the existence and co-existence of *G. pallidipes* and *G. morsitans centralis*

More flies, for both *G. pallidipes* and *G. morsitans* were caught inside the park (21,003 and 8016, respectively) compared to the interface (8118 and 2379, respectively). The chance of co-existing between the two species reduced outside the protected area (0.021 times). The probability to catch *G. morsitans centralis* was 1.412 times more in the dry season than *G. pallidipes* (0.899 times). The co-existence of both species increased 2.860 times more in the dry season than it was during the wet season (Table 3.2.). For months, the comparison reference was made to August. *G. pallidipes* were more collected in June (1.628 times), April (1.512 times), and March (1.416 times).

Table 3. 2: Multivariate logistic regression analysis for occurrence and co-occurrence of *Glossina pallidipes* and *G. morsitans centralis* according to the study area and district, sampling season, month and ecosystem.

Predictor	*G. pallidipes*			*G. morsitans centralis*			Coexistence		
	Fly catch	OR (95% CI)	P	Fly catch	OR (95% CI)	P	Fly catch	OR (95% CI)	P
Study area									
Akagera N.P.	21003	1.00		8016	1.00		29019	1.00	
Interface	8118	1.275 (1.216-1.336)	0.000	2379	0.218 (0.113-0.423)	0.000	10497	0.021 (0.007-0.065)	0.000
Sampling season									
Dry	6055	0.899 (0.858-0.942)	0.000	2166	1.412 (1.263-1.578)	0.000	8221	2.860 (1.781-4.593)	0.000
Wet	23066	1.00		8229	1.00		31295	1.00	
Sampling month									
March	4275	1.416 (1.264-1.586)	0.000	1399	0.648 (0.469-0.897)	0.009	5674	0.312 (0.163-0.596)	0.000
April	13903	1.512 (1.365-1.675)	0.000	4589	0.547 (0.432-0.691)	0.000	18492	0.002 (0.001-0.004)	0.000
May	4888	1.093 (0.980-1.219)	0.111	2241	0.670 (0.515-0.872)	0.003	7129	0.023 (0.011-o.48)	0.000
June	2763	1.628 (1.437-1.843)	0.000	812	0.507 (0.375-0.685)	0.000	3575	0.073 (0.034-0.153)	0.000
July	1955	1.283 (1.128-1.459)	0.000	733	0.664 (0.486-0.907)	0.010	2688	0.070 (0.27-0.180)	0.000
August	1337	1.00		621	1.00		1958	1.00	
Ecosystem									
Mountain	5097	0.823 (0.775-0.873)	0.000	2207	1.215 (1.145-1.289)	0.000	7304	1.000 (0.00 -.[b])	1.000
Savannah	1231	0.756 (0.582-0.839)	0.000	580	1.322 (1.192-1.467)	0.000	1811	ND	ND
Swampy	14675	1.00		5229	1.00		19904	1.00	
District									
Kayonza	1663	2.335 (1.447-3.769)	0.001	603	0.428 (0.265-0.691)	0.001	2266	99.360 (63.481-155.517)	0.000
Gatsibo	159	0.770 (0.691-0.857)	0.000	19	1.299 (1.167-1.446)	0.000	178	13.090 (9.096-18.836)	0.000
Nyagatare	6296	1.00		1757	1.00		8053	1.00	

OR=Odds Ratio; CI=Confidence Interval, P= P-value; ND= Not Determined; Reference categories: Akagera NP for the area, Wet for seasons, August for months, Swampy for ecosystems, Nyagatare for districts and Yes for co-existence

Inside the park ecosystems, *G. pallidipes* occurred less in the mountain (0.823 times less) and savannah ecosystems (0.756 times less) than it was in swampy. However, *G. morsitans centralis* increased 1.215 times more in mountains and 1.322 times more in the savannah. The co-existence of both species was the same in mountains and swamps. With reference to

Nyagatare District, *G. pallidipes* occurred 2.335 times more in Kayonza and 0.770 times less in Gatsibo. The occurrence of *G. morsitans centralis* was 0.428 times less in Kayonza and 1.299 times more in Gatsibo. The co-existence of both species increased 13.090 times in Gatsibo and enormously in Kayonza (99.360 times) compared to the situation in Nyagatare.

3.6. Discussion

Results from this study clearly show that the Akagera NP remains a favorable refuge for tsetse populations in the area, and therefore contributing to the constant risk of trypanosomosis transmission in the neighbourhood. Tsetse habitat in the eastern region of Rwanda has reduced greatly due to the demographic pressure. The remaining savannah habitat seems to favour *G. pallidipes*, which tends to dominate other savannah species in the region (Ciosi *et al.*, 2014; Saarman *et al*. 2019 ; Ngari *et al*., 2020). The high tsetse abundance in Akagera NP was found all year round with seasonal variations from wet to dry season. The abundance was much associated with Acacia swampy ecosystem as demonstrated in Tanzania by Ngonyoka (Ngonyoka *et al.*, 2017). The higher abundance of tsetse flies during the rainy season is explained by their behavioural activities. Tsetse flies customarily aggregate in dense vegetation in the dry season and disperse remarkably into areas that are more open during the rainy season (Camberlin and Wairoto, 1997; Leak *et al.*, 2008). This behaviour affects the fly catches (Nnko *et al*., 2017), one of the reasons why traps deployed during the sunny period (June, July and August) caught fewer flies than those deployed in the wet season (March, April and May) according to the local climatic conditions. Nevertheless, the combined effects of hosts, vegetation, climate, and human settlements affect the abundance and distribution of flies.

Among the three previously reported species (G. *pallidipes, G. morsitans centralis* and *G. brevipalpis*), this study only found two species, G. *pallidipes* and *G. morsitans centralis*. Their distribution is fairly homogenous for both species across all the vegetation types. *Glossina pallidipes* were numerically dominant in all surveyed localities. It had densities three times

higher than *G. morsitans centralis*. Most traps that captured zero *G. morsitans centralis* also did not get any *G. pallidipes.* There was a relatively higher population of *G. morsitans centralis* in this study compared to earlier findings by Mihok *et al*., (1992). In that study, *Glossina pallidipes* were 82.6% whereas *G. morsitans centralis* were 15.2%. However, the later work was area-limited and did not include Akagera NP, so comparisons may be misleading. In particular, the difference could be linked to the adjacent game reserve in Tanzania, which was reported to be infested with *G. morsitans* (Daffa *et al*., 2013), though the traps in these areas did not catch any tsetse fly during this study.

This study did not find any *G. brevipalpis* though it was previously reported. A study by Mihok *et al*., (1992) found 2.2% of *G. brevipalpis* in a total fly catch of 312,801 tsetse flies. As stated above, this work was undertaken a few kilometres away from this study area but did not include Akagera NP. Another monitoring work done in 2013 by Oloo ‘’unpublished report’’ in Akagera NP found only one female *G. brevipalpis* in one site of the swamp ecosystem where traps were deployed. It is a forest-type species, frequently associated with waterside evergreen thickets, and forest islands in savannah habitat. The host preferences are mostly the hippo and the bushpig (Pollock, 1982a). This indicates that its conducive habitat in the Akagera region could be the lakeshore swampy forests of the park where it was not seen during the survey. The species also has both poor responses to traps and odour bait attractants (Leak *et al*., 2008). However, *G. brevipalpis* is in most cases found in few numbers when co-existing with the *Morsitan*s (savannah) group (Auty *et al.*, 2012). The absence of tsetse flies in a trap catch does not necessarily mean their absence in the area (Bouyer *et al*., 2010;Chikowore *et al*., 2017). Many factors are involved and the behaviour of some species differs in response to the trapping strategy.

Higher apparent density was found in Nyagatare district compared to Kayonza and Gatsibo. I attribute this to the feeding patterns and preferences of tsetse flies. Wild hosts are concentrated

in the north of the park (Macpherson, 2013; Macpherson, 2019), which is entirely located in Nyagatare District. In terms of livestock density, high cattle densities in the country are found in Nyagatare (NISR, 2020).

Tsetse data collection was solely based on stationary traps. No fly rounds were carried out, and it is known that certain savannah species (i.e. *G. morsitans*) are more attracted by moving targets. We used baited traps only, which may affect the abundance of some species such as the forest type associated with waterside evergreen thickets and the swamp habitat. These species (i.e. *G. brevipalpis*) have a poor response to both traps and baits.

3.7. Conclusion and recommendations

1. Tsetse occurrence seems to be limited to the protected Akagera NP and a narrow band around it. The infestation was significantly lower in the buffer area and reduced quickly to zero at a few kilometre distance away from the park border in both seasons.
2. This study did not find any *Glossina brevipalpis* in its normal suitable habitat where *G.pallidipes* was outnumbering.

The investigator recommends the following:

1. The use of different entomological methodologies could help to better understand the behaviour of tsetse populations in the study area, as tsetse species respond differently in the field.
2. There is a need to investigate whether the *morsitans* species hamper the survival and the distribution of *G. brevipalpis*, especially when it has a conducive environment, or simply confirm its disappearance from the area.
3. Conducting a nationwide monitoring to have a national level picture of the tsetse and trypanosomosis challenge and confirm the absence of the vector from other areas that were historically or are still potentially at risk.

CHAPTER FOUR: *TRYPANOSOMA* SPECIES, BLOOD MEAL SOURCES AND THE ENDOSYMBIONTS COMMUNITY FOR *GLOSSINA* AT THE HUMAN-WILDLIFE-LIVESTOCK INTERFACE OF AKAGERA NATIONAL PARK IN RWANDA

4.1. Introduction

African trypanosomes are linked with tsetse-infested game reserves and parks, which shelter various wild species that maintain the parasites. Akagera NP is one example of such an area in Rwanda. A number of wild animals do not suffer from trypanosomosis as they have an inherent capability of surviving the *Trypanosoma* infections and therefore some serve as the disease reservoirs (Baker *et al*., 1967**;** Connor, 1994**;** Reichard, 2002**;** Mbaya *et al*.,2009)**.** Uncovering the diversity of trypanosomes in tsetse vectors is crucial to understanding the dynamics between trypanosome species, their hosts, and tsetse flies (Garcia *et al*., 2018).

The tsetse blood meal source is dependent on the availability of hosts in an area and the preferences of the tsetse flies for the available hosts (Rodrigues *et al*., 2019). Tsetse fly blood meal analysis is a valuable approach to estimate the risk of parasite transmission between domestic and wild hosts at the wildlife-human-livestock interface (Auty *et al*., 2016). Tracking the feeding patterns of tsetse flies and their host choice is a key element in understanding the vector-host interactions, and subsequently predicting the reservoirs of trypanosomes in an area (Odeniran *et al*., 2019).

The symbionts-trypanosomes relationship modulates the vector competence of tsetse flies and has therefore the potential for vector and /or disease control (Weiss *et al*., 2013; Geiger *et al*., 2018; Herren *et al*., 2020). *Sodalis* is a bacteria in the family Pectobacteriaceae, known as a symbiont that favours trypanosome infection in tsetse fly (Farikou *et al*., 2010; Hamidou *et al*., 2013; Wamwiri *et al*., 2014; Rio *et al*., 2019). *Sodali*s could potentially be used as a target for vector control (Geiger *et al*., 2015; De Vooght *et al*., 2018; Roma *et al*., 2019). On the other hand in infected arthropods, *Wolbachia*, a *Rickettsia* organism in the family Anaplasmataceae,

causes disorders in reproduction such as parthenogenesis and especially cytoplasmic incompatibility (CI) (Alam *et al*., 2011), thus impairing host fertility, lifespan, immunity and development (Glaser & Meola, 2010; Kambris *et al*., 2010). *Spiroplasma,* which is a Mollicute, a group of small bacteria without cell wall, has been demonstrated to decrease vector competence in tsetse flies (Schneider *et a*l., 2019). It has been found in different tsetse fly species in various African countries (Doudoumis *et al*., 2017). The Salivary Gland Hypertrophy Virus (SGHV, Hytrosaviridae family) is an important insect virus that decimates tsetse colonies, hence hampering the production of sterile male tsetse in the control of tsetse-transmitted trypanosomes (Lietze *et al*., 2011). Its use as a biological control agent for tsetse is not yet clear (Demirbas-uzel *et al*., 2018). This chapter aimed at determining the trypanosome diversity, the hosts' preference and the endosymbionts in tsetse flies. It therefore shows their relationship in the epidemiology of tsetse-transmitted trypanosomes in Akagera region of Rwanda.

4.2. Materials and Methods

4.2.1. Study area and collection of flies

This study was a continuation of the one reported in Chapter 3. The methodology for fly collection is described in sections 3.3.1 and 3.3.2. Briefly, 20 sites (10 in Akagera NP and 10 at the interface) were randomly selected for this study (Fig 4.1). Collected undamaged and blood fed flies (selected from field-deployed traps- Fig 4.2), were individually preserved as dry carcasses in 2ml Eppendorf tubes (Eppendorf AG, Hamburg-Germany) containing pieces of silica gel - coarse 6-20 mesh (Vardaan House, New Delhi, India) and separated by cotton wool. Tubes were labelled according to the trap, location, species and sex of the fly, month, and date after identification. Tubes with flies were then transported to the laboratory of the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi-Kenya for molecular analysis.

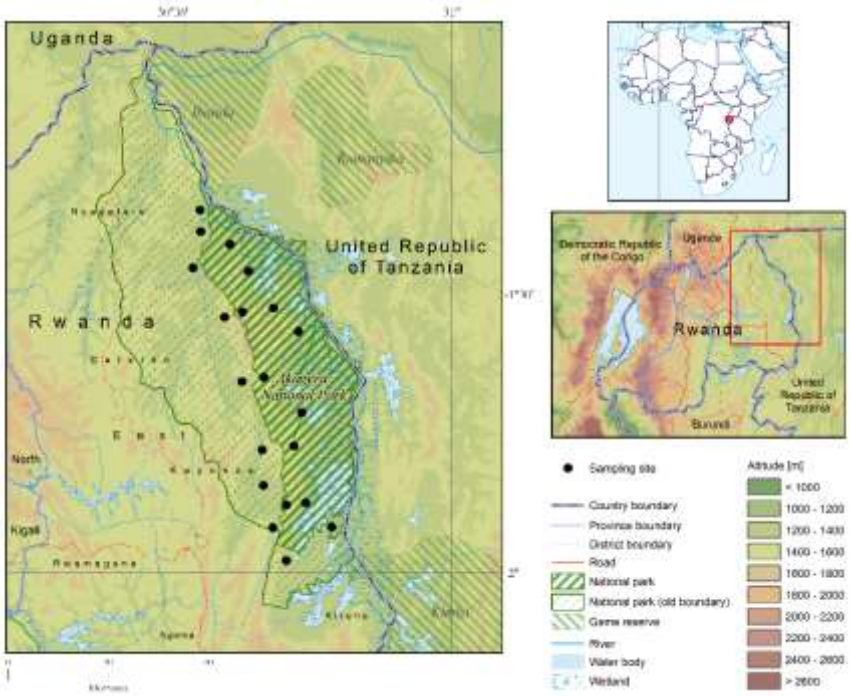


Figure 4.1: Map showing the collection sites for tsetse flies in Akagera National Park and the wildlife-human-livestock interface

Figure 4.2: Fly nets containing Tsetse flies caught in a trap during the study

4.2.2. DNA extraction from *Glossina*

To determine which tissue type is most reliable for detecting trypanosomes and assessing the impact of the fly infections, each tsetse fly was cut into two parts comprising of the head and proboscis (H+P) and the thorax plus abdomen (T+A) (Odeniran *et al*., 2019). Each cut part was separately handled, but remained with the identification of the fly. The surface of each fly was sterilised by quick soaking in 1% bleach, then in 70% ethanol and allowed to dry on a paper towel to avoid external contaminants including DNA particles. To cut, the scalpel blade was soaked in 70% ethanol, and then wiped with a paper towel containing 2% bleach to avoid any contamination between flies. Respective cut parts were placed in 1.5 ml PCR clean Eppendorf tubes (Eppendorf AG, Hamburg-Germany) with 2.0 mm zirconia beads (Stratech, UK) and crushed using Tissue Lyser II (Qiagen, Hilden- Germany) for 30 seconds at a frequency of 3000 revolutions per minute. A simplified arthropod genomic-DNA extraction protocol was used to isolate DNA from homogenates of respective tsetse fly cut parts as described by Margam *et al*., (2010). Eppendorf BioSpectrometer (Enfield, CT, USA) was used to determine the purity and quantity of the DNA, whereas the quality was determined using 1.5 % agarose gel electrophoresis and visualisation under UV light.

4.2.3. Detection and identification of *Trypanosoma* species

Tsetse DNA was first analysed in pools of equal individual DNA volumes. A pool was made of three flies of the same trap, collection time, species and sex. In cases where a pool turned positive, the individual DNAs making the pool were re-examined to determine the individual infection rate. Trypanosomes were detected using ITS1_CF and ITS1_BR universal primer (Table 4.1) that targets the trypanosomal internal transcribed spacer region (Njiru *et al*., 2005). A conventional PCR was performed in 9800 Fast and Gene Amp PCR system 9700 thermocyclers (Applied biosystems by life technologies). The reaction was in 10µL volume containing 3µL of nuclease free water, 5µL of 2X DreamTaq Green Master Mix (Thermo

Fisher Scientific), 0.5µL of each primer at 10 mM concentrations and 1µL of DNA template. The cycling conditions are detailed in table 4.1. *Trypanosoma* species were identified according to their respective band sizes. Cuts parts were analysed individually for trypanosomes.

4.2.4. Determination of human infective *Trypanosoma brucei rhodesiense*

All the samples positive for *Trypanozoon* were further subjected to supplementary PCR with TBR primers (Welburn *et al.*, 2001). The TBR positive samples were subsequently tested by amplifying the Serum Resistance-Associated (SRA) gene that is specific for *T. brucei rhodesiense,* and confers resistance to survive in human serum (Radwanska *et al*., 2002). The gene was amplified using B537/537 (Welburn *et al*., 2001) and SRA A/E primers (Gibson *et al*., 2002) (Table 4.1). The PCRs were performed in a ProFlex thermocycler (Applied Biosystems by Life technologies) in a 10µL volume reaction containing 3µL of nuclease free water, 5µL of 2X DreamTaq Green Master Mix (Thermo Fisher Scientific), 0.5µL of each primer at 10 mM concentrations and 1µL of DNA template.

4.2.5. Density of endosymbionts in *Glossina*

Screening for *Sodalis glossinidius, Wolbachia, Spiroplasma,* and salivary gland hypertrophy virus (SGHV) was done in the tsetse genomic DNA. The detection of endosymbionts followed the same pooling approach as for trypanosomes. Abdomen (A+T) DNA was used for *Sodalis, Wolbachia,* and *Spiroplasma* while both parts were used for SGHV since salivary glands are found both in the abdomen and mouthparts. The PCR amplification used the GPO1F/R primer for *Sodalis,* WspF1/R1, and wspecF/R primers for *Wolbachia,* RPOB primer for *Spiroplasma* and P74 primer for SGHV. For each primer, the reaction was made of 10 microliters comprising 6µL nuclease free water, 2µL of 5x HOT FIREPol Blend Master Mix (Solis BioDyne, Estonia), 0.5µL forward primer, 0.5µL reverse primer and 1µL template). The amplifications were run by conventional PCRs in Gene Amp PCR system 9700 and 9800 Fast thermocyclers (Applied

biosystems by life technologies). Positive controls for each symbiont were used. For SGHV, the positive control used was a synthetic P74 gene plasmid standard of SGHV (GenScript Inc. NJ, USA).

4.2.6. Identification of tsetse blood meal sources

Only abdomen (A+T) DNA templates were considered for blood meal analysis because it is the site for blood meal digestion. Real-time quantitative PCR (qPCR) and Two-Gene High-Resolution Melting Analysis were used to detect the blood meal source in flies. Primers targeting the genes for Cytochrome B (Cyt B) and 16S rRNA markers were used (Table 4.1.). DNA from abdomens were used for blood meal analysis and reactions were prepared in a volume of 10 µL consisting of 6 µL of nuclease free water, 2 µL of 5X Hot FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia), 0.5 µL of each primer at 10 mM concentrations and 1 µL of DNA template. The Thermo-cycling and high-resolution melting (HRM) analysis were performed in Quant studio™ 3 system (Applied biosystems by Thermo Fisher Scientific). QuantStudio™ Design & Analysis Software was used to analyse the melt curves and select the representatives for sequencing.

Table 4. 1: Primers used for the detection of Trypanosome species, endosymbionts and blood meal sources for Glossina species using PCR, and their PCR cycling conditions

SN	Target gene		Primer sequence (5'-3')	Target size (bp)	Reference
1	ITS 1	CF	CCGGAAGTTCACCGATATTG	250 to 700	(Njiru *et al*., 2005)
		BR	TTGCTGCGTTCTTCAACGAA		
		Cycling Conditions: 95^0C for 3min, 35 cycles: 95^0C for 30 sec, 60^0C for 30 sec, 72^0C for 1min, final extension at 72^0C for 10 min			
2	*T. brucei*	TBR 1	CGA ATG AAT ATT AAA CAA TGC GCA GT	177 (repetitive)	(Welburn *et al*., 2001)
		TBR 2	AGA ACC ATT TAT TAG CTT TGT TGC		
		PCR conditions as follows: 95°C for 3 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final elongation step of 10 min at 72°C.			
3	SRA	SRA A	GACAACAAGTACCTTGGCGC	460	(Gibson *et al*., 2002)
		SRA E	TACTGTTGTTGTACCGCCGC		

		The PCR conditions for SRA A/E were as follows: 95°C for 3 min, 40 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 1 min, with a final elongation step of 10 min at 72°C.			
4	SRA	B537	CCATGGCCTTTGACGAAGAGCCCG	743	(Welburn *et al*., 2001)
		B538	CTCGAGTTTGCTTTTCTGTATTTTTCCC		
		The touchdown PCR conditions were 95°C for 3 min, followed by 10 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 min, followed 25 cycles of 94°C for 320 seconds, annealing at 63.8°C for 30 seconds and extension at 72°C for 1 min per cycle. The final extension was set at 72°C for 7 min			
Blood meal source					
5	**Vertebrate 16S**	Vert 16S F	GAGAAGACCCTRTGGARCTT	250	(Omondi *et al*., 2015)
		Vert 16S R	CGCTGTTATCCCTAGGGTA		
		The PCR conditions were as follows: 95˚C for 15 minutes followed by 40 cycles of at 95˚C for 20 sec, 56 ˚C for 20 sec and 72˚C for 30 sec. The final elongation was at 72˚C for 5 min. Dissociation was done from 70 ^{0}C to 99.9 ^{0}C at the rate of 0.5^{0}C/ sec			
6	**Vertebrate cytochrome *B***	Cyt b F	CCCCTCAGAATGATATTTGTCCTCA	380	(Omondi *et al*., 2015) (Mejı *et al*., 2012) (Boakye *et al*., 1999)
		Cyt b R	CATCCAACATCTCAGCATGATGAAA		
		The PCR conditions were as follows: 95˚C for 15 minutes followed by 40 cycles of at 95˚C for 20 sec, 56 ˚C for 20 sec and 72˚C for 30 sec. The final elongation was at 72˚C for 5 min. Dissociation was done from 70 ^{0}C to 99.9 ^{0}C at the rate of 0.5^{0}C/ sec			
Endosymbionts					
7	**GPO gene**	GPO l F	TGAGAGGTTCGTCAATGA	564	(Dale and Maudlin, 1999)
		GPO I R	ACGCTGCGTGACCATTC		
		Cycling Conditions: 95^{0}C for 15min, 35 cycles: 95^{0}C for 1min, 55^{0}C for 1min, 72^{0}C for 1 min, and final extension at 72^{0}C for 10 min			
8	***Wolbachia* surface protein gene**	wsp_F1	GTCCAATARSTGATGARGAAAC	714	(Baldo *et al*., 2006); (Zhou and Neill, 1998)
		wsp_R1	CYGCACCAAYAGYRCTRTAAA		
		Cycling Conditions: 94^{0}C for 15min, 37 cycles: 94^{0}C for 30 sec, 59^{0}C for 45 sec, 72^{0}C for 1 min 30 sec, and final extension at 72^{0}C for 10 min			
9	***Wolbachia* 16S rRNA gene**	Wspec For	CATACCTATTCGAAGGGATAG	438	(Werren and Windsor, 2000)
		Wspec Rev	AGCTTCGAGTGAAACCAATTC		
		Cycling Conditions: 95^{0}C for 15 min, 2 cycles of 2 min at 95^{0}C, 1min at 60^{0}C and 1min at 72^{0}C, followed by 35 cycles of 30sec at 95^{0}C, 1min at 60^{0}C and 45 s at 72^{0}C and a final extension at 72^{0}C for 5 min			
10	***Spiroplasma RPOB***	RPOB3044F_ALL	ARTHTTACCADTDGAAGATATGCC	300	(Chepkemoi *et al.*, 2017)
		RPOB3380R_ALL	TGTARTTTRTCATCWACCATGTG		
		Cycling Conditions: 95^{0}C for 15min, 35 cycles: 95^{0}C for 30 sec, 55.9^{0}C for 30 sec, 72^{0}C for 30 sec, and final extension at 72^{0}C for 10 min			
11	**SGHV P74 gene**	P74 2F	TGTCARATWAATTATCCMCGYGGTAA	373	(Abd-alla *et al*., 2011)
		P74 2R	AARTCATCGCAATARTAYTTRTT		

		Cycling Conditions: 95^0C for 15min, 35 cycles: 95^0C for 30 sec, 60^0C for 30 sec, 72^0C for 30 sec, and final extension at 72^0C for 7 min		

4.2.7. Data analysis

The analysis was performed by SPSS software (SPSS Inc., IL, USA). Pearson chi-square ($\chi 2$) was used to determine the trypanosome infection rates and blood meal sources between different variables such as area and seasons. Logistic regression was used to determine the correlations between endosymbionts and trypanosome infections in flies. The significance ($p < 0.05$) at 95% confidence interval was considered. *T. vivax* has a lifecycle that occurs completely in tsetse fly mouthparts, and was therefore not included in analysis of symbionts-trypanosome associations.

Calculation of blood meal feeding frequency and feeding indices

The feeding frequency was calculated as a proportion of blood meals from a particular host out of the total number of blood meals examined. The feeding index or selection index (Wi) is a population density-based selection ratio (probability of selection), computed using previously reported methods based on forage ratios (Kilpatrick *et al*., 2006; Lardeux *et al.*, 2007; Hamer *et al*., 2009; Auty *et al.*, 2016). The aerial population count estimates that are conducted every 2 years in Akagera NP by Akagera management company (AMC) were used.

$$Wi = \frac{Oi}{Pi}$$

Where Wi = feeding index (selection index) of a particular host i; oi =proportion of blood meal from a particular host i out of the total blood meals from an area; and Pi= density of that host i out of the total density of hosts found in an area. The Wi value above 1 designates the host is more frequently selected than it would be through random selection. Hosts with Wi values between 0 and 1 are avoided, or less frequently fed on than expected by chance.

4.2.8. Gene sequence analysis

PCR amplicons for selected representative positive samples were re-amplified in larger PCR reaction volumes and run in 2% ethidium stained agarose gel electrophoresis. The target ones with a correct single band were purified by Exo1-rSAP (New England BioLabs, inc. MA, US) as instructed in the guidelines. The products with more than one band were excised and then purified by QIAquick PCR purification kit (Qiagen, Germany). The purified amplicons were sent to Macrogen Inc. (Holland) for unidirectional Sanger sequencing. The resultant sequence chromatograms were processed using Geneious prime 20.2.2 (Biomatters, New Zealand). All sequences were edited and aligned using Geneious Prime version 2020.2.2 software (Biomatters). BLASTN searches was used to identify homologous sequences of reference and sequence entries closely related with each of the individual sequences from this study (Altschul *et al*., 1990). Maximum likelihood phylogenies were inferred using PhyML version 3.0. An Akaike information criterion for automatic selection for an appropriate model of evolution was employed during the phylogeny construction. The generated tree was visualized and edited in Figtree 1.4. Pairwise genetic distances were conducted in MEGA software version 7 using Tajma-Nei model (Kumar *et a*l., 2016).

4.3. Results

4.3.1. Trypanosome infections in flies

A total of one thousand and one hundred and one (1101) tsetse flies (771 *Glossina pallidipes* and 330 *Glossina morsitans centralis*) were analyzed. The overall infection rate was 13.9% (153/1101) in the Head and proboscis (HP) and 24.3% (268/1101) in Thorax and Abdomen (TA) (Table 4.2.). Eight species of trypanosomes were identified. For each species, Head +proboscis and Thorax+ Abdomen were analyzed in parallel as HP/TA. Of these species, *T.brucei brucei* accounted for 4.1/7.1%, *T.congolense* Kilifi (2.2/2.1%), *T.congolense* savannah (1.6/1.2%), *T. evansi* (0/0.9%), *T.godefreyi* (1.2/3.1%), *T. grayi* (0/1.08%), *T.simiae*

(2.08/3.7%), *T.theileri* (0/2.08%) and *T.vivax* (5.2/3.7%) (Appendices 1&2). Mixed infections were 2.2/0.8% (25/9). Table 4.3 shows the variations in trypanosome infections between different predictors.

Table 4. 2: Comparison of infections between tsetse fly species from Akagera region

Fly species	**Sex**	**NE**	***All*** *HP/TA*	***Tbb*** *HP/TA*	***Tck*** HP/TA	***Tcs*** HP/TA	***Te*** HP/TA	***Tgod*** HP/TA	***Tgr*** HP/TA	***Tsim*** HP/TA	***Tth*** HP/TA	***Tv*** HP/TA	***Mixed*** HP/TA
Gp	F	456	66/113	17/37	13/11	8/4	0/0	5/19	0/4	8/15	0/8	24/18	8/3
	M	315	45/92	15/33	6/6	3/5	0/2	6/11	0/6	6/13	0/12	16/8	5/4
	Σ (%)	**771**	**111/205 (14.4/25.6%)**	**32/70 (4.1/9%)**	**19/17 (2.4/2.2%)**	**11/9 (1.4/1.1%)**	**0/2 (0/0.2%)**	**11/30 (1.4/3.9%)**	**0/10 (0/1.3%)**	**14/28 (1.8/3.6%)**	**0/20 (0/2.6%)**	**40/26 (5.2/3.4%)**	**13/7 (1.7/0.9%)**
	p value		***0.915/0.172***										
Gmc	F	195	23/39	6/8	3/3	2/2	0/6	3/3	0	5/8	0	11/9	7/0
	M	135	19/23	7/0	3/3	5/3	0/2	0/2	0/2	4/5	0/3	7/6	5/2
	Σ (%)	**330**	**42/63 (12.7/19%)**	**13/8 (3/2.4%)**	**6/6 (1.8/1.8%)**	**7/5 (2.1/1.5%)**	**0/8 (0/2.4%)**	**3/5 (0.9/1.5%)**	**0/2 (0/0.6%)**	**9/13 (2.7/3.9%)**	**0/3 (0/0.9%)**	**18/15 (5.4/4.5%)**	**12/2 (3.6/0.6%)**
	p value		***0.168/0.064***										
Total (*Gp+Gmc*) (%)		**1101**	**153/268 (13.9/24.3%)**	**45/78 (4.1/7.1%)**	**25/23 (2.2/2.1%)**	**18/14 (1.6/1.2%)**	**0/10 (0/0.9%)**	**14/35 (1.2/3.1%)**	**0/12 (0/1.08%)**	**23/41 (2.08/3.7%)**	**0/23 (0/2.08%)**	**58/41 (5.2/3.7%)**	**25/9 (2.2/0.8%)**
p value			***0.962/0.089***										

Gp=Glossina pallidipes, Gmc=Glossina morsitans centralis, NE= Number examined, , *HP/TA=Head+Proboscis / Thorax+Abdomen, F=Female, M=Male, Tbb=Trypanosoma brucei brucei, Tck=Trypanosoma congolense* kilifi, *Tcs=Trypanosoma congolense* savannah, *Te=Trypanosoma evansi*,, *Tgod=Trypanosoma godefreyi, Tgr=Trypanosoma grayi, Tsim=Trypanosoma simiae, Tth=Trypanosoma theileri, Tv=trypanosoma vivax.*

There were slightly higher infections in *Glossina pallidipes* (14.4/25.6%) than in *Glossina morsitans centralis* (12.7/19%, p=0.962/0.089).

25 mixed infections were identified in HP *(Tv+Tbb=8, Tv+Tsim=7, Tck+Tbb=3, Tck+Tv=2, Tcs+Tv=2, Tbb+Tsim=1, Tcs+Tbb=1* and *Tv+Tgod=1),* and 9 mixed infections in TA *(Tbb+Tgod=3, Tck+Te=1, Tck+Tgr=1, Tck+Tth=1, Tck+Tv=1, Tgod+Tsim=1* and *Tgod+Tsim=1).*

Table 4. 3: Comparison of infections between different predictors

Predictor	**Variable**	**NE**	***All*** HP/TA	***Tbb*** HP/TA	***Tck*** HP/TA	***Tcs*** HP/TA	***Te*** HP/TA	***Tgod*** HP/TA	***Tgr*** HP/TA	***Tsim*** HP/TA	***Tth*** HP/TA	***Tv*** HP/TA	**Mixed** HP/TA
Season	Dry	429	**43/92** **(10.02/21.4%)**	13/31 (3.03/7.2%)	9/10 (2.09/2.3%)	3/7 0.6/1.6%	0/0 0%	3/11 (0.6/2.5%)	0/2 (0/0.4%)	6/11 (1.3/2.5%)	0/8 (0/1.8%)	18/18 (4.1%)	6/6 (1.3%)
	Wet	672	**110/176** **(16.3/26.2%)**	32/47 (4.7/7%)	16/13 (2.3/1.9%)	15/7 (2.2/1%)	0/10 (0/1.4%)	11/24 (1.6/3.5%)	0/10 (0/1.4%)	17/30 (2.5/4.4%)	0/15 (0/2.2%)	40/23 (5.9/3.4%)	19/3 (2.8/0.4%)
	p value		***0.078/0.018***										
Area	Akagera NP	600	**95/155** **(15.8/25.8%)**	35/53 (5.8/8.8%)	16/13 (2.6/2.1%)	9/8 (1.5/1.3%)	0/8 (0/1.3%)	10/21 (1.6/3.5%)	0/12 (0/2%)	13/27 (2.1/4.5%)	0/12 (0/2%)	37/4 (6.1/0.6%)	20/3 (3.3/0.5%)
	Interface	501	**58/113** **(11.5/22.5%)**	10/25 (2/5%)	9/10 (1.8/2%)	9/6 (1.8/1.2%)	0/2 (0/0.4%)	4/14 (0.8/2.8%)	0/0 0%	10/14 (2/2.8%)	0/11 (0/2.2%)	21/37 (4.2/7.3%)	5/6 (1/1.2%)
	p value		***0.025 / 0.000***										
District	Gatsibo	15	**2/3** **(13.3/20%)**	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/3 (6.6/20%)	0/0 (0%)	1/0 (6.6/0%)	0/0 (0%)
	Kayonza	201	**29/74** **(14.4/36.8%)**	5/12 (2.4/6%)	6/5 (3/2.5%)	3/3 (1.5%)	0/0 (0%)	4/14 (2/7%)	0/0 (0%)	6/7 (3/3.5%)	0/8 (0/4%)	7/31 (3.5/15.4%)	2/6 (1/3%)
	Nyagatare	285	**27/36** **(9.5/12.6%)**	5/13 (1.7/4.5%)	6/5 (2.1/1.7%)	6/3 (2.1/1.05%)	0/2 (0%)	0/0 (0%)	0/0 (0%)	3/4 (1.05/1.4%)	0/3 (0%)	13/6 (4.5//2.1%)	3/0 (0%)
	p value		***0.645 / 0.033***										

Gp=Glossina pallidipes, Gmc=Glossina morsitans centralis, NE= Number examined, , *HP/TA=Head+Proboscis / Thorax+Abdomen, F=Female, M=Male, Tbb=Trypanosoma brucei brucei, Tck=Trypanosoma congolense* kilifi, *Tcs=Trypanosoma congolense* savannah, *Te=Trypanosoma evansi,, Tgod=Trypanosoma godefreyi*, *Tgr=Trypanosoma grayi*, *Tsim=Trypanosoma simiae, Tth=Trypanosoma theileri, Tv=trypanosoma vivax.*

More trypanosome infections were observed in the wet season (16.3/26.2%) than in the dry season (10.02/21.4%), although no statistical significance was found in HP (p= 0.078) rather for TA (p=0.018). Akagera NP accounted for (15.8/25.8%), compared to the interface area (11.5/22.5%), p=0.025 / 0.000. At the interface, higher tsetse trypanosome infections rates were observed in Kayonza district (14.4/36.8%). The infections prevalence varied across individual trypanosome species) (Fig 4.3.).

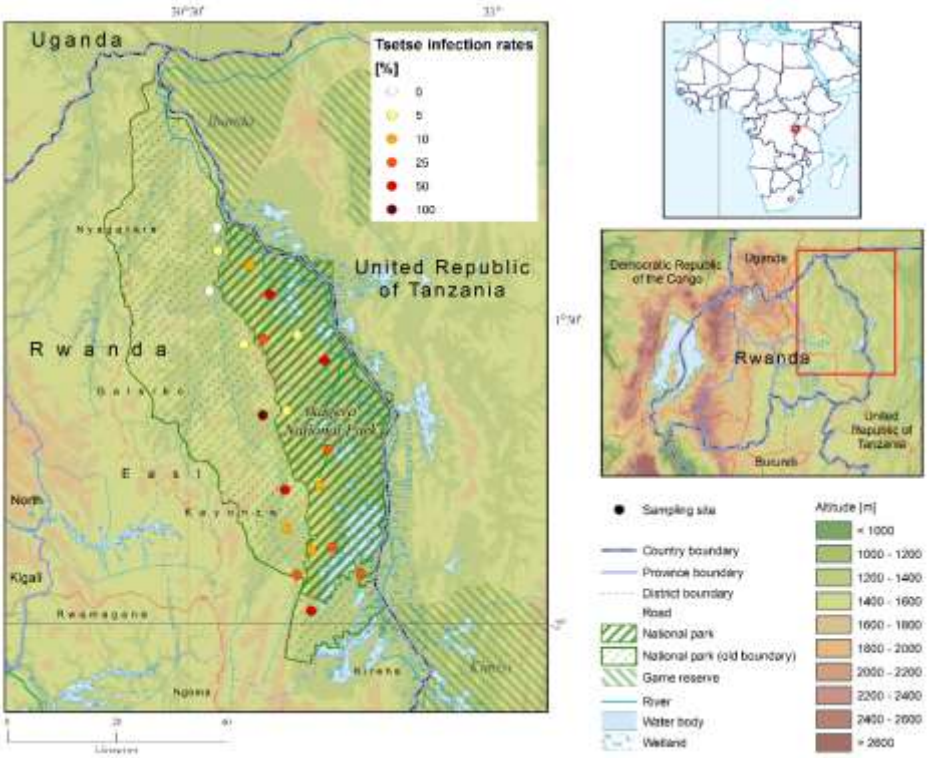


Figure 4.3: Prevalence of infection in different locations of the study area

4.3.1.1. Detection of SRA gene in tsetse flies

Forty-five (45) samples of Head and proboscis (HP) and 78 from Thorax and abdomen (TA) that were positive for *T.brucei brucei* were subjected to further TBR primer screening. All of them turned positive to TBR, however, none tested positive for the SRA gene by using either SRA A/E or B537/538 primers. This indicates that no *T.brucei rhodesiense* was found in tsetse flies analyzed.

4.3.1.2.. Phylogenetic analysis of trypanosome species

Trypanosoma (T.) congolense sequences from this study showed similarity with 2 different strains. 25 sequences of *T.congolense* had BLAST results of between 97.85-98.43% with GenBank accession MK756200 (*G.palpalis*, Nigeria) and 97% with U22317.1 (Rat, Kenya), both accession numbers being *Trypanosoma congolense* Kilifi-type. Eighteen sequences showed 99.68% similarity with LC492130.1 (Donkey, Sudan) and 91.68-91.24% with U22315.1 (IL1180) (Rat, Kenya), both being *Trypanosoma congolense* type Savannah. *T.brucei brucei* sequences had 95-98% with KR092353 (Uganda), KR092361 (DR Congo), and KR092362 (Ivory Coast). *T.evansi* had 98.07% with KX898423.1 (Iran). *T.godefreyi* had 97.87% and 100.00% respectively with MK131967.1 and MK131839.1 both from Zambia. *T.grayi* sequences had 95.85% and 99.44% respectively with MG255205.1 and MK656903.1 (Cameroon), and 96.60%with MZ147878.1 (Chad). The BLAST for *T. simae* sequences showed 98.97%, 98.85%, and 97.59% with JN673387.1 (Tanzania), MK132108.1 (Zimbabwe), and AB742533.1 (Ghana) respectively. *T.theileri* got a similarity of 98.11% with JN673396.1 (Zambia). *T. vivax* representatives showed 97.99-96.82% similarity with GenBank DQ316037, clone of IL3905 (Kenya), 93.13% with KX584844.1 (Mozambique), 92.68% with KM391825.1 (Ethiopia) and 91.67-93.13% with MW689625 (Kenya). The accession numbers of nucleotide sequences from this study were deposited to the GenBank database (Appendix 3). Figure 4.4. illustrates the phylogenetic tree resulting from the representative sequences generated by this study.

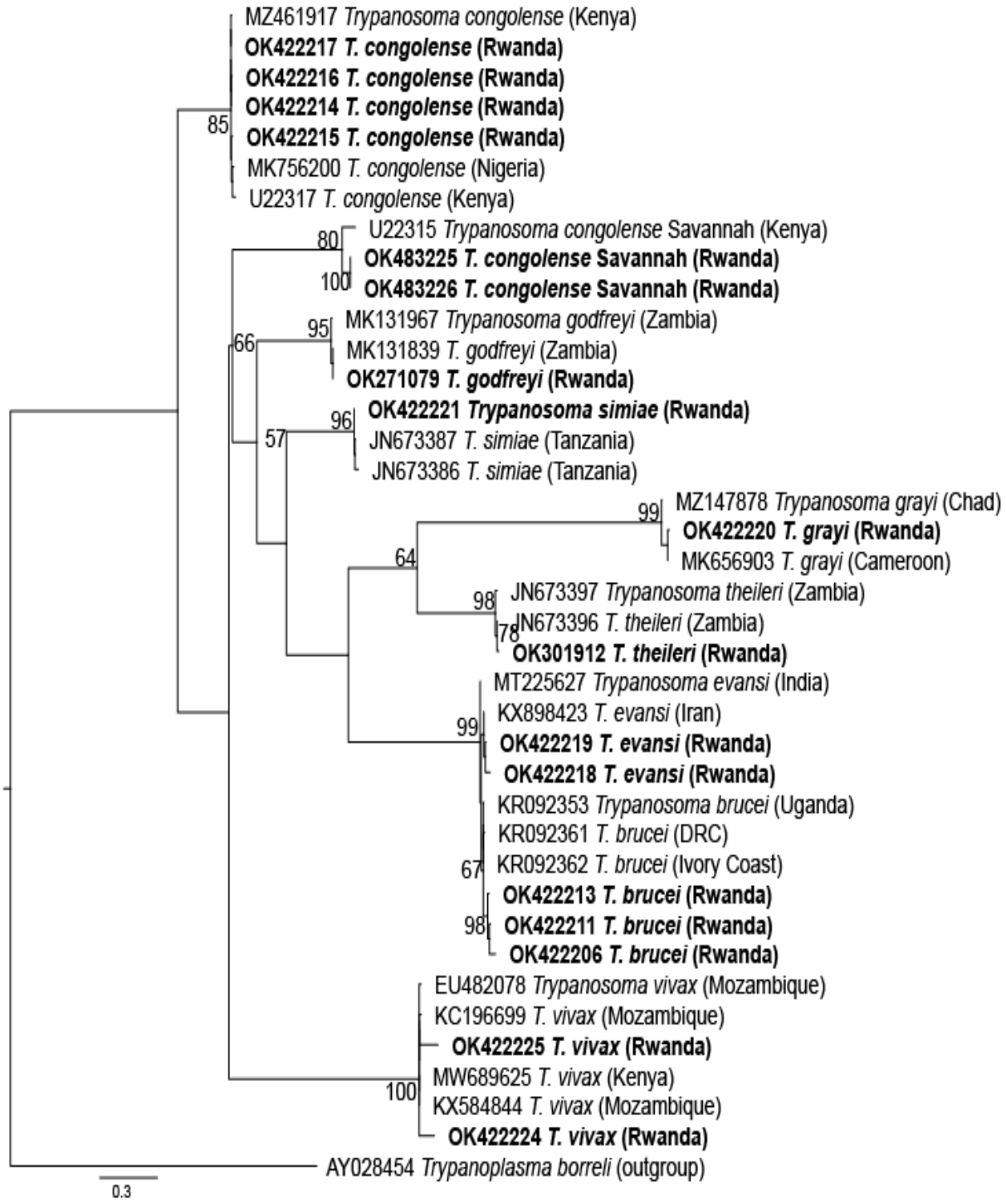


Figure 4.4: Maximum likelihood phylogeny of Trypanosoma spp. based on ITS1

GenBank accession numbers and country of origin are indicated for each sequence. Sequences from this study are in bold. Bootstrap values at the major nodes are of percentage agreement among 1,000 replicates. The tree is rooted to out-group sequence AY028454 (in bracket at bottom of the tree).

4.3.2. Blood meal analysis results

Host DNA was found in 312 samples (312/367=85%). These comprised of 50 cases of multiple feeding (47 double and 3 triple feeding) and 262 single feedings. Host DNA was missed in 25 samples (25/367=6.8%). Eighteen species of hosts were identified (Table 4.4.).

Table 4. 4: Hosts identified and their feeding frequency

SN	Hosts fed on	Feeding frequency	NCBI Genbank closest match & % identity (Vert 16S)	NCBI Genbank closest match & % identity (Cytochrome B)
1	African buffalo (*Syncerus caffer*)	134/367 (36.5%)	JQ235547.1 (100%) JQ235542.1 (99.44%)	KX697546.1 (100%) KX697546.1 (99.67%) KX697512.1 (96.61%)
2	Common warthog (*Phacochoerus africanus*)	52/367 (14.1%)	DQ409327.1 (99.46%) KJ193171.1 (97.88%) KJ193171.1 (86.97%)	-
3	Cattle (*Bos Taurus*)	39/367 (10.6%)	-	AY682375.1 (96.1%)
4	African savannah elephant (*Loxodonta africana*)	32/367(8.7%)	AB443879.1 (96.33%)	KX697470.1 (97.88%)
5	Bushbuck (*Tragelaphus scriptus*)	27/367 (7.3%)	JN632706.1 (87.68%)	HQ641317.1 (92.86%)
6	Human (*Homo sapiens*)	21/367 (5.7%)	MK248422.2 (98.89%) MN687316.1 (98.51%) MT511085.1 (88.42%)	MT568795.1 (100%) LC088149.1 (100%) KX697544.1 (99.66%)
7	Olive baboon (*Papio Anubis)*	17/367 (4.6%)	-	-
8	Black rhinoceros (*Diceros bicornis*)	9/367 (2.4%)	MK909143.1 (97.28%)	-
9	Hippopotamus (*Hippopotamus amphibious*)	7/367 (1.9%)	AP003425.1 (92.49%)	-
10	Impala (*Aepyceros melampus*)	7/367 (1.9%)	-	-
11	Goat (*Capra hircus*)	6/367 (1.6%)	-	-
12	Eland (*Tragelaphus oryx*)	5/367 (1.3%)	KX697487.1 (82.12%)	-
13	Blue monkey (*Cercopithecus mitis)*	4/367 (1.08%)	-	-
14	Giraffe (*Giraffa camelopardalis tippelskirchi*)	4/367 (1.08%)	-	-
15	Topi (*Damaliscus lunatus*)	3/367 (0.8%)	-	-
16	Common Duiker *(Sylvicapra grimmia)*	3/367 (0.8%)	-	-
17	Nile Tilapia (*Oreochromis niloticus*)	2/367 (0.5%)	-	XM 003447436.5 (100%)
18	Plain Zebra (*Equus quagga*)	2/367 (0.5%)	-	-
19	Defassa waterbuck (*Kobus ellipsiprymnus defassa*)	1/367 (0.27%)	-	-

No similarity match with NCBI blast was found in 23 pools (25/367=6.2%), and the blood source could not be identified in 7 samples (7/367= 1.9%). Table 4.5 shows how hosts were preferred in comparison with the season, area, and tsetse fly species.

Table 4. 5: Feeding preference associated with the season, area, and tsetse species

SN	Hosts fed on	Feeding frequency	Season		Area		Tsetse species	
			Dry	Wet	Interface	Park	*Gmc*	*Gp*
1	Buffalo	134/367	48	86	57	77	44	90
2	Warthog	52/367	17	35	16	36	13	39
3	Cattle	39/367	20	19	39	0	11	28
4	Elephant	32/367	14	18	24	8	7	25
5	Bushbuck	27/367	7	20	4	23	6	21
6	Human	21/367	9	12	15	6	4	17
7	Baboon	17/367	7	10	2	15	7	10
8	Rhinoceros	9/367	4	4	0	9	4	5
9	Hippo	7/367	2	5	1	6	1	6
10	Impala	7/367	1	6	0	7	2	5
11	Goat	6/367	5	1	5	1	1	5
12	Eland	5/367	2	3	3	2	3	2
13	Monkey	4/367	1	3	0	4	3	1
14	Giraffe	4/367	0	4	1	3	2	2
15	Topi	3/367	2	1	0	3	0	3
16	Duiker	3/367	2	1	3	0	1	2
17	Nile Tilapia	2/367	1	1	0	2	0	2
18	Plain Zebra	2/367	2	0	0	2	0	2
19	Waterbuck	1/367	0	1	0	1	0	1
		P value	0.571		0.000		0.090	

There was no statistical significance in feeding patterns between the seasons (p=0.571). However, variations exist with host species. Cattle and goats were more fed on during the dry season, even for Topi, duiker and zebra. The park animals contributed a lot more than the interface (p = 0.000), however, it is evident that cases of feeding on cattle, elephants, humans, goat and some wild animals like eland and duiker were increased at the interface than inside the park. Both *G.m.centralis* and *G.pallidipes* feed on the same hosts, *G.pallidipes* showed an increased feeding frequency, although not statistically significant (p=0.090).

Figures 4.5 and 4.6 show the blood-feeding preference in different localities on the park and the interface area, respectively.

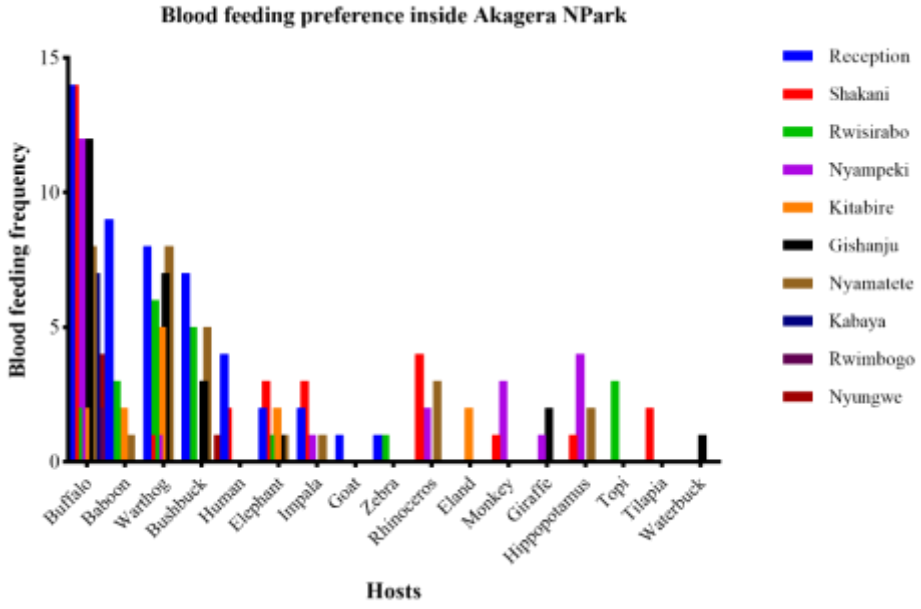


Figure 4.5: Hosts preference of tsetse flies inside Akagera National Park

There was an increased feeding frequency mainly on buffalo, warthog, and bushbuck in localities of park reception site, Shakani, Nyampeki, Nyamatete and Gishanju.

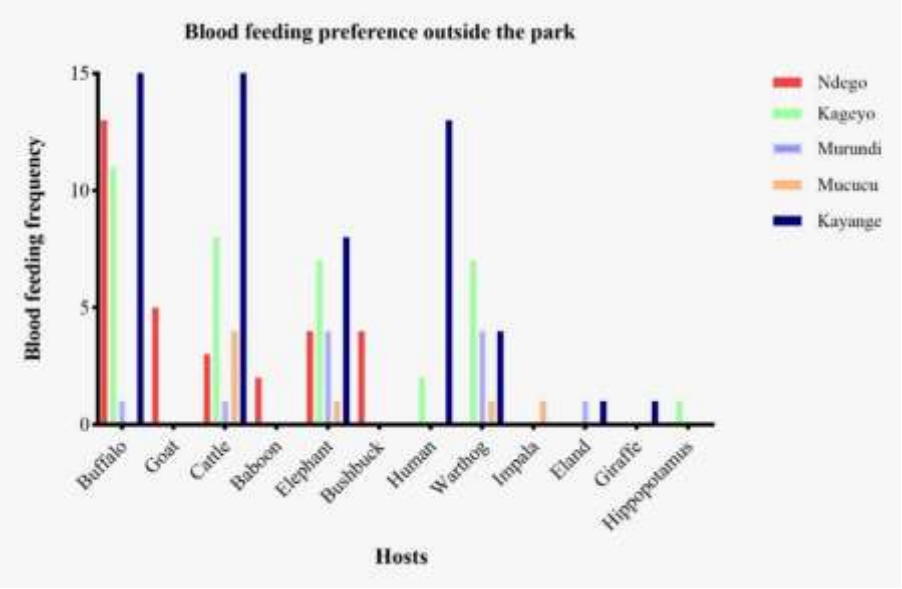


Figure 4.6: Hosts preference of tsetse flies in localities around Akagera National Park

At the interface, the buffalo, cattle, elephant, human, and warthog were most preferred frequently in localities of Kayange, Kageyo, and Ndego. Based on the population densities of

the animals as per the aerial census, the feeding indices were analysed to see the most likely hosts to be preferred by tsetse flies.

According to the feeding indices calculated based on each species population density, the rhino, elephant, bushbuck, warthog, giraffe, eland, buffalo and duiker were the most likely preferred wild hosts. The cattle, humans, goat, and Nile tilapia were not included as their population estimates were not availed during the study time. Table 4.6 shows the order of preference according to the calculated feeding indices.

Table 4. 6: Order of preference according to the feeding indices analysis

SN	Hosts fed on	NE	Feeding frequency	*G.m.c.*	*G.p.*	Population by aerial count estimation	Feeding index
1	Rhinoceros	367	9 (2.4%)	4/9	5/9	25	16.2
2	Elephant	367	32 (8.7%)	7/32	25/32	109	13.2
3	Bushbuck	367	27 (7.3%)	6/27	21/27	121	10.04
4	Warthog	367	52(14.1%)	13/52	39/52	871	2.69
5	Giraffe	367	4 (1.08%)	2/4	2/4	78	2.3
6	Eland	367	5 (1.3%)	3/5	2/5	120	1.87
7	Buffalo	367	134 (36.5%)	44/134	90/134	3456	1.74
8	Duiker	367	3 (0.8%)	1/3	2/3	97	1.4
9	Monkey	367	4 (1.08%)	¾	1/4	455	0.4
10	Olive baboon	367	17 (4.6%)	7/17	10/17	3255	0.23
11	Topi	367	3 (0.8%)	0/3	3/3	682	0.2
12	Hippopotamus	367	7 (1.9%)	1/7	6/7	1838	0.17
13	Impala	367	7 (1.9%)	2/7	5/7	2414	0.13
14	Zebra	367	2 (0.5%)	0/2	2/2	1936	0.05
15	Waterbuck	367	1 (0.27%)	0/1	1/1	1050	0.04
16	Cattle	367	39(10.6%)	11/39	28/39	-	-
17	Human	367	21 (5.7%)	4/21	17/21	-	-
18	Goat	367	6 (1.6%)	1/6	5/6	-	-
19	Nile Tilapia	367	2 (0.5%)	0	2/2	-	-
						16507	

NE= Number examined; *Gmc= Glossina morsitans centralis*; *Gp=Glossina pallidipes*

4.3.2.1. Trypanosome infections in relationship to tsetse feeding preference

Eight species of trypanosomes and nineteen hosts DNA were identified in tsetse flies' abdomen samples. Flies infected with *T.b.brucei* had mainly fed on the buffalo, elephant, and warthog. Flies infected with *T. congolense* strains had predominantly fed on the buffalo. *T.simiae*-infected flies fed primarily on the warthog while flies infected with *T.vivax* had fed on a wide host range including mainly the buffalo, warthog, elephant, and cattle. The detailed feeding frequency of infected flies on hosts is shown in table 4.7.

Table 4. 7: Host feeding frequency in relation to trypanosome infections

SN	Host/trypanosome species	Freq	*Neg*	*Tbb*	*Tck*	*Tcs*	*Te*	*Tgod*	*Tgr*	*Tsim*	*Tth*	*Tv*
1	Buffalo	134	81	12	9	10	4	4	1	3	3	7
2	Warthog	52	23	4	2	0	0	4	1	10	1	7
3	Cattle	39	32	1	1	0	0	0	0	0	1	4
4	Elephant	32	18	5	1	1	0	2	0	0	0	5
5	Bushbuck	27	11	5	3	0	1	3	0	3	1	0
6	Human	21	15	1	0	1	0	1	0	1	1	1
7	Baboon	17	12	1	0	0	0	3	0	0	0	1
8	Rhinoceros	9	6	0	0	1	0	0	2	0	0	1
9	Hippopotamus	7	4	0	1	0	0	0	2	0	0	0
10	Impala	7	5	2	0	0	0	0	0	0	0	0
11	Goat	6	6	0	0	0	0	0	0	0	0	0
12	Eland	5	5	0	0	0	0	0	0	0	0	0
13	Monkey	4	3	1	0	0	0	0	0	0	0	0
14	Giraffe	4	3	1	1	0	0	0	1	0	1	0
15	Topi	3	2	1	0	0	0	0	0	0	0	0
16	Duiker	3	3	0	0	0	0	0	0	0	0	0
17	Nile Tilapia	2	2	0	0	0	0	0	0	0	0	0
18	Zebra	2	1	0	0	1	0	0	0	0	0	0
19	Waterbuck	1	1	0	0	0	0	0	0	0	1	0

Freq= feeding frequency, Neg= Negative for trypanosomes, T= Trypanosoma, Tbb=T. brucei brucei, Tck=T. congolense kilifi, *Tcs=T. congolense* savannah, *Te=T. evansi,, Tgod=T. godefreyi, Tgr=T.grayi, Tsim=T.simiae, Tth=T. theileri, Tv=T. vivax.*

Infected flies fed less on some hosts such as duiker, Tilapia, topi, zebra, and waterbuck. The frequent associations between host feeding and trypanosome infections were selected and presented in Figure 4.7 below

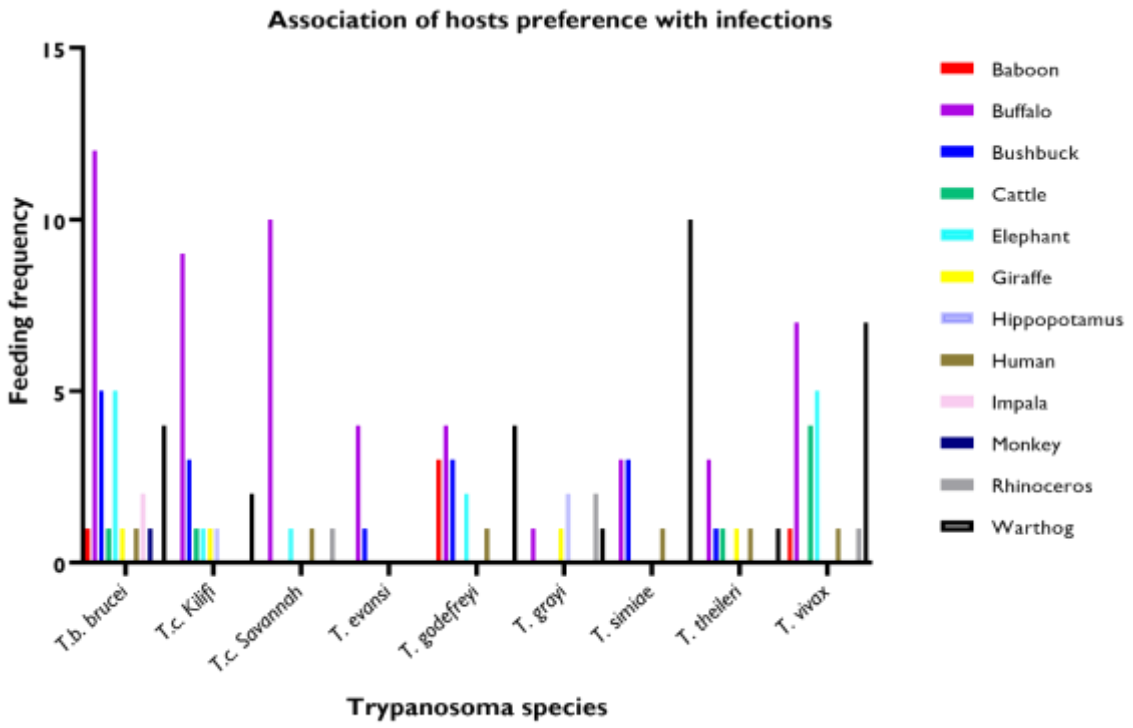


Figure 4.7: Association between tsetse trypanosome infections with the host preference

The infections with *T.b.brucei* were much associated with tsetse blood meal from the buffalo, elephant and bushbuck. *T. congolense* strains and *T. evansi* were related to the buffalo blood meal whereas *T. godefreyi* was associated with buffalo, warthog and bushbuck. *T. simiae* was much linked to the warthog blood meal, while *T. vivax* was related to the blood meals from the buffalo, warthog, elephant and cattle. *T.vivax* has a wide range of hosts followed by *T.b.brucei*. The buffaloes contributed almost all the trypanosome species detected.

4.3.3. Endosymbionts and their relationship with trypanosome infections in flies

The number of flies screened for the four endosymbionts (*Sodalis, Wolbachia, Spiroplasma,* and SGHV) were 1011. A total of 31 flies (31/1101, 2.8%) [(21 (2.7%) *G.pallidipes* and 10 (3%) *G.m.centralis*)], among which only 18 flies (14 *G.pallidipes* and 4 *G.m.centralis*) were positive for trypanosome infections and other 13 were trypanosome negative. Contrary to our expectations, more *Sodalis* negative flies were found positive to trypanosomes (250/268, 93.3%) compared to *Sodalis* positive flies having trypanosome infections (18/268, 6.7%).

This difference was highly statistically significant at p=0.000, indicating that there was no relationship between *Sodalis* and trypanosome infections in this study (Table 4.8).

Table 4. 8: Overview of the symbionts-trypanosomes association

Symbiont	*G.pallidipes*			*G.morsitans centralis*			Total		
	Tryps +	Tryps -	P value	Tryps +	Tryps -	P value	Tryps +	Tryps -	P value
Sodalis +	14	7	0.000	4	6	0.102	18	13	0.000
Sodalis -	191	559		59	261		250	820	
Wolbachia +	11	23	0.275	4	15	0.508	15	38	0.294
Wolbachia -	194	543		59	252		253	795	

G. = *Glossina*, Tryps= Trypanosome, += positive, - =negative

For *Wolbachia*, 53 flies out 1101 were positive (53/1101, 4.8%) [(34 (4.4%) *G.pallidipes* and 19 (5.7%) *G.m.centralis*)]. Of these, 15 flies were *Trypanosoma*-positive (11 *G.pallidipes* and 4 *G.m.centralis*) and 38 flies were Trypanosoma-negative (23 *G.pallidipes* and 15 *G.m.centralis*). No statistical significance was found (p=0.294) between *Wolbachia*-positive flies with and without trypanosomes. 2 cases of co-infection of *Sodalis* and *Wolbachia* were observed, both of them were trypanosome-positive, one for *T.simiae* and another for *T.b.brucei.* Figure 4.8 shows how the *Sodalis* and *Wolbachia* were distributed in the study area. No *Spiroplasma* and SGH Virus were found in all samples analyzed.

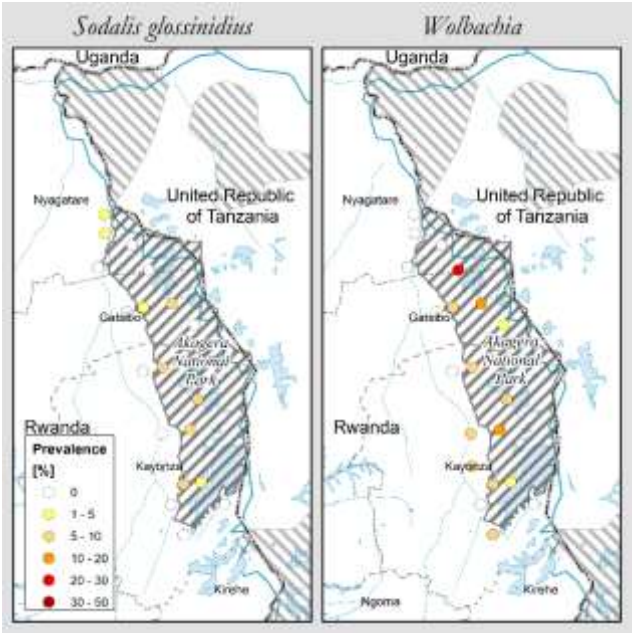


Figure 4.8: Distribution of detected endosymbionts

4.4. Discussion

The most prevalent species in Head+Proboscis was *T.vivax,* followed respectively by *T.b.brucei, T.congolense* Kilifi, *T.simiae, T.congolense* savannah and *T.godefreyi.* The overall infection rate from this study (13.9 %HP) is higher than the 3.7% that was reported by Mihok *et al*., 1992). However, the latter study used dissection and microscopy only and was carried on a small area outside our study area. The infection rate in the Thorax+Abdomen (TA) was higher than in Head+Proboscis (HP). This finding is in agreement with Isaac *et al*., (2016), Odeniran *et al*., (2019), Weber *et al*., (2019) and Signaboubo *et al*., (2021). This can be attributed to the fact that the abdomen is the site for the establishment of trypanosomes before maturation in the mouthparts (Maudlin *et al*., 2004). Depending on the Trypanosome species, a parasite may be found in the midgut and/or the salivary glands, both being anatomically located in the abdomen (Leak, 1999). Another key reason is the presence in the abdomen, of the blood meal that might come from different infected hosts. The majority of infections are limited to the midgut and therefore unable to reach the maturation in the mouthparts (Nayduch & Aksoy, 2007, Roditi & Lehane, 2008). Nevertheless, I could not determine whether the infections are immature since we did not search specifically for the trypanosome in their respective sites in the abdomen. Despite the above explanation, the study found higher proportions of *T. congolense* infection in the proboscis than in the abdomen. This could be attributed to the clearing of the parasite in the mid-gut by the tsetse fly and its persistence in proboscis (Lehane *et al*.,2000; Mariani *et al*.,2004).

The infection rates obtained after crushing the whole fly exaggerate and /or underestimate the proportions. A case in point is the number of *T. vivax* present in the abdomen, which would have been counted as the infections if the whole fly was crushed. Another example is an underestimation of *T. congolense* infections after considering the proboscis of only infected

mid-guts. This may have an impact on the determination of the fly vector competence. The interpretation of tsetse trypanosome infections should be wisely done depending on the location in the fly and the method used. Not all the infections established in the fly mid-gut will be transmitted to the susceptible hosts as many of them will not be able to mature in the mouthparts (Farikou *et al*., 2010). The molecular detections of trypanosomes in tsetse flies target the parasite DNA, however, the technique is likely to overestimate the fly infection rate (Farikou *et al*., 2010; Simo *et al*., 2012) as It amplifies even the DNA of dead parasites. The DNA does not discriminate between infective and immature forms, therefore, the presence of a trypanosome DNA in a fly does not certainly designate an infection (Mekata *et al.*, 2008). A recent infected blood meal preceding the examination could be the source and it does not necessarily mean a mature or an established infection (Macleod *et al.*, 2007).

This study supports the findings of Garcia *et al*., (2018) that *T. congolense* Kilifi is widespread in East Africa. *T. vivax* was found in the abdomen of many tsetse flies, a species that is both cyclically and mechanically transmitted, but completes in its lifecycle in the proboscis of a tsetse fly. The same findings were reported by Simo *et al*., (2012) and Simo *et al*., (2015). Such presence is attributable to the ingestion of an infected blood meal during feeding by tsetse flies. Nevertheless, the short passage in the abdomen towards the mouthparts where its lifecycle occurs is also possible (Ooi *et al.*, 2016). *T.evansi* is usually unable to undergo the development in tsetse flies due to the loss of maxicircles in the mitochondrial (kinetoplast) DNA (Borst *et al*.,1987), and therefore mechanically transmitted by biting flies. Its presence in tsetse flies was probably ascending from a recent infected blood meal. *T. theileri* was found in tsetse flies, which is in agreement with other studies (Votýpka *et al*., 2015; Garcia *et al*., 2018; Rodrigues *et al*., 2019). The presence of *T.theileri* in the tsetse abdomen could be due to a recent infected blood meal as well. Nevertheless, its ability to be transmitted either cyclically or mechanically by tsetse flies and its pathogenic potential remain uncertain. *T. grayi* is a parasite of reptiles

(Minter-Goedbloed *et al*., 1993**;** Hamilton *et al*., 2009**;** Fermino *et al.*, 2013**;** Kelly *et al*., 2014) and it was found in riverine tsetse flies associated with water bodies (Bourzat & Gouteux, 1990**,** Gouteux & Gibson, 1996)**.** It was found in the tsetse gut and we think it should not be considered as unusual to find it in *G. pallidipes* since Akagera NP has several internal lakes harboring crocodiles within the large swampy ecosystem. Additionally, the area is only colonized by the savannah group of tsetse i.e. *G. pallidipes* and *G. morstitans centralis* (Gashururu *et al*., 2021). These flies might be feeding on crocodiles, although the infected fly was found to have fed on a giraffe and not a crocodile. Similarly, Ngomtcho *et al.*, (2017) found it in the proboscis of *G. m. submorsitans* which is a savannah species as well as *G.pallidipes.* Further investigations are required to know if *T.grayi* can really mature in mouthparts and be transmitted to other hosts via blood feeding or mechanically.

Blood meal sources and their relationship with tsetse trypanosome infection

Few samples could not get similarity matches on NCBI Blast. The reason could be the sequences of bad quality resulting from the mixture of different host DNAs. This study findings on main feeding preference by *G.pallidipes* in Akagera park is in agreement with Auty *et al*., (2016) who found the same in Serengeti National Park of Tanzania. Again in the Nguruman game reserve of Kenya, *Glossina pallidipes* fed mainly on the African elephant, warthogs, African buffalo, and baboons (Nyawira, 2009; Muturi *et al*., 2011). The findings of this study show that Tsetse blood feeding is much associated with wildlife animals, even at the interface. Although Akagera NP is entirely fenced along its interface with livestock farms and human settlements, few wild animals were observed outside the park. Thus, it is clear that tsetse flies collected at the interface feed on both wild and domestic hosts. Transmission of trypanosomes is therefore most likely to occur by sylvatic and domestic cycles between hosts. As discussed earlier, we recall that trypanosomes from the sylvatic cycle are the most pathogenic to domestic animals (Van den Bossche *et al.*, 2010).

Livestock such as cattle and goats contributed few blood meals at the interface as opposed to the wild animals. The same scenario was found in Kenya by Channumsin *et al.*, (2021). This could be linked to the farming practices being implemented in the area such as reducing the unwanted vegetation in farms; which could be the resting places for tsetse flies. On top of that, farmers are aware of the active hours of tsetse flies and try to avoid taking their animals to high-risk areas during that time. Furthermore, this reduced feeding success to livestock could also be explained by the decreased density of tsetse flies preferring to feed on hosts that are outside the protected area (Lord *et al.*, 2018). It is uncertain whether this is simply because tsetse flies avoid livestock when more preferred hosts are available.

Human DNA was found in tsetse blood meals as is the case for many other regional similar studies (Bett *et al*., 2008; Makhulu *et al*., 2021). However, it is believed that feeding on humans is difficult for a tsetse fly based on its size and the buzzing sound. Other reasons include the reaction to the bite, odor camouflage, and use of repellents (Hargrove, 1976; Baylis & Nambiro, 1993; Baylis, 1996). Human DNA might have come from manipulating flies or simply because flies fed on humans frequenting or residing in the area. There is a risk of being bitten during traps deployment, collection, or DNA extraction (England & Baldry, 1972). Whatever the case, these findings were observed in human-dwelling localities and it shows how humans are in close contact with tsetse flies and therefore the risk of contracting trypanosome infections. Some Nile Tilapia blood meals were found, a fish species that is locally reared in inland lakes. Fish are aquatic and almost impossible to be fed on by a tsetse fly. We attribute this to the presence of a fishing site and fish cleaning process in Shakani around lake Ihema within the park. Flies may have touched fish during those activities.

Multiple feeding may have resulted from pooling or using two different gene markers in this case. However, it was observed in other studies with individual flies of the same tsetse species in protected areas (Channumsin *et al*., 2021). Whatever the case, feeding on multiple hosts

increases the exposure to a diverse range of trypanosomes, therefore a high transmission of the disease. Despite the mixed blood meal sources, we predict that the likely important reservoirs for trypanosomes in the area are the buffalo, the warthog, the elephant, and the bushbuck. These associations are in agreement with trypanosome infections in wild hosts/ reservoirs in various studies (Baker *et al*., 1967**;** Connor, 1994**;** Reichard, 2002**;** Mbaya *et al.*,2009)**.**

Symbionts densities and their relationship with tsetse trypanosome infection

Regionally, the *Sodalis* prevalence of 2.8% in *Pallidipes* is lower than the 16% prevalence of *Sodalis* in *G. Pallidipes* of Shimba hills (Wamwiri *et al.,* 2013), and 6.3% for *G. pallidipes* in Masai Mara National Reserve (Makhulu *et al*., 2021) both from Kenya. In *G. morsitans centralis*, the prevalence was 3.08%, which is lower compared to the prevalence of 57.7% (*Sodalis*) found in Kafue Zambia (Griffith *et al*., 2018; Gaithuma *et al.,* 2020). It is worth recalling that the *Sodalis* density is known to vary according to the tsetse species and *Sodalis* genotype in question (Geiger *et al.,* 2006; Farikou *et al*., 2011). The prevalence of 4.8% (*Wolbachia)* from this study is also lower compared to 95.3% that was found by Griffith *et al*., (2018) in Zambia. Further identification of *Sodalis* genotypes circulating in tsetse populations of the Akagera region would decipher the trypanosomes-symbionts associations in the area. Though unusual, we found co-infections of *Wolbachia* and trypanosomes, implicating the possibility of coexistence. The same was also reported in Cameroon by Kanté *et al*., (2018).

No relationship between trypanosome infection in tsetse flies and *Sodalis* was found in this study. The same was observed in *G. brevipalpis*, *G.morsitans morsitans,* and *G. pallidipes* of Zambia (Dennis *et al*., 2014), *G. morsitans submorsitans* and *G. tachinoides* of Cameroon (Kame-ngasse *et al*., 2018) and *G.pallidipes* in Shimba hills of Kenya (Channumsin *et al*., 2018). However, this is not in agreement with the positive relationship that was reported in Cameroon (Farikou *et al*., 2010, Farikou *et al*., 2011) and in Kenya (Wamwiri *et al*., 2013; Hamidou *et al*., 2013; Wamwiri *et al*., 2014; and Makhulu *et al*., 2021). There was no

association between *Wolbachia* and trypanosome infection in flies as was the case in Cameroon (Kanté *et al*., 2018). Nevertheless, the ecological circumstances of the studied tsetse populations can influence the *Wolbachia* densities (Mouton *et al*., 2007, Yun *et al*., 2011). Further investigation with different markers would maximize the detection. A deep identification of various *Wolbachia* haplotypes would help in better understanding their relationship with trypanosomes.

We did not find any *Spiroplsma* and SGH virus positives in all flies. Our findings for *G. pallidipes* and *G. morsitans centralis* are comparable to the work of Doudoumis *et al.*, (2017) who found zero prevalence in *G. pallidipes* and *G. morsitans centralis.* In the latter study, a higher prevalence was observed in *G.f.fuscipes, G.tachnoides,* and *G.p.palpalis*. *Spiroplasma* tends to occur in specific populations, varying in locations and seasons (Schneider *et al*., 2019). Additionally, it was demonstrated that *Wolbachia* infection in flies impedes the presence of *Spiroplasma* (Doudoumis *et al*., 2012a; Doudoumis *et al*., 2013). It could have contributed to the *Spiroplasma* absence in tsetse populations of the studied area.

4.5. Conclusions

1. Eight species of trypanosomes are circulating in tsetse flies, viz. *Trypanosoma congolense* savannah, *T. congolense* Kilifi, *T.vivax*, *T.brucei brucei*, *T.simiae*, *T.godefreyi, T.grayi, T. evansi* and *T. theileri*.
2. Out of 8 species identified in flies, except *T.grayi* and *T.theileri*, the remaining six species are pathogenic to livestock. *T.grayi* is stercorarian and is therefore posteriorly transmitted by the tsetse fly.
3. *T.evansi* and *T.theileri* are normally not tsetse-transmitted, rather transmitted mechanically by other biting flies. Their presence in tsetse fly could be due to recent infected blood meals.

4. No human infective *T.brucei rhodesiense* was detected in tsetse fly vectors.
5. The molecular tools are good for the identification of trypanosomes in tsetse flies, however, are likely to overestimate the fly infection rate.
6. This study confirms that infection rates obtained after crushing the whole fly would exaggerate and /or underestimate the proportions. This may have an impact on the determination of the fly vector competence. On the same note, the interpretation of tsetse trypanosome infections should be wisely done depending on the location in the fly and the method used.
7. Only two endosymbionts were found in tsetse flies (*Sodalis* and *Wolbachia*). No relationship was found between endosymbionts' presence and trypanosome infections in tsetse flies, implicating further investigation.
8. The preferred hosts for tsetse blood meal are buffalo, warthog, cattle, savannah elephant and bushbuck. Livestock is less preferred even in livestock-rearing areas.

The following recommendations are formulated:

1. Cases of human DNA was found in tsetse blood meal. The sleeping sickness surveillance should target especially human dwelling areas for regular monitoring.
2. The use of different markers is recommended to maximize the detection of symbionts. A deep identification of various *Wolbachia* haplotypes is recommended to better understand their relationship with trypanosomes.
3. We recommend further identification of *Sodalis* genotypes circulating in tsetse populations of the Akagera region to decipher the trypanosomes-symbionts associations in the area.

CHAPTER FIVE: OCCURRENCE, DIVERSITY AND DISTRIBUTION OF *TRYPANOSOMA* SPECIES CIRCULATING IN CATTLE AT THE HUMAN-WILDLIFE-LIVESTOCK INTERFACE OF AKAGERA NATIONAL PARK IN RWANDA

5.1. Introduction

African trypanosomiasis is associated with rural areas in low resource settings. The tsetse and trypanosomiasis challenge to livestock and humans is often linked to the infested protected areas (Lord *et al*., 2018). This situation increases the exposure to tsetse bite in farming zones around the infested protected areas (Van den Bossche *et al*., 2010). In Rwanda, the same challenge is reported around the Akagera region of Rwanda near the border with Tanzania (Chatikobo *et al*., 2009; Mazimpaka *et al*., 2017). African Animal Trypanosomiasis (AAT) was detected in cattle and tsetse flies vectors in Bukora (current Kirehe district) by Mihok *et al*., (1992). Sporadic cases of *T. b. rhodesiense* sleeping sickness were last diagnosed in Rwanda around the year 1990 (Clerinx *et al*., 1998). Over the last 20 years, there are no reported cases of *T. b. rhodesiense* sleeping sickness in the country (Franco *et al*., 2020; Simarro *et al*., 2010; WHO, 2020). However, the area is still considered at marginal risk. In neighbouring Uganda, it has been shown that cattle present the risk of transmission and contribute to the spread of *T. b. rhodesiense* HAT (Welburn *et al*., 2001; Fèvre *et al*., 2005; Jenna *et al*., 2016). Several diagnostic tests are used to detect trypanosomes, i.e. parasitological (Murray, 1977), Immunological (OIE, 2013, Boulangé *et al*., 2017) and molecular techniques (Simwango *et al*., 2017). Although different diagnostic tests for trypanosomes differ in sensitivity, each technique has its advantages and drawbacks (Paguem *et al*., 2019, OIE, 2013, Ng'ayo *et al*., 2005; Kivali *et al*., 2020).

However, their respective results may serve diversely according to the purpose. The parasitological methods are already in use and the immunological rapid test (VerY Diag) is

commercialised in the area. PCR has not been systematically used for the detection of trypanosomes in Rwanda. This work was the first large-scale study to map *Trypanosoma* occurrence in cattle around the tsetse–infested Akagera National Park. The study determined the disease status, identified the genetic diversity and distribution of trypanosomes circulating in cattle blood by using microscopy, immunological rapid tests and molecular techniques. The generated findings are crucial for designing evidence-based strategies for AAT control in the area.

5.2. Materials and Methods

5.2.1. Study area

The three districts i.e. Kayonza, Gatsibo and Nyagatare, were the main target areas for this study. However, some data were also collected from the Kirehe District further south (Fig 5.1), in areas bordering Kimisi game reserve of Tanzania.

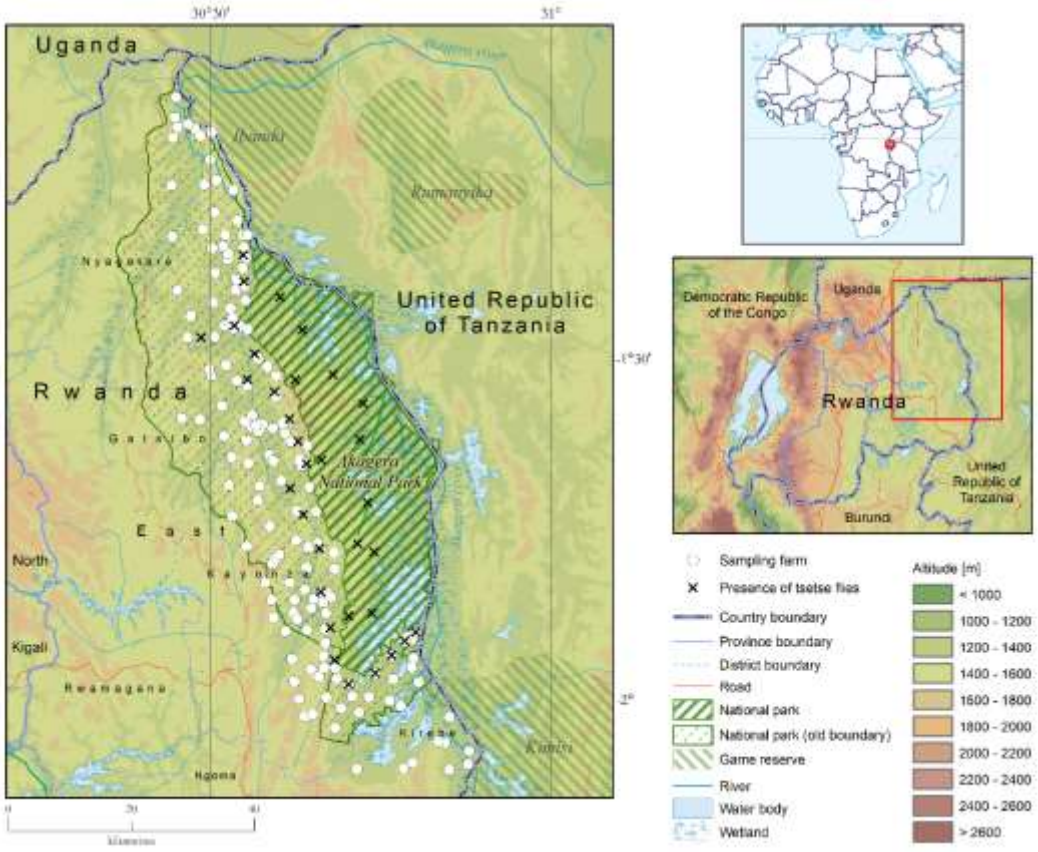


Figure 5.1: Sampling sites for blood collection

5.2.2. Study design and sample size determination

A cross-sectional study was undertaken between March and July 2019, using a stratified multistage random sampling method. The sample size was calculated using the formula for indefinite population (Thrusfield, 2007). There was no reliable data on farm records, farming households and cattle population in the area. It was determined based on the baseline-estimated prevalence of 50%, with an absolute desired precision of 5% at the confidence interval of 95%. To increase the chance of sampling many animals in the whole study area, the same calculations were made for each of the three main target districts (stage 1): Kayonza (n=384), Nyagatare (n=384) and Gatsibo (n=236). The target sample size in Gatsibo District was not reached because consent from some farmers vould not be obtained. The study purposively selected sectors (stage 2) based on their proximity to Akagera Park, its connections and adjacent game reserves in Tanzania. In that way, one sector of the fourth District (Kirehe) was later included (n=33), thus making a total sample size of 1037 cattle. These comprised 521 (50.24%) Ankole, 514 (49.56%) Ankole x Friesian and 2 (0.19%) pure Friesian cattle. There were more females (n=946) than males (n=91) and the majority were above 2 years of age (n=876). In total,12 sectors (administrative entities under the District) were included in the study (6 in Kayonza, 3 in Nyagatare, 2 in Gatsibo and 1 in Kirehe Districts), and therefore considered as the strata.

Information such as location, cattle population, herd size, communal watering and gathering points was obtained from the local Veterinary Services. At the farm level, individual Ankole and Ankole x Friesian cattle of above 6 months of age and both sexes were randomly selected. Calves aged less than 6 months were excluded because they are less likely to be exposed to AAT, considering the adopted local management system. Farmers do not take the young calves to risky areas for grazing. Cattle below 2 years of age were considered as young and those above 2 years as adults according to cattle owners' information. All sampling sites were georeferenced.

5.2.3. Blood collection

Blood samples from 1,037 cattle were collected from four districts (Figure 5.1). Before blood collection, informed oral consent was obtained from farmers. About four (4) mL of blood was collected from the coccygeal vein of each animal using sterile needles, and ethylene diamine tetraacetic acid (EDTA) vacutainer tubes (Vacutest Kima, Italy), and each tube was given a unique identifier code. Thin blood smears were immediately prepared on-site as described by Woo, (1970) , and the remaining blood was transported in cool boxes containing ice blocks to the laboratory of the Rwanda Agriculture Board. At the same laboratory, the buffy coat technique (BCT) (Murray, 1977) was carried out and the specimens for PCR were prepared. An aliquot of 500 µl of blood was transferred into cryovials and mixed with Lysis, Storage and Transportation (LST) buffer (Schultz *et al*., 1999) at a ratio of 1:1, and then transported to the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi-Kenya for molecular analysis.

5.2.4. Parasitological examination - thin smear and buffy coat technique

The smears that were prepared, fixed in methanol and stored after blood collection were Giemsa stained and examined for trypanosomes (Murray, 1977) by light microscope at a magnification of 100 (Opta-Tech Ltd, Poland). For the buffy coat technique (BCT), haematocrit tubes were sealed with Cristaseal (Hawksley, UK) and centrifuged at 13,000 revolutions per minute (rpm) for 5 min. Hawksley reader was used to determine the packed cell volumes (PCV) levels of each animal and the Buffy coat smears made. The PCV was measured to check correlations of anaemia with trypanosome infections. The Buffy coat smear slides were prepared and observed under a light microscope (Opta-Tech Ltd, Poland) for the presence of trypanosomes as described by Murray *et al*,. (1977).

5.2.5. Nucleic acid extraction

Genomic DNA was extracted from 1037 blood samples using two methods. The DNA from the first batch of samples was extracted using Bioline Isolate II genomic DNA kit (Meridian Life Science company, Memphis, TN, USA) as described by the manufacturer's instructions. Genomic DNA from another batch of samples was extracted by the Non-enzymatic salting-out method as described by Suguna *et al*., (2014). Thereafter, the purity and quantity of the DNA were measured by Eppendorf BioSpectrometer (Enfield, CT, USA); while the quality was measured by 1.5% agarose gel electrophoresis and visualised under UV light.

5.2.6. Molecular detection of trypanosomes

PCR combined with High-Resolution Melting (HRM) analysis using 18S generic primers (Table 3.2.) were carried out in a volume of 10 µL reaction consisting of 6 µL of nuclease-free water, 2 µL of 5X Hot FIREPol EvaGreen HRM Mix (Solis BioDyne, Tartu, Estonia), 0.5 µL of each primer at 10 mM concentrations and 1 µL of DNA template. The PCR conditions were as follows: initial enzyme activation at 95˚C for 12 minutes followed by 40 cycles of denaturation at 95˚C for 30 seconds, annealing at 60 ˚C for 30 sec and elongation at 72˚C for 1 minute. The final elongation step was set at 72˚C for 10 minutes. Separation was done after PCR amplification from 70 ^{0}C to 99.9 ^{0}C at the rate of 0.5^{0}C/ sec**.** Each run comprised of known positive controls and negative control (PCR mix without nucleic acid template) on which the analysis of amplified PCR products and melt profiles were based. The PCR/HRM analysis was performed on a QuantStudio 3 system (Applied biosystems by Thermo Fisher Scientific).

5.2.7. Detection of *T. evansi* from *Trypanozoon* positive samples

After the initial molecular screening by PCR/HRM, all *Trypanozoon* - positive samples were subsequently analysed to detect *T. evansi.* Subtypes A and B were targeted by PCR in a ProFlex thermocycler (Applied Biosystems by Life technologies). *Trypanosoma evansi* Subtype A was

screened using ILO F/R primers. 10 µL volume reaction containing 3 µL of nuclease-free water, 5 µL of 2X DreamTaq Green Master Mix (Thermo Fisher Scientific), 0.5 µL of each primer at 10 mM concentrations and 1 µL of DNA template. The PCR conditions were as follows: 95°C for 1 min, 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final elongation step of 5 min at 72°C. Eva B1/B2 primers were used to detect *T. evansi* Subtype B in a 10 µL volume reaction containing 3 µL of nuclease-free water, 5 µL of 2X DreamTaq Green Master Mix (Thermo Fisher Scientific), 0.5 µL of each primer at 10 mM concentrations and 1 µL of DNA template. The PCR conditions were 95°C for 1 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, with a final elongation step of 10 min at 72°C.

5.2.8. Tests for human infective *T. brucei rhodesiense*

To assess the presence of human-infective trypanosomes in the cattle blood, all the samples positive for *Trypanozoon* were further subject to PCR with TBR primers (Welburn *et al*., 2001) to confirm their identity. The TBR positive samples were subsequently tested by amplifying the Serum Resistance-Associated (SRA) gene using B537/537 (Welburn *et al*., 2001) and SRA A/E primers (Gibson *et al*., 2002) (Table 3.1). SRA gene is specific for *T. brucei rhodesiense and* confers resistance to survive in human serum. The PCRs were performed in a ProFlex thermocycler (Applied Biosystems by Life technologies) in a 10 µL volume reaction containing 3 µL of nuclease-free water, 5 µL of 2X DreamTaq Green Master Mix (Thermo Fisher Scientific), 0.5 µL of each primer at 10 mM concentrations and 1 µL of DNA template. The PCR conditions for TBR were as follows: 95°C for 3 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, with a final elongation step of 10 min at 72°C. The touchdown PCR was used to amplify the SRA gene with B537/538. The conditions were 95°C for 3 min, followed by 10 cycles of 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 1 min, followed 25 cycles of 94°C for 320 seconds, annealing at 63.8°C for 30 seconds and extension

at 72°C for 1 min per cycle. The final extension was set at 72°C for 7 min. The PCR conditions for SRA A/E were as follows: 95°C for 3 min, 40 cycles of 95°C for 1 min, 68°C for 1 min and 72°C for 1 min, with a final elongation step of 10 min at 72°C.

Table 5. 1: Primers used for the detection of Trypanosoma species from blood samples from cattle and their sources

Primer name	Target gene / specificity	Sequence (5' to 3')	product size / range (bp)	Reference
18S – 3F 18S- 4R	18S rRNA Trypanosomes	GACCRTTGTAGTCCACACTG CCCCCTGAGACTGTAACCTC	199–241	(Hamilton *et al*., 2007)
ILO 7957 ILO 8091	RoTat1.2 VSG *T.evansi* subtype A	GCC ACC ACG GCG AAA GAC TAA TCA GTG TGG TGT GC	530	(Urakawa *et al*., 2001)
Eva B1 EVA B2	*T.evansi* subtype B	CACAGTCCGAGAGATAGAG CTGTACTCTACATCTACCTC	436	(Njiru *et al*., 2006)
TBR 1 TBR 2	*T. brucei*	CGA ATG AAT ATT AAA CAA TGC GCA GT AGA ACC ATT TAT TAG CTT TGT TGC	177 (repetitive)	(Welburn *et al*., 2001)
B537 B538	SRA gene	CCATGGCCTTTGACGAAGAGCCCG CTCGAGTTTGCTTTTCTGTATTTTTCCC	743	(Welburn *et al*., 2001)
SRA A SRA E	SRA gene	GACAACAAGTACCTTGGCGC TACTGTTGTTGTACCGCCGC	460	(Gibson *et al*., 2002)

5.2.9. Sequence analysis

As described under section 4.2.8. Gene sequence Analysis. Briefly, the representative positive amplicons were re-amplified, purified and sent to Macrogen Inc. (Holland) for unidirectional Sanger sequencing. The sequence chromatograms were processed using Geneious prime 20.2.2. NCBI BLASTn was used to identify homologous sequences.

5.2.10. Immunological rapid diagnostic test (VerY Diag)

Among the 1,037 blood samples collected, two hundred ninety-nine (299) were randomly selected and used for a rapid test called VerY Diag (Boulangé *et al*., 2017). The test was done on fresh blood samples after collection. The number of samples was limited to 299 because of the cost of the test kit ($6 per single test). VerY Diag is a lateral flow rapid field test with immune-chromatography designed to detect *T. congolense* and *T. vivax* species. It was

developed using recombinant antigens TcoCB1 (test line Tc, 0.65 mg/mL) and TvGM6 (test line Tv, 1.2 mg/mL) (Boulangé *et al*., 2017). With the help of a pipette supplied in the kit, a drop of whole blood (20 µL) was deposited into the specimen well of the cassette. Immediately, another drop (40 µL) of the dilution buffer was added to the same well, avoiding to drop any solution in the cassette observation window. Results were read after 10 minutes.

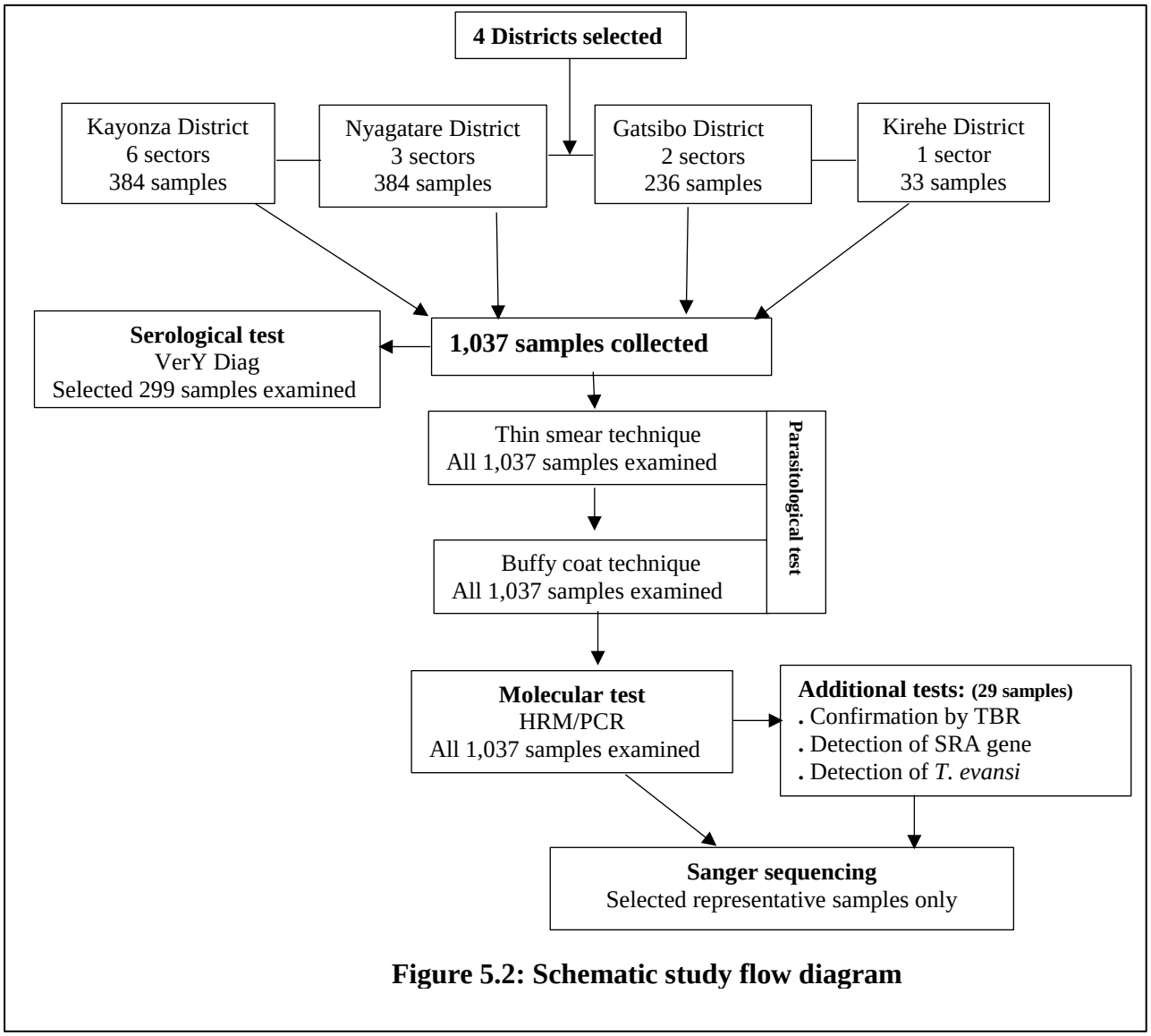


Figure 5.2: Schematic study flow diagram

5.2.11. Data analysis

The data was analysed by descriptive statistics in SPSS software (SPSS Inc., IL, USA). Parametrical tests (ANOVA) were used to compare the Mean PCV and disease prevalence between different areas. The Cohen Kappa test was used to compare the level of agreement between the different diagnostic methods. The significance threshold was fixed at 5% precision, 95% of confidence and a p value of less than 0.05 were considered.

5.3. Results

5.3.1. *Trypanosoma* species detected by parasitological and molecular methods

The overall prevalence of *Trypanosoma* infections by thin smear was 5.6%, of which *T. congolense* accounted for 3.5% (n=37/1037*), T. vivax* 1.9% (n=20/1037), *Trypanozoon* 0.09% (n=1/1037) and the inconclusively identified trypanosomes 0.28% n=3/1037). The Buffy coat technique increased the overall prevalence to 7.1% of which 5.1% (n=53/1037) were *T. congolense*, 2.4% (n=25/1037) of *T. vivax*, 0.09% (n=1/1037) of *Trypanozoon* and 0.86% (n=9/1037) of inconclusive trypanosomes. All the inconclusive trypanosomes were suspected to be non-pathogenic, and they were later identified as *T. theileri* by PCR and by sequencing. No mixed infections were detected by microscopy. The overall prevalence of pathogenic trypanosomes detected by PCR/HRM was 18.7% (n=194/1,037). Of these, *T. congolense* represented 10.7% (n=111/1,037), *T. vivax* 5.2% (n=54/1,037), *Trypanozoon* 2.8% (n=29/1,037). *Trypanozoon* - positive samples tested by PCR/HRM were subjected to specific primers for *T. brucei brucei* and *T. evansi*, which later gave *T. brucei brucei* 2% (n=21/1,037) and *T. evansi* 0.7% (n=8/1,037) (Table 5.2). The 8 samples positive to *T. evansi* were detected by ILO primer, and are therefore *T. evansi* sub-type A. All samples were negative to Eva B1/B2 primer targeting sub-type B, meaning no *T. evansi* sub-type B was detected in samples examined. The non-pathogenic *T. theileri* represented 8% (n=83/1,037).

Among the above infections, eight mixed infections (0.7%; n=8/1037) were found, comprising four infections of *T. brucei brucei* and *T. congolense,* 2 infections of *T. vivax* and *T. theileri,* one infection of *T. congolense* and *T. vivax,* and one infection of *T. congolense* and *T. evansi*. As per PCR/HRM results, *T. congolense* and *T. vivax* were dispersed throughout the three districts. No *T. vivax* was found in Kirehe and *T. brucei brucei* infections were concentrated in Nyagatare. The non-pathogenic *T. theileri* was more prevalent in Gatsibo and Kayonza. Figures 5.3 and 5.4 show the spatial distribution of trypanosomal infections by diagnostic methods across the study area.

Table 5. 2: Comparative trypanosomal infections by different diagnostic tests

District	Sector	NE	Thin smear						Buffy coat technique						PCR/HRM						
Kayonza			*Tc*	*Tv*	*Tz*	**Over. Prev.**	U	*Mixed*	*Tc*	*Tv*	Tz	**Over. Prev.**	*U*	*Mixed*	*Tc*	*Tv*	*Tbb*	Te	**Over. Prev.**	*T. th*	Mixed
	Ndego	70	5	2	0	**(10%)**	0	0	7	2	0	**(12.8%)**	0	0	10	6	0	2	**(25.7%)**	0	1(Tv+T.th)
	Kabale	6	0	1	0	**(16.6%)**	0	0	0	1	0	**(16.6%)**	0	0	0	1	0	0	**(16.6%)**	0	-
	Rwinkwavu	10	0	1	0	**(10%)**	0	0	0	1	0	**(10%)**	0	0	0	4	0	0	**(40%)**	0	-
	Mwiri	80	1	1	0	**(2.5%)**	2	0	1	1	0	**(2.5%)**	3	0	3	7	0	0	**(12.5%)**	16 (20%)	-
	Gahini	89	3	0	0	**(3.3%)**	0	0	5	1	0	**(6.7%)**	1	0	8	2	0	1	**(12.3%)**	5 (5.6%)	1 (Tc+Te)
	Murundi	129	6	1	0	**(5.4%)**	0	0	16	1	0	**(13.1%)**	0	0	42	8	1	1	**(40.3%)**	12 (9.3%)	1(Tc+Tv)
	subtotal	***384***	***15***	***6***	***0***	***(5.4%)***	***0***	***0***	***29***	***7***	***0***	**(9.3%)**	***4***	***0***	***63***	***28***	***1***	**4**	**(25%)**	***33 (8.6%)***	
Gatsibo	Rwimbogo	190	1	3	0	**(2.1%)**	0	0	2	6	0	**(4.2%)**	0	0	3	12	0	0	**(7.9%)**	18 (9.4)	1(Tv+T.th)
	Kabarore	46	0	0	0	**(0%)**	1	0	0	0	0	**(0%)**	3	0	0	0	2	0	**(4.3%)**	19 (41.3%)	-
	Subtotal	***236***	***1***	***3***	***0***	***(1.7%)***	***1***	***0***	***2***	***6***	***0***	**(3.4%)**	***3***	***0***	***3***	***12***	***2***	**0**	**(7.2%)**	***37 (15.6%)***	
Nyagatare	Karangazi	174	18	9	0	**(15.5%)**	1	0	17	10	0	**(15.5%)**	1	0	37	13	8	4	**(35.6%)**	4 (2.3%)	4 (Tb+Tc)
	Rwimiyaga	186	3	2	1	**(3.2%)**	1	0	5	2	1	**(4.3%)**	1	0	7	1	5	0	**(7%)**	5 (2.7%)	-
	Matimba	24	0	0	0	**(0%)**	0	0	0	0	0	**(0%)**	0	0	1	0	4	0	**(20.8%)**	0	-
	Subtotal	***384***	***21***	***11***	***1***	***(8.6%)***	***2***	***0***	***22***	***12***	***1***	**(9.1%)**	***2***	***0***	***45***	***14***	***17***	**4**	**(20.8%)**	***9 (2.3%)***	
Kirehe	Mpanga	33	0	0	0	**(0%)**	0	0	0	0	0	**(0%)**	1	0	0	0	1	0	**(3%)**	4 (12.1%)	-
	Subtotal	***33***				**(0%)**		**0**				**(0%)**		**0**			**1**	**0**	**(3%)**	**4 (12.1%)**	
	Total	**1037**	**37**	**20**	**1**	**(5.6%)**	**3**	**0**	**53**	**25**	**1**	**(7.1%)**	**9**	**0**	**111**	**54**	**21**	8	**(18.7%)**	**83(8%)**	**8 (0.7%)**

NE=Number of animals examined; Tc=*Trypanosoma congolense*; Tv = *Trypanosoma vivax*; Tz=Trypanozoon; U=unidentified; *T. brucei brucei = Trypanosoma brucei brucei, Te= Trypanosoma evansi, T.th= Trypanosoma theileri, Over. Prev.* = Overall prevalence. The overall prevalence does not include infection with the non-pathogenic *T. theileri.* Mixed infections are included in overall prevalence as single infections

Non-pathogenic trypanosomes were mostly found in areas of Gatsibo 15.6% and Kirehe (12.1%) districts, but also some parts of Kayonza (8.6%).

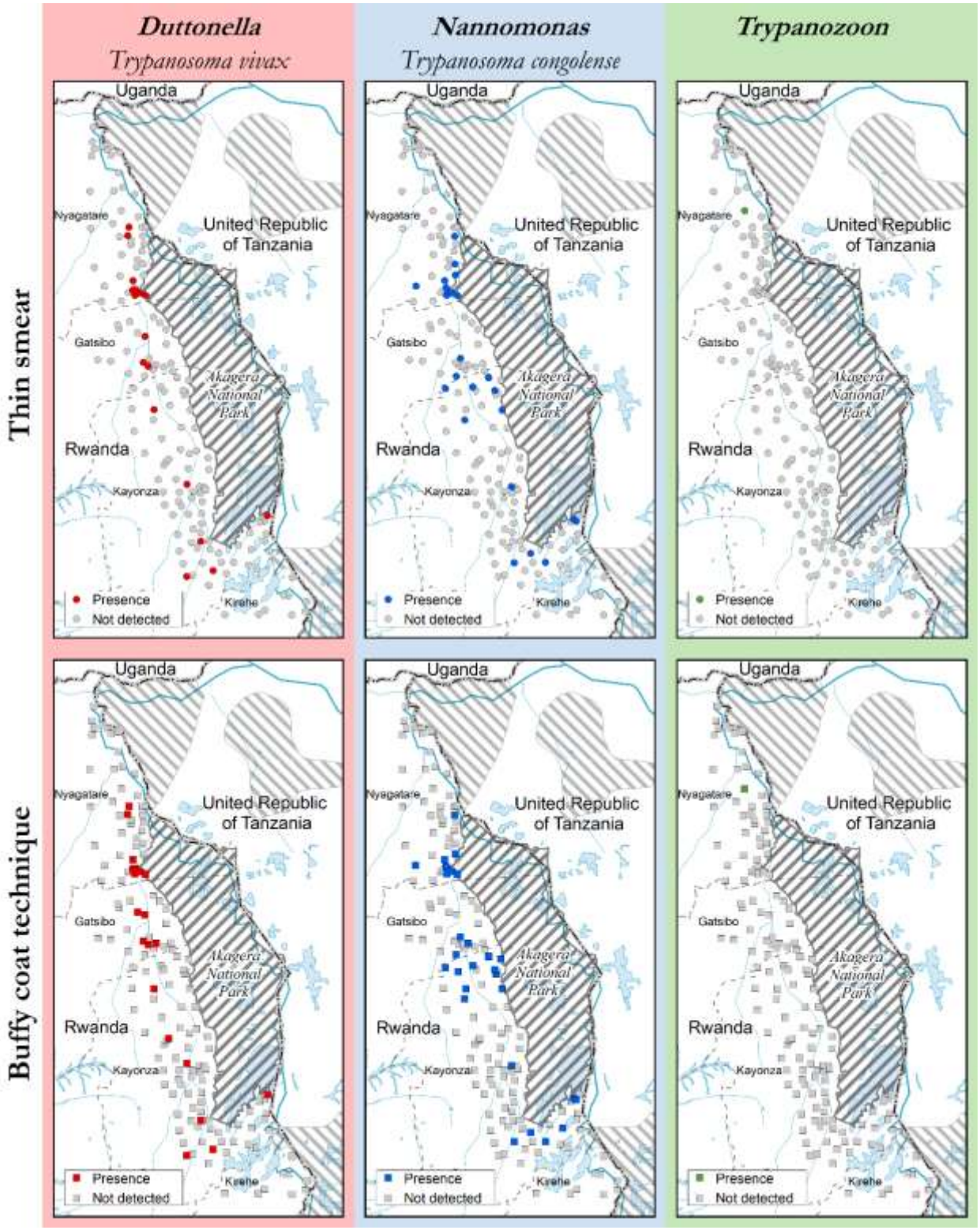


Figure 5.3: Distribution of trypanosomes detected by thin smear and Buffy coat technique

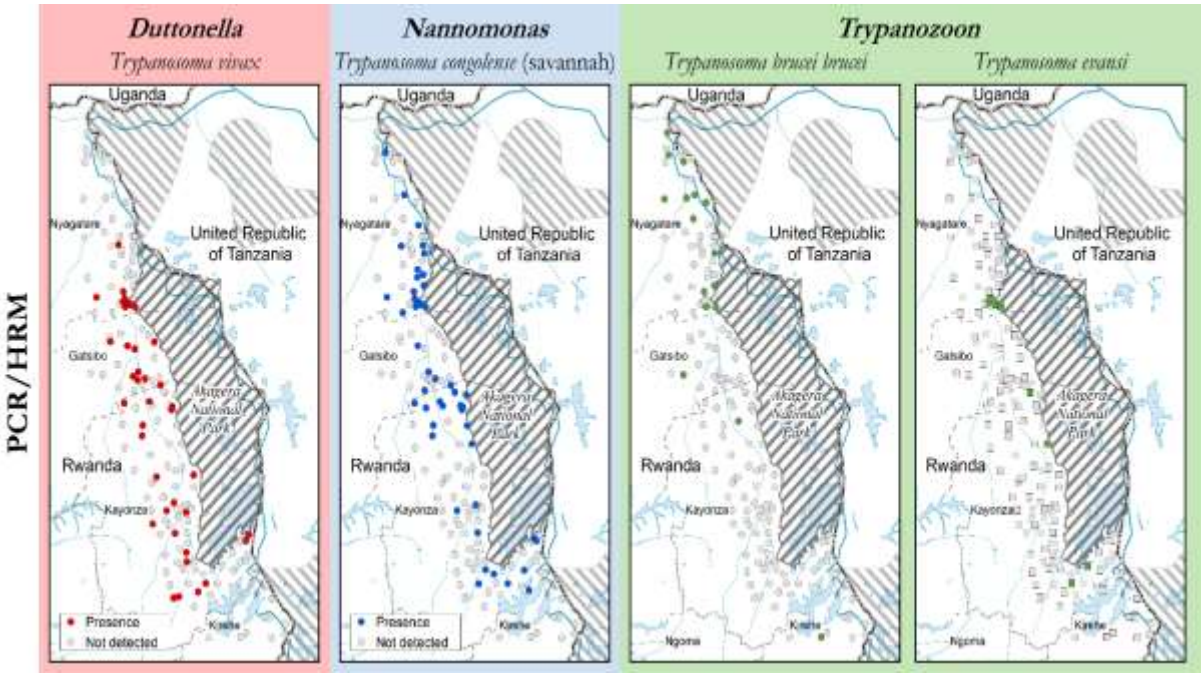


Figure 5.4: Distribution of pathogenic trypanosomes detected by PCR/HRM

By comparing infection status between breeds, indigenous Ankole cattle were more infected by *T. congolense* (15.7%; 82/521) and *T. vivax* (7.3%; 38/514) than crossbreed Ankole x Friesians (p=0.000). However, more *Trypanozoon* species were found in crossbreed Ankole x Friesians than indigenous Ankole (Table 5.2). The occurrence of non-pathogenic infections (*T.theileri*) between the two breeds was very similar. The number of pure Friesian cattle was too small to be considered in comparison, therefore not included in this table.

Table 5. 3: Other predictors of infection with different trypanosome species [Positive by HRM-PCR]

Predictor	**NE**	**Pathogenic infections**					**Non-pathogenic**	**Mixed infections**			
Breed		***Tc***	***Tv***	***Tbb***	***Te***	***Total***	***T.th***	***Tbb +Tc***	***Tc+Tv***	***Tc+Te***	***Tv+T.th***
Ankole	521	82 (15.7%)	38 (7.3%)	8 (1.5%)	4 (0.7%)	132 (25.3%)	42 (8%)	2 (0.38%)	1 (0.2%)	1 (0.2%)	0
Ankolex Friesian	514	29 (5.6%)	16 (3.1%)	13 (2.5%)	4 (0.7%)	62 (12%)	41 (7.97%)	2 (0.38%)	0	0	1 (0.2%)
Friesian	2	0	0	0	0	0	0	0	0	0	0
Sex											
Female	946	105 (11.1%)	43 (4.5%)	20 (2.1%)	8 (0.8%)	176 (18.6%)	78 (8.2%)	4 (0.4%)	1 (0.1%)	1 (0.1%)	2 (0.2%)
Male /Neutered	91	6 (6.5%)	11 (12.1%)	1 (1.08%)	0	18 (19.8%)	5 (5.4%)	0	0	0	0
Age											
< 2 years [Young]	161	11 (6.8%)	16 (9.9%)	1 (0.6%)	0	28 (17.4%)	10 (6.2%)	0	0	0	0
>2years [Adults]	876	100 (11.4%)	38 (4.3%)	20 (2.2%)	8 (0.9%)	166 (18.9%)	73 (8.3%)	4 (0.4%)	1 (0.1%)	1 (0.1%)	2 (0.2%)
Overall	**1037**	**111 (10.7%)**	**54 (5.2%)**	**21 (2%)**	**8 (0.7%)**	**194 (18.7%)**	**83 (8%)**	**4 (0.38%)**	**1 (0.1%)**	**1 (0.1%)**	**1 (0.1%)**

NE=Number of animals examined; Tc=*Trypanosoma congolense*; Tv = *Trypanosoma vivax*; *Tbb = Trypanosoma brucei brucei; Te= Trypanosoma evansi, T.th= Trypanosoma theileri.* Non- pathogenic *T. theileri* cases are counted separately. Mixed infections are counted in single infections.

The overall prevalence in Ankole breeds was 25.3% and 12% for crossbreed Ankole x Friesians.

As shown in Fig 5.5, non-pathogenic trypanosomes were more prevalent in Gatsibo and Kayonza districts than in the north (Nyagatare district) and south (Kirehe district). HRM melt curve profiles and their alignment (Figure 5.6) were the basis of the identification of trypanosomes.

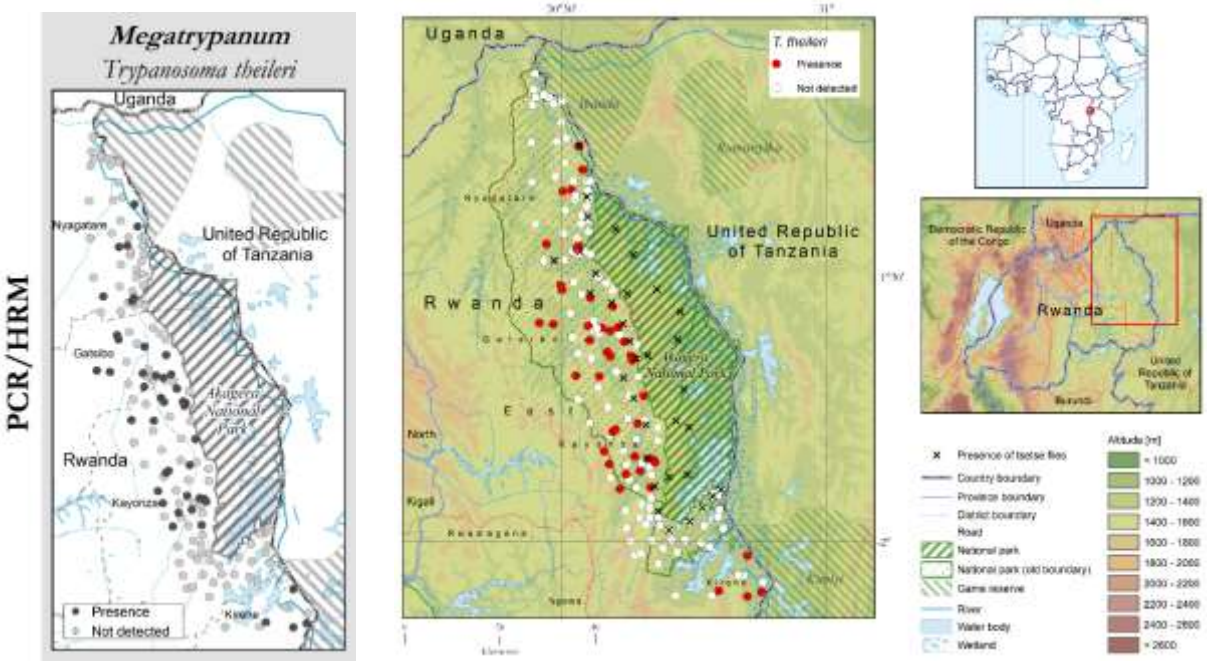


Figure 5.5: Distribution of non-pathogenic trypanosomes across the study area

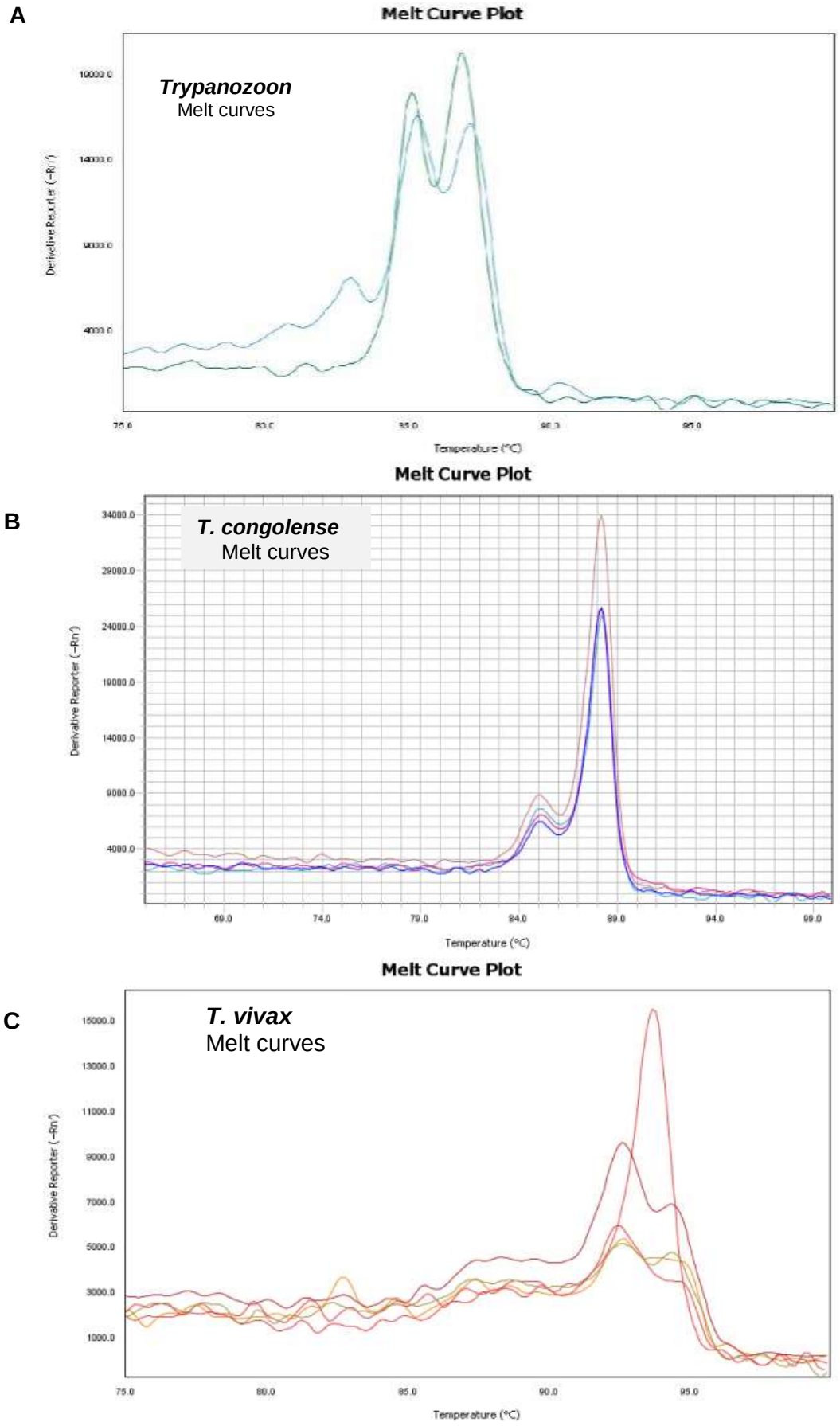


Figure 5. 6: Melt curve plots for representative positives

A: T. brucei s.l. *B: T. congolense* *C: T. vivax*

5.3.2. Correlation of packed cell volume with *Trypanosoma* infections

The overall mean PCV of infected animals was 29.5 for PCR and 28.5 for microscopy compared to 30.4 and 30.3 observed in non-infected animals, respectively for PCR and microscopy. This difference did not show any significant effect on the trypanosome infection for PCR (p>0.164) and microscopy results (p>0.212). There was even a negative Pearson correlation between the PCV values of negative and the positive results (r= -0.007). PCV was not determined for 2 animals due to the poor quality of the blood after centrifugation, however, no trypanosomes were detected in those animals for all the methods used. Lower and higher PCV values were seen in either group. Looking at individual trypanosome species, the PCV values of infected animals were grouped in thresholds where the PCV of 26% and less was considered anaemic (Table 4). For many positive cases, the PCV was above the threshold of 26%. There was no statistical difference between the two groups of infected animals (p>0.162) and (p>0.212), respectively for PCR and microscopy.

Table 5. 4: Correlation between cattle PCV and trypanosome species according to thresholds

PCR/HRM				
Species	**Mean PCV**	**<26%**	**>26%**	**Sum**
T. brucei brucei	29.7	7	14	21
T. evansi	28.2	3	5	8
T. congolense	29.2	39	72	111
T. vivax	28.9	18	36	54
T. theileri	30.4	19	64	83
Mixed infections	27.3	3	5	-
Total	*NA*	86	191	277
P value	0.162			
Microscopy (Buffy coat technique)				
T. congolense	28.4	22	31	53
T. vivax	27.2	11	14	25
Trypanozoon	31	0	1	1
Unidentified	32.1	1	9	10
Mixed infections	-	0	0	
Total	NA	34	55	89
P value	0.212			

NA=Not applicable, mixed infections were counted in single infections

Animals with mixed infections seem to have a lower mean PCV (27.3), followed by the single infections of *T. vivax* (28.9) even though this difference was not statistically significant. For microscopy, 34 positives were below 26 and 55 positives above 26 of PCV.

5.3.3. Genetic diversity of trypanosome species

The 18S rRNA study sequences of *T. congolense* showed similarity of between 98.71-99.56% with GenBank accession: AJ223563.1(Cattle) and 98.24 – 99.14% with AJ009146.1 (Goat) and U22315.1 - IL1180 (Cattle), all of them being *T. congolense savannah* from Kenya. The study found no *T. congolense* forest and *T. congolense* Kilifi subspecies. *T. vivax* representatives from this study showed 100.00% similarity with IL3905 GenBank DQ317414 (cattle, Kenya) and 100% with GenBank: KM391821 (cattle, Ethiopia). The two GenBank similarities correspond to the TvL1-G genotype (West Africa & East Africa) of *T.vivax*. *Trypanosoma theileri* representatives showed 100.00% similarity with the GenBank accession: KF924256 (cattle, Poland) and AJ009163 (UK). The BLAST results of the representative sequences from *Trypanozoon* gave the highest similarity (100%) to 2 or more species of the group such as *T. brucei brucei* XR002989632 from the UK and *T. evansi* MN446740.1 from China. The amplicons size was not long enough to definitely resolve them. The nucleotide sequences from this study were deposited to the GenBank database under the following accession numbers OK264415, OK264416, OK264417 (*T.brucei*), OK264418, OK264419 (*T.congolense*), OK264420, OK264421 (*T.theileri*), OK264422, OK264423 (*T.vivax*) (Figure 5.7). Divergence estimates are shown in Table 5.5.

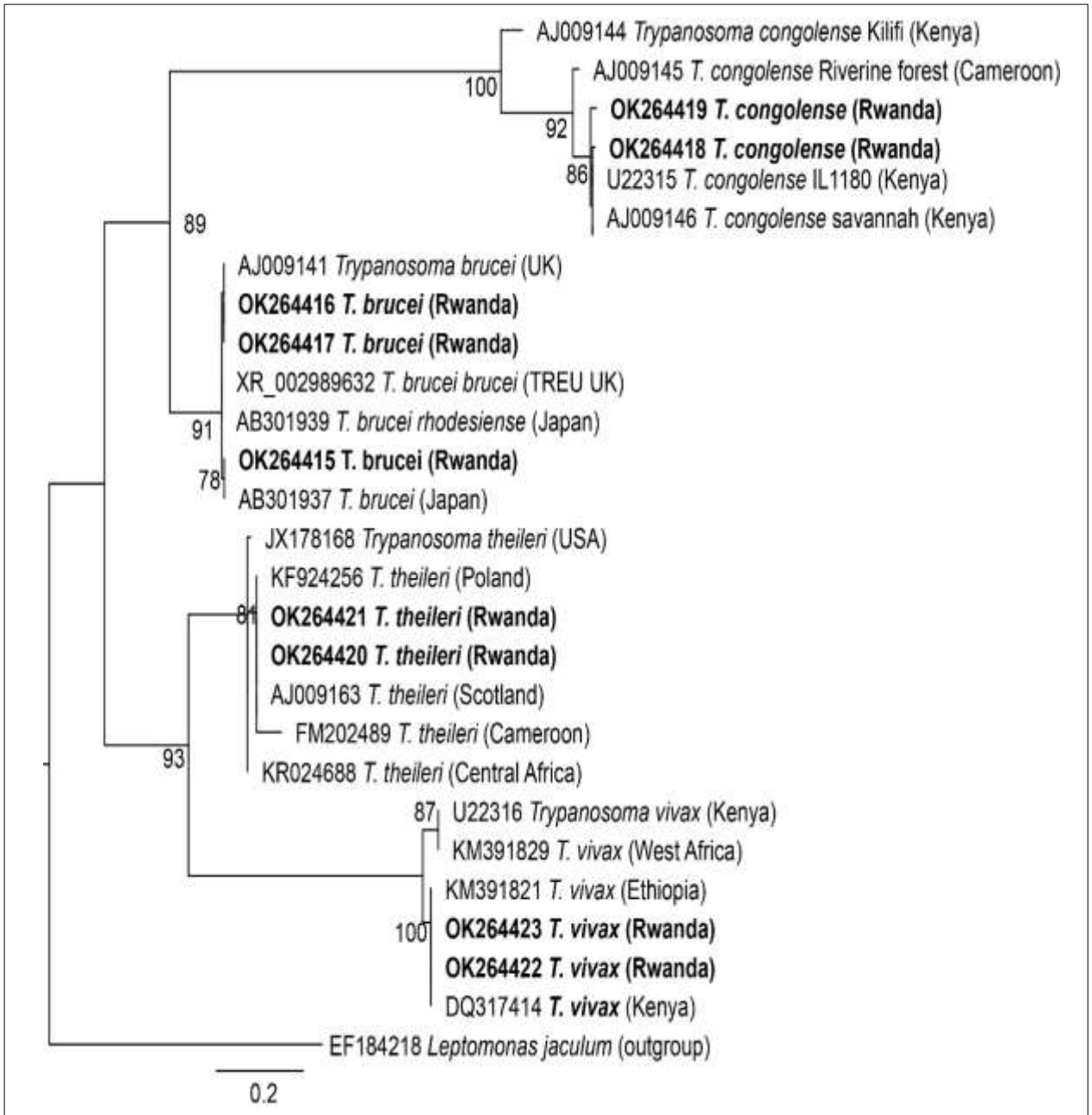


Figure 5.7: Maximum likelihood phylogeny of *Trypanosoma spp*. based on partial 18S rRNA gene.

GenBank accession numbers and country of origin are indicated for each sequence. Sequences from this study are in bold. Bootstrap values at the major nodes are of percentage agreement among 1,000 replicates. The tree is rooted to outgroup sequence EF184218.

Table 5. 5: Evolutionary divergence estimates between *Trypanosoma spp*. of this study and the sequences and related GenBank sequences

Species	Generated sequences	GenBank similarity ID (references)	p-distance
Trypanosoma brucei	OK264415	AB301937 - Japan	0.000
Trypanosoma brucei	OK264416	AJ009141 - UK	0.000
Trypanosoma brucei	OK264417	XR002989632 - UK	0.000
Trypanosoma congolense	OK264418 & OK264419	U22315 (Savannah – IL1180) - Kenya	0.000
		AJ009146 (Savannah) - Kenya	0.000
		AJ009145 (Riverine forest) - Cameroon	3.000*
		AJ009144 (Kilifi) - Kenya	17.000*
Trypanosoma theileri	OK264420 & OK264421	AJ009163 – Scotland	0.000
		FM202489 – Cameroon	0.000
		KF924256 - Poland	0.000
Trypanosoma vivax	OK264422 & OK264423	DQ317414 (*TvL1-G*enotype) - Kenya	0.000
		KM391821 (*TvL1-G*enotype) - Ethiopia	0.000
		U22316 (*Tvv1-G*enotype) - Kenya	5.000*
		KM391829 – West Africa	5.000*

*****The p-value above 1 mentioned here reflects a strain of species that is a mismatch with our sequences

5.3.4. Detection of SRA gene in cattle

Out of 29 samples tested positive for *Trypanozoon* by PCR/HRM, 21 were positive to the TBR primer, however negative for *T. evansi*. They were, therefore, classified as *T. brucei brucei*. None of the 21 TBR positive samples tested positive for the SRA gene by using either SRA A/E or B537/538 primers. This means that no causative agent of *rhodesiense* sleeping sickness was found in the cattle blood analysed.

5.3.5. Immunological rapid test results (VerY Diag)

Out of 299 animals examined using the VerY Diag rapid test, 296 showed conclusive results while 3 cassettes showed indecisive results. 19 (6.4%) showed antibodies to *T. congolense*, 77 (26%) to *T. vivax* and while 88 (29.7%) animals showed antibodies to both *T. congolense* and *T. vivax*. 112 samples (37.8%) did not show trypanosome antibodies (Table 5.6). Fig 5.8 shows

the example for the results of the rapid diagnostic test (VerY Diag), and their distribution is shown in Fig. 5.9. The VerY Diag cassettes technology does not cross-react with *Trypanozoon* species.

Table 5. 6: Summary of VerY Diag test results

VerY Diag test	**NE**	***T.co***	***T. vivax***	***T.co +T. vivax***	**Total infections**	**Negative**	**Not specific**
	299	19	77	88	184	112	3
%		6.4%	26%	29.7%	62.1%	37.8%	1%

NE=Number of animals examined; T.co=*Trypanosoma congolense*; T. vivax = *Trypanosoma vivax*; Mixed *infections counted separately from single infections*

(A) No Ab detected **(B) *T. congolense* Ab** **(C) *T. vivax* Ab** **(D) Cross reaction: Ab for *T. congolense* and for *T. vivax***

Figure 5.8: Rapid test cassettes used in the field

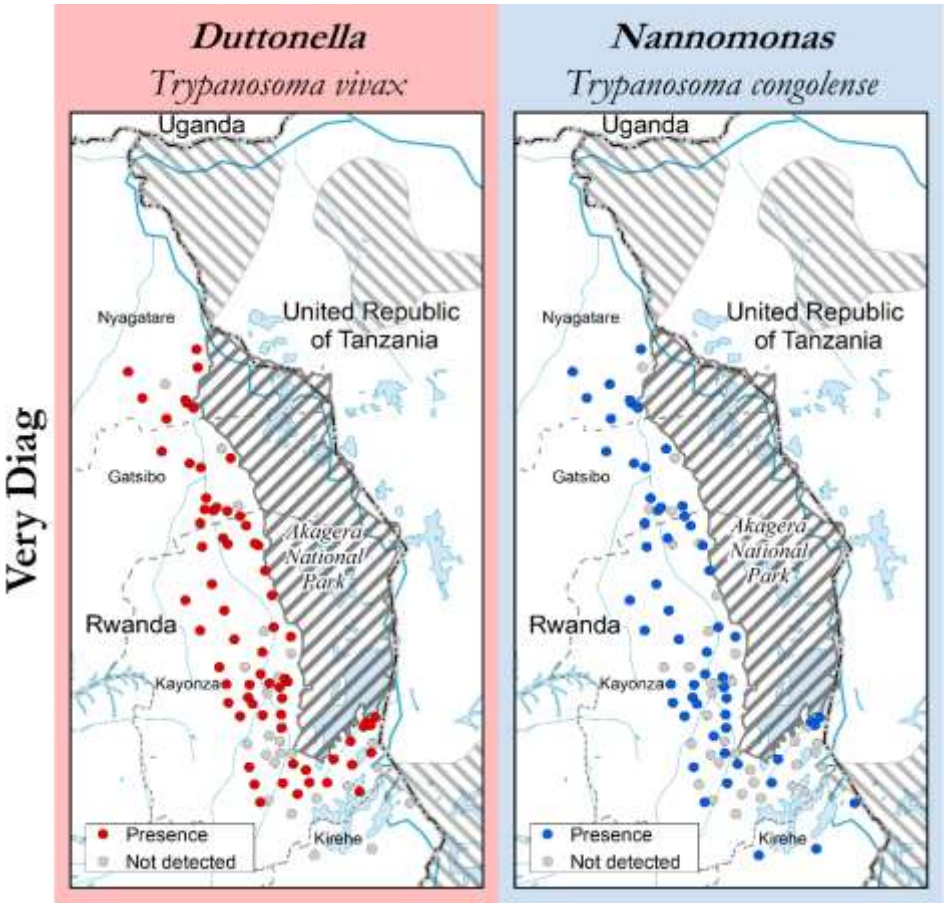


Figure 5.9: Distribution of trypanosomes antibodies detected by VerY Diag

5.3.6. Comparison between diagnostic tests

The thin smear and Buffy coat technique detected few trypanosome infections, however, the buffy coat technique showed a higher sensitivity as compared to the thin smear. Apart from the increased number of positive cases, PCR/HRM detected mixed infections and considerably more *Trypanozoon* infections. Using the PCR/HRM results as a reference, the sensitivity and specificity of the other diagnostic tests are shown in Table 5.7.

Table 5. 7: Sensitivity and specificity of different detection tests used

Test	NE	Positives (n)	Negatives (n)	Infection rate %	Sensitivity % (95% CI)	Specificity % (95% CI)
Thin smear	1037	61	976	5.9	28.9	99.1
Buffy coat	1037	88	949	8.4	40	98.6
VerY Diag	299	184	115	61.5	86	32.5
qPCR/HRM (Reference test)	1037	277	767	26.7	NA	NA

All positive cases are inclusive (pathogenic and non-pathogenic) N=number, NA= not applicable

Thin smear and Buffy coat technique increase the false negatives hence the low sensitivity while VerY Diag resulted in poor specificity. The Cohen Kappa test showed an increased level of agreement between the thin smear and Buffy coat technique (K= 0.807), and a low agreement between thin smear and PCR/HRM (K=0.310). An agreement coefficient of K=0.424 was found between the Buffy coat technique and PCR/HRM. The immunological rapid test (VerY Diag) only detects antibodies of *T. congolense* and *T. vivax*. The Cohen Kappa test was, therefore, run merely for the two species when compared with other methods. There was a very low agreement of K=0.037, K=0.042, K=0.031 between the thin smear, Buffy coat technique, and PCR/HRM respectively.

5.4. Discussion

This study determined the diversity of trypanosomes circulating in cattle around the Akagera NP in Rwanda and found common pathogenic trypanosomes for cattle (i.e. *T. congolense, T. vivax*, *T. brucei* and *T. evansi*) and the non-pathogenic *T. theileri*. The findings on pathogenic trypanosomes are in line with what was reported in the area by Mihok *et al*., (1992). This is the first report of *T. evansi* in cattle in Rwanda. *Trypanosoma congolense* was the most abundant species, followed by *T.vivax* and Trypanozoon as the least abundant. In particular, *T. congolense* and *T. vivax* seem to be closely associated with the park and its tsetse-infested boundaries (Gashururu *et al*., 2021).

The study noted a cluster of *Trypanozoon* species in the north of Akagera NP (Figure 5.3). The north of the park has a higher concentration of wild animals (Macpherson, 2013; Macpherson, 2019) and the same is the case for livestock around the interface. A concomitant study found higher densities of tsetse flies (Gashururu *et al*., 2021) and other biting flies (stomoxys, etc) were observed in the same area during the data collection, which suggests increased transmission in the north. There might be some preferred hosts harbouring *Trypanozoon* species on which the tsetse flies and other biting flies are feeding.

Trypanosoma evansi has a wide host range (OIE, 2013) and similar sub-type A was isolated from camels and buffalo in the region (Kamidi *et al*., 2017). Regionally, this trypanosome species was extensively found and studied in Kenya (Kamidi *et al*., 2017, Njiru *et al*., 2004). The non-pathogenic *T. theileri* was found in cattle reared around Akagera NP, as it was also reported previously by Mihok *et al*., (1992). This benign parasite was found in cattle in Uganda (Matovu et al., 2020) and other African regions (Ngomtcho *et al*., 2017). The parasite was mainly detected in Gatsibo and Kayonza districts (Figure 5.4). *Trypanosoma theileri* is in the stercorarian group, under the subgenus *Megatrypanum*. The *Trypanosoma theileri* group comprises three species hardly discernible but which are host specific: *T. theileri* for bovine, *T. melophagium* for ovine and *T. cervi* for deer. The host specificity helps, in addition to the sequencing data to identify these parasites (Calzolari *et al*., 2018). *Trypanosoma theileri* is transmitted between wild and domestic animals by biting flies such as tabanids and *Stomoxys*. Despite the presence of mechanical vectors in the area, there should be increased interaction between livestock and some *T. theileri*-specific wild hosts from the park side. Further investigation on this is recommended to evaluate the current pathogenic effect of *T. theileri* group on cattle and /or other livestock species' health.

Apart from tsetse flies, biting flies such as tabanids and *Stomoxys* were observed and are good mechanical vectors of some *Trypanosoma* species. The presence of *T. evansi* and *T. theileri* in

cattle blood showed the importance of other blood-feeding flies in mechanically transmitting the trypanosomes in the area. *Trypanosoma vivax* can also be transmitted in this way. This indicates a possible role of AAT mechanical transmission in the area, even though the mechanically transmitted trypanosomes cannot survive long outside the host (OIE, 2013). Biting flies were collected in the area and the related data will be presented elsewhere.

As in many similar studies, the microscopy showed low sensitivity in detecting trypanosomes as compared to molecular techniques (Paguem *et al*., 2019). However, the microscopy specificity is still high. The low sensitivity results from subclinical infections expressing low levels of parasitaemia in infected animals, which are not detected by microscopy. Notwithstanding the consistency, the buffy coat technique confirms its higher sensitivity in that it detected more infections than a thin smear. This was slightly more evident for *T. congolense* infections. Microscopy failed to detect almost all the *Trypanozoon* and *T. theileri*. This could be because of very low parasitemia and affinity for tissues by *T. brucei*, while *T. congolense* and *T. vivax* are mainly intravascular parasites (OIE, 2013). At microscopy, infections of *T. theileri* were suspected and not identified. However, their identity was later confirmed as *T. theileri* by HRM and sequencing.

Although sensitive, PCR missed some positive cases of trypanosomes that were detected by microscopy. This could be due to the quantity and or quality of parasitic nucleic acid extracted. PCR detects trypanosomes DNA in a sample and is much sensitive compared to other routine diagnostic techniques, of clinical, subclinical and chronic infections for both pathogenic and non-pathogenic trypanosomes (Simwango *et al*., 2017).

A higher infection rate was found in Kayonza. This is probably because the district has the longest interface area with ANP compared to other districts. There were lower infections in Ankole x Friesians than Ankole cattle. Farmers tend to care more for the improved cattle than

Ankole ones, hence spending more money and time on disease treatment and prevention. This could be the reason why the trypanosome infections were lower in Ankole x Friesians in the study area. However, no previous data showed different susceptibility to trypanosome infection between these types of cattle breeds in the area to compare with our findings.

The PCV values were not linked to trypanosome infections. Some cattle with PCV below 26 were negative while others with the PCV values higher than 26 were positive for trypanosomes. The same observation was reported in Uganda (Matovu *et al*., 2020). Contrarily in endemic areas, cattle with the PCV of 26 and below are usually considered infected as a result of anaemia associated with the disease severity (Marcotty *et al*., 2008). This could be due to low parasitaemia or simply other health conditions and malnutrition (Simukoko *et al*., 2011), (Takeet *et al*., 2013). The infection cases found below the threshold could be associated with severe disease (high parasitaemia). Again, the constant use of trypanocides by farmers (own observations and personal communication with farmers) was observed in the area during the study and could prevent the disease severity, hence the absence of anaemia.

This study had the following limitations:

1. The study was designed to target mainly the risk areas for *Trypanosoma* infections around Akagera NP and a few areas around the adjacent game reserves in Tanzania. A broader study area targeting distant localities and including others along the game reserves would help to understand the impact of the distance to Akagera NP. The role of the nearby game reserves in the transmission of trypanosomes in the area would be understood as well.
2. Although this study serves as a basis, it was only limited to one livestock species (cattle). Further investigations in other animal species and research on transmission dynamics would shed light on the full picture of trypanosomes circulating. This will contribute to a better understanding of the disease epidemiology in this setting.

3. The study was also limited by the lack of data on trypanosome infections for the rest of Rwandan territory. This affected the comparison of the current findings with the previous at a national level.

5.5. Conclusions

1. Four pathogenic species of *Trypanosoma* (*T. congolense, T. vivax*, *T. brucei* and *T. evansi*) and one non-pathogenic species (*T. theileri)* were detected in cattle in the study area.
2. The most prevalent species was *T. congolense*, which is considered the most pathogenic for cattle in sub-Saharan Africa.
3. No human-infective *T. b. rhodesiense* were detected in the study area.
4. There was a poor correlation between PCV and *Trypanosoma* infections in cattle, which indicates that estimation of PCV may not always be a good indicator of the infections as it could be linked to other health conditions, which should be studied.
5. The presence of *T. evansi* and *T. theileri*, which are *Trypanosoma* species transmitted mechanically by blood-feeding flies other than *Glossina*, showed the possible importance of such flies in the study area. This requires further investigation.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1. General discussion

This study was undertaken to determine (i) the distribution of species of *Glossina* (ii) the *Trypanosoma* species circulating in tsetse flies, their infection rate, and the endosymbionts, (iii) the hosts' preference for the tsetse flies, and (iv) the *Trypanosoma* species circulating in cattle, at the wildlife-livestock interface of Akagera National Park in Rwanda. It was hypothesised that (1) Tsetse flies are abundant in the study area and *Trypanosoma* infections are prevalent in cattle around the park, (2) Tsetse flies harbour human infective trypanosomes, but cattle do not, and (3) cattle is the most preferred host for blood meals of the tsetse flies.

This study shows an important shrink in tsetse distribution outside the current park as opposed to the old distribution maps (Evens and Meyus, 1957; Ford, 1977; Mihok *et al*., 1992). One of the major reasons for this is that the majority of the previously reported infested area and half of Akagera Park was rezoned for human settlement and farming activities (Hajabakiga, 2004; Sun *et al*., 2018). This has led to changes in land use, habitat break-up, and increased human activities which in turn reduce tsetse distribution in an area, especially for species of the savannah group (Cecchi *et al*., 2008; Lord *et al*., 2018). This situation has been the norm for many areas in sub-Saharan Africa, where the edges of protected areas are experiencing demographic pressure (Wittemyer *et al*., 2008). A similar reduction in *G. morsitans* populations was reported in eastern and southern Africa, including Zambia (Ducheyne *et al*., 2009; Mweempwa *et al*., 2015) and Malawi (Gondwe *et al*., 2009). The lack of suitable habitats across the interface could explain such decline around Akagera NP (Lord *et al*., 2018). This pattern is also apparent in western Africa, where tsetse flies of the *morsitans* group are increasingly found only in remnant populations, mostly associated with protected areas (Diarra *et al*., 2019; de Gier *et al*., 2020).

In a survey conducted in 2012 in Tanzania, the game reserves of Ibanda and Kimisi were found to be infested with *Glossina morsitans* and characterized as high-risk tsetse infested areas. The same survey characterized an area neighbouring Kimisi, a game-controlled area as a Non-Tsetse infested area, possibly due to a lower concentration of wild animals, the source of blood meals for tsetse flies (Daffa *et al*., 2013). All three areas are at the border with Rwanda and adjacent to Akagera NP. The linkage between the protected areas in Tanzania and Akagera NP in terms of tsetse infestation is a reminder of the transboundary nature of the trypanosomosis problem. The tsetse challenge to livestock and humans remains at the interface with the park where the community of farmers is settled and cattle are reared in big numbers. Van den Bossche *et al*., (2010) described a similar scenario, and the same was found around Serengeti National Park in Tanzania (Lord *et al*., 2018). The distance to the Akagera NP boundary, type of vegetation, and land use seemed to be the factors determining the abundance of tsetse flies at the interface area as was confirmed by Salekwa *et al*., (2014). Among the three strata used at the interface, large numbers of *Glossina* were observed in Nyagatare District. The latter district is located in the north of the park where there is a high concentration of wild animals (Macpherson, 2019) and high densities of livestock, which contributes to making the area a more suitable tsetse habitat.

The abundance of *G. pallidipes* goes in line with the situation in several other countries in the eastern and southern Africa regions (Ngari *et al*., 2020; Daffa *et al*., 2013; Shereni *et al*., 2016). This species is the most widely distributed tsetse species in those regions, hence the main vector of AAT and potentially the vector of *T. b. rhodesiense* sleeping sickness (Bateta *et al*., 2020). However, recent studies highlight the genetic modifications amid *Glossina* populations (Okeyo *et al*., 2017) due to environmental changes (Moore and Messina, 2010; Malele *et al*., 2011a; Wamwiri and Changasi, 2016), which could lead to the dominance and adaptations of certain species in a region. This needs to be assessed in the Akagera region as well to locate isolated

tsetse populations. This knowledge could help to assess the risk of reinvasion and inform about the feasibility and desirability of eliminating tsetse-transmitted trypanosomes (Adam *et al*., 2014; Bouyer *et al*., 2015).

G. pallidipes was more infected (14.4/25.6%) than *G. m. centralis* (12.7/19%). Regionally, this infection rate is higher compared to the neighbouring Tanzania: 6% in Rufiji district (Malele *et al*., 2011b), 3% in Simanjiro for *G. swynertonni, G. m. morsitans* and *G. pallidipes*. and 8.8% in Maasai steppe (highest in *G. swynnertoni* and lowest in *G. pallidipes*) (Ngonyoka *et al*., 2017). It is still higher to 10.7% *G. fuscipes fuscipes* in North-Western Uganda (Opiro *et al*., 2021) and 2.4% (1.8% for *pallidipes*) in Maasai Mara National Reserve, Kenya (Makhulu *et al.*, 2021). However, these findings are lower when compared to two game reserves of Mozambique by Garcia *et al*., (2018). *G.morsitans morsitans* had 77.6%/90.5% and *Glossina pallidipes* with 22.4%/9.5% respectively for Gorongosa National Park and Niassa National Reserve.

This study provides the first report of *T. evansi* in cattle in Rwanda. The park shelters the known reservoirs of trypanosomes such as buffaloes and warthogs. Tsetse flies freely feed on both wild animals and livestock. The open grazing management adopted favours the transmission of trypanosomes. The park can be considered presumably as a block of tsetse infestation. However, tsetse-transmitted trypanosomes may be found in tsetse-free areas due to the movement of animals (Ahmed *et al*., 2016) through sales, family migrations or other livestock programmes. This area shares a border with the Ibanda game reserve in Tanzania, which could contribute to the transmission of trypanosomes in the area. There is a need to investigate this situation, although no tsetse flies were collected around this game reserve during the entomological study (Gashururu *et al*., 2021).

However, the PCR-positive cases do not necessarily mean the clinical disease. Therefore, the results cannot be directly associated with the disease impact on the production and health status

of the cattle in the study area (Ng'ayo *et al*., 2005; Kivali *et al*., 2020). Nevertheless, it should be noted that carrier animals affect the smooth running of control programmes (Wissmann *et al*., 2011). Additionally, PCR is expensive and less applicable in rural settings where farmers are concerned to know infected animals for treatment. Molecular detection should be recommendable in case the goal is to maximize the detection and describe the diversity of trypanosomes. However, if the goal is to find animals for treatment and to minimise diagnostic cost, the buffy coat technique could be an option.

VerY Diag detects circulating antibodies for *T. congolense* and *T. vivax* and does not discriminate between active, recent and past infections following treatment. The VerY Diag detects more *T. vivax* than *T. congolense* for it has a sensitivity of 92.0 % against *T. congolense* and 98.2 % against *T. vivax* (Boulangé *et al*., 2017). The test does not show cross-reactivity with *Trypanozoon* or non-pathogenic trypanosomes (Boulangé *et al*., 2017). Nevertheless, it is not yet known whether the test may cross-react with other antigens not yet identified or not. Farmers often use Diminazene aceturate and Isometamedium chloride upon clinical presentation, and a good number of cattle can still be positive to the test after treatment due to the relatively long half-life of circulating immunoglobulins. Trypanosome antibodies usually last 3-4 months on average after curative treatment or host self-cure, but they can last up to 13 months in some cases (OIE, 2013). Another reason could be the extremely low parasitaemia, a situation in which few trypanosomes present are hidden in the blood capillaries, in the dermis and fatty tissues, but rarely occurring in the main bloodstream and still stimulate the immune system. Tests that detect antibodies are helpful in epidemiological research but not reliable for a diagnostic purpose (Masake and Nantulya, 1991). The test should be more useful for presumptive diagnosis of Trypanosomiasis (OIE, 2013), especially in low endemic areas.

The parasitological methods are already in use and the immunological rapid test (VerY Diag) is commercialised in the area. HRM-PCR was used as a confirmatory and most reliable method.

Nevertheless, results from each diagnostic technique would purposively inform farmers and other relevant stakeholders according to their needs. Infections of *T. evansi* might be mistaken with other *Trypanozoon* species. Specific, cheaper field serological test CATT/*T.evansi* is recommended in the area, because PCR is expensive and therefore, not affordable locally. The SRA gene was not found circulating in cattle examined in this study. The SRA gene is specific for *T. b. rhodesiense* and confers resistance to survive in human serum. It serves to differentiate human infective trypanosomes and animal infective *T. b. brucei* (Radwanska *et al*., 2002). SRA gene is expressed by trypanosomes found in both humans and animals. Therefore, cattle can serve as a reservoir for *rhodesiense* sleeping sickness. Our observations in cattle corroborate the notion that *rhodesiense* sleeping sickness may be absent from the area. The notion stems from the lack of reported cases of *rhodesiense* HAT in Rwanda for over 20 years, and the existence of an adequate surveillance system (Franco *et al*., 2020; Simarro *et al*., 2010). Currently, WHO declared Rwanda free from sleeping sickness. However, surveillance should be maintained to confirm the absence of *rhodesiense* HAT or detect the potential re-emergence of the disease in this historically endemic area. The data presented in this study provide useful information for the validation of HAT elimination as a public health problem at the national level.

At the end of this study, the first hypothesis that tsetse flies are abundant in the study area and that *Trypanosoma* infections are prevalent in cattle around the park is confirmed. However, Tsetse flies and cattle do not harbour human infective trypanosomes. The second hypothesis is, therefore, partially rejected and partially confirmed. The third hypothesis, that cattle are not the most preferred host, rather the buffalo is totally rejected. However, cattle were among the top three preferences for tsetse flies.

6.2. Conclusions

1. *G. pallidipes* and *G. morsitans centralis* are sympatric in the area and are both infected with trypanosomes. They are therefore all potential vectors of Trypanosomes to livestock reared around Akagera NP. However, G. *pallidipes* could be the more important vector due to their higher density.
2. The Tsetse challenge (occurrence, density, trypanosome infections), in the area is increased during the rainy season, inside the park than outside. At the interface, tsetse flies are abundant in Nyagatare District, while the trypanosome infections are increased in Kayonza District.
3. Tsetse blood feeding is much associated with wildlife animals both inside the park and at the interface. The likely important reservoirs for trypanosomes in the area are the buffalo, the warthog, the elephant, and the bushbuck. However, the buffalo seems to be the most frequent and therefore the main preferred host on either side.
4. Only *Sodalis* and *Wolbachia* endosymbionts were found and were both more concentrated in Akagera NP than outside. Their prevalence was too low to evaluate efficiently the relationship with trypanosome infection, and subsequently their role on tsetse competence.
5. The presence of tsetse-transmitted trypanosomes shows a continuous contact between tsetse vectors and animals. The presence of mechanically transmitted *T. evansi* and *T. theileri* shows the importance of biting flies in the epidemiology of African Trypanosomes in the area.
6. The linkage of findings on trypanosome infections in tsetse flies and cattle suggests the high transmissibility of *T.congolense* savannah strain in the area.

6.3. Recommendations

1. The study recommends the regular surveillance of both animal and human trypanosomiasis at the buffer zone even though the tsetse distribution rapidly decreases outside the park.
2. Given that the two adjacent game reserves in Tanzania are infested and tsetse flies are dynamic and conducive environment-dependent. Regionally coordinated control efforts and regular monitoring in the predictive areas is recommended to keep the distribution updated.
3. The vector control should focus on the spatial targeting of interventions in some areas of the park and the buffer zone around it. Despite the current efforts, there is a need for a more coordinated control mechanism using a multidisciplinary approach involving all the concerned parties. A combination of vector control, community engagement and improved farming practices, are suggested, as tsetse flies are limited to a known area.
4. An assessment of the impact of trypanosomiasis on livestock production is recommended, including a survey on the use of trypanocidal drugs. This will help farmers and policymakers rationalize control strategies and prioritise the intervention areas.
5. Cases of human DNA in tsetse blood meals were detected in some localities. HAT needs to be monitored closely, surveillance should target those areas and actions to promote the progressive reduction or the elimination of the AAT burden in the area can be envisaged
6. Further investigations are recommended to know the endosymbionts community in the Akagera region and their association with tsetse fly and trypanosomes. The use of different markers is recommended to maximize the detection. A deep identification of various *Wolbachia* haplotypes is recommended.

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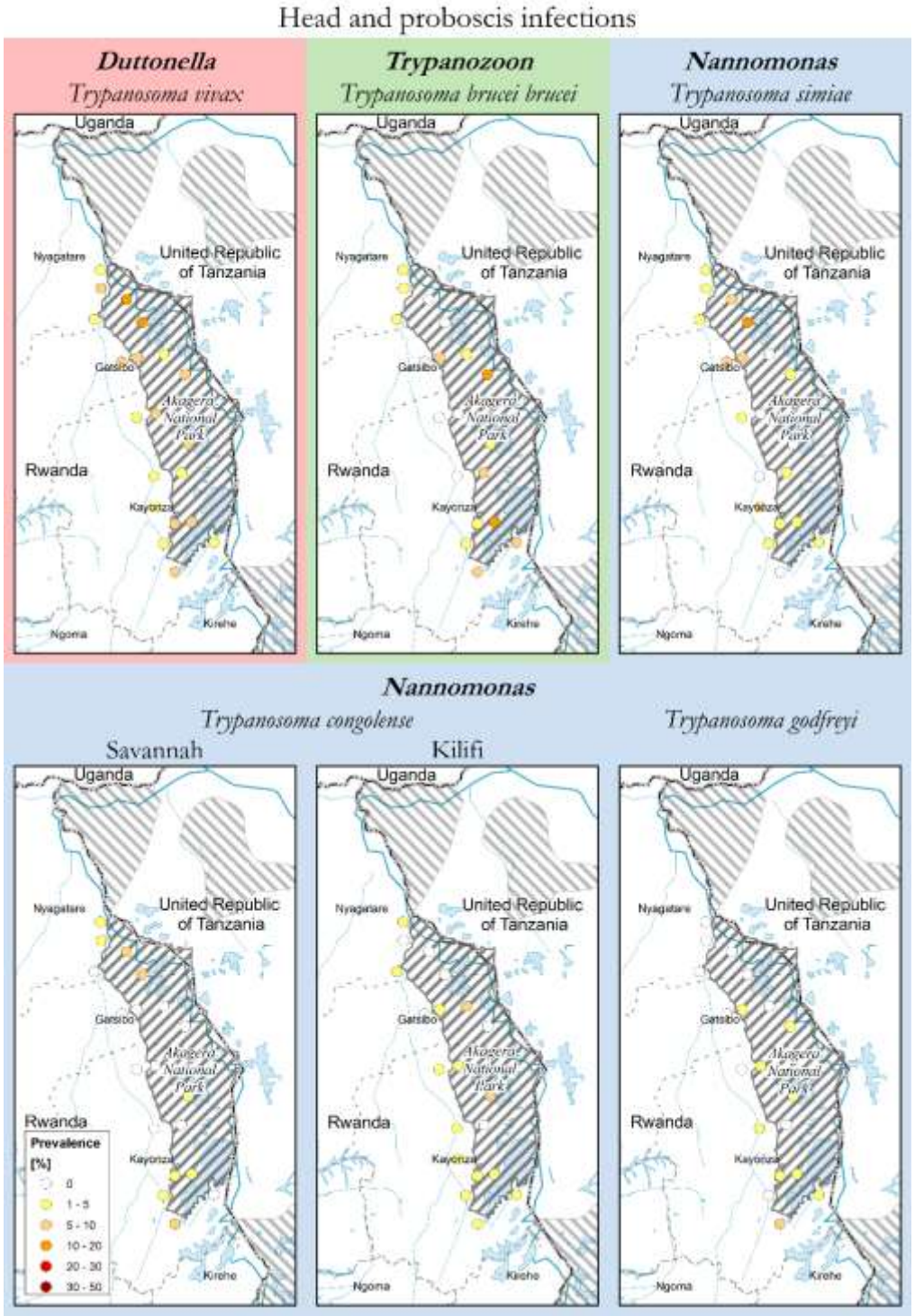
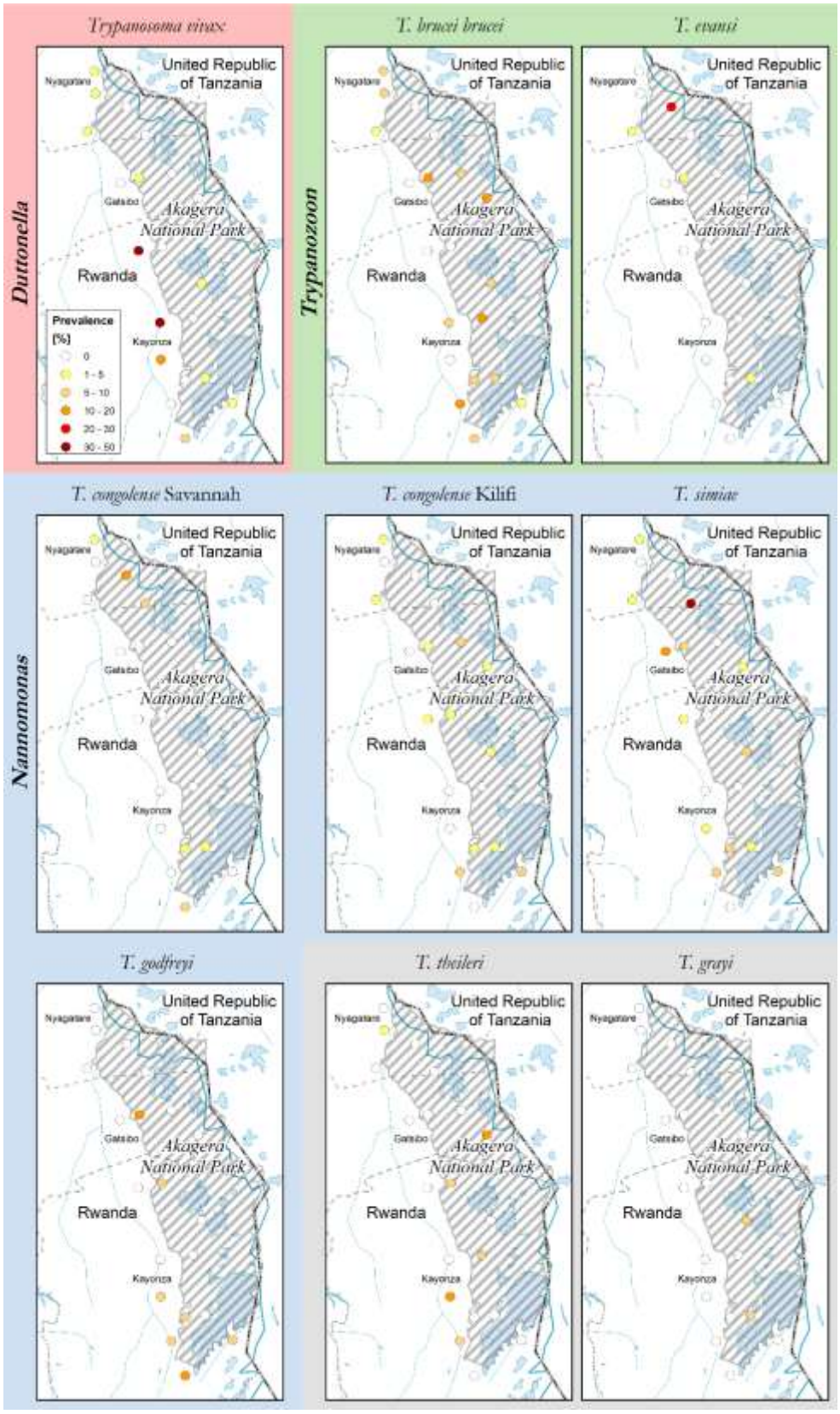
APPENDICES

Appendix 1: Distribution of *Trypanosoma* infections detected in Head and Proboscis (HP) of tsetse flies



Appendix 2: Distribution of *Trypanosoma* infections detected in Thorax and Abdomen (TA) of tsetse flies



Appendix 3: NCBI GenBank accession numbers generated from this study

- **Trypanosomes - internal transcribed spacer 1 (ITS1) Primer**

OK301912 *Trypanosoma theileri* isolate AP168
OK301913 *Trypanosoma vivax* isolate KP47
OK301914 *Trypanosoma vivax* isolate KP65
OK301915 *Trypanosoma vivax* isolate NP49
OK301916 *Trypanosoma vivax* isolate NP79
OK301917 *Trypanosoma vivax* isolate NP88
OK301918 *Trypanosoma vivax* isolate NP99
OK301919 *Trypanosoma vivax* isolate AP 59
OK301920 *Trypanosoma vivax* isolate AP193
OK301921 *Trypanosoma vivax* isolate AP21
OK301922 *Trypanosoma vivax* isolate AP54
OK301923 *Trypanosoma vivax* isolate AP77
OK301924 *Trypanosoma vivax* isolate AP31
OK301925 *Trypanosoma vivax* isolate AP15
OK301926 *Trypanosoma vivax* isolate AP188B
OK301927 *Trypanosoma vivax* isolate AP197
OK301928 *Trypanosoma vivax* isolate KP03
OK301929 *Trypanosoma vivax* isolate KP28B
OK301930 *Trypanosoma vivax* isolate KP41

OK271079 *Trypanosoma godfreyi* isolate AP17

OK422206 *Trypanosoma brucei* isolate KP07
OK422207 *Trypanosoma brucei* isolate KP13
OK422208 *Trypanosoma brucei* isolate NP33
OK422209 *Trypanosoma brucei* isolate NP57
OK422210 *Trypanosoma brucei* isolate AP07
OK422211 *Trypanosoma brucei* isolate AP45
OK422212 *Trypanosoma brucei* isolate AP102
OK422213 *Trypanosoma brucei* isolate AP137
OK422214 *Trypanosoma congolense* isolate KP59
OK422215 *Trypanosoma congolense* isolate NP24
OK422216 *Trypanosoma congolense* isolate AP122
OK422217 *Trypanosoma congolense* isolate AP188A
OK422218 *Trypanosoma evansi isolate* AP 122
OK422219 *Trypanosoma evansi isolate* AP 197
OK422220 *Trypanosoma grayi isolate* AP148
OK422221 *Trypanosoma simiae* isolate KP28A
OK422222 *Trypanosoma simiae* isolate NP62
OK422223 *Trypanosoma simiae* isolate AP194
OK422224 *Trypanosoma vivax* isolate AP20
OK422225 *Trypanosoma vivax* isolate AP50

OK483225 *Trypanosoma congolense* type Savannah isolate 41A

OK483226 *Trypanosoma congolense* type Savannah isolate AP 125

- **Blood meal - Vertebrate 16S Primer**

OK271430 *Loxodonta africana* voucher KP60 (savannah elephant)

OK271431 *Phacochoerus africanus* voucher P17 (common warthog)

OK271432 *Phacochoerus africanus* voucher P114 (common warthog)

OK271433 *Homo sapiens* voucher N47 (Human)

OK271434 *Homo sapiens* voucher P185 (Human)

OK092311 *Syncerus caffer* P94 (African buffalo)

OK092312 *Phacochoerus africanus* P42 (common warthog)

OK092313 *Phacochoerus africanus* voucher P45 (common warthog)

OK092314 *Phacochoerus africanus* voucher P67 (common warthog)

- **Blood meal – Cytochrome B**

OK625555 *Syncerus caffer* isolate P08 (African buffalo)

OK625556 *Syncerus caffer* isolate P198 (African buffalo)

OK625557 *Syncerus caffer* isolate N09B (African buffalo)

OK625558 *Bos taurus* isolate K04 (Cattle)

OK625559 *Tragelaphus scriptus* isolate P20A (Bushbuck)

OK625560 *Loxodonta africana* isolate P20B (savannah elephant)

OK625561 *Homo sapiens* isolate P119 (Human)

OK625562 *Homo sapiens* isolate P11 (Human)

OK625563 *Homo sapiens* isolate P31 (Human)

- **Endosymbiont - *Wolbachia* – wsp**

OK625564 *Wolbachia* endosymbiont isolate N15A

Appendix 4: Ethical approvals

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,
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Tel: 4449004/4442014/8
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Direct Line: 4449048

REF: FVM BAUEC/2019/246

Dr. Richard Gashururu Simba,
University of Nairobi
Dept. VMP & Parasitology
25/10/2019

Dear Dr. Simba

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Epidemiology of Trypanosomiasis in cattle at the wildlife-livestock interface of Akagera National Park, Rwanda.

Dr. Richard Gashururu Simba J87/ 52130/2017

We refer to your revised PhD. proposal submitted to our committee for review and your application letter dated 9th September 2019. . We have reviewed your application for ethical clearance for the study on Epidemiology of Trypanosomiasis in cattle at the wildlife-livestock interface of Akagera National Park, Rwanda.

We have noted that you have revised all sections as suggested by the Faculty Biosafety Animal Use and Ethics committee.

The proposed protocols in cattle and numbers of mice to be used in the study meets minimum standards of the Faculty ethical regulation guidelines.

We have also noted that registered veterinary surgeons will supervise the work.

We hereby give approval for you to proceed with the project as outlined in the submitted proposal.

Yours sincerely

Dr. Catherine Kaluwa, BVM, MSc, Ph.D
Chairperson,
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine

UNIVERSITY of RWANDA

COLLEGE OF AGRICULTURE, ANIMAL SCIENCES & VETERINARY MEDICINE

OFFICE OF THE DIRECTOR OF RESEARCH & INNOVATION

Our ref: 080./ 19 /DRI September 2,2019

Mr. Richard Gashururu,
Tel: *+250788556595*
E-mail: gasirich@yahoo.fr
School of Veterinary Medicine
UR-CAVM

Dear Gashururu,

RE: **Ethical Clearance for your research project title "Epidemiology of Trypanosomosis in Cattle at the Wildlife-Livestock Interface of Akagera National Park, Rwanda''**

On behalf of the Research Screening and Ethical Clearance Committee of the College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda, I am pleased to inform you that your request for the ethical clearance for the project entitled **"Epidemiology of Trypanosomosis in Cattle at the Wildlife-Livestock Interface of Akagera National Park, Rwanda''** has been approved.

You are recommended to follow the approved protocol during the conduct of your research; any amendment should be communicated to the Committee for review and approval. At the completion of the project you are requested to submit the report to Directorate of Research and Innovation.

Congratulations to you team, we are looking forward to a successful implementation of the project.

Sincerely,

Dr. Guillaume NYAGATARE
Acting Director of Research and Innovation
Chairperson of the College Research Screening and Ethical Clearance Committee
UR-CAVM

Cc: - Dean of the School of Veterinary Medicine