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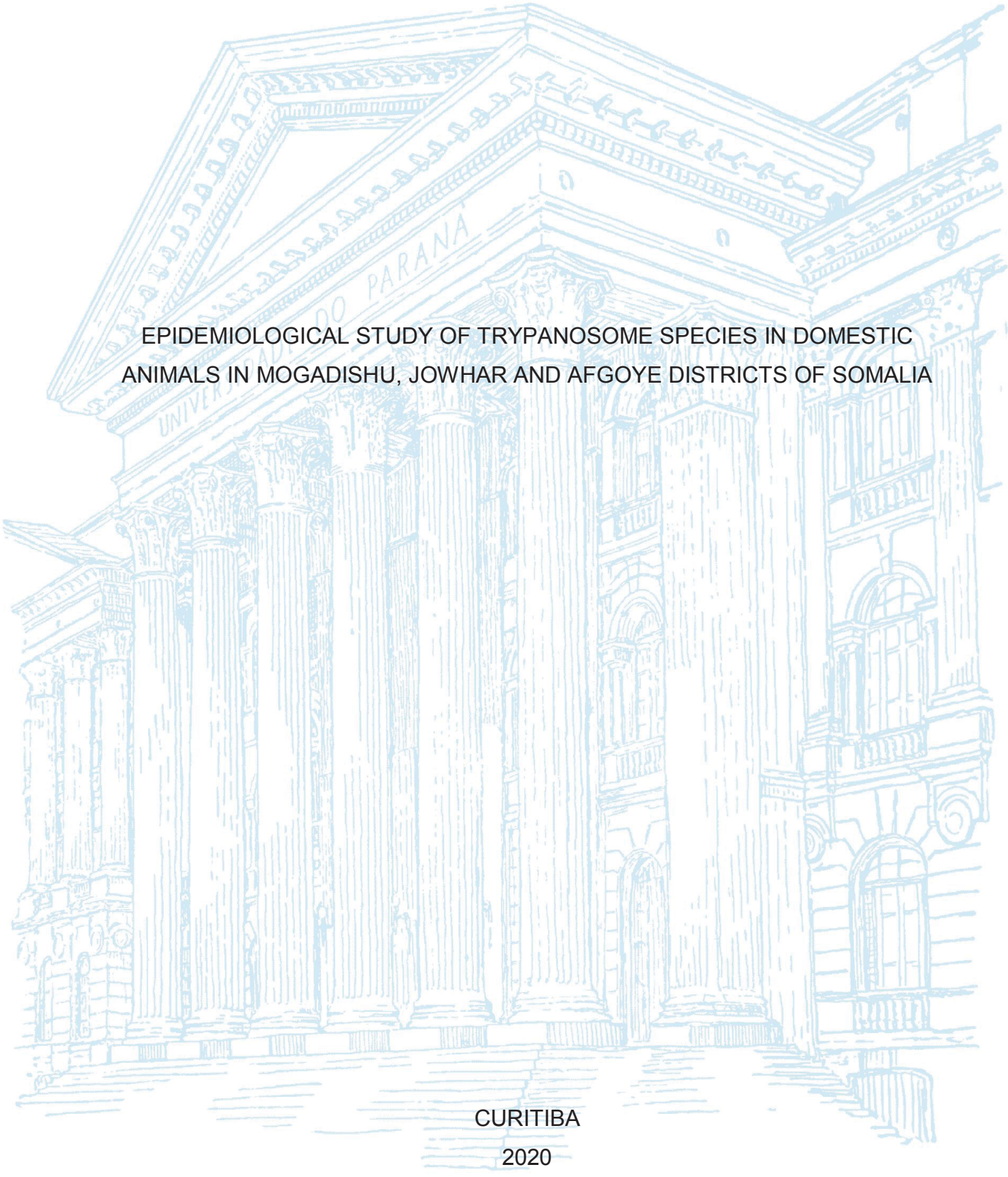

EPIDEMIOLOGICAL STUDY OF TRYPANOSOME SPECIES IN DOMESTIC ANIMALS IN MOGADISHU, JOWHAR AND AFGOYE DISTRICTS OF SOMALIA

CURITIBA

2020

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EPIDEMIOLOGICAL STUDY OF TRYPANOSOME SPECIES IN DOMESTIC ANIMALS IN MOGADISHU, JOWHAR AND AFGOYE DISTRICTS OF SOMALIA

Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como um requerimento parcial para obtenção do título de doutor em Ciências Veterinárias

Orientador: Prof. Dr. Rafael Felipe da Costa Vieira
Co-orientador: Prof. Dr. Abdalla Mohamed Ibrahim

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APPROVAL MINUTE

The Examining Board is designated by the Faculty of the Graduate Program of VETERINARY SCIENCES at the Universidade Federal do Paraná where invited to argue the THESIS of DOCTOR IN PHILOSOPHY by **AHMED ABDULKADIR HASSAN**, entitled: **EPIDEMIOLOGICAL STUDY OF TRYPANOSOME SPECIES IN DOMESTIC ANIMALS IN MOGADISHU, JOWHAR AND AFGOYE DISTRICTS OF SOMALIA**, under the supervision of Dr. RAFAEL FELIPE DA COSTA VIEIRA, which and after assessment of the candidate and the work, the Examining Board decided for the **APPROVE** in the present rite.

The granting of the title of doctor in philosophy is contingent upon the fulfillment of all the requirements indicated by the Examining Board and terms determined in the regulation of the Graduate Program.

CURITIBA, July 01st, 2020.

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DEDICATION

I dedicate this work with great appreciation

To my father and mother,

To my brothers and sisters,

To my wife and children,

To my friends and mentors

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“Science is a way of thinking much more than it is a body of knowledge”.

(**Carl Sagan**)

RESUMO

Tripanossomose Africana Animal e Humana (TAA e TAH, respectivamente) continua sendo uma preocupação econômica e de saúde significativa na África. Na Somália, a guerra civil da década de 1990 resultou na destruição de estruturas educacionais, de pesquisa, econômicas e sociais, tornando as pontuações do país muito baixas para a maioria dos indicadores humanitários. Estudos anteriores sobre a detecção de espécies de *Trypanosoma* em animais domésticos da Somália só foram realizados durante a década de 1990, usando métodos padrão de detecção de tripanossomas (MPDT), esfregaço de sangue e técnica Woo. Existem poucas informações sobre o status da Tripanossomose na Somália, especialmente em relação à epidemiologia molecular. Desta forma, o objetivo deste estudo foi avaliar a prevalência e a caracterização molecular de tripanossomas em animais domésticos da Somália. Esta tese de doutorado apresenta dois manuscritos e revisão de literatura sobre Tsé-tsé e Tripanossomose. O primeiro manuscrito é um levantamento parasitológico, sorológico e molecular de *Trypanosoma* spp. em camelos (*Camelus dromedarius*) da Somália. Um total de 182 amostras de sangue de *Camelus dromedarius* de nômades e de fazendas leiteiras foram avaliadas usando os métodos MPDT, sorológico (CATT/*T. evansi*) e molecular (ITS1-PCR). Todas as amostras de camelos foram negativas para *Trypanosoma* spp. por STDM. Um total de 125/182 (68,7%, 95% CI: 61,4-75,3%) camelos foram soropositivos para *T. evansi* por CATT / *T. evansi*. Os camelos criados no sistema nômade apresentaram mais chance de serem soropositivos para *T. evansi* do que aqueles sob sistema de produção leiteira (OR: 5,6, IC 95%: 2,1–15,2, P = 0,0001). Cinco dos 182 (2,7%, IC 95%: 0,9-6,3%) camelos apresentaram resultado positivo para *Trypanosoma* sp. por ITS1-PCR. O sequenciamento da região ITS1 da espécie *Trypanosoma* detectada revelou que os camelos estavam infectados com *T. evansi* e *T. simiae*. O segundo manuscrito versa a respeito da detecção parasitológica e molecular de *Trypanosoma* spp. em bovinos, caprinos e ovinos da Somália. Um total de 614 amostras de sangue de bovinos (n = 202), caprinos (n = 206) e ovinos (n = 206) foram avaliadas usando a técnica de papa leucocitária (TPL) e ensaios moleculares incluindo SRA-PCR para detectção de *T. brucei rhodesiense*, agente causador da TAH. Um total de 21/614 (3,4%; IC95%: 2,1-5,2%) e 101/614 (16,4%; IC95%: 13,6-19,6%) ruminantes foram positivos para *Trypanosoma* spp. por BCT e PCR, respectivamente. Por PCR, a maior prevalência

de espécies de *Trypanosoma* foi observada em bovinos (23,8%; IC: 18,4-30,1%), seguidos por caprinos (17,5%; IC: 12,9-23,3%) e ovinos (8,3%; 95% IC: 5,1-12,9%). Um total de 74/101 (73,3%; 95% IC: 63,5-81,6%) ruminantes mostraram coinfecção com pelo menos duas espécies de *Trypanosoma*. *Trypanosoma evansi*, *T. godfreyi*, *T. vivax*, *T. brucei*, *T. simiae* e *T. congolense* foram as espécies de *Trypanosoma* encontradas no presente estudo. Todas as amostras positivas para *T. brucei* pelo TBR-PCR foram negativas para *T. b. rhodesiense*, pelo ensaio SRA-PCR. Este é o primeiro estudo sobre a detecção molecular de *Trypanosoma* spp. em animais domésticos na Somália. Mais investigações epidemiológicas e medidas de controle sustentável são necessárias para gerenciar infecções por *Trypanosoma* spp. e sua propagação no país. Além disso, os estudos também devem se concentrar na detecção de *T. b. rhodesiense*, agente causador do TAH, no país.

Palavras-chave: Tripanossomose, *Glossina* spp., AAT, HAT, Saúde Única, Somália

ABSTRACT

Animal and Human African trypanosomiasis (AAT and HAT, respectively) remain significant health and economic concern in Africa. In Somalia, the civil war of the 1990s resulted in the destruction of educational, research, economic and social structures, making the country scores very low for most humanitarian indicators. Previous studies on the detection of *Trypanosoma* species in Somali domestic animals have only been performed during the 1990s using standard trypanosome detection methods (STDM), blood smear and Woo techniques. Scanty information exists about the trypanosomiasis status in Somalia, especially regarding molecular epidemiology. Accordingly, the aim of this thesis was to evaluate the prevalence and molecular characterization of trypanosomes in domestic animals from Somalia. This doctoral thesis presents two manuscripts and a general literature review on Tsetse and Trypanosomiasis. The first manuscript is a parasitological, serological and molecular survey of *Trypanosoma* spp. in camels (*Camelus dromedarius*) in Somalia. A total of 182 blood samples from *Camelus dromedarius* from nomadic and dairy farms were evaluated using STDM, serological (CATT/*T. evansi*) and molecular (ITS1-PCR) methods. All camel samples were negative for *Trypanosoma* spp. by STDM. A total of 125/182 (68.7%, 95% CI: 61.4–75.3%) camels were seropositive for *T. evansi* by CATT/*T. evansi*. Camels reared in the nomadic system were more likely to be seropositive for *T. evansi* than those under dairy production system (OR: 5.6, 95% CI: 2.1–15.2, *P* = 0.0001). Five out of 182 (2.7%, 95% CI: 0.9–6.3%) camels tested positive for *Trypanosoma* sp. by ITS1-PCR. Sequencing of the ITS1 region of the *Trypanosoma* species detected herein revealed that camels were infected with *T. evansi* and *T. simiae*. The second manuscript deals with the parasitological and molecular detection of *Trypanosoma* spp. in cattle, goats and sheep from Somalia. A total of 614 blood samples from cattle (n = 202), goats (n = 206) and sheep (n = 206) were evaluated using the buffy coat technique (BCT) and molecular assays including SRA-PCR performed for *T. b. rhodesiense*, HAT causative agent. Twenty-one out of 614 (3.4%; 95% CI: 2.1-5.2%) and 101/614 (16.4%; 95% CI: 13.6-19.6%) ruminants were positive for *Trypanosoma* spp. by BCT and PCR, respectively. Using PCR, the highest *Trypanosoma* species prevalence was observed in cattle (23.8%; 95% CI: 18.4-30.1%) followed by goats (17.5%; 95% CI: 12.9-23.3%) and sheep (8.3%; 95% CI: 5.1-12.9%). A total of 74/101 (73.3%; 95% CI: 63.5-81.6%) ruminants were shown

coinfection with at least two Trypanosome species. *Trypanosoma evansi, T. godfreyi, T. vivax, T. brucei, T. simiae* and *T. congolense* were the *Trypanosoma* species found in the present study. All *T. brucei*-positive samples by the TBR-PCR have tested negative for *T. b. rhodesiense,* by the SRA-PCR assay. This is the first study on the molecular detection of *Trypanosoma* sp. in domestic animals in Somalia. Further epidemiological investigations and sustainable control measures are needed to manage infections of *Trypanosoma* spp. and its spreading in the country. In addition, studies should also focus on the detection of HAT causative agents in the country.

Keywords: Trypanosomiasis, *Glossina* spp., AAT, HAT, One Health, Somalia

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Literature Review

Parasitological, serological and molecular survey of camel trypanosomiasis in Somalia

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LIST OF ABREVIATIONS

AAT	- African Animal Trypanosomiasis
ARTC	- Abrar Research and Training Centre
AU	- Abrar University
BCT	- Buffy Coat Techniques
BLAST	- Basic local alignment search tool
CAPES	- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CATT	- Card agglutination test for trypanosomiasis
CI	- Confidence interval
CNPq	- Conselho Nacional de Desenvolvimento Científico e Tecnológico
CNS	- Central Nervous System
CSF	- Cerebrospinal Fluid
DNA	- Deoxyribonucleic acid
ELISA	- Enzyme linked immunosorbent assay
ETS	- External Transcribed Spacer
GOHi	- Global One Health initiative
HAT	- Human African Trypanosomiasis
HCT	- Haematocrit Centrifuge Technique
ICRC	- International Committee of the Red Cross
IFAT	- Indirect Fluorescent Antibody Test
ITS	- Internal transcribed spacer
kDNA	- kinetoplast DNA
LAMP	- Loop-mediated isothermal amplification
MDGs	- Millennium Development Goals
MGE-PCR	- Mobile Genetic Elements PCR
ML	- Maximum likelihood
NASBA	- Nucleic Acid Sequence-Based Amplification
nPCR	- nested-PCR
NTTCP	- National Tsetse and Trypanosomiasis Control Project
OIE	- Office International des Epizooties (now World Organisation for Animal Health)
OR	- Odds ratio
PCR	- Polymerase chain reaction
PCR-RFLP	- PCR Restriction Fragment Length Polymorphism

PCV	- Packed cell volume
RBCs	- Red Blood Cells
rDNA	- ribosomal DNA
RNA	- Ribonucleic acid
rRNA	- ribosomal RNA
SAT	- Sequential Aerosol Technique
SIT	- Sterile Insect Technique
SPSS	- Statistical package for social sciences
SRA gene	- Serum Resistance Associated gene
SSA	- Sub-Saharan Africa
STDM	- Standard Trypanosome Detection Methods
T & T	- Tsetse and Trypanosomiasis
UFPR	- Universidade Federal do Paraná
UV	- Ultraviolet
VBD	- Vector-borne diseases
VBP	- Vector-borne pathogen
VSG	- Variant Surface Glycoproteins
VVBDI	- Vector and Vector Borne Diseases Institute
WBCs	- White Blood Cells

TABLE OF CONTENT

1 GENERAL INTRODUCTION

1.1 BACKGROUND

Trypanosomes are protozoan parasites found worldwide, infecting animals and humans, most often transmitted by blood-sucking insects (Hoare, 1972; Truc *et al.,* 2013). The typical pathogenic human trypanosomiasis are sleeping sickness, or human African trypanosomiasis (HAT), and the Chagas disease, American human trypanosomiasis (Truc *et al.,* 2013). The HAT is a fatal disease occurring in sub-Saharan Africa (SSA) and transmitted by tsetse flies, caused by two subspecies of trypanosomes: *T. brucei gambiense* (the chronic form) or *T. b. rhodesiense* (the acute form), which is derived from the animal parasite *T. b. brucei* that has acquired the ability to infect humans. On the other hand, Chagas disease is caused by *T. cruzi* and transmitted mainly by triatomine bugs (Truc *et al.,* 2013). Apart from these species or sub-species, most trypanosomes are infective only to animals, such as *T. b. brucei*, *T. congolense*, and *T. vivax*, the agents of the complex animal trypanosomiasis called “nagana” in Africa and responsible for important economic losses (Paul *et al.,* 2018). *T. evansi* has the widest host range and geographical distribution for a disease called “surra” around the world (Desquesnes *et al.,* 2013). Humans possess an innate protection against most *Trypanosoma* species. However, cases of atypical human trypanosomiasis caused by *T. b. brucei*, *T. vivax*, *T. congolense*, *T. evansi* and *T. lewisi*, which were all considered non-infective to humans, have been reported (Truc *et al.,* 1998; Joshi *et al.,* 2005; Deborggraeve *et al.,* 2008; Haridy *et al.,* 2011; Truc *et al.,* 2013).

Trypanosomiasis directly constrains the productivity of livestock by reducing birth rates, increasing abortion rates, and increasing mortality rates (Swallow, 2000). In Africa, it has been estimated that AAT cause economic losses of 4.5 billion US dollars per year (Oluwafemi *et al.,* 2007), as a result of direct (mortality, production losses, costs of prophylactic and curative trypanocidal drugs) and indirect losses due to crop production decay and agricultural workers’ involvement (deficiency of animal protein diets) (Harberd 1988, Angara *et al.* 2014). Trypanosomiasis is also one of the factors that constrain the development of specialized dairy enterprises in the sub-humid and humid lowlands of the continent (Swallow, 2000). In Somalia, the economic impact of AAT has been estimated in 88 million US dollars (Mohamed and Dairri, 1987). Although the National Tsetse and

Trypanosomiasis Control Project (NTTCP) has been established in the 1980s (Mohamed and Dairri 1987), and a tsetse and trypanosomiasis (T & T) control project has been funded by the International Committee of the Red Cross (ICRC) in some villages of Shabelle and Jubba regions (ICRC, 2017), no other control measures or wide coverage area to reduce the losses from trypanosomiasis have been implemented following the collapse of the central government of Somalia in 1991 (Salah, 2016). Effective and sustainable T & T control projects will contribute in improving livestock health which enhances the production and livelihood of dependent families (Swallow, 2000).

Methods of epidemiological screening include direct parasite examination using thick and thin peripheral blood films stained with Giemsa stains or fresh wet blood or buffy coat smears and, more recently, molecular methodologies based on the polymerase chain reaction (PCR) (Rosenblatt 2009; OIE Manual, 2013). Parasitological techniques are labour intensive and can lack sensitivity under field conditions due to routinely low peripheral parasitaemia in infected livestock (Mattioli and Faye, 1996; Picozzi *et al.,* 2002). PCR based diagnostic methods have largely overcome difficulties associated with sensitivity and specificity, and they can provide multi-species-specific detection of trypanosomes in a single PCR (Cox *et al.,* 2005; Salim *et al.* 2011) and have been used in epidemiological studies (Picozzi *et al.* 2002; Desquesnes and Dávila 2002; Kouadio *et al.* 2014).

In Somalia, AAT have been reported in domestic animals by standard trypanosome detection methods (STDM) (Mohamed and Dairri 1987, Macchioni and Abdullatif, 1985; Dirie *et al.* 1988a, b; Ainanshe *et al.,* 1992). A previous study has found *T. congolense* (Schoepf *et al.* 1984; Dirie *et al.*, 1988b) and *T. vivax* infecting sheep (Dirie *et al.*, 1988b). Both trypanosome species have also been reported in cattle from southern Somalia (Moggridge, 1936; Dirie *et al.,* 1988a). Additionally, *T. simiae*, *T. congolense*, *T. brucei* and *T. evansi* have also been detected in Somali camels by STDM (Baumann and Zessin, 1992; Pellegrini, 1948; Dirie *et al.* 1989). However, no data are available regarding the prevalence of *T. b. rhodesiense*, the HAT causative agent, in Somalia (Harberd 1988; WHO 2007), despite the presence of the disease in ecologically similar areas of neighbouring Kenya and Ethiopia (Baker *et al.,* 1970; Rutto and Karuga, 2009), with the practice of transhumance, and the presence of the vector, *Glossina spp.*, in the

country (Harberd 1988). Effective disease control and management depends heavily upon knowledge of the epidemiology of the disease, which in turn relies upon methods that incorporate screening of both animal and human populations (Cox *et al.,* 2005).

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2 LITERATURE REVIEW

2.1 IMPORTANCE OF LIVESTOCK

Livestock species play a pivotal role in supporting livelihoods of communities in Africa. They are a source of livelihood, income, nutrition, agricultural traction, soil productivity, transport and pride (Herrero *et al.,* 2012). Any factor that can affect the health and productivity of livestock also constraints the development and wellbeing of livestock dependent families. Therefore, diseases like trypanosomiasis is of a major socioeconomic importance, hence its control is vital (Baral, 2010).

2.2 TRYPANOSOMES

2.2.1 Taxonomy

Trypanosomes are classified under the Kingdom Protista, sub-kingdom Protozoa, phylum Sarcomastigophora, class Zoomastigophora, order Kinetoplastida, family Trypanosomatidae, and the genus *Trypanosoma* (Hoare, 1972; Baral, 2010; Wilkowsky, 2018). The classification of mammalian trypanosomes is shown in figure 2.1.

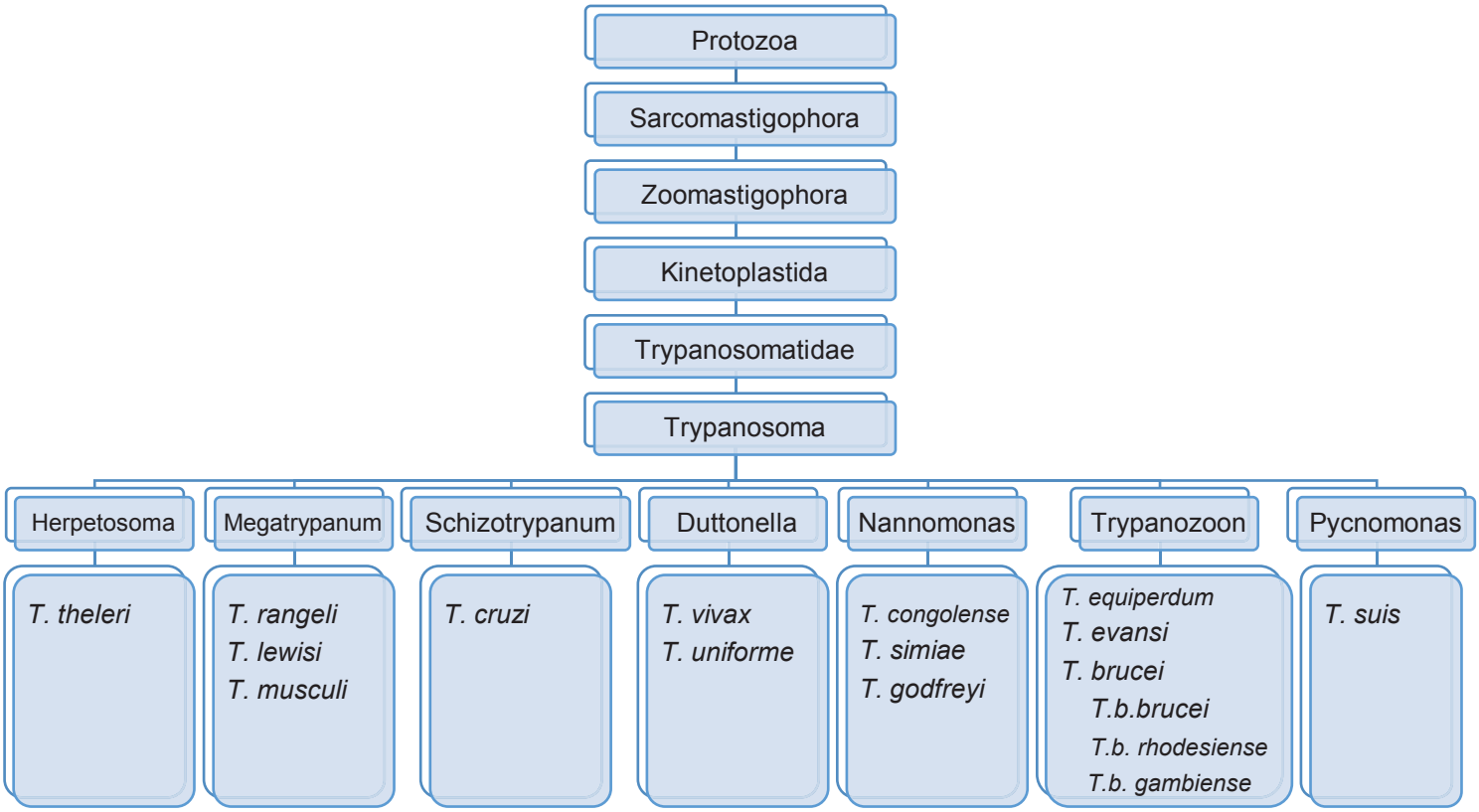

Figure (2.1) Classification of the major mammalian trypanosomes (Adapted from Baral, 2010)

According to the mechanism of transmission by the insect vector, the trypanosomes are divided into two groups, Stercoraria and Salivaria parasites (Wilkowsky, 2018). The development of stercoraria parasites takes place in the intestinal tract of the invertebrate vector and the infection to the vertebrate is transmitted via faecal contamination, while salivarian parasites develop in the salivary gland and infection occurs via inoculation (Baral, 2010; Wilkowsky, 2018). Most of the pathogenic trypanosomes of domestic animals fall into the Salivaria group (Uilenberg, 1998; Wilkowsky, 2018).

2.2.2 Morphology

Trypanosomes are single-cell flagellated protozoa (figure 2.2), of 8-40 μm in size depending on the species, with an undulating membrane and flagellum at the anterior end (Wilkowsky, 2018). They are motile and possess a writhing movement among red blood cells (RBCs) of vertebrates when observed in wet blood smears (Wilkowsky, 2018). In addition, there is a kinetoplast at the base of the flagellum, located towards the posterior end of trypomastigote forms of the parasite (Uilenberg, 1998; Wilkowsky, 2018). The kinetoplast is an enlarged region of the mitochondrion which contains condensed mitochondrial DNA forming a network of interlocked circular molecules of different size: minicircles of about 1kb and maxicircles of 22kb (Baral, 2010; Wilkowsky, 2018). Unlike other trypanosomes, the kinetoplast DNA (kDNA) of *T. evansi* does not contain maxicircle, and they are no longer able to undergo their cycle in *Glossina* spp. (Baral, 2010; Wilkowsky, 2018). Different morphological forms appear in the life cycle of trypanosomes, distinguished mainly by the position, length and the cell body attachment of the flagellum. Trypanosomes present two main morphologically distinguishable forms: epimastigote and trypomastigote, in which kinetoplast of the epimastigote located anterior to the nucleus (Wheeler *et al.,* 2013; Kaufer *et al.,* 2017). Trypanosomes possess protective cell coat of antigens called variant surface Glycoproteins (VSG) which is a major antigen of the parasite whose antigenicity is in continuous changes to escape the immune system of the host (Baral, 2010; Wilkowsky, 2018).

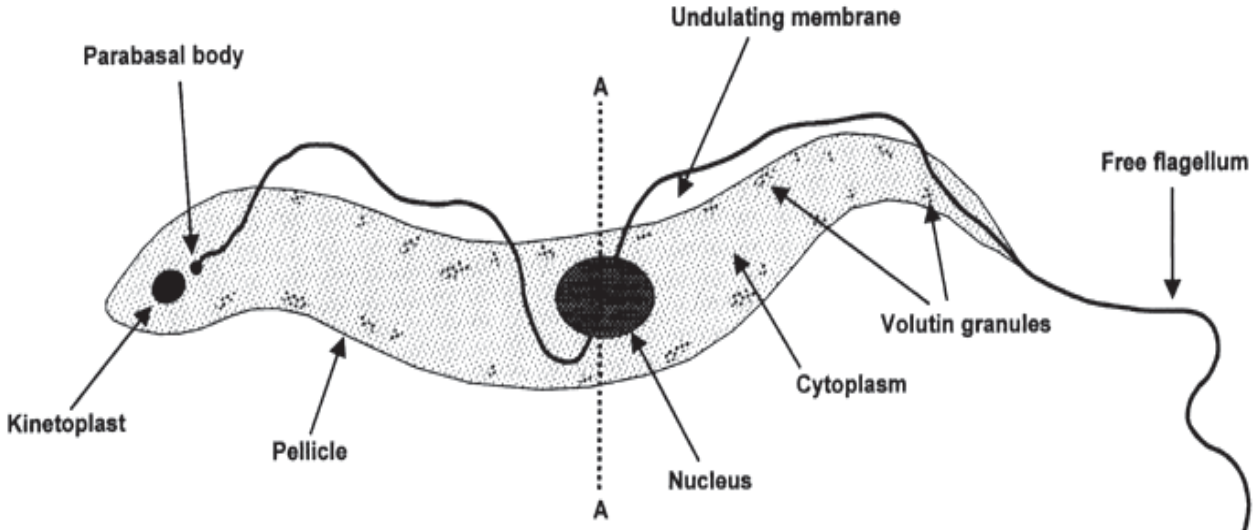

Figure (2.2) A simplified sketch of a trypanosome (Uilenberg, 1998)

2.2.3 Reproduction

Trypanosomes reproduce asexually by binary fission; two daughter cells arise from an existing one (Uilenberg, 1998). The division into two daughter cells (binary fission) follows the sequence of events illustrated in figure 2.3. In addition to the binary fission, a sexual reproduction involving meiotic division and production of haploaid gametes have been reported in *Trypanosoma* spp., but is not an obligatory part of the life cycle (Jenni, 1990; Gibson, 2015).

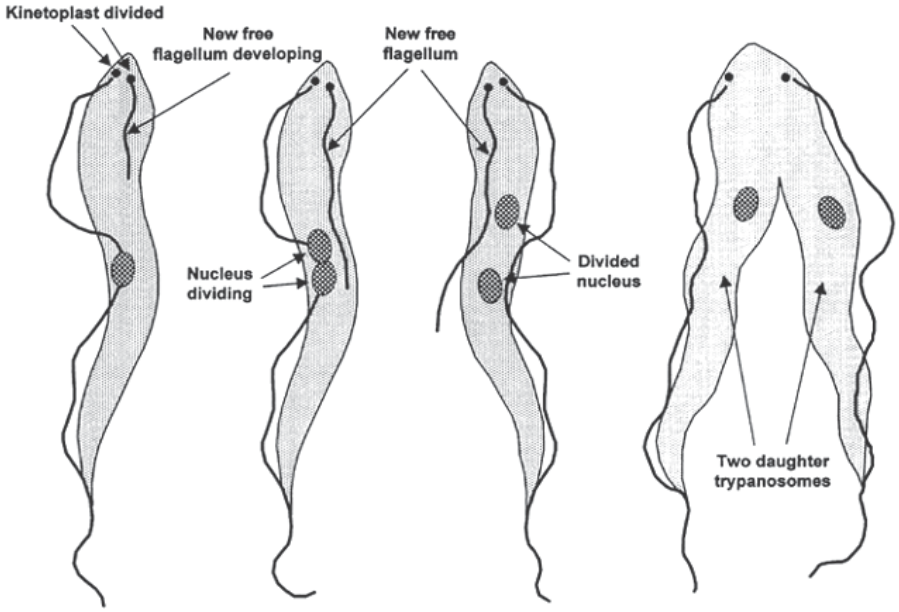

Figure (2.3) A simplified sketch of a trypanosome division (Uilenberg, 1998)

2.2.4 Life Cycle

The life cycle of most trypanosomes involves an insect vector and a mammalian host. The two major patterns of life cycles are related to whether the trypanosome belongs to the Salivarian or Stercorarian section (Wilkowsky, 2018). With the exception of *T. evansi* and *T. equiperdum*, all the pathogenic animal trypanosomes are cyclically transmitted by tsetse flies, *Glossina* species (Baral, 2010). However, *T. vivax* and *T. congolense* can also be mechanically transmitted by haematophagous insects (Desquesnes and Dia, 2003; 2004).

In the cycle of infection by salivarian trypanosomes (figure 2.4), a blood-feeding vector become infected after ingesting blood containing trypomastigotes from the infected host. Then, parasites divide by the binary fission in the gut and sometimes by sexual reproduction in the salivary gland and eventually migrates to the salivary gland or proboscis and transform into epimastigotes which later transform into metacyclic trypomastigotes, the infective stage of the trypanosome, that can infect a new mammalian host (Jenni, 1990; Baral, 2010; Gibson, 2015; Wilkowsky, 2018). Different trypanosome species use different parts of the alimentary system of the fly for their development: midgut and proboscis for subgenus *Nannomonas*, midgut and salivary glands for subgenus *Trypanozoon*, proboscis for subgenus *Duttonella* (Malele *et al.,* 2003). The parasites enter the lymphatic system and pass into the bloodstream. After inoculation into the bloodstream of a new vertebrate host, the cycle starts again and the parasite continues to divide in this form (Baral, 2010; Wilkowsky, 2018).

Trypanosoma equiperdum, the causative agent of a sexually transmitted disease of equids called “dourine,” is the only trypanosome that is not transmitted by an invertebrate vector, and it differs from other trypanosomes in that it is primarily a tissue parasite that rarely invades the blood (Wilkowsky, 2018).

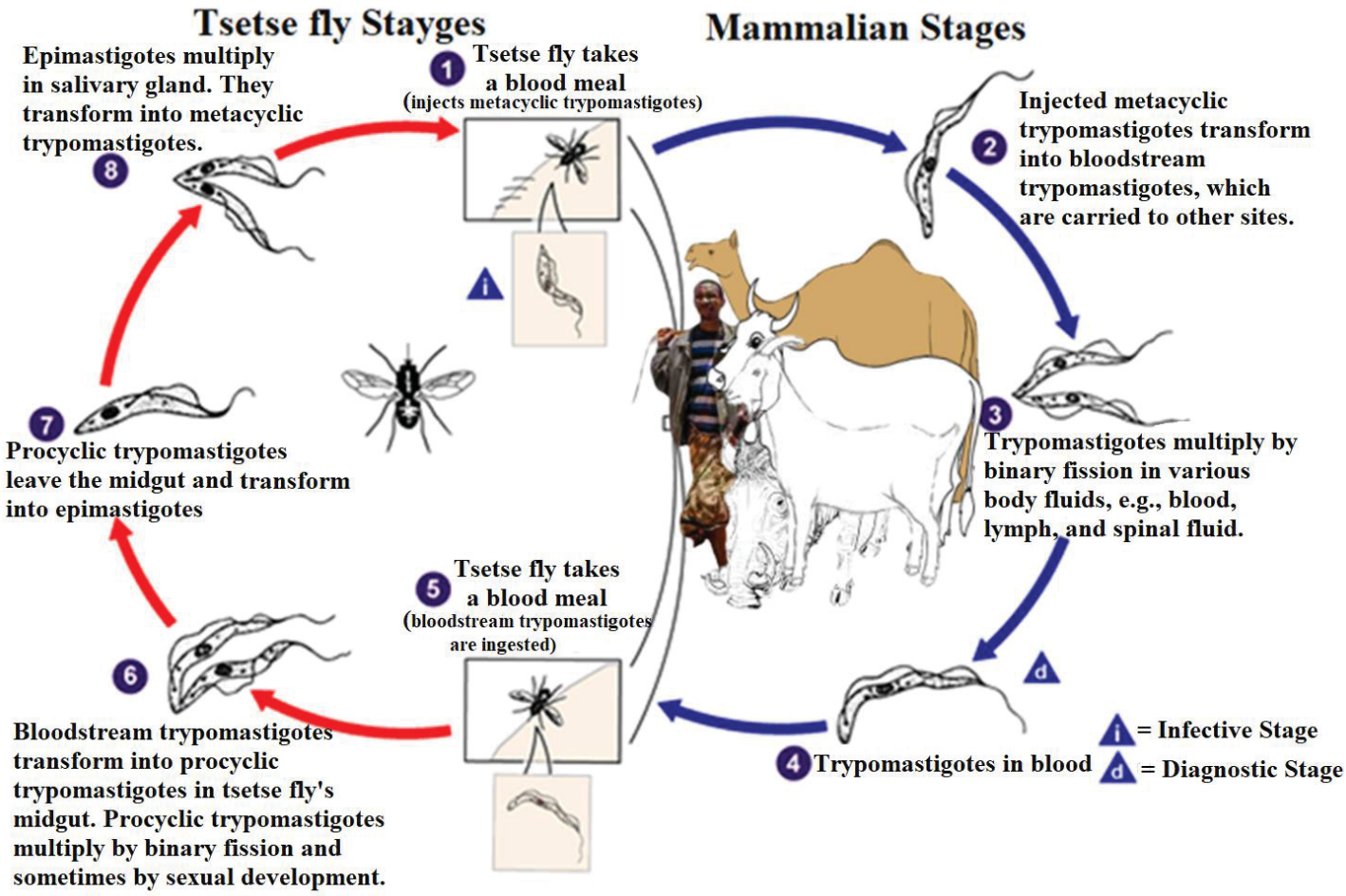

Figure (2.4) A simplified sketch of Life cycle of African trypanosomes (modified from DPDx, 2019)

2.2.5 Transmission

Various transmission methods were reported for different *Trypanosoma* species. Transmission of trypanosomes by insects may occur through cyclical or mechanical means. In cyclical transmission, the Trypanosomes go through specific life cycle stages in the vector before they develop the ability to infect the next host and this depends on the biochemical and physiological interactions that occur between the parasite and its insect host (Geiger *et. al.,* 2015; Wilkowsky, 2018), while mechanical transmission occurs when they are transferred directly from the blood of an infected host to another in its original form without undergoing development in the insect (Hall and Wall, 2004).

The majority of African trypanosomes are cyclically transmitted by tsetse flies (*Glossina* spp.). This biological transmission is restricted to the tsetse belt (15^{o}N to 26^{o}S), the endemic region of tsetse flies (Urquhart *et al.,* 1996; Wilkowsky, 2018; Krinsky, 2019). Cyclical transmission occurs with *T. congolense*, *T. vivax*, *T. simiae*, *T. suis*, *T. godfreyi*,

and *T. brucei* and the trypanosomes responsible for human sleeping sickness, *T. b. rhodesiense* and *T. b. gambiense* (Wells, 1972; McNamara *et. al.,* 1994; Wilkowsky, 2018). However, *T. evansi* and *T. vivax* are transmitted mechanically by biting flies, such as *Tabanus, Haematopota, Chrysops* and *Stomoxys* spp. and this has enabled these *Trypanosoma* spp. to spread widely beyond Africa into Asia and Latin America (Desquesnes and Dia, 2003; 2004; Luckins and Dwinger, 2004; Desquesnes *et al.,* 2013; Wilkowsky, 2018). Tsetse flies themselves can also act as mechanical vector in Africa (Wilkowsky, 2018). In South and Central America, *T. evansi* can also be transmitted by the vampire bats (*Dosmodus rotundus*), which act as both reservoirs and vectors (Baral, 2010). Besides mechanical transmission by insects and vampire bats, *T. evansi* can be transmitted through milk, iatrogenic, per-oral, and possibly sexually (Brun *et al.,* 1998; Baral, 2010).

Experimental transmission of *T. evansi* has been successful with mosquitos such as *Aedes aegypti*, *A. Argenteus*, and *Anopheles fuliginosus*; However, the epidemiological significance has not been demonstrated (Desquesnes *et al.,* 2013). Several experimental studies have indicated that *T. brucei* and *T. congolense* may be mechanically transmitted by biting insects in the absence of tsetse enabling a global spread of the infection by the movement of infected animals (Wells, 1972; Mihok *et al.,* 1995; Sumba *et al.,* 1998; Desquesnes and Dia, 2003).

The natural transmission of *T. equiperdum* occurs only during copulation of equines; However, experimental infections inoculating parasites by the intravenous or intraperitoneal route indicate that mechanical transmission by blood-feeding flies cannot be excluded as a possible route (Gizaw *et al.,* 2017; Wilkowsky, 2018).

In Latin America, *Trypanosoma cruzi*, the causative agent of Chagas disease (American Human Trypanosomiasis), is transmitted cyclically by Triatomine bugs (kissing bugs), through the insect faecal contamination (Hoare, 1972; Luckins and Dwinger, 2004; Baral, 2010). Besides classical vector-borne transmission, congenital, transfusion-associated, and oral/foodborne transmission is also possible (Wilkowsky, 2018).

The nonpathogenic *Trypanosoma theileri,* which under some circumstances found to be pathogenic, is transmitted by *Tabanus* spp. and ticks (Uilenberg, 1998; Latif *et al.*, 2004; Luckins and Dwinger, 2004; Sood *et. al.,* 2018; Zeb *et al.,* 2019). Although

most *Trypanosoma* spp. are transmitted by haematophagous insects, ticks are also likely to be vectors of some members of this genus as the cases of *Trypanosoma theileri, T. vivax* and *T. evansi* (Marlon *et al.,* 2013; Abdullahi *et al.,* 2016; Zeb *et al.,* 2019). However, another study attempted transmission of *Trypanosoma evansi* to rats and mice by direct ingestion of engorged ticks but the possible role of ticks could not be demonstrated (Vergne *et al.,* 2011). Further studies on tick-infecting trypanosomes are needed in order to evaluate the potential role of ticks as passive vectors of trypanosomes.

2.3 TSETSE FLIES

Tsetse flies (*Glossina* spp.) are obligate bloodsucking flies of medical and veterinary importance for transmitting trypanosomes that cause African sleeping sickness and African animal trypanosomiasis (Krinsky, 2019). After trypanosomes develop in the tsetse host, the fly remains infective over its life span, which is around five to six weeks in the field (Abd-Alla, 2009).

2.3.1.1 Taxonomy

Tsetse flies belong to the order of the Diptera, family Glossinidae, genus *Glossina* with 31 species and subspecies (figure 2.5), which are grouped into three subgenera (fusca, palpalis and morsitans groups) found respectively in forests, riverine and savannah areas (Urquhart *et al.,* 1996; Rogers and Robinson, 2004; Krinsky, 2019).

All species of tsetse flies are probably capable of transmitting pathogenic trypanosomes. However, only a few species (such as *Glossina morsitans morsitans, G. m. centralis, G. pallidipes, G. palpalis palpalis, G. fuscipes fuscipes,* and *G. tachinoides*) are major vectors of trypanosomes that affect humans and domestic animals (Gooding and Krafsur, 2005).

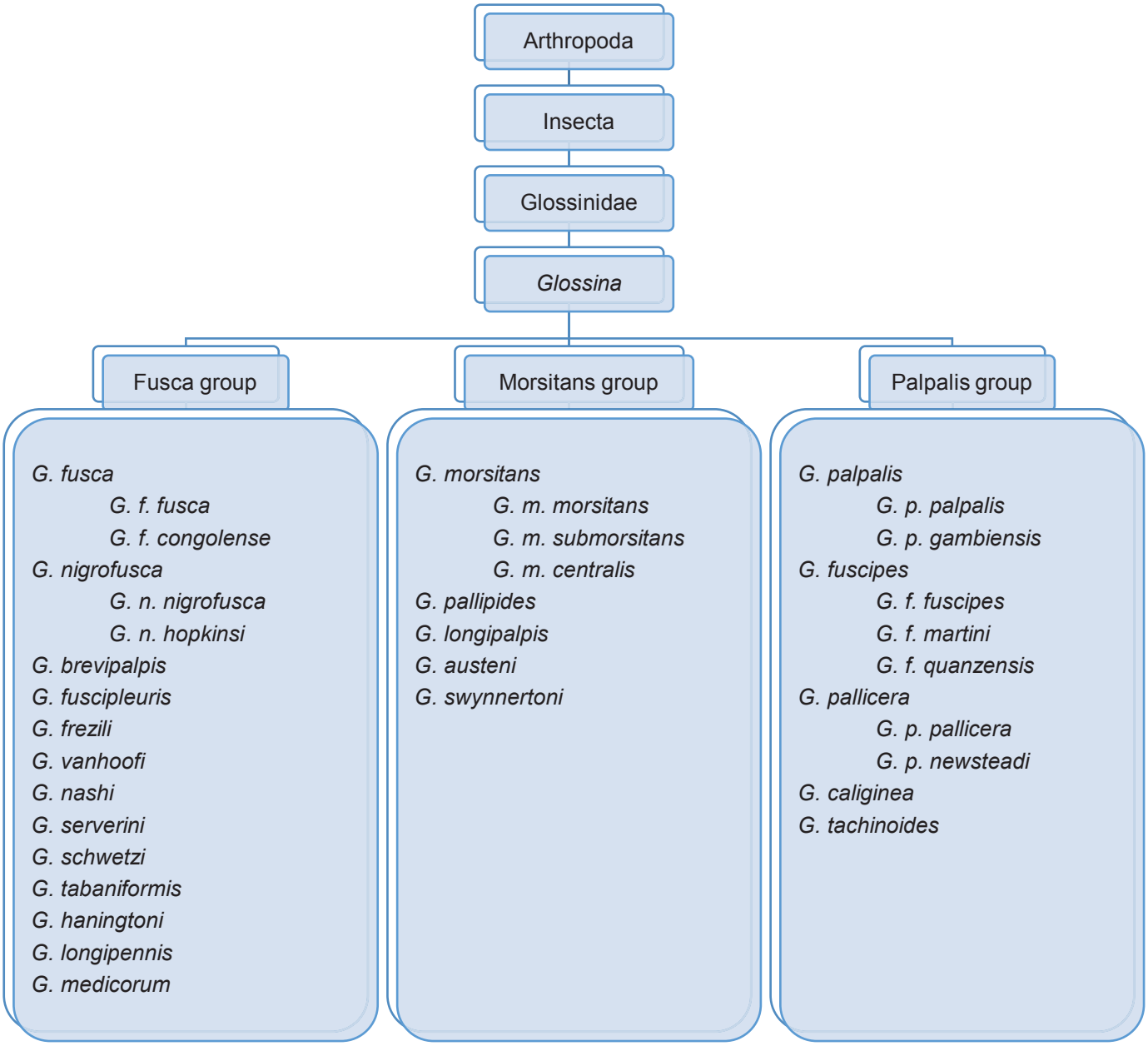

Figure (2.5) Classification of the *Glossina* sp. (Adapted from Rogers and Robinson, 2004)

2.3.1.2 Morphology

Tsetse adults (figure 2.6) are characterized by several distinctive morphological features. These include the long, rigid and forward proboscis; the position and branching of the fringe on the arista of their antenna; the folding pattern when at rest, which looks like a closed pair of scissors; and wing venation with characteristic cleaver (hatchet) cell present in the centre of the wing that can be easily distinguished from all other flies (Pollock, 1992; Urquhart *et al.,* 1996; Krinsky, 2019).

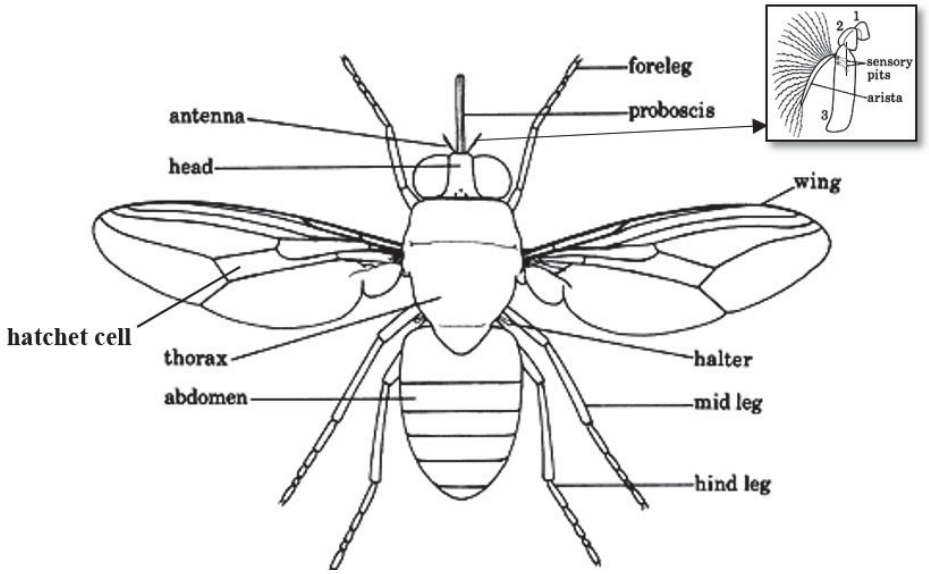

Figure (2.6) A simplified sketch of *Glossina* spp. (Tsetse fly), dorsal view (modified from Pollock, 1992)

2.3.1.3 Geographic Distribution

Tsetse flies now occur in over 10 million square kilometers in the tropical and subtropical regions of sub-Sahelian Africa (about 15ºN to 26ºS) (Urquhart *et al.,* 1996; Krinsky, 2019). Two species of tsetse flies, *Glossina fuscipes fuscipes* and *G. morsitans submorsitans*, have been recorded in southwestern Saudi Arabia (Elsen *et al.,* 1990). The geographical distributions of members of the three taxonomic groups of *Glossina* sp. are shown in figure 2.7. The palpalis group occurs primarily along watercourses in western and central Africa, while morsitans group of savanna species is primarily central and southeastern in distribution. The fusca group is found in forested areas that overlay most of the western and central African distribution of the palpalis group (Krinsky, 2019). The wide distribution of the *Glossina* vector is one of the greatest obstacles to livestock development and has prevented the establishment of sustainable agricultural systems in many areas of enormous potential (Wilkowsky, 2018). Accurate information on geographic distribution of the tsetse fly is of vital importance to better control African trypanosomiasis.

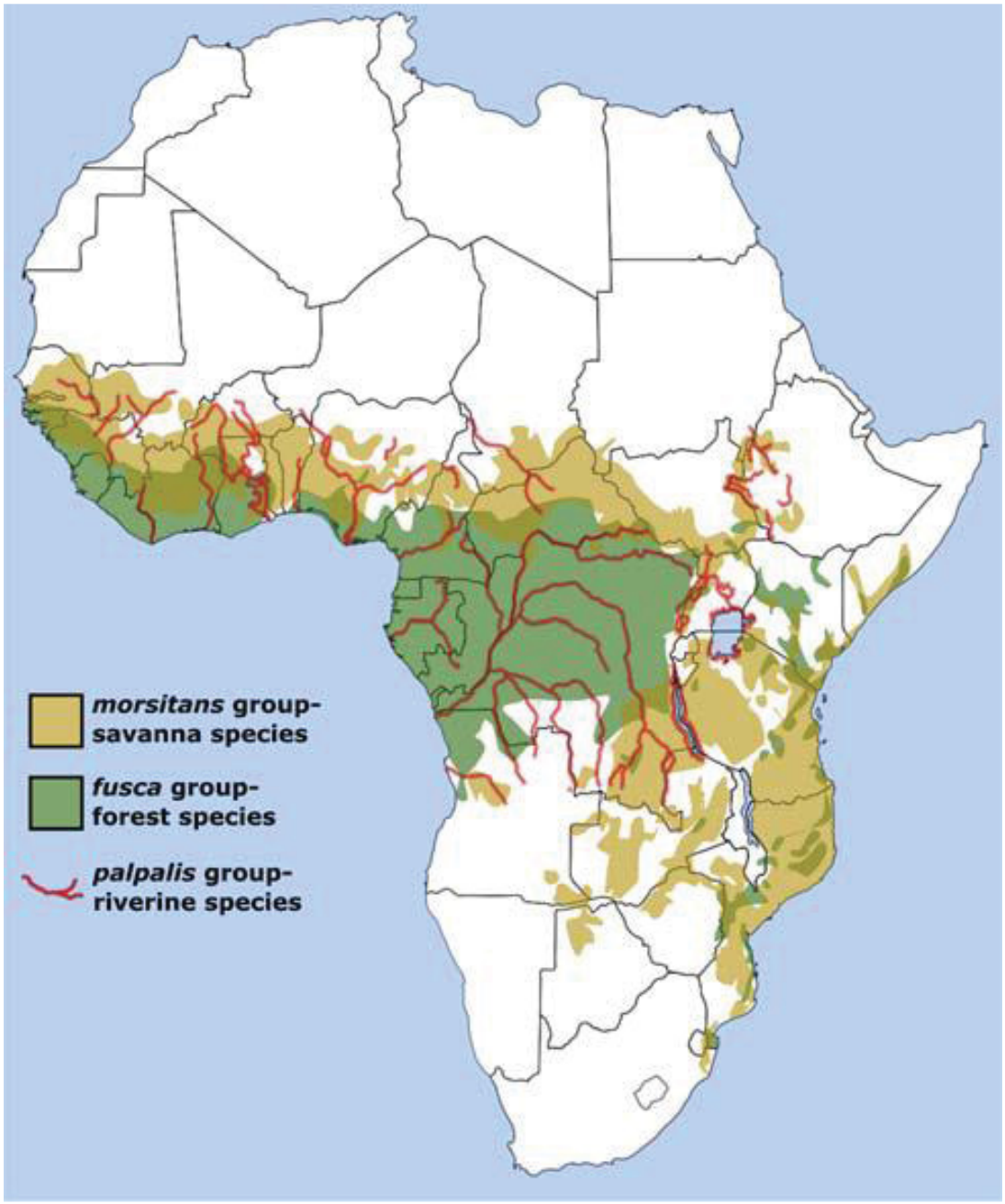

Figure (2.7) Distribution of *Glossina* spp. in Africa (Adopted from Krinsky, 2019).

2.3.1.4 Life Cycle

Tsetse adults of both sexes bite vertebrates and imbibe blood, the fly's only food (Krinsky, 2019). The females, in contrast to other muscids, are viviparous and produce only one larva at a time, up to a total of 8-12 larvae, hence, the rate of reproduction is extremely slow compared to other diptera (Urquhart *et al.,* 1996). The larva moves actively in the soil for pupation and this posteriorly has a pair of prominent dark polypneustic lobes. Adult flies emerge about 30 days after formation of the puparium (Krinsky, 2019). Breeding generally continues throughout the year with peak fly numbers occurring at the end of the rainy season, while the longevity of adult flies in nature is

variable, ranging from a few days to several months (Urquhart *et al.,* 1996). The entire process of tsetse life cycle is shown in figure 2.8 and in the available multimedia developed by Wellcome Trust Film Unit (1987). The low reproductive rate in tsetse is compensated by the extreme protection given to each larva by the female, by virtue of the viviparous mode of development. However, the low reproductive rate makes the impact of any loss of female flies greater than in species that mass-produce eggs (Krinsky, 2019). Hence, effective tsetse control reduces or eliminate the fly population in the African continent.

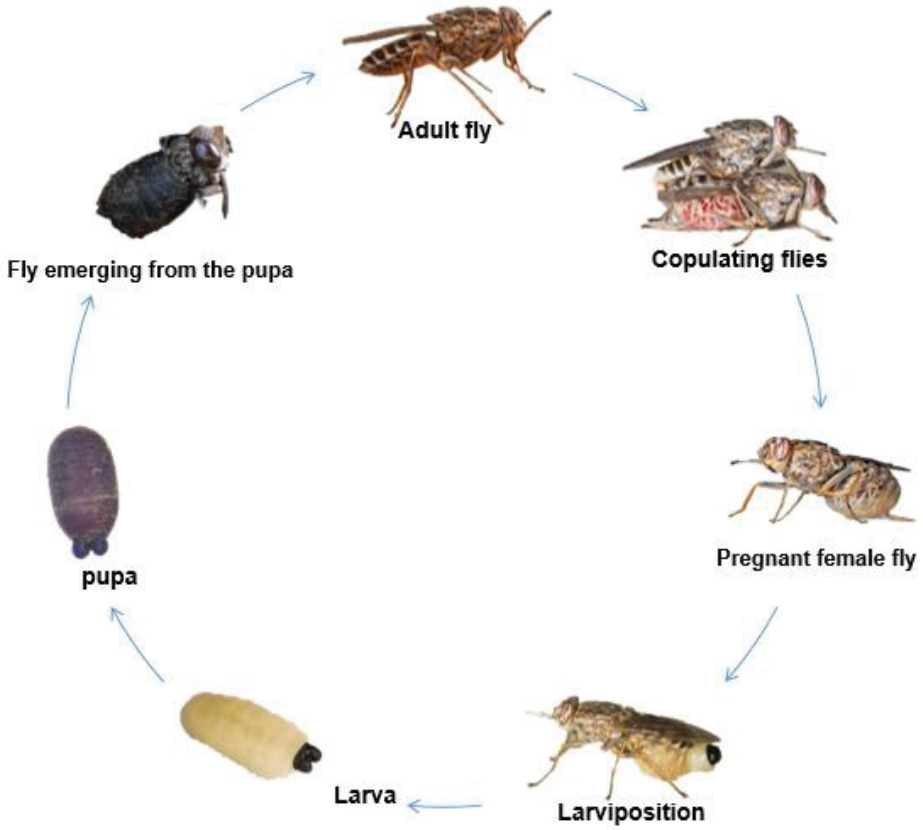

Figure (2.8) Life cycle of a Tsetse fly (modified from Leak, 1999).

2.3.1.5 Pathogenic Significance

Although the bites of tsetse flies are very painful and cause marked irritation, tsetse flies have had a great impact on human health in Africa, both as efficient vectors of trypanosomes that cause extreme human suffering in the form of African sleeping sickness and as vectors of trypanosomes that kill nonnative animals, preventing the development of animal domestication in the continent (Urquhart *et al.,* 1996; Krinsky, 2019).

2.4 TRYPANOSOMIASIS

Trypanosomiasis is a fatal disease of both animals and humans with a heavy economic loss mainly in Africa (Baral, 2010). The African trypanosomes lead into two different diseases; African Animal Trypanosomiasis (AAT) and Human African Trypanosomiasis (HAT) or sleeping sickness (Wilkowsky, 2018).

2.4.1.1 Pathogenesis

When the tsetse fly injects the infective trypomastogotes into the skin of the host, the animal triggers the immune system to synthesize antibodies but through VSG the trypanosomes escape from the host immune system to establish and progress into the disease. The metacyclic trypomastigote divide and multiply and give rise to the typical blood forms that invade the lymphatics and lymph nodes, and then the blood stream (Uilenberg, 1998). Lymphoid enlargement and splenomegaly develop associated with plasma cell hyperplasia and hypergammaglobulinaemia, which is primarily due to an increase in IgM (Urquhart *et al.,* 1996). Fever is highest at the first peak of parasitaemia and fluctuates thereafter with parasitaemia waves (Taylor and Authie, 2004). Anaemia is a cardinal feature of the disease and is proportional to the degree of parasitaemia. It is haemolytic in that the RBCs are removed from the circulation by the expanded mononuclear phagocytic system (Urquhart *et al.,* 1996). The virulence of the infecting parasite population and the age, nutritional status and breed of the host influence the severity of anaemia (Taylor and Authie, 2004). General lesions are congestive, inflammatory and degenerative, and sometimes haemorrhagic. They may affect various organs: heart, central nervous system (CNS), eyes, testes, ovaries and the pituitary gland. Congestive heart failure is an important cause of death in chronic cases and is related to the combined effects of prolonged anaemia, myocardial damage and increased vascular permeability (Taylor and Authie, 2004).

2.4.1.2 Clinical Findings

The clinical sign of a trypanosome infection varies considerably according to the species of trypanosome involved and a number of host factors (Wilkowsky, 2018). Depending on the affected species and the genetic background of the animal or breed, the outcome will fluctuate in either susceptibility or relative resistance to infection, which has been termed “trypanotolerance” (Uilenberg, 1998; Wilkowsky, 2018). However,

general clinical signs of AAT are intermittent fever, anaemia, oedema, generalized enlargement of the superficial lymph nodes, abortion, decreased fertility, reduced productivity, emaciation and often mortality (Urquhart *et al.,* 1996; Wilkowsky, 2018; OIE Manual, 2018). Ruminants die, usually of congestive heart failure, or gradually recover if the level of challenge is low, while stress results in relapse (Wilkowsky, 2018).

Post-mortem signs may include emaciation, enlarged lymph nodes, liver and spleen, excessive fluid in the body cavities, and petechial haemorrhages. Neither clinical nor post-mortem signs of trypanosomiasis are pathognomonic (Uilenberg, 1998; Taylor and Authie, 2004; OIE Manual, 2018).

Domestic and wild pigs can become infected with various species of trypanosomes but infrequently show symptoms or disease pathology unless coinfected with *T. simiae*, which is highly pathogenic for domestic pigs (Hamill *et al.* 2013; Wilkowsky, 2018). According to these authors, pigs constitute a significant reservoir of trypanosome infection in cattle, sheep, and goats. *Trypanosoma simiae* and *T. godfreyi* have been reported in livestock, but their specific role in clinical disease in ruminants remains unclear (Auty *et al.*, 2015; Simwango *et al.,* 2017).

Equines are considered to be the only natural host of *T. equiperdum*, which causes a chronic disease in horses, while most infections in donkeys seem to be mild or asymptomatic. The first stage of the disease in horses is characterized by oedema and damage of the genitalia. The second stage is pathognomonic for dourine, where periodical, typical cutaneous plaques or skin thicknesses can occur, with sizes ranging from extremely small to hand-sized. The third phase of dourine is characterized by progressive anaemia, disorders of the nervous system mainly paralysis of the hind legs, paraplegia and, finally, death (Wilkowsky, 2018).

Owing to these varied clinical manifestations, diagnosis of trypanosomiasis cannot be based on clinical signs alone, hence, laboratory confirmation of the diagnosis is an absolute necessity (Nantulya, 1990).

2.4.1.3 Laboratory Diagnosis

A variety of diagnostic tests are available and researchers are still working to improve existing tests and develop new ones (OIE Manual, 2018). Diagnosis of trypanosomes in animals can be achieved by parasitological, immunological, and

molecular methods. The choice of a particular test will be guided by economic principles and the availability of expertise in the laboratory (Wilkowsky, 2018). Reliable interpretation of results from diagnostic tests will depend on test validity as well as on proper sample selection/collection, the sample size, and the way the diagnostic tests are conducted (OIE Manual, 2018). The clinical signs are not pathognomonic and the standard techniques for the detection of trypanosomes are not sufficiently sensitive, hence, a high proportion of infections still remain undetected (Nantulya, 1990). Molecular diagnostics was developed with unique potential for sensitive and specific detection of animal and human trypanosomiasis (Büscher, 2002).

2.4.1.3.1 Parasitological Methods

Direct evidence for trypanosome infection is based on demonstrating the presence of trypanosomes in blood, lymph or cerebrospinal fluid from the putative host under the microscope (Eisler *et al.,* 2004; Wilkowsky, 2018). The simplest microscopic methods are wet and thick or thin Giemsa-stained blood films, but sensitivity depends on the blood volume used, the level of parasitaemia and the operator expertise (Wilkowsky, 2018). Therefore, these techniques have been modified using concentration methods to improve the sensitivity of the microscopic based methods (Wilkowsky, 2018; OIE Manual, 2018). Techniques for concentration of the trypanosomes by centrifugation of a blood sample are still the most widely applied in animal trypanosomiasis (Büscher, 2002). After centrifugation of blood in microhaematocrit capillary tube, motile trypanosomes can be detected directly under the microscope at the level of white blood cells (WBCs) layer and plasma, this method is so-called haematocrit centrifuge technique (HCT) or Woo method (Woo, 1970; Büscher, 2002; OIE Manual, 2018). Another concentration technique is the Murray method or buffy coat techniques (BCT), in which the capillary tube is cut 0.5 mm below the buffy coat, and this coat and the uppermost layer of RBCs are extruded on to a clean microscope slide for examination of the presence of motile trypanosomes using dark-ground or phase-contrast microscope (Murray *et al.* 1977; Wilkowsky, 2018; OIE Manual, 2018). A previous study found that the BCT was the most sensitive method for the diagnosis of African trypanosomiasis followed by HCT, wet and thick and thin Giemsa-stained blood films (Paris *et al.* 1982).

The HCT and BCT are particularly useful in that the packed cell volume (PCV) can be assessed at the same time. Measuring the PCV by placing the microhaematocrit capillary tube containing blood to a haematocrit reader is valuable for determining the degree of anaemia, which is a useful indicator of trypanosome infection (Marcotty *et al.,* 2008; OIE Manual, 2018).

2.4.1.3.2 Serological Methods

Although direct demonstration of trypanosomes in the infected animal gives conclusive proof of infection, the limitations of parasitological diagnosis in terms of low sensitivity has been the driving force for a great deal of research into alternative techniques that provide indirect evidence of infection, namely immunodiagnostic techniques (Luckins, 1992). Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomiasis, with variable sensitivity and specificity (OIE Manual, 2018).

Detection of antibodies against the parasites is usually performed by indirect fluorescent antibody test (IFAT) that uses whole parasites fixed on a slide, or Enzyme linked immunosorbant assay (ELISA), which uses trypanosome lysates as target (Wilkowsky, 2018). Both antibody detection tests have high sensitivity and genus specificity, but the species specificity is low. Another simple antibody detection method that is particularly suitable for field use in Africa is the card agglutination test for trypanosomiasis (CATT) (Eisler *et al.,* 2004). The test is based on detection of antibodies against predominant surface antigens of *T. brucei gambiense* (Büscher, 2002). A similar test exists for *T. evansi* (CATT/*T.evansi*) based on the native variant surface glycoprotein (VSG) of the predominant variable antigen type (VAT) RoTat 1.2 of *T. evansi* (Büscher, 2002; Wilkowsky, 2018). The diagnostic specificity of antibody detection tests is largely defined by the antigen preparation. Therefore, the use of crude antigen preparations should be discouraged, and initiatives to replace them with recombinant or synthetic peptides should be supported (Büscher *et al.,* 2019). Since the recombinant antigen can be expressed and easily produced in large batches in the laboratory, the recombinant fragments of *T. vivax* cathepsin L-like peptidase, *T. congolense* Cathepsin B1 and the cytoskeleton associated protein GM6 of species of subgenus *Trypanozoon*, *T. vivax* and *T. congolense* could be considered as a useful and reliable supplementary diagnostic

technique to microscopy and PCR for the detection of trypanosomiasis (Nguyen *et. al.,* 2015; Boulangé *et. al.,* 2017; Büscher *et al.,* 2019).

2.4.1.3.3 Molecular Methods

Accurate diagnosis of animal trypanosomiasis and definitive identification of the causative trypanosome species are clearly useful objectives that may contribute to the epidemiology and ultimately the long-term control of the disease (Eisler *et al.,* 2004). While these two simple objectives remain elusive with all the aforementioned parasitological and immunological technologies, molecular methods based on the detection and amplification of nuclear DNA certainly have the technical potential to achieve them. The kinetoplast DNA (kDNA) mini and maxi-circles of trypanosomes have also been used as a source of DNA probes for their specific identification (Eisler *et al.,* 2004). Several DNA sequences have been used for the sensitive and specific PCR-based detection of livestock trypanosome DNA in vertebrate host blood and/or in tsetse flies (Desquesnes *et al.,* 2001). However, accurate species/subspecies differentiation requires up to eight different PCRs per sample, which increases the costs and impacts on the practical application of the technique for large-scale epidemiological studies, with a final result often indicating a limited range of positives within the sample set (Cox *et al.,* 2005). Attempts to combine already available primers into a single multiplex PCR have been discouraging due to lower sensitivity compared to individual species-specific PCR tests and the appearance of non-specific and non-expected PCR products with some combinations of primers (Desquesnes *et al.,* 2001; Thumbi *et al.,* 2008). In order to maximize the potential of PCR while limiting the actual number of amplifications required, researches have focused on multiple species identification with a single primer set (Desquesnes *et al.,* 2001; Eisler *et al.,* 2004; Njiru *et al.,* 2005). Given the heterogeneity in the nucleotide sequence composition of both the internal transcribed spacer (ITS) and the external transcribed spacer (ETS) of ribosomal DNA (rDNA) and the conserved nature of ribosomal RNA (rRNA) gene sequences, this locus is ideal for designing oligonucleotide primers, for the discrimination of the different trypanosomes by PCR (Eisler *et al.,* 2004).

A universal PCR test (Kin-primers) have been designed to react with kinetoplastid species, annealing in the conserved regions of the 18S and 5.8S rRNA to amplify the

ITS1, the sequence of which is usually 300-800 bp in length (McLaughlin *et al.* 1996; Desquesnes *et al.,* 2001). In the ITS-1 generic PCR test, the primers are chosen in the 18S and 5.8S rRNA genes such that they match all trypanosome species of interest; the size of the single PCR fragment amplified from a trypanosome sample is then measured by gel electrophoresis against a DNA size marker (Njiru *et al.,* 2005; Gibson, 2009). The kin-primers showed a high potential for multiple species-specific diagnosis of the main livestock trypanosomes through a single PCR; however, the ITS1 based primers showed a higher diagnostic sensitivity than that previously described using the kin-primers (Njiru *et al.,* 2005). Additional test, so-called nested ITS-PCR, which detects the inter-specific length variation of the ITS regions of ribosomal genes producing a unique size of PCR product for each species has been developed by Cox *et al.* (2005). This nested ITS-PCR technique can be used to screen large numbers of biological samples directly, quickly, and accurately, making it a simple, cost effective, robust and reliable tool for investigating the complex epidemiology of African trypanosomiasis (Cox *et al.,* 2005). Table 2.1 summarises the sequences of primers for KIN (Desquesnes *et al.,* 2001), ITS1 (Njiru *et al.* 2005) and nested-ITS (Cox *et al.* 2005) PCR assays, and the amplified product size for each trypanosome.

Table (2.1) PCR primers and Trypanosome band sizes

Taxa	**Band size**	**PCR Assay**	**Specific primers**	**Primer sequence (**5′-3′)	**Reference**
T. congolense savannah	1413	Nested-ITS PCR	ITS1 outer forward ITS2 outer reverse ITS3 inner forward ITS4 inner reverse	GATTACGTCCCTGCCATTTG TTGTTCGCTATCGGTCTTCC GGAAGCAAAAGTCGTAACAAGG TGTTTTCTTTTCCTCCGCTG	Cox *et al.* (2005)
T. congolense forest	1513				
T. congolense kilifi	1422				
T. simiae Tsavo	951				
T. simiae	847				
Trypanozoon	1207 – 1224				
T. vivax	611				
T. godfreyi	–				
T. theileri	998				
T. congolense savannah	750	KIN PCR	Kin1 forward Kin2 reverse	CCTGATAAAACAAGTATCGGCAGCAA AATGTGTTCGAGTACTTCGGTCACGCT	Desquesnes *et al.* (2001)
T. congolense forest	780				
T. congolense kilifi	680				
T. simiae Tsavo	–				
T. simiae	435				
Trypanozoon	540				
T. vivax	305				
T. godfreyi	–				
T. theileri	455				

T. congolense savannah	700				
T. congolense forest	710				
T. congolense kilifi	620				
T. simiae Tsavo	370	ITS1 PCR	ITS1 forward ITS2 reverse	CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAACGAA	Njiru *et al.* (2005)
T. simiae	400				
Trypanozoon	480				
T. vivax	250				
T. godfreyi	300				
T. theileri	–				

Mini-chromosomes of the nuclear DNA contain satellite DNA (sat-DNA) which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA (Desquesnes and Dávila, 2002). The success of species-specific PCR tests prompted the development of tests to identify the human infective trypanosomes, *T. b. rhodesiense* and *T. b. gambiense*, to allow the incrimination of reservoir hosts and vectors of these pathogens (Gibson, 2009). Satellite based *Trypanosoma brucei* subspecies PCR- developed by (Moser *et al.*, 1989) identifies *T. brucei* subspecies from the *Trypanozoon* subgenus, *T. brucei rhodesiense* PCR identifies the pathogen of HAT by targeting the serum resistance associated (SRA) gene that is absent from other *T. brucei* subspecies (Radwanska *et al.,* 2002, Maina *et al.,* 2007), and this single gene PCR test has proved invaluable for identifying this zoonotic subspecies in its animal reservoir hosts (Njiru *et al.,* 2004). Furthermore, Masiga *et al.* (1992) developed PCR assays that identifies *T. congolense* sub groups; *T. congolense* savannah, *T. congolense* forest, *T*. *congolens*e Kilifi, and *T. simiae* with primers targeting sat-DNA. In addition, there exists a PCR assays that detects *T. godfreyi* (Masiga *et al.*, 1996) based on primers targeting sat-DNA, *T. vivax* (Masake *et al.*, 1997) based on primers targeting the 400 bp fragment of the species-specific antigen (ILO) and *T. evansi* (Urakawa *et al.,* 2001) targeting RoTat 1.2 VSG gene. The main primers available for species-specific PCR assays are presented in table 2.2.

Table (2.2) Species-specific PCR primers and Trypanosome band sizes

Taxa	**Band size**	**Specific primers**	**Target**	**Primer sequence** (5′-3′)	**Reference**
T. evansi	488	RoTat 1.2 forward RoTat 1.2 reverse	RoTat1.2 VSG	GCCACCACGGCGAAAGAC TAATCAGTGTGGTGTGC	Urakawa *et al.* (2001)
T. simiae	437	TSM1 forward TSM2 reverse	Sat-DNA	CCGGTCAAAAACGCATT AGTCGCCCGGAGTCGAT	Masiga *et al.* (1992)
T. brucei	173	TBR1 forward TBR2 reverse	Sat-DNA	CGAATGAATATTAAACAATGCGCAGT AGAACCATTTATTAGCTTTGTTGC	Moser *et al.* (1989)
		SRA forward	SRA gene	ATAGTGACAAGATGCGTACTCAACGC	

T. b. rhodesiense	284	SRA reverse		AATGTGTTCGAGTACTTCGGTCACGCT	Radwanska *et al*. (2002)
T. godfreyi	149	DGG1 forward DGG2 reverse	Sat-DNA	CTGAGGCTGAACAGCGACTC GGCGTATTGGCATAGCGTAC	Masiga *et al.* (1996)
T. congolense savannah	316	TCS1 forward TCS2 reverse	Sat-DNA	CGAGAACGGGCACTTTGCGA GGACAAACAAATCCCGCACA	Masiga *et al.* (1992)
T. congolense forest	350	TCF1 forward TCF2 reverse	Sat-DNA	GGACACGCCAGAAGGTACTT GTTCTCGCACCAAATCCAAC	Masiga *et al.* (1992)
T. congolense kilifi	294	TCK1 forward TCK2 reverse	Sat-DNA	GTGCCCAAATTTGAAGTGAT ACTCAAAATCGTGCACCTCG	Masiga *et al.* (1992)
T. vivax	400	ILO1264 forward ILO1265 reverse	Gene encoding specific antigen	CAGCTCGGCGAAGGCCACTTCGCTGGGGTG TCGCTACCACAGTCGCAATCGTCGTCTCAAGG	Masake *et al.* (1997)

The ITS1 and nested ITS based PCR assays detects higher numbers of positive cases, and reduced the number of PCR reactions per sample to one and two respectively, compared to the species specific primers (Thumbi *et al.,* 2008). The ITS based PCR assays definitely make PCR diagnosis more accurate, faster and less costly to carry out for large epidemiological studies on African trypanosomiasis (Cox *et al.,* 2005; Thumbi *et al.,* 2008).

Nonetheless, there are other molecular methods that can be used to detect trypanosomes and these include mobile genetic elements PCR (MGE-PCR), loop-mediated isothermal amplification (LAMP), PCR restriction fragment length polymorphism (PCR-RFLP) and nucleic acid sequence-based amplification (NASBA), however, these tests are not yet suitable for routine diagnosis (Büscher, 2002; Njiru *et al.,* 2008; Njiru *et al.,* 2011; Odongo *et al.,* 2016; Wilkowsky, 2018).

Based on molecular phylogenetic studies of the last 25 years, the classification of protists has been and continues to be extensively modified (Schnittger and Florin-Christensen, 2017). The number of phylogenetic studies of African trypanosomes has increased (Haag, 1998; Gibson *et al.,* 1999; Hughes and Piontkivska, 2003; Maganga *et al.,* 2017). This technique has yet to be applied to mixed infections, which require a more involved analysis of minisatellite markers for effective separation (Eisler *et al.,* 2004). However, molecular phylogenetic analysis is still lacking for Somali livestock trypanosomes. These limitations render necessary a molecular revision of the profusion of livestock trypanosome species, including all of those described in Somalia before this study.

2.4.1.4 Control of Trypanosomiasis

Strategies for the prevention and control of trypanosomiasis are based on reducing infection by area-wide vector control and suppression of disease in infected animals by use of curative and prophylactic trypanocidal drugs (Urquhart *et al.,* 1996; Wilkowsky, 2018). Vaccines against trypanosomes are still not available due to the antigenic variation of the trypanosomes surface coat that the trypanosomes have ability to produce a large number of antigenically different populations throughout the course of infection in the hosts (Uilenberg, 1998; Baral, 2010; 1998; Wilkowsky, 2018). A major factor compromising the establishment of effective trypanosomiasis control is the heavy economic burden this places on impoverished African countries, which already have scarce resources and often further depleted by civil unrest (Balakrishnan and Zumla, 2001).

2.4.1.4.1 Vector Control

Tsetse flies are responsible for transmission of trypanosomes that cause AAT and HAT in Africa, and are thus of economic importance in the regions they inhabit (Bateta *et al.,* 2020). The goal of programmes aimed to controlling the tsetse fly population is to reduce their numbers to a level where transmission is greatly diminished or interrupted (Balakrishnan and Zumla, 2001). Several techniques to control the transmitting tsetse fly vector have been developed and tried with varying degrees of success, including the sterile insect technique (SIT), the destruction of fly habitat, the use of insect traps, insecticide-treated animals – live baits, and Sequential Aerosol Technique (SAT) (Wilkowsky, 2018). Although habitat destruction or elimination of animal reservoirs have been used successfully in the past to control tsetse flies, they are no longer used due to their environmental implications (Grant, 2001; Wilkowsky, 2018). Biological control techniques have been found to be ineffective, hence, the mainstay of tsetse fly control is now based on the use of insecticides either through aerial spraying, ground spraying, insecticide-treated traps or screens, or insecticide-treated animals (Balakrishnan and Zumla, 2001). These techniques, albeit successful in suppressing tsetse populations, failed to eliminate the tsetse fly (Adam *et al.,* 2013). The SAT, which can effectively clear large areas of tsetse in a relatively short time, requires substantial economic and infrastructure support and this operational demands are beyond the means of most

African governments (WHO, 2020). Another method for tsetse control is the use of sterile insect technique that depends on the release of irradiated sterile males into the environment. The sterile males mate normally with females preventing them from producing any offspring resulting in reduction of the tsetse population (Abd-Alla, 2009). This technique has been successfully used in the island of Zanzibar, although the situation in Zanzibar differs from the other African countries because this island is geographically isolated from other countries reducing the chance of re-infestation from neighbouring areas (Bailey, 1998; Abd-Alla, 2009).

Another approach is the use of traps or insecticide-impregnated targets/screens. Traps are enclosures which may be hung from posts, into which tsetse flies enter and then die, either by contact with insecticide or sun exposure, while impregnated screens or targets are flat pieces of blue and black cloth suspended by wooden posts and trap flies in flight (Balakrishnan and Zumla, 2001). The olfactory attractivity of traps and targets for tsetse flies depends on their shape, size, colour and colour pattern, and this differs from species to species (Uilenberg, 1998). Different attractants such as carbon dioxide, acetone, butanone, 4-methylphenol, 3-n propylphenol, bovine or buffalo urine have been used to increase tsetse flies trapping (Uilenberg, 1998; Abd-Alla, 2009). The targets have the advantage of being relatively more effective and cheaper than traps, although traps have the distinct advantage of displaying dead tsetse flies, which helps to motivate local people to participate in schemes using this technique and counting of caught flies (Uilenberg, 1998; Grant, 2001; Abd-Alla, 2009). Similar to the concept of traps and targets, the live bait technique involves treating cattle with appropriate insecticide formulations, usually by means of cattle dips, or as pour-on, spot-on, or spray-on veterinary formulations (Abd-Alla, 2009). Pyrethroides are the chemical of choice for this application, the scheme has been proved to be successful and major reduction in tsetse populations have been achieved in different areas such as Tanzania and Burkina Faso (Fox et al., 1993; Bauer *et al.*, 1995).

In Africa, tsetse control efforts have been undertaken as individual national projects without recourse to the trans-boundary nature of the disease and the problem has always been a relapse after any control effort due to the complex mitigating factors such as rural poverty, civil strives and the lack of a concerted efforts to sustain the

individual national control efforts (Adam, 2014). Vector control efforts have succeeded in reducing infection rates, but recent resurgence in tsetse fly population density raises concerns that vector control programs require improved strategic planning over larger geographic and temporal scales (Bateta *et al.,* 2020).

2.4.1.4.2 Pathogen Control

The drugs currently in use are diminazene aceturate, which is purely curative in action, homidium bromide and chloride, which are curative with some prophylactic activity, and isometamidium which is curative but also has a strong prophylactic action (Wilkowsky, 2018). These are usually successful except where trypanosomes have developed resistance to the drug or in some very chronic cases (Urquhart *et al.,* 1996). The treatment with suramin, quinapyramine sulphate and cymelarsan are mainly used against *T. evansi* in camels (Uilenberg, 1998).

2.4.1.4.3 Use of Trypanotolerant Breed

Trypanotolerance, the innate ability of certain livestock breeds to tolerate African trypanosomes and remain productive, has been described as an economical and sustainable option for combating trypanosomiasis (Wilkowsky, 2018). Currently, trypanotolerant livestock are playing significant roles in the control of tsetse-trypanosomiasis in that their use in tsetse-affected areas allows livestock production and related development to occur that would otherwise not be possible with other breeds (Agyemang, 2005). It has previously been reported that increased bovine resistance to trypanosomosis is associated with more control over parasitemia and related anemia, two of the main pathogenic effects of trypanosome infections (Chamond *et al.,* 2010). Eastern Africa contains cattle that possess some limited degree of trypanotolerance, notably the Boran (Njogu *et al.*, 1985). Since Somali livestock are not registered to have a trypanotolerant breeds, one can conclude that if trypanosomiasis is not controlled in the country, farmers will always experience great losses of livestock during outbreaks.

2.5 ZOONOTIC POTENTIAL OF TRYPANOSOMIASIS

Humans have always depended on animals as sources of food, income, transport, employment and companionship. However, animals are also important sources of various diseases transmitted directly or indirectly to humans (Seimenis, 1998). It has been estimated that 60% of all human pathogens and 75% of emerging infectious

diseases are classified as zoonosis (Gebreyes *et al.,* 2014; Klous *et al.,* 2016). The HAT, sleeping sickness, remains a significant public health problem in Africa caused by *T. brucei rhodesiense* in East and South Africa, and *T. brucei gambiense* in West and Central Africa (Radwanska *et al.,* 2002). Following the bite of a tsetse fly, the early stage of the disease, haemolympatic phase, appears with clinical symptoms of fever, headache, joint pains and itching, while late stage of the disease, neurological phase, is characterized by the presence of the trypanosome in the cerebrospinal fluid (CSF) with typical signs of the disease: confusion, disturbed sleep pattern, sensory disturbances, extreme lethargy, poor condition, coma and death if left untreated (Steverding, 2008). Other clinical signs include adenopathy especially enlarged neck lymph nodes causing Winterbottom's sign mainly in gambiense type; this distinctive sign is diagnostic for the disease in a person who has been exposed to tsetse (Ormerod, 1991). Early-stage disease is treated with an intravenous injection of suramin in rhodesiense disease and with an intramuscular pentamidine in gambiense disease, while the arsenical melarsoprol is the only effective drug for the late-stage disease in both forms of the HAT, as the drug crosses the blood brain barrier (Baral, 2010). Furthermore, atypical human infections caused by *T. b. brucei*, *T. vivax*, *T. congolense*, *T. evansi* and *T. lewisi*, that normally are restricted to animals have been reported (Truc *et al.,* 2013). Only a few cases of atypical human trypanosomiasis have been fully confirmed, especially in Asia, leading to the hypothesis that the actual prevalence is probably underestimated (Truc *et al.,* 2013). Hence, further epidemiological studies and effective control are needed in African people; However, comprehensive national approaches supported by the international technical collaboration will alleviate or eliminate the public health and economic impact of zoonotic diseases (Seimenis, 1998).

2.6 ECONOMIC IMPORTANCE OF AFRICAN TRYPANOSOMIASIS

Trypanosomiasis, both of humans and of livestock, is one of the most important factors restricting economic development in Africa (Wilson, *et al.,* 1963). While the depredations caused by African trypanosomiasis in both people and animals were clearly identified and recognized by the beginning of the 20th century, concerted attempts to quantify and analyse its economic impact on African agriculture really only began in the 1970s (Shaw, 2004). The problem of trypanosomiasis lies at the heart of Africa's struggle

against poverty and dealing with this disease has the potential to impact on all eight United Nations Millennium Development Goals (MDGs) (Shaw, 2009). Direct impacts include low production, mortality and trypanocidal costs, while indirect impacts are aggregated into four groups: crop production, land use, ecosystem structure and function, and human welfare (Swallow, 2000). Moreover, HAT socio-economic impacts includes: disruption of daily activities, food insecurity, neglect of homestead, poor academic performance/school drop-outs and death (Bukachi *et al.,* 2017).

Animal reared under the trypanosomiasis challenge have a 11-20% lower calving rates, 10-20% higher calf mortality in susceptible breeds of cattle and 20% decrease in milk yield (Swallow, 2000; Shaw, 2004). Up to 38% weight loss and a reduction in work efficiency of oxen used to cultivate the land are additional direct effects of the disease (Shaw, 2004). Studies on sheep and goats also indicate that the main impacts of trypanosomiasis are on lambing rates (reduced by 4-38%) and kidding rates (reduced by 37%) (Swallow, 2000). In addition, the annual loss directly attributed to trypanosomiasis, in terms of reduced meat and milk production and in terms of cost related to treating the disease or controlling the vector, has recently been estimated at US $1.2 billion; However, this figure rises to over US $4.5 billion per year, if losses in potential crop and livestock production attributable to the disease are considered, and excludes the losses attributable to the effects of sleeping sickness in humans (Oluwafemi *et al.,* 2007). The lack of productive livestock in the tsetse infested area, is a key barrier in Africa to significantly improve agriculture and therefore the achievement of sustainable development (Swallow, 2000). Hence, the tsetse and trypanosomiasis control would be essential to the alleviation of hunger, food insecurity and poverty in the Africa continent.

2.7 TSETSE AND ANIMAL TRYPANOSOMIASIS SITUATION IN SOMALIA

African trypanosomiasis is a neglected trans-boundary disease transmitted mainly by the tsetse flies. Previous researches on trypanosomiasis in Somalia have been evaluated mainly by parasitological (Pellegrini, 1948; Macchioni and Abdullatif 1985; Mohamed and Dairri 1987, Dirie *et al.* 1989; Dirie *et al.* 1988a, b; Ainanshe *et al.,* 1992; Baumann and Zessin, 1992) and serological tools (Schoepf *et al.* 1984; Baumann and Zessin, 1992; Mohamoud, 2017), which are less accurate. Previous studies performed in ruminants from Somalia have shown prevalence rates ranging from 4% to 28.6% by

STDM (Bernacca, 1967; Schoepf *et al.* 1984), while in camels the prevalence ranges between 1.7% and 56.4% by STDM (Baumann and Zessin, 1992; Dirie *et al.* 1989). There are 11 different pathogenic trypanosomes species known to exist in Africa (Nakayima *et al.,* 2012). A previous study has found *T. congolense* and *T. vivax* infecting sheep (Schoepf *et al.* 1984; Dirie *et al.*, 1988b) and cattle (Moggridge, 1936; Dirie *et al.,* 1988a) from southern Somalia. Additionally, *T. simiae*, *T. congolense*, *T. brucei* and *T. evansi* have also been detected in Somali camels by STDM (Baumann and Zessin, 1992; Pellegrini, 1948; Dirie *et al.* 1989).

The Human African trypanosomiasis (sleeping sickness) is unknown in Somalia, despite the presence of *Glossina pallidipes*, a known vector of the disease in other areas of East Africa with the practice of transhumance (Harberd 1988; WHO 2007). Since domestic and wild animals play a major role as a reservoir for the human infections with trypanosomes (Hamill *et al.,* 2013; Ruiz *et al.,* 2015) with a prevalence of *Trypanosoma brucei* of 0.8% by STDM and 3.3% by PCR among domestic ruminants in Somalia (Schoepf *et al.* 1984; Hassan-Kadle *et al.*, unpublished), large epidemiological studies should focus on the detection of *T. b. rhodesiense,* HAT causative agent, in both humans and reservoir animals in the country as the movement of animals from endemic areas of Kenya (Rutto and Karuga, 2009) and Ethiopia (Baker *et al.,* 1970) to Somalia may introduce the disease into the country previously free of the zoonotic species where tsetse flies were already present (Harberd 1988).

Several insect species have been implicated in the transmission of *Trypanosoma* species in Somalia including, *Glossina* spp. (Harberd,1988) and *Tabanus* spp. (Dirie *et al.* 1989), and the relative distribution and abundance of these Trypanosome vectors is critical to the development of control options for AAT in the country. The tsetse fly distribution in Somalia appears to be restricted to the riparian and thicket vegetation of the Shabelle and Jubba river valleys with an area of about 9,407 km^2 and to a separate higher rainfall area in the South-West which is contiguous with the Kenya coastal tsetse belt with an area of about 3,070 km^2 (Harberd,1988). Four species of flies are epidemiologically important in the country: *G. austeni, G. brevipalpis, G. longipennis* and *G. pallidipes* (Moggridge 1936, Hursey 1985, Mohamed and Dairri 1987, Torr *et al.* 1989). In Somalia, tsetse populations are believed to be comparatively isolated and confined:

tsetse is absent from the upper reaches of the Shabelle river above Nuur Fanax and do not extend above Baardheere on the Jubba river. Also, tsetse infestation of the lower Jubba is separated from the Kenya salient infestation by a tsetse-free "corridor" of some 80 km width (Harberd, 1988). The author highlighted the possibility of achieving complete eradication of tsetse fly from the Shabelle and Jubba river system since the tsetse flies are in comparatively isolated and restricted area. Although extensive T and T control operations have been running since the beginning of the 20th century, African Animal Trypanosomiasis is still a major constraint of livestock production in sub-Saharan Africa (Meyer *et al.,* 2016). Control of trypanosomiasis in animals involves disease monitoring and the use of curative and prophylactic trypanocidal drugs although drug resistance is becoming increasingly common including studies from Somalia (Schönefeld *et al.,* 1987; Ainanshe *et al.,* 1992; Wilkowsky, 2018).

The economic importance of the tsetse flies and trypanosomiasis problem in Somalia is further appreciated when the importance of livestock to the Somali economy is considered. Initial estimates of the mortality losses due to trypanosomiasis, based on herd productivity surveys in the river valley areas of Somalia, showed that trypanosomiasis caused 22%, 8% and 2% of the recorded deaths and emergency slaughter of cattle, sheep and goats respectively, ranging to over 50% of the deaths of cattle in heavily tsetse-infested areas with an estimated economic impact of about 88 million US dollars (Mohamed and Dairri 1987; Harberd, 1988). Realising the huge economic losses due to animal trypanosomiasis and the risk of introduction of sleeping sickness in the country, the Somali Government created the National Tsetse and Trypanosomiasis Control Project (NTTCP) in 1980s (Mohamed and Dairri 1987). Although early T & T control campaigns were initially successful (LRDC, 1986), civil war of 1990s has disturbed the operations and discontinued the project completely from the country (Salah, 2016) and this allowed re-infestation of areas where eradication had been achieved. However, T & T control project were implemented by an international agency, International Committee of the Red Cross (ICRC), in limited areas in Shabelle and Jubba regions (ICRC, 2017) but the achievement was not sustained. Effective and sustainable T & T control projects will contribute in improving livestock health which enhances the production and livelihood of dependent families (Swallow, 2000). Furthermore, accurate

and efficient identification of the trypanosome species present in the vertebrate hosts and fly vectors is vital to assess the disease risk in Somalia.

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3 RATIONALE AND OBJECTIVES

3.1 RATIONALE

In Somalia, the widespread infestation of trypanosome vectors is one of the main obstacles to the development of the livestock industry which supports the livelihood of 12.3 million Somali communities living in an area of 640,000 km^2 (Bernacca, 1967; UNFPA, 2014). In addition, there is a lack of data on AAT after the civil war of 1990s. Moreover, the transhumance practiced by Somali livestock keepers to Ethiopia and Kenya, where the HAT is reported (Baker *et al.,* 1970; Rutto and Karuga, 2009), may introduce the HAT pathogen in the country. Therefore, there is a need to generate information about the epidemiological situation and molecular characterization of *Trypanosoma* spp. including *T. b. rhodesiense* in domestic animals (camel, cattle, goats and sheep) from Somalia.

3.2 HYPOTHESIS

- *Trypanosoma* spp. is prevalent in camels, cattle, goats and sheep in Somalia.

3.3 OBJECTIVES

3.3.1 General Objective

- To contribute to the animal diseases management in Somalia by availing recent epidemiological data on African Animal Trypanosomiasis using parasitological, serological and molecular techniques.

3.3.2 Specific Objectives

- To determine the prevalence of *Trypanosoma* spp. in camels in Somalia, using STDM, serological and molecular methods.
- To determine the prevalence of *Trypanosoma* spp. in cattle, goats and sheep in Jowhar and Afgoye districts of Somalia, using BCT and molecular methods;
- To identify *Trypanosoma* species infecting camels in Somalia by sequencing of the ITS-1 region;
- To determine factors associated with exposure/infection by *Trypanosoma* spp. in ruminants in Somalia.

3.4 REFERENCES

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4 MANUSCRIPT: PARASITOLOGICAL, SEROLOGICAL AND MOLECULAR SURVEY OF CAMEL TRYPANOSOMIASIS IN SOMALIA

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4.1 ABSTRACT

Background: Camel trypanosomiasis or surra is of great concern in Somalia, since the country possesses the largest one-humped camel (*Camelus dromedarius*) population in the world. Civil war in Somalia has resulted in the destruction of educational, research, economic and social structures, making the country scores very low for most humanitarian indicators. Previous studies on detection of *Trypanosoma* species in Somali camels have only been performed during the 1990s using standard trypanosome detection methods (STDM). Considering the lack of state-of-the-art knowledge on camel trypanosomiasis in Somalia, the present study aimed to assess the prevalence of *Trypanosoma* spp. in three districts of Somalia.

Methods: A total of 182 blood samples from *C. dromedarius* from nomadic and dairy farms were evaluated using STDM, serological (CATT/*T. evansi*) and molecular (ITS1-PCR) methods.

Results: All samples were negative for *Trypanosoma* spp. by STDM. A total of 125/182 (68.7%, 95% CI: 61.4–75.3%) camels were seropositive for *T. evansi* by CATT/*T. evansi*. Camels reared in nomadic system were more likely to be seropositive for *T. evansi* than those under dairy production system (OR: 5.6, 95% CI: 2.1–15.2, *P* = 0.0001). Five out of 182 (2.7%, 95% CI: 0.9–6.3%) camels tested positive for *Trypanosoma* sp. by ITS1-PCR. Sequencing of the ITS1 region of the *Trypanosoma* species detected herein revealed that camels were infected with *T. evansi* and *T. simiae*.

Conclusions: *Trypanosoma evansi* is highly prevalent in camels from the Banadir region of Somalia, particularly in nomadic herds. To our knowledge, this is the first study to confirm infections with *T. evansi* and *T. simiae* in Somali camels through DNA sequencing. Our data highlight the need for implementation of adequate control measures aiming to reduce the impact on camel production in the country.

Keywords: CATT/*T. evansi*, Dromedary, ITS1-PCR, *Trypanosoma evansi*, *Trypanosoma simiae*.

4.2 BACKGROUND

Trypanosomiasis are vector-borne diseases (VBD) that causes noticeable economic losses [1–3] and affects the development of both livestock and human health in Africa [4]. In Somalia, camel trypanosomiasis or surra is of great concern since the country possesses the largest one-humped camel (*Camelus dromedarius*) population in the world, estimated at nearly 8,000,000 heads [5–7]. The economic importance of camels for Somalia is due to their role as a food source, as currency, as a means of transporting milk and water as well as an indicator of social issues [7]. Camels are uniquely adapted to survive and produce under extreme arid and semi-arid conditions of Somalia [7], with the majority of animals kept by nomadic pastoralists in the country [6–7].

Civil war in Somalia has resulted in the destruction of educational, research, economic and social structures, making the country score very low for most humanitarian indicators [8]. Currently, Somali communities and their livestock are experiencing a famine and suffering from preventable diseases, due to geographical and political isolation and lack of state-of-the-art knowledge. Recently, after some security and political settlement in the country, camels are kept around urban areas as a semi-intensive dairy farming system.

In Africa, camels may be affected by tsetse-transmitted *Trypanosoma* species, including *T. simiae*, *T. brucei*, *T. congolense* and *T. vivax* [9–10]. In Somalia, previous studies on detection of *Trypanosoma* species were performed during the 1990s and reported *T. evansi* prevalence rates ranging from 1.7% to 56.4% in camels by standard trypanosome detection methods (STDM) [7, 11] and complement fixation test [7].

Additionally, *T. simiae* [9], *T. congolense* and *T. brucei* have also been detected in Somali camels by STDM [11].

Clinical signs of trypanosomiasis may be absent in camels, and thus, laboratory diagnosis should be carried out for confirmation of infection. Several methods with varying degrees of sensitivity and specificity may be used for the diagnosis of trypanosomiasis. Standard trypanosome detection methods, such as microscopical examination of fresh or stained blood-smears, has been historically used in the identification of *Trypanosoma* spp. Unfortunately, this technique lacks sensitivity and specificity. A serological assay, the card agglutination test for *T. evansi* (CATT/*T. evansi*) is a rapid diagnostic test and currently recommended by the World Organization for Animal Health [2, 12]. Additionally, molecular analysis targeting the internal transcribed spacer 1 (ITS1) region provides multi-species-specific detection of trypanosomes in a single PCR [13], and has been used in epidemiological studies.

Although the National Tsetse and Trypanosomiasis Control Project (NTTCP) was established in the 1980s in Somalia, no control measures have been implemented to date. Accordingly, considering the lack of state-of-the-art knowledge on camel trypanosomiasis in Somalia, the present study aimed to assess the prevalence of camel trypanosomiasis in three districts of Somalia using STDM, serological (CATT/*T. evansi*) and molecular (ITS1-PCR) methods.

4.3 METHODS

4.3.1 Study area

Banadir region is one of the eighteen regions of the Federal Republic of Somalia. The region itself is coextensive with Mogadishu city, the capital of the country. It consists of 17 districts, and three of them were included in this study: Kahda (2°4'4.17"N, 45°14'16.16"E), Daynile (2°4'24.61"N 45°16'48"E) and Yaqshid (2°4'3.97"N, 45°21'35.9"E). These districts are the main camel rearing areas in the investigated region.

4.3.2 Study animals and blood sampling

From December 2015 to March 2016, which represents the dry season in Somalia, a total of 182 *C. dromedarius* (176 females and 6 males) ≥ 2 years-old from nomadic (*n* = 49) and dairy (*n* = 133) farms in the Kahda (*n* = 72), Daynile (*n* = 87) and Yaqshid (*n* = 23) districts were evaluated. Blood samples were collected by jugular venipuncture.

Three millilitres were placed into tubes without anticoagulant and kept at room temperature (25 °C) until visible clot retraction; the samples were then centrifuged at 1500× *g* for 5 min, serum separated and kept at -20 °C for serological studies. One ml was placed into EDTA tubes for packed cell volume (PCV) measurement, microscopical detection of trypanosomes and preparation of blood spots on filter paper (Whatman no.4, Whatman, Springfield Mill, United Kingdom) for PCR analysis. A PCV of 0.26 l/l or less was used as an indicator of anaemia [14].

4.3.3 Parasitological diagnosis of *Trypanosoma* spp.

All camel blood samples were evaluated for the presence of *Trypanosoma* spp. by STDM. Briefly, a drop of fresh whole blood (after gentle mixing) was placed on a clean microscope slide, covered with coverslip and examined for the motile parasites, as previously described [15]. Giemsa-stained thin blood and buffy coat smears were also examined for the presence of *Trypanosoma* spp., as described elsewhere [2].

Detection of *T. evansi* antibodies by card agglutination test (CATT/*T. evansi*)

Camel serum samples were tested for the presence of *T. evansi* antibodies using the card agglutination test (CATT/*T. evansi*) [16], according to the manufacturer's instructions (Institute of Tropical Medicine, Antwerp, Belgium).

4.3.4 DNA extraction and PCR for *Trypanosoma* spp.

Genomic DNA was extracted from all 182 dried blood spots by Chelex-100 (Sigma-Aldrich, St. Louis, USA), as previously described [17]. The DNA samples were evaluated by a PCR assay targeting the ITS1 region of *Trypanosoma* species using previously described primers [18]. The PCR amplifications were performed in a total reaction volume of 25 µl containing 0.5 µl of 10 pM of each primer, 12.5 µl of 2× master mix (New England BioLabs, Ipswich, MA, USA), 9.5 µl of PCR water and 2 µl of each DNA template. PCR amplifications were performed with a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems®, Foster City, CA, USA). The amplification conditions used included an initial denaturation at 94 °C for 30 s, followed by 30 cycles of 94 °C for 30 s, 58 °C for 40 s, 68 °C for 1 min, with a final extension step at 68 °C for 5 min and cooling at 4 °C. Nuclease-free water and a *T. evansi*-positive sample (kindly donated by the VVBDI, Tanga, Tanzania) were used as negative and positive control, respectively, in all PCR runs. The amplified PCR products were analysed by electrophoresis in a 1.5% agarose

gel at 100–120V for 60 min. Quick Loading 100 bp DNA ladder (New England BioLabs) was included on each gel, stained with ethidium bromide, and finally visualized under ultraviolet (UV) illuminator (UVITEC™, Cambridge, UK).

4.3.5 Sequencing and phylogenetic analysis

Amplicons (~400 bp) obtained from two *Trypanosoma*-positive samples were sequenced in both directions by the Sanger method at Bioneer Corporation in Daejeon, South Korea and were assembled using Geneious Prime® 2019.1. Consensus sequences were subjected to BLASTn analysis [19] for determining the identity with the sequences deposited in the GenBank database.

The *Trypanosoma* ITS1 region sequences (GenBank: MH885470, MH885471) were aligned with sequences from GenBank using ClustalW [20] and alignments were improved using GUIDANCE2 [21]. The best-fit model of nucleotide substitution was determined using jModeltest v.2.1.10 [22] and was set as F81+G in the maximum likelihood (ML) phylogenetic estimation on the CIPRES Science Gateway [23], including 1000 bootstrap replicates. The resulting tree was visualized using FigTree software version 1.4.3 [24] and the final layout was rendered using Inkscape version 0.92.3 [25].

4.3.6 Data management and analysis

The PCV data were not normally distributed (Shapiro-Wilk normality test, $W = 0.98$, $P = 0.018$). Therefore, a non-parametric Mann-Whitney test was used to compare the PCV concentration between *Trypanosoma*-seropositive and seronegative camels. Either Chi-square or Fisher's exact test was used to assess association of the individual variables (district and production system) with *Trypanosoma* spp. infection. Odds ratio (OR), 95% confidence intervals (95% CI) and *P*-values were calculated, and results were considered significant when $P < 0.05$. Data were compiled and analysed by Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA).

4.4 RESULTS

All samples were negative for *Trypanosoma* spp. by STDM. A total of 125/182 (68.7%, 95% CI: 61.4–75.3%) camels were seropositive for *T. evansi* by CATT/*T. evansi*. Camels reared in the nomadic system were more likely to be seropositive to *T. evansi* than those reared under the dairy production system (OR: 5.6, 95% CI: 2.1–15.2, $P = 0.0001$). No associations between seropositivity for *T. evansi* and the three districts of

Somalia evaluated were found ($P > 0.05$). The prevalence of *Trypanosoma* sp. for each variable evaluated is summarized in Table 1.

Five out of 182 (2.7%, 95% CI: 0.9–6.3%) camels tested positive for *Trypanosoma* spp. by ITS1-PCR. Concordant results for *Trypanosoma* spp. presence determined by CATT/*T. evansi* and ITS1-PCR were found in three of 182 camels (1.6%, 95% CI: 0.3–4.7%).

Table 1 Prevalence of camel trypanosomiasis within each variable studied

Variable		CATT/*T. evansi*				ITS1-PCR			
		+/*n*	**Prevalence (%) (95% CI)**	***P*-value**	**OR (95% CI)**	**+/*n***	**Prevalence (%) (95% CI)**	***P*-value**	**OR (95% CI)**
Production system	Nomadic	44/49	89.8 (77.8–96.6)	0.0001 (χ^2 = 13.9)	5.6 (2.1–15.2)	2/49	4.1 (0.5–14)	0.408 (χ^2 = 0.45)	1.8 (0.3–11.4)
	Dairy	81/133	60.9 (52.1–69.2)			3/133	2.3 (0.5–6.5)		
District	Daynile	62/87	71.3 (60.6–80.5)	0.242 (χ^2 = 1.38)	1.5 (0.8–2.9)	2/87	2.3 (0.3–8.1)	0.502 (χ^2 = 0.45)	0.5 (0.09–3.33)
	Yaqshid	18/23	78.3 (56.3–92.5)	0.164 (χ^2 = 1.94)	2.2 (0.7–6.5)	0/23	0.0 (0.0–14.8)	0.989 (χ^2 = 0.32)	0.0
	Kahda	45/72	62.5 (50.3–73.6)			3/72	4.2 (0.9–8.8)		

Abbreviations: +, number of positive animals; *n*, number of samples; 95% CI, 95% confidence interval; OR, odds ratio

The mean PCV concentration for camels was 0.27 l/l. A total of 61/182 (33.5%, 95% CI: 26.7–40.9%) camels were anaemic. No statistical difference (U = 2944, Z = -1.89, P = 0.059) was found in mean PCV between *Trypanosoma*-seropositive (0.27 l/l) and *Trypanosoma*-seronegative camels (0.28 l/l). Association between *Trypanosoma* infection and anaemia was not found (χ^2 = 1.93, df = 1, P = 0.165).

Five *Trypanosoma*-positive samples were sequenced; however, only two sequences yielded consistent data. One *Trypanosoma*-positive sample (GenBank: MH885471) sequenced showed 99.78% (460/461 bp) identity to *T. evansi* ITS1 region sequences detected in camels from Iran (GenBank: KX898422, KX898423). The other sequence obtained (GenBank: MH885470) showed 99.25% (398/401 bp) and 98% (393/401 bp) identity with *T. simiae* ITS1 region sequence from warthogs (*Phacochoerus africanus*) of Tanzania and Zambia, respectively (GenBank: JN673387 and JN673386). The phylogenetic tree based on sequences of the ITS1 region indicated that *T. evansi* obtained herein was closely related to *T. evansi* detected in camels from Iran, whereas *T. simiae* detected in the present study was closely related to *T. simiae* detected in warthog from Zambia (Fig. 1).

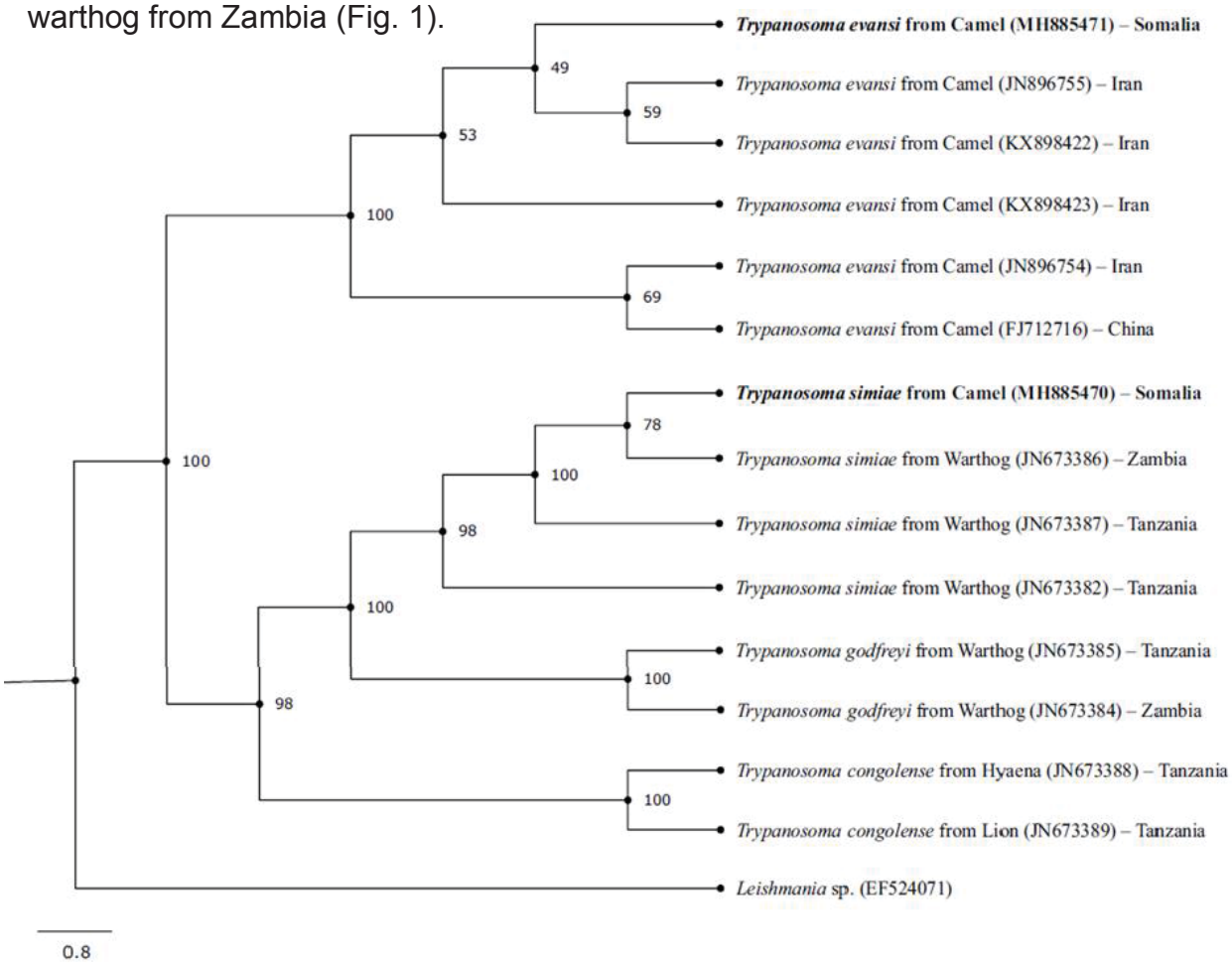

Fig. 1 Phylogenetic relationships of *Trypanosoma* spp. evaluated in camels based on ITS1 region sequence with selected sequences from GenBank (Accession numbers in the figure). The ITS1 tree was rooted with *Leishmania* sp. (GenBank: EF524071)

4.5 DISCUSSION

To the author's knowledge, this is the first study to combine STDM, serological and molecular detection of *Trypanosoma* and assess these results for potential associations with epidemiological data collected from camels in Somalia. Herein, overall 69.8% camels from the Banadir region of Somalia were positive for *Trypanosoma* spp. Interestingly, *Trypanosoma* prevalence found herein was higher than previous studies performed in camels from Somalia which have shown prevalence rates ranging from 1.7% to 56.4% by STDM [7, 11] and complement fixation test [7]. Differences among the prevalence of *Trypanosoma* may be explained by the camel population and management, diagnostic test used, and tsetse seasonal dynamics (rainy *vs* dry season).

Sequencing of the ITS1 region of the *Trypanosoma* species detected herein revealed that camels were infected with *T. evansi* and *T. simiae* (Fig. 1). Although previous studies in Somalia have reported *T. evansi*, *T. simiae*, *T. brucei brucei* and *T. congolense* infecting camels by STDM, the present study is the first to confirm infections with *Trypanosoma* spp. in camels in this country by DNA sequencing.

In the present study, all animals were negative for *Trypanosoma* by STDM, while the molecular and serological prevalence were 2.7% and 68.7%, respectively. Combining CATT/*T. evansi* and ITS1-PCR has increased the prevalence of *Trypanosoma* to 69.8%, corroborating with previous studies suggesting that improved sensitivity and specificity for detection of VBD pathogens can be achieved using different diagnostic methods [26]. The difference between the ratio of ITS1-PCR and parasitological method found herein may be explained by low parasitaemia which is typical for the chronic phase of infection [27]; this is also supported by the high seroprevalence observed indicating that a large proportion of camels are exposed to the parasite.

Herein, the *T. evansi* seroprevalence rate was significantly higher in nomadic camels as compared to dairy farm camels (P = 0.0001). Previous studies on Somali camels have shown that animals living in riverine zones of the country were more likely to acquire infection by *Trypanosoma* species than those living in inland zones [7]. The camels' role in the subsistence sector is not primarily for supply of meat and money, but mainly for provision of milk [7]. Considering that nomadic herders usually depend on traditional ethno-veterinary remedies to treat and prevent diseases in their camels [28,

29], the high *T. evansi* seroprevalence found herein highlights the need for implementation of adequate control measures aiming to reduce the impact of trypanosomes on camel production in Somalia. On the other hand, dairy animals evaluated in the present study were frequently examined by veterinary practitioners and treated with Suramin (data not shown), which may explain the low seroprevalence found.

4.6 CONCLUSIONS

Trypanosoma spp. is highly prevalent in camels from the Banadir region of Somalia, particularly in nomadic herds. To our knowledge, this is the first study to confirm infections with *T. evansi* and *T. simiae* in Somali camels by DNA sequencing. Our data highlight the need for implementation of adequate control measures aiming to reduce the impact of trypanosomes on camel production in the country, which possesses the largest one humped camel population in the world.

Abbreviations

ARTC: Abrar Research and Training Centre; AU: Abrar University; BLAST: basic local alignment search tool; CATT: card agglutination test for trypanosomiasis; CI: confidence interval; DNA: deoxyribonucleic acid; GOHi: Global One Health initiative; ITS: internal transcribed spacer; ML: maximum likelihood; NTTCP: National Tsetse and Trypanosomiasis Control Project; OR: odds ratio; PCR: polymerase chain reaction; PCV: packed cell volume; SPSS: statistical package for social sciences; STDM: standard trypanosome detection methods; UV: ultraviolet; VBD: vector-borne Diseases; VVBDI: Vector and Vector Borne Diseases Institute.

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Ethics approval and consent to participate

This study was approved by the ethical committee of Abrar University, Somalia (reference number AU/ARTC/EC/02/2015). All camel owners gave consent to sample their animals.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this article are provided within the article. Sequences have been submitted in the GenBank database under the following accession numbers: MH885470 and MH885471 for *Trypanosoma* ITS1 regions sequences of *T. simiae* and *T. evansi*, respectively.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AAHK, AMI and RFCV designed the study. AAHK, AMI and AAY collected the data. AAHK and AMI curated the data. AAHK, AMI, AAY, HSN, TSWJV and RFCV carried out the methodology. AAHK, AMI, AAY and TSWJV performed the data analysis. AAHK, AMI, HSN and RFCV drafted the manuscript. All authors read and approved the final manuscript.

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5 PARASITOLOGICAL AND MOLECULAR DETECTION OF *TRYPANOSOMA* SPP. IN CATTLE, GOATS AND SHEEP FROM SOMALIA

Manuscript submitted to the Parasitology Journal.

5.1 ABSTRACT

African animal trypanosomiasis (AAT) affects the livestock of 12.3 million Somalis and constrains their development and wellbeing. There is missing data on AAT in the country after the civil war of the 1990s. Therefore, this study has aimed to assess the prevalence of *Trypanosoma* spp. in 614 blood samples from cattle (n = 202), goats (n = 206) and sheep (n = 206) in Afgoye and Jowhar districts, Somalia using parasitological and molecular methods. Twenty-one out of 614 (3.4%; 95% CI: 2.1-5.2%) and 101/614 (16.4%; 95% CI: 13.6-19.6%) ruminants were positive for *Trypanosoma* spp. by BCT and ITS1-PCR, respectively. Using ITS1-PCR, the highest prevalence was observed in cattle (23.8%; 95% CI: 18.4-30.1%) followed by goats (17.5%; 95% CI: 12.9-23.3%) and sheep (8.3%; 95% CI: 5.1-12.9%). A total of 74/101 (73.3%; 95% CI: 63.5-81.6%) ruminants were shown coinfection with at least two Trypanosome species. The four *T. brucei*-positive samples have tested negative for *T. b. rhodesiense,* by the SRA-PCR. *Trypanosoma evansi, T. godfreyi, T. vivax, T. brucei, T. simiae* and *T. congolense* were the *Trypanosoma* species found in this study. This is the first study on the molecular detection of *Trypanosoma* sp. in ruminants in Somalia. Further investigations and control measures are needed to manage Trypanosomiasis spreading in the country. Studies should also focus on the detection of *T. b. rhodesiense* in the country.

Keywords: Trypanosomiasis, *Glossina* spp., AAT, HAT, Southwest, Hirshabelle, Somalia.

KEY FINDINGS

- *Trypanosoma* spp. were detected in cattle, goats and sheep in Afgoye and Jowhar districts of Somalia by parasitological and molecular methods.
- This is the first study on the molecular detection of *Trypanosoma* sp. in ruminants in Somalia.
- *Trypanosoma evansi, T. godfreyi, T. vivax, T. brucei, T. simiae* and *T. congolense* were the *Trypanosoma* species found in this study.
- *T. simiae* and *T. godfreyi* were detected in cattle and goats from Somalia for the first time.
- The four *T. brucei*-positive samples have tested negative for *T. b. rhodesiense,* by the SRA-PCR.

5.2 INTRODUCTION

African animal trypanosomiasis (AAT) is a parasitic disease caused by protozoans of the genus *Trypanosoma* (OIE Manual, 2013) and causes economic losses of 4.5 billion US dollars per year (Oluwafemi *et al*., 2007), as a result of direct (mortality, production losses, costs of prophylactic and curative trypanocidal drugs) and indirect losses due to crop production decay and agricultural workers' involvement (deficiency of animal protein diets) (Harberd, 1988; Angara *et al*., 2014). In Somalia, the economic impact of AAT has been estimated in 88 million US dollars (Mohamed and Dairri, 1987). Although the National Tsetse and Trypanosomiasis Control Project (NTTCP) has been established in the 1980s (Mohamed and Dairri, 1987), and a tsetse and trypanosomiasis (T & T) control project has been funded by the International Committee of the Red Cross (ICRC) in some villages of Shabelle and Jubba regions (ICRC, 2017), no other control measures or wide coverage area to reduce the losses

from trypanosomiasis have been implemented following the collapse of the central government of Somalia in 1991 (Salah, 2016). Effective and sustainable T & T control projects will contribute to improving livestock health that enhances the production and livelihood of dependent families (Swallow, 2000).

Examination of thick and thin peripheral blood or buffy coat films stained with Giemsa stains or fresh wet blood or buffy coat smears has been used for the detection and identification of Trypanosome species (Rosenblatt, 2009; OIE Manual, 2013). However, these direct parasitological techniques lack sensitivity and specificity (Mattioli and Faye, 1996; Picozzi *et al*., 2002). Therefore, molecular methods provide multi-species-specific detection of trypanosomes in a single PCR (Salim *et al*., 2011) and have been used in epidemiological studies (Picozzi *et al*., 2002; Desquesnes and Dávila 2002; Kouadio *et al*., 2014; Hassan-Kadle *et al*., 2019). Moreover, the pan-PCR techniques for Trypanosomes would reduce the cost of PCR three to five times (Njiru *et al*., 2005).

In Somalia, AAT has been reported in different domestic ruminants by standard trypanosome detection methods (STDM) (Mohamed and Dairri, 1987; Macchioni and Abdullatif, 1985; Dirie *et al*., 1988a, b; Ainanshe *et. al*., 1992). A previous study has found *T. congolense* (Schoep *et al*. 1984; Dirie *et al*., 1988b) and *T. vivax* infecting sheep (Dirie *et al*., 1988b). Both Trypanosome species have also been reported in cattle from southern Somalia (Moggridge, 1936, Dirie *et al*. 1988a). Additionally, a recent study has confirmed infections with *Trypanosoma evansi* and *Trypanosoma simiae* in camels through DNA sequencing (Hassan-Kadle *et al*., 2019). Moreover, the human African trypanosomiasis (sleeping sickness) is unknown in Somalia, despite the presence of *Glossina pallidipes*, a known vector of the disease in other areas of East Africa with the practice of transhumance (Harberd, 1988). The widespread infestation of Trypanosome vectors in Somalia is one of the main obstacles to the development of the livestock industry which supports the livelihood of 12.3 million Somali communities living in an area of 640,000 km^2 (Bernacca, 1967; UNFPA, 2014). However, there is a lack of data on AAT after the civil war of the 1990s. Hence, the

present study aimed to assess the prevalence of *Trypanosoma* spp. in cattle, goats and sheep from Afgoye and Jowhar districts of Somalia.

5.3 MATERIALS AND METHODS

5.3.1 Study Area

The present study was carried out in Afgoye (2°08'47.67"N 45°07'08.11"E) and Jowhar (2°46'38.72"N 45°30'05.85"E) districts, Somalia, areas known to be infested by tsetse and other biting flies (Hursey, 1985; Mohamed and Dairri, 1987; Dirie *et al.* 1989).

5.3.2 Sampling

From November 2017 and February 2018, which represents the dry season in Somalia, a total of 614 ruminants from Afgoye (n = 304) and Jowhar (n = 310) were evaluated. Blood samples were collected by jugular venipuncture of cattle (n = 202; 183 females and 19 males), sheep (n = 206; 190 females and 16 males) and goats (n = 206; 191 females and 15 males). Five millilitres were placed into EDTA tubes for microscopical detection of Trypanosomes and preparation of blood spots on filter paper (Whatman no.4, Whatman, Springfield Mill, United Kingdom) for PCR analysis (Ahmed *et al.*, 2011).

5.3.3 Parasitological diagnosis of *Trypanosoma* spp.

All blood samples were evaluated for the presence of *Trypanosoma* spp. by the buffy coat technique (BCT), as previously described (Murray *et al.*, 1977). The BCT was considered positive when Trypanosomes could be visually detected regardless of the species.

5.3.4 DNA extraction and PCR for *Trypanosoma* spp.

DNA was extracted from all 614 blood spots by Chelex® 100 (Sigma–Aldrich, St. Louis, USA), as previously described (Ahmed *et al*., 2011). Five different PCR assays were used to detect *Trypanosoma* spp.: (i) ITS1-PCR that amplifies the ITS1 region (Njiru *et al*., 2005), which is known to vary in size within trypanosome species, except members of *Trypanozoon* species (*T. brucei brucei*, *T. brucei rhodesiense*, *T. brucei gambiense*, *T. evansi*, and *T. equiperdum*), and therefore differentiates Trypanosomes by their ITS sizes, (ii) TBR-PCR specific for *T. brucei* (Moser *et al.,* 1989), (iii) SRA-PCR targeting the human-serum-resistance-associated (SRA) gene uniquely specific for *T. brucei rhodesiense* (Radwanska *et. al.,* 2002), (iv) RoTat 1.2 PCR which amplifies the Rode Trypanozoon antigenic type (RoTat) 1.2 variable surface glycoprotein (VSG) gene fragment specific for the detection of *T. evansi*

(Urakawa *et al.,* 2001), and (v) TSM-PCR for detection of *T. simiae* (Masiga *et al.,* 1992).

All samples were initially screened for *Trypanosoma* spp. by the ITS1-PCR assay (Njiru *et al*., 2005). Samples positive for *Trypanozoon* subspecies were further tested for *T. brucei* using TBR-PCR (Moser *et al.* 1989) and RoTat 1.2 PCR for the presence of *T. evansi* (Urakawa *et al.* 2001). Positive samples on the TBR-PCR were further screened for *T. brucei rhodensiense* by SRA-PCR (Radwanska *et. al.,* 2002). Finally, all *T. simiae* positive samples on the ITS1-PCR assay were further subjected to the TSM-PCR (Masiga *et al.,* 1992). The PCR primers used in this study are shown in table 1. A camel sample known to be positive for *T. evansi* (Hassan-Kadle *et. al.,* 2019) was used as a positive control, in all ITS1, TBR and TSM PCR runs, while. *brucei rhodesiense* DNA (kindly donated by Dr. Enock Matovu, Makerere University, Uganda) was used in SRA-PCR assay. Nuclease-free water was used as a negative control in all reactions. The amplified PCR products were analysed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and visualized by UV illuminator (UVITEC™ Cambridge, UK).

Table (1). PCR primers used in the present study

Taxa	Specific primers	Primer sequence	Reference
Trypanosoma spp.	ITS1 outer forward	5′-GATTACGTCCCTGCCATTTG-3′	[23]
	ITS2 outer reverse	5′-TTGTTCGCTATCGGTCTTCC-3′	
	ITS3 inner forward	5′-GGAAGCAAAAGTCGTAACAAGG-3′	
	ITS4 inner reverse	5′-TGTTTTCTTTTCCTCCGCTG-3′	
Trypanosoma spp.	ITS1 forward	5′-CCGGAAGTTCACCGATATTG-3′	[22]
	ITS2 reverse	5′-TTGCTGCGTTCTTCAACGAA-3′	
T. evansi	RoTat 1.2 forward	5′-GCCACCACGGCGAAAGAC-3′	[35]
	RoTat 1.2 reverse	5′-TAATCAGTGTGGTGTGC-3′	
T. simiae	TSM1 forward	5′-CCGGTCAAAAACGCATT-3′	[36]
	TSM2 reverse	5′-AGTCGCCCGGAGTCGAT-3′	
T. brucei	TBR1 forward	5′-CGAATGAATATTAAACAATGCGCAGT-3′	[33]
	TBR2 reverse	5′-AGAACCATTTATTAGCTTTGTTGC-3′	
T. b. rhodesiense	SRA forward	ATAGTGACAAGATGCGTACTCAACGC	[34]
	SRA reverse	AATGTGTTCGAGTACTTCGGTCACGCT	

5.3.5 Data management and analysis

Either Chi-square or Fisher's exact test was used to assess association of the individual variables (district and species) with *Trypanosoma* spp. infection. Odds ratio (OR), 95% confidence intervals (95% CI) and *P*-values were calculated, and results were considered significant when $P \leq 0.05$. Data were compiled and analysed by Statistical Package for Social Sciences (SPSS) version 25 (IBM Corp., Armonk, NY, USA).

5.4 RESULTS

5.4.1 Parasitological diagnosis of *Trypanosoma* spp.

A total of 21/614 (3.4%; 95% CI: 2.1-5.2%) ruminants were positive for Trypanosome species by BCT. Cattle were more likely to be infected by *Trypanosoma* spp. than sheep (OR: 3.8; $\chi^2 = 6.0$, $P = 0.01$). Ruminants reared in Afgoye district were more likely to be infected to *Trypanosoma* spp. than those reared in Jowhar district (OR: 3.4, 95% CI: 1.2-9.4, $P = 0.01$) (table 2).

Table 2: Prevalence of *Trypanosoma* spp. in cattle, goats and sheep from Afgoye and Jowhar districts, Somalia

Variable		BCT				ITS1-PCR			
		+/*n*	Prevalence (%) (95% CI)	*P*-value	OR (95% CI)	+/*n*	Prevalence (%) (95% CI)	*P*-value	OR (95% CI)
Afgoye	Cattle	10/100	10 (4.9–17.6)	0.089 ($\chi2 = 2.9$)	2.7 (0.8–8.9)	35/100	35 (25.7-45.2)	< 0.001 ($\chi2 = 18.5$)	4.9 (2.3-10.7)
	Goat	2/102	1.9 (0.24-6.9)	0.407 ($\chi2 = 0.7$)	0.5 (0.1-2.7)	14/102	13.7 (7.7-21.9)	0.385 ($\chi2 = 0.8$)	1.5 (0.6-3.5)
	Sheep	4/102	3.9 (1.1-9.7)			10/102	9.8 (4.8-17.3)		
Jowhar	Cattle	4/102	3.9 (1.1-9.7)	0.041 ($\chi2 = 4.2$)	0.0	13/102	12.7 (6.9-20.8)	0.145 ($\chi2 = 2.1$)	2 (0.8-5.3)
	Goat	1/104	0.9 (0.02-5.2)	0.318 ($\chi2 = 1.0$)	0.0	22/104	21.2 (13.8-30.3)	0.003 ($\chi2 = 9$)	3.7 (1.5-9.1)
	Sheep	0/104	0.0 (0.0-3.5)			7/104	6.7 (2.7-13.4)		
Species	Cattle	14/202	6.9 (3.8-11.4)	0.014 ($\chi2 = 6.0$)	3.8 (1.2-11.6)	48/202	23.8 (18.4-30.1)	< 0.001 ($\chi2 = 18.3$)	3.5 (1.9-6.3)
	Goat	3/206	1.5 (0.3-4.2)	0.703 ($\chi2 = 0.15$)	0.7 (0.2-3.4)	36/206	17.5 (12.9-23.3)	0.005 ($\chi2 = 7.8$)	2.4 (1.3-4.3)
	Sheep	4/206	1.9 (0.5-4.9)			17/206	8.3 (5.1-12.9)		
District	Afgoye	16/304	5.3 (3.0-8.4)	0.013 ($\chi2 = 6.2$)	3.4 (1.2-9.4)	59/304	19.4 (15.3-24.2)	0.005 ($\chi2 = 3.8$)	1.5 (0.9-2.4)
	Jowhar	5/310	1.6 (0.5-3.7)			42/310	13.6 (10.2-17.8)		

Abbreviations: +, number of positive animals; *n*, number of samples; 95% CI, 95% confidence interval; OR, odds ratio

5.4.2 PCR for *Trypanosoma* spp.

Overall, 101/614 (16.4%, 95% CI: 13.6-19.6%) ruminants tested positive for *Trypanosoma* spp. by the ITS1-PCR assay.

The highest prevalence of *Trypanosoma* spp. was observed in cattle (48/202; 23.8%) followed by goats (36/206; 17.5%) and sheep (17/206; 8.3%) (table 2). The

ITS1-PCR assay has detected 27/101 (26.7%; 95% CI: 18.4-36.5%) ruminants single infected by Trypanosomes. Additionally, coinfections with different trypanosomes were found in 74/101 (73.3%; 95% CI: 63.5-81.6%) ruminants, with coinfections mainly observed in cattle (35/48; 72.9%) and goats (26/36; 72.2%). The prevalence of *Trypanosoma* spp. by ITS1-PCR within each ruminant species evaluated are summarized in table 3.

Table (3). Single and coinfections with *Trypanosoma* spp. in cattle, goats and sheep in Somalia.

Single/Coinfections	**Species**			**Districts**		**Total +/ITS1-PCR assay (%)**
	Cattle +/n (%)	**Goat +/n (%)**	**Sheep +/n (%)**	**Afgoye +/n (%)**	**Jowhar +/n (%)**	
Single infection	13/202(6.4%)	10/206 (4.9%)	4/206 (1.9%)	9/304 (2.9%)	18/310 (5.8%)	27/101 (26.7%)
Trypanozoon	7/202 (3.5%)	9/206 (4.4%)	4/206 (1.9%)	4/304 (1.3%)	16/310 (5.2%)	20/101 (19.8%)
T. congolense	3/202 (1.5%	0/206 (0%)	0/206 (0%)	3/304 (0.9%)	0/310 (0%)	3/101 (2.9%)
T. godfreyi	3/202 (1.5%)	0/206 (0%)	0/206 (0%)	2/304 (0.7%)	1/310 (0.3%)	3/101 (2.9%)
T. simiae	0/202 (0%)	1/206 (0.5%)	0/206 (0%)	0/304 (0%)	1/310 (0.3%)	1/101 (0.9%)
Two species	22/202(10.9%)	16/206 (7.8%)	5/206 (2.4%)	30/304 (9.9%)	13/310 (4.2%)	43/101 (42.6%)
T. v & Trypanozoon	4/202 (1.9%)	11/206 (5.3%)	3/206 (1.5%)	16/304 (5.3%)	2/310 (0.6%)	18/101 (17.8%)
T. g & Trypanozoon	8/202 (3.9 %)	1/206 (0.5%)	2/206 (0.9%)	6/304 (1.9%)	5/310 (1.6%)	11/101 (10.9%)
T. s & Trypanozoon	3/202 (1.5%)	3/206 (1.5%)	0/206 (0%)	3/304 (0.9%)	3/310 (0.9%)	6/101 (5.9%)
T. c & Trypanozoon	4/202 (1.9%)	1/206 (0.5%)	0/206 (0%)	3/304 (0.9%)	2/310 (0.6%)	5/101 (4.9%)
T. v & T. c	1/202 (0.5%)	0/206 (0%)	0/206 (0%)	0/304 (0%)	1/310 (0.3%)	1/101 (0.9%)
T. g & T. c	2/202 (0.9%)	0/206 (0%)	0/206 (0%)	2/304 (0.7%)	0/310 (0%)	2/101 (1.9%)
Three species	10/202 (4.9%)	6/206 (2.9%)	8/206 (3.9%)	17/304 (5.6%)	7/310 (2.3%)	24/101 (23.8%)
T. v, T. g & Trypanozoon	7/202 (3.5%)	1/206 (0.5%)	7/206 (3.4%)	12/304 (3.9%)	3/310 (0.9%)	15/101 (14.9%)
T. v, T. s & Trypanozoon	0/202 (0%)	4/206 (1.9%)	0/206 (0%)	1/304 (0.3%)	3/310 (0.9%)	4/101 (3.9%)
T. c, T. g & Trypanozoon	2/202 (0.9%)	0/206 (0%)	0/206 (0%)	2/304 (0.7%)	0/310 (0%)	2/101 (1.9%)
T. v, T. c & Trypanozoon	0/202 (0%)	0/206 (0%)	1/206 (0.5%)	1/304 (0.3%)	0/310 (0%)	1/101 (0.9%)
T. c, T. s & Trypanozoon	1/202 (0.5)	0/206 (0%)	0/206 (0%)	1/304 (0.3%)	0/310 (0%)	1/101 (0.9%)
T. g, T. s & Trypanozoon	0/202 (0%)	1/206 (0.5%)	0/206 (0%)	0/304 (0%)	1/310 (0.3%)	1/101 (0.9%)
Four species	3/202 (1.5%)	4/206 (1.9%)	0/206 (0%)	3/304 (0.9%)	4/310 (1.3%)	7/101 (6.9%)
T. v, T. s, T. g &Trypanozoon	2/202 (0.9%)	3/206 (1.5%)	0/206 (0%)	2/304 (0.7%)	3/310 (0.9%)	5/101 (4.9%)
T. v, T. c, T. s &Trypanozoon	0/202 (0%)	1/206 (0.5%)	0/206 (0%)	1/304 (0.3%)	0/310 (0%)	1/101 (0.9%)
T. v, T. c, T. g & Trypanozoon	1/202 (0.5)	0/206 (0%)	0/206 (0%)	0/304 (0%)	1/310 (0.3%)	1/101 (0.9%)

Abbreviations: +, number of positive animals; *n*, number of samples; *T.v, T. vivax; T.c, T. congolense; T.g, T. godfreyi; T.s, T. simiae.*

Cattle (OR: 3.5, 95% CI: 1.9-6.3, $P < 0.001$) and goats (OR: 2.4, 95% CI: 1.3-4.3, $P = 0.005$) were more likely to be infected with *Trypanosoma* spp. than sheep. Ruminants reared in Afgoye district were likely to be infected by *Trypanosoma* spp. than those in Jowhar district (OR: 1.5, 95% CI: 0.9-2.4, $P = 0.005$). The highest

prevalence of Trypanosome infection was recorded in cattle and goats from Afgoye and Jowhar districts, respectively (table 2).

Ninety-one ITS1-PCR *Trypanozoon*-positive samples were further tested by species-specific PCR to characterize the *T. evansi* and *T. brucei.* As a result, four out of 91 (4.4%) ruminants tested positive for *T. brucei* by the TBR-PCR assay, while the remaining 87/91 (95.6%) ruminants tested positive for *T. evansi* by the RoTat 1.2 PCR assay. All the four *T. brucei*-positive samples have tested negative for *T. b. rhodesiense* by the SRA-PCR assay. Additionally, two PCR assays were used to detect *T. simiae* in the present study: the ITS1-PCR was able to detect 19/101 (18.8%) ruminants, while 12/19 (63.2%) ruminants tested positive by the TSM-PCR assay.

5.5 DISCUSSION

To the author's knowledge, this is the first study to combine BCT and molecular detection of Trypanosome species in ruminants from Somalia. The overall prevalence recorded by ITS1-PCR was significantly higher than the one observed with the BCT technique ($P<0.001$, $\chi^2 = 58.3$). This was expected due to the known higher sensitivity of molecular methods, as previously reported (Angwech *et al.,* 2015; N'Djetchi *et al.,* 2017).

Herein, overall 3.4% ruminants were positive to *Trypanosoma* spp. by using the BCT technique. Previous studies performed in ruminants from Somalia have shown prevalence rates ranging from 4% to 28.6% by STDM with a record of *T. congolense, T. vivax and T. brucei* (Moggridge, 1936; Schoepf *et al.,* 1984; Dirie *et al.,* 1988a, b; Ainanshe *et al.,* 1992). Considering that *T. brucei* and *T. evansi* are morphologically indistinguishable (Gibson 2003), it is important to consider that previous studies performed in Somalia may have misclassified the *Trypanosoma* species detected in ruminants.

In the present study, 16.4% ruminants tested positive for *Trypanosoma* spp. by the ITS1-PCR assay, which is in agreement with a previous study that has reported a prevalence of 17.2% in Tanzania (Simwango *et al.*, 2017). However, our finding is lower than that reported in Sudan (57.7%) (Osman *et al.*, 2016) and Uganda (41%) (Angwech *et al.*, 2015), but it is higher than that reported in Zambia (7.5%) (Laohasinnarong *et al.*, 2015). Differences among the prevalence of Trypanosome species may be explained by the population studied and management, and tsetse seasonal dynamics (rainy vs dry season).

Previous studies have shown that sheep is more frequently infected by *Trypanosoma* species than goats under natural conditions, suggesting that goats are more refractory to Trypanosome infections than sheep (Masiga *et al.*, 2002; Ng'ayo *et al.*, 2005). In the present study, *Trypanosoma* spp. infection rates were significantly higher in cattle (23.8%; $P<0.001$, $\chi^2 = 18.3$) and goats (17.5%; $P=0.005$, $\chi^2 = 7.8$) than in sheep (8.3%), with goats being 2.4 times more likely to be infected by these protozoa than sheep. This may be attributed by the host susceptibility to Trypanosome infections and the skin coat suitability for fly feeding (Murray *et al.,* 1982; Wilkowsky, 2018).

Trypanosoma evansi (86.1%) followed by *T. vivax* (45.5%) were the predominant *Trypanosoma* species detected in ruminants from the studied areas. This may be due to the camel grazing in the area and the presence of other biting flies which may accelerate the mechanical transmission of the parasite (Mossaad *et. al.* 2020). The *T. simiae* and *T. godfreyi* were detected in cattle and goats, which may be linked to the presence of warthog (*Phacochoerus africanus*) and bushpig (*Potamochoerus larvatus somaliensis*) in the studied districts (data not shown).

Although a previous study has detected *T. simiae* in sheep from Kenya (Ng'ayo *et al.*, 2005), herein all sheep tested negative for this protozoon. Wild pigs constitute a reservoir for *T. simiae* and *T. godfreyi* infections to ruminants (Hamill *et al.,* 2013). To the author's knowledge, this is the first study to detect *T. simiae* and *T. godfreyi* in cattle and goats from Somalia.

In the present study, ruminants reared in Afgoye district were 1.5 times more likely to be infected by *Trypanosoma* spp. than those in Jowhar district ($P = 0.005$, $\chi^2 = 3.8$). Both districts are located along with the Shabelle river, where tsetse and other biting flies are often abundant (Hursey, 1985; Mohamed and Dairri, 1987; Dirie *et al.* 1989). However, in Jowhar, livestock owners repel flies with smoke from smouldering cattle dung around the animal shelters with cattle being grazed during the night, when flies are inactive, while goats and sheep are grazed during the day. This probably explains why cattle (12.7%) Trypanosome infection rate was lower than goats (21.2%), with goats being 3.7 times more likely to be infected by *Trypanosoma* spp. than sheep ($P = 0.003$, $\chi^2 = 9$). Conversely, in Afgoye, cattle, goats and sheep are grazed during the day, when flies are active, and no other local strategies for controlling T & T have been implemented. This possibly explains why cattle Trypanosome infection rate (35%) was higher than goats (13.7%) and sheep (9.8%), with cattle being 4.9 times

more likely to be infected by *Trypanosoma* spp. than sheep (P<0.001, χ^2 = 18.5). Thus, this may explain why the overall Trypanosome prevalence rate in Afgoye was higher than in Jowhar district.

Small ruminants constitute a key component of livestock in many regions of East Africa. A previous study has shown that sheep and goats may harbour *T. b. rhodesiense*, the causative agent of sleeping sickness or human African trypanosomiasis (Ng'ayo *et al.*, 2005). Additionally, previous studies have also implicated that cattle may act as a reservoir of Trypanosomes to humans (Onyango *et al.*, 1966; N'Djetchi *et al.,* 2017). In the present study, all animals tested with the SRA primer set were tested negative for *T. b. rhodesiense*. Considering that the *T. b. rhodesiense* vector, *Glossina pallidipes*, has been reported in Somalia (Mohamed and Dairri, 1987), associated to the fact that this Trypanosome species is present in ecologically similar areas of neighbouring Kenya (Rutto and Karuga, 2009) and Ethiopia (Baker *et al.,* 1970), further studies should focus on the detection of *T. b. rhodesiense* in the country.

5.6 CONCLUSION

The present study has shown that AAT is prevalent in cattle, goats and sheep from Afgoye and Jowhar districts of Somalia, with the identification of *Trypanosoma evansi, T. brucei, T. vivax, T. congolense*, *T. godfreyi* and *T. simiae*. Coinfections with at least two Trypanosome species were also reported in all ruminants in this study. This is the first study on the molecular detection of *Trypanosoma* spp. in Somali ruminants as well as detection of *T. simiae* and *T. godfreyi* in cattle and goats in Somalia. Further large-scale studies and sustainable control programmes against trypanosomes and its vectors are needed in the country. In addition, studies should also focus on the possibility of detecting of *T. brucei rhodesiense,* HAT causative agent, in the country.

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Ethics statement

This study was approved by the ethical committee of Abrar University, Somalia (reference number AU/ARTC/EC/04/2017). All cattle, goats and sheep owners gave consent to sample their animals.

Conflict of interest

None

5.7 REFERENCE

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6 GENERAL CONCLUSIONS

African trypanosomes cause significant disease in humans and animals in Africa. The present study has shown that animal trypanosomiasis are prevalent in camels, cattle, goats and sheep from Mogadishu city, and Afgoye and Jowhar districts of Somalia. This is the first study on the molecular detection of *Trypanosoma* sp. in ruminants in Somalia, with the identification of *Trypanosoma evansi, T. brucei, T. vivax, T. congolense*, *T. godfreyi* and *T. simiae.* Coinfections with at least two Trypanosome species were also reported in the present study. Further epidemiological investigations and sustainable control measures are needed to manage infections of *Trypanosoma* spp. and its spreading in the country. In addition, studies should also focus on the detection of *T. b. rhodesiense,* HAT causative agent, in the country.

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ANNEX 1 – ETHICS COMMITTEE APPROVAL (N^{O} AU/ARTC/EC/02/2015)

Abrar University

ETHICS COMMITTEE CERTIFICATE OF APPROVAL

Ethical reference number: AU/ARTC/EC/02/2015

Date: 28/6/2015

This ethical certificate is issued by the Abrar University Research Ethics Committee in respect of the research project outlined below on the recommendations of the College of Veterinary Medicine Committee.

Title of Research Project: Parasitological, serological and molecular detection and characterization of camel Trypanosomiasis in Banadir region, Somalia

Investigator: Dr. Ahmed Abdulkadir Hassan-Kadle.

Supervisor: Prof. Dr. Rafael Felipe da Costa Vieira. Department of Veterinary Medicine, Federal University of Parana/ Brazil.

Co-Supervisor: Prof. Dr. Abdalla Mohamed Ibrahim. College of Veterinary Medicine, Abrar University/ Somalia.

The Clearance is given on the following conditions:

(a) Any significant changes in the conditions or undertaking or outline in the approved research proposal must be communicated to the Committee.

(b) Any breach of ethical undertaking or practices that have an impact on ethical conduct of research must be reported to the Committee.

(c) Principal researcher must report through his Supervisors and Committee issues of ethical compliance at the end of the research project.

(d) The Committee retains the right to withdraw or amend the ethical clearance if any breach of ethical practices as outlined in the ethical Policy and Abrar University Research Guidelines is detected or suspected.

Dr. Hassan Mohamed A
Chair, AU Research Ethics Committee
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ANNEX 2 – ETHICS COMMITTEE APPROVAL (N^{O} AU/ARTC/EC/04/2017)

Abrar University

ETHICS COMMITTEE CERTIFICATE OF APPROVAL

Ethical reference number: AU/ARTC/EC/04/2017

Date: 15/10/2017

This ethical certificate is issued by the Abrar University Research Ethics Committee in respect of the research project outlined below on the recommendations of the College of Veterinary Medicine Committee.

Title of Research Project: Parasitological and molecular detection of *Trypanosoma* species from domestic animals in the tsetse infested areas of Somalia

Investigator: Dr. Ahmed Abdulkadir Hassan-Kadle.

Supervisor: Prof. Dr. Rafael Felipe da Costa Vieira. Department of Veterinary Medicine, Federal University of Parana/ Brazil.

Co-Supervisor: Prof. Dr. Abdalla Mohamed Ibrahim. College of Veterinary Medicine, Abrar University/ Somalia.

The Clearance is given on the following conditions:

(a) Any significant changes in the conditions or undertaking or outline in the approved research proposal must be communicated to the Committee.

(b) Any breach of ethical undertaking or practices that have an impact on ethical conduct of research must be reported to the Committee.

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