Characterization of genetic diversity of bovine trypanosomes in African and Middle Eastern countries

(アフリカおよび中近東におけるウシ感染性トリパノソーマ原虫の遺伝的多様性に関する研究)

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List of publications related to the dissertation

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List of abbreviations

AAT	African animal trypanosomiasis
apoL-I	apolipoprotein L-I
CATT	Card agglutination test
DADO	District Agriculture Development Office
DAHLDO	District Animal Health and Livestock Development Officer
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ESAG	Expression site associated genes
HAT	Human African trypanosomiasis
ITS1	Internal transcribed spacer 1
kDNA	kinetoplast DNA
LAMP	Loop-mediated isothermal amplification
NGS	Next generation sequencing
OIE	World Organization for Animal Health
PCR	Polymerase chain reaction
PCV	Packed cell volume
rDNA	ribosomal DNA
RoTat1.2	*Rode Trypanozoon antigen* type 1.2
rRNA	ribosomal RNA
SRA	Serum resistance associated
Tb	*Trypanosoma brucei*
VSG	Variant surface glycoprotein
WHO	World Health Organization

1. General introduction

1.1 Classification of trypanosomes

Trypanosome parasites are the member of the sub-kingdom *Discoba*, phylum *Euglenazoa*, class *Kinetoplastea*, order *Metakinetoplastina*, family *Trypanosomatidae*, and genus *Trypanosoma* (NCBI, 2020) (Figure1).

The family *Trypanosomatidae* (phylum *Euglenozoa*) are uniflagellate protozoa found in vertebrates and also in insects and plants (Cayla *et al*., 2019). Trypanosomatids belong to the kinetoplastids which are defined by the unique mitochondrial genome of a kinetoplast (Vickerman *et al*., 1965; Lukes *et al*., 2005). The kinetoplast contains "minicircles" and "maxicircles" DNA (Shapiro *et al*., 1995; Ryan *et al*., 1998), which vary in number and sequence according to the species (Borst *et al*., 1985). *Trypanosoma* is a genus that causes the medical and veterinary concern in the family *Trypanosomatidae* (Giordani *et al*., 2016; Büscher *et al*., 2017).

The species from the *Trypanosoma* genus are classified in several subgenera; *Duttonella, Herpetosoma, Nannomonas, Pycnomonas, Schizotrypanum, Trypanozoon*, or unclassified subgenus. *Trypanosoma* genus is also divided into two sections, *salivaria* and *stercoraria* according to the part of the digestive tract of the vector where the parasitic cycle occurred (Desquesnes *et al*., 2016) (Figure 1). *Salivaria* trypanosomes develop to the infective form in the salivary glands, thus the parasites are mostly transmitted to the mammals by feeding behavior of the vectors. On the other hand, *stercoraria* trypanosomes grow in the posterior part of the midgut, thus the parasites are mostly transmitted to the mammals through contact with vector feces (Desquesnes *et al*., 2016).

Salivarian pathogenic trypanosomes consists of the subgenera *Trypanozoon*, *Duttonella*, *Nannomonas*, and *Pycnomonas* (NCBI, 2020). They cause diseases in mammalian hosts

including humans, livestock, and wildlife affecting their health status in various ways. *Trypanozoon* consists of human and animal infective trypanosomes. *Trypanosoma evansi* belongs to the sub-genus *Trypanozoon,* which is the pathogenic mammalian trypanosome first discovered in the world at 1880 from the Indian horses and camel blood (Hoare, 1972). *Trypanosoma evansi* is commonly observed as a long slender form of the parasite in the bloodstream with very limited polymorphism (Hoare, 1972; Misra *et al*, 2016). It is discriminated from the others by a reduced (lack of maxi-circles with identical mini-circles) or a total absence of kinetoplast DNA (kDNA) (Borst *et al*., 1987). Owing to the loss of kinetoplast DNA which is required for the oxidative phosphorylation in the tsetse fly, *T. evansi* is "trapped" in the bloodstream form (Paris *et al*., 2011). The change in kDNA characteristics could be utilized to characterize *T. evansi* from other *Trypanozoon* species. *Trypanosoma evansi* has long been considered to be a monophyletic species independent from *Trypanosoma brucei*. However, the recent genome-scale sequence studies suggested a more complex relationship between *T. evansi* and *T. brucei* parasites. According to a series of genome-wide analyses, *T. evansi* and *T. brucei* are not monophyletic, and *T. evansi* is sometimes clustered together with *T. brucei* from the same geographical area (Carnes *et al*., 2015; Cuypers *et al*., 2017; Richardson *et al*., 2017). These data indicate that *T. evansi* emerged multiple times from different *T. brucei* ancestors and should be classified as a subspecies of *T. brucei*. Nevertheless, the nomenclature of the *Trypanozoon* parasites is still under debate. A similar argument, whether *Trypanosoma equiperdum* should be classified as a separate species from *T. brucei*, has long been discussed until now (Claes *et al*, 2005). The other *Trypanozoon* species is *T. brucei* which only exists in sub-Saharan Africa. *Trypanosoma brucei* is further classified into three morphologically similar subspecies; *Trypanosoma brucei rhodesiense, Trypanosoma brucei gambiense,* and *Trypanosoma brucei brucei*.

Dutonella, also classified as salivarian trypanosomes, includes two species of *Trypanosoma vivax* and *Trypanosoma uniforme*. The studies for this subgenus are often conducted on *T. vivax* due to its economic importance in the field of livestock particularly in South America. However, despite its economic significance, *T. vivax* remains as the less studied parasite compared to the other pathogenic trypanosomes due to the limitation on experimental animal model and *in-vitro* culturing. The last pathogenic salivarian trypanosomes discussed is *Nannomonas*. This subgenus includes *Trypanosoma simiae, Trypanosoma godfreyi*, and *Trypanosoma congolense*, which are the common trypanosomes infecting livestock and wildlife. *T. simiae* and *T. godfreyi* usually infect *Suidae* family, such as: warthogs, domestic pigs, etc. *Trypanosoma congolense* infects a broader range of animals including domestic animals and wildlife. These *Nannomonas* species are considered to be non-infective to humans.

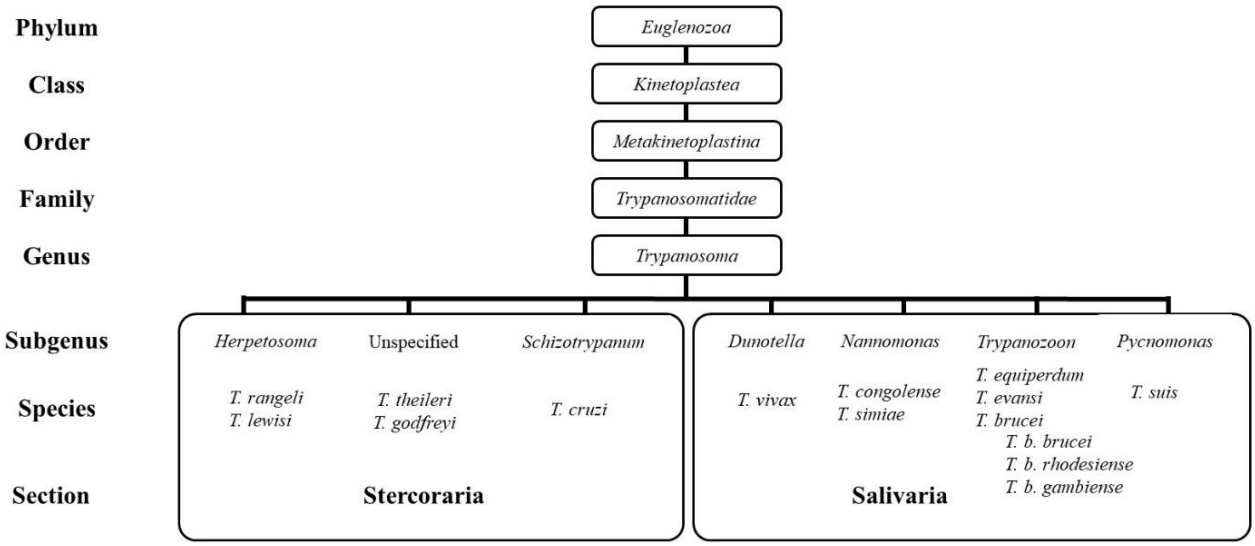


Figure 1. Classification chart of trypanosomes.

The taxonomic rank is shown on the left. Representative species in each subgenus are shown. Sections are defined based on where the parasitic cycle occurred in the digestive tract of the vector. Sections are shown at the bottom.

Source: The NCBI Taxonomy database

1.2 Human and Animal Trypanosomiasis: distribution, pathogenesis, and clinical signs

The major human trypanosomiases caused by the human infective trypanosomes is sleeping sickness, or human African trypanosomiasis (HAT) (Hoare, 1972). HAT is a disease in sub-Saharan Africa which is transmitted by tsetse flies (Hoare, 1972). It occurs in two forms with distinct disease features; gambiense HAT caused by *T. b. gambiense* and rhodesiense HAT caused by *T. b. rhodesiense*. Gambiense HAT is an anthroponotic disease that mostly transmitted from human-to-human, therefore humans play a role as the main reservoir and animals play a minor one (Franco *et al*., 2014). It is distributed widely in west and central Africa and responsible for the 98% of reported cases of HAT (WHO, 2019) (Figure 2). It causes a chronic infection that may be largely asymptomatic over a long period. When the clinical signs get more obvious, the patient is usually in the late disease stage, and the central nervous system is already affected (Brun *et al*, 2010). In contrast, rhodesiense HAT is a zoonotic disease, mainly transmitted from animals (wildlife and livestock) to humans (Franco *et al*., 2014). It has spread in 13 countries in eastern and southern Africa, which accounts for under 2% of reported cases (WHO, 2019) (Figure 2). Rhodesiense HAT is an acute disease, which takes only a few weeks or months after the tsetse bite for the parasite to invade the central nervous system (Brun *et al*, 2010).

On the other hand, most trypanosomes infect only non-primate animals which cause the complex domestic animal trypanosomiasis called African animal trypanosomiasis (AAT), or “nagana” in Africa. AAT is the disease which is characterized by the high mortality in the domestic animals and causes serious economic losses. AAT is caused by trypanosomes belong to the subgenera *Nannomonas* (*T. congolense*, *T. simiae*), *Trypanozoon* (*T. b. brucei*, *T. b. rhodesiense*) and *Dutonella* (*T. vivax*). They are endemic in sub-Saharan Africa, except *T. vivax*, which can be also found outside Africa (Figure 3).

Infections in cattle by African animal trypanosomes cause a diverse clinical sign of disease from acute, sub-acute, to chronic. The bovine trypanosomiasis is recognized by anemia, fever, diarrhea, extreme weight loss, and even death (Mare, 2004). The major clinical signs in acute forms include anemia, intermittent fever, lacrimation, swollen in lymph nodes, oedema, abortion and decreased fertility, loss of appetite, body condition and productivity, early death. Especially in cattle, the disease is usually chronic, but it will relapse when cattle are stressed (Bekele *et al*., 2015). Some indigenous breed of cattle is known to be genetically resistant to AAT including West African taurine breeds of cattle, N'Dama and West African Shorthorn (Murray *et al*., 1984).

Trypanosoma evansi causes a globally distributed disease "Surra" in livestock and wildlife (Gutierrez *et al*., 2010). Surra is reported in North and Northeast Africa, Latin America, the Middle East and Asia, and Europe (Gutierrez *et al*., 2010) (Figure 3). This disease has two clinical forms acute form found in horses and camels, and a chronic form found in cattle and water buffalo. Animals suffer from fever, anaemia, extreme weight loss and exhaustion, and eventual death in acute infections, while in chronic infections, irregular fever during parasitaemia peaks occurred. Surra in bovids is generally known to be a chronic, mild, or asymptomatic disease (Gill, 1977). Fever, abortion, and decreased milk production are frequently reported in dairy cattle (Kashiwazaki *et al*., 1998; Pholpark *et al*., 1999) while in beef cattle, high mortality was observed when surra occurs for the first time in a new area (Chobjit *et al*., 2006).

Trypanosoma theileri is commonly found in healthy cattle and establish a fine host-parasite relationship. Although the infection of *T. theileri* is considered as non-pathogenic trypanosomiasis which is usually exhibit low parasitemia, infected animals may develop increased parasitemia and clinical disease when they are stressed and immune-suppressed (Connor *et al*., 2004).

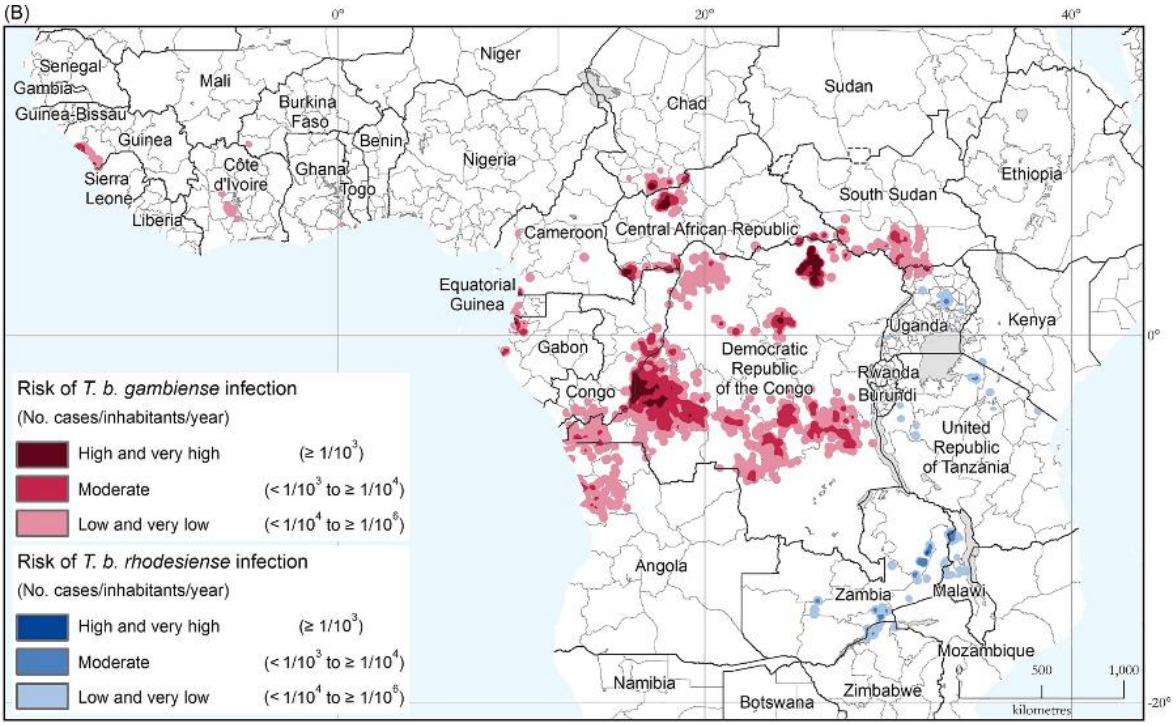


Figure 2. The reported cases of human African trypanosomiasis in tsetse-belt Africa during period 2012-2016.

Red and blue color indicates the *T. b. gambiense* and *T. b. rhodesiense* infections, respectively. Higher infection cases are shown with higher intensity of color. Gambiense HAT is endemic in west and central Africa, while rhodesiense HAT is endemic in eastern and southern Africa. Source: Franco *et al.*, 2017.

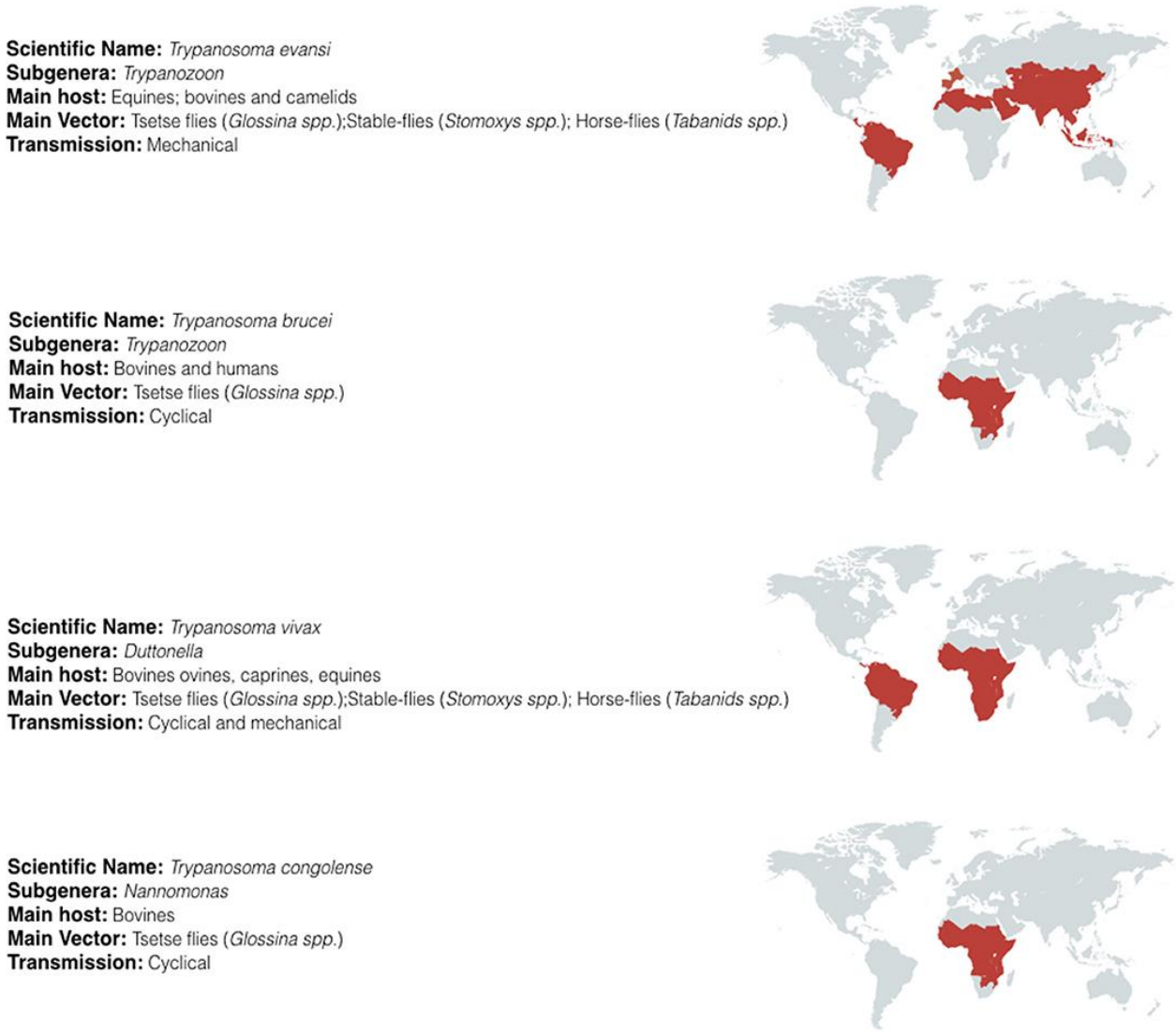


Figure 3. Distribution map of four trypanosome parasites causing bovine trypanosomiasis.

The distribution of *T. evansi*, *T. brucei*, *T. vivax*, and *T. congolense* are shown. The red colors indicate the regions where the parasites are commonly found. The parasite distributions highly depend on the endemicity of vectors.

Source: Radwanska *et al*., 2018

1.3 Transmission and life cycle of trypanosomes

Transmission and life cycle of trypanosomes are mostly investigated using *T. brucei* as a model. The short stumpy form parasite is taken up by the fly, progress through the body of the insect vector passing via the midgut, proventriculus and salivary gland, to be re-injected through the proboscis as infective metacyclic trypomastigotes into a new target (Vickerman *et al*., 1985) (Figure 4).

Trypanosoma evansi transmission in livestock usually relies on mechanical transmission by haematophagous flies, especially by tabanids and *Stomoxys* flies where the parasites survive for short time in the vector. The contamination of a wound could also possible to facilitate the transmission of *T. evansi*. Besides those factors, iatrogenic transmission due to the use of nonsterile surgical instruments or needles especially during mass treatments and vaccination campaigns might spread the disease too (Davila *et al*.,2000). In Latin America, a biological transmission by the vampire bat has also been suggested (Hoare *et al*., 1972). Vampire bats usually get the infection by the oral route when they leak blood from an infected prey, mostly cattle and horses. As a host of *T. evansi*, bats may show clinical symptoms and die within one month during the initial phase of the disease (Desquesnes, 2004). Similar to *T. evansi, T. vivax* is also mechanically transmitted by tabanids and *Stomoxys*. It spreads in Africa (Desquesnes *et al*., 2003; Desquesnes *et al*., 2004) and Latin America (Silva *et al*., 1998; Jones *et al*., 2001; Desquesnes, 2004), which significantly affects cattle breeding. At present, *T. vivax* has not yet invaded Asia and Europe. Nevertheless, *T. vivax* seems to have the same potential for the geographical spread with *T. evansi*.

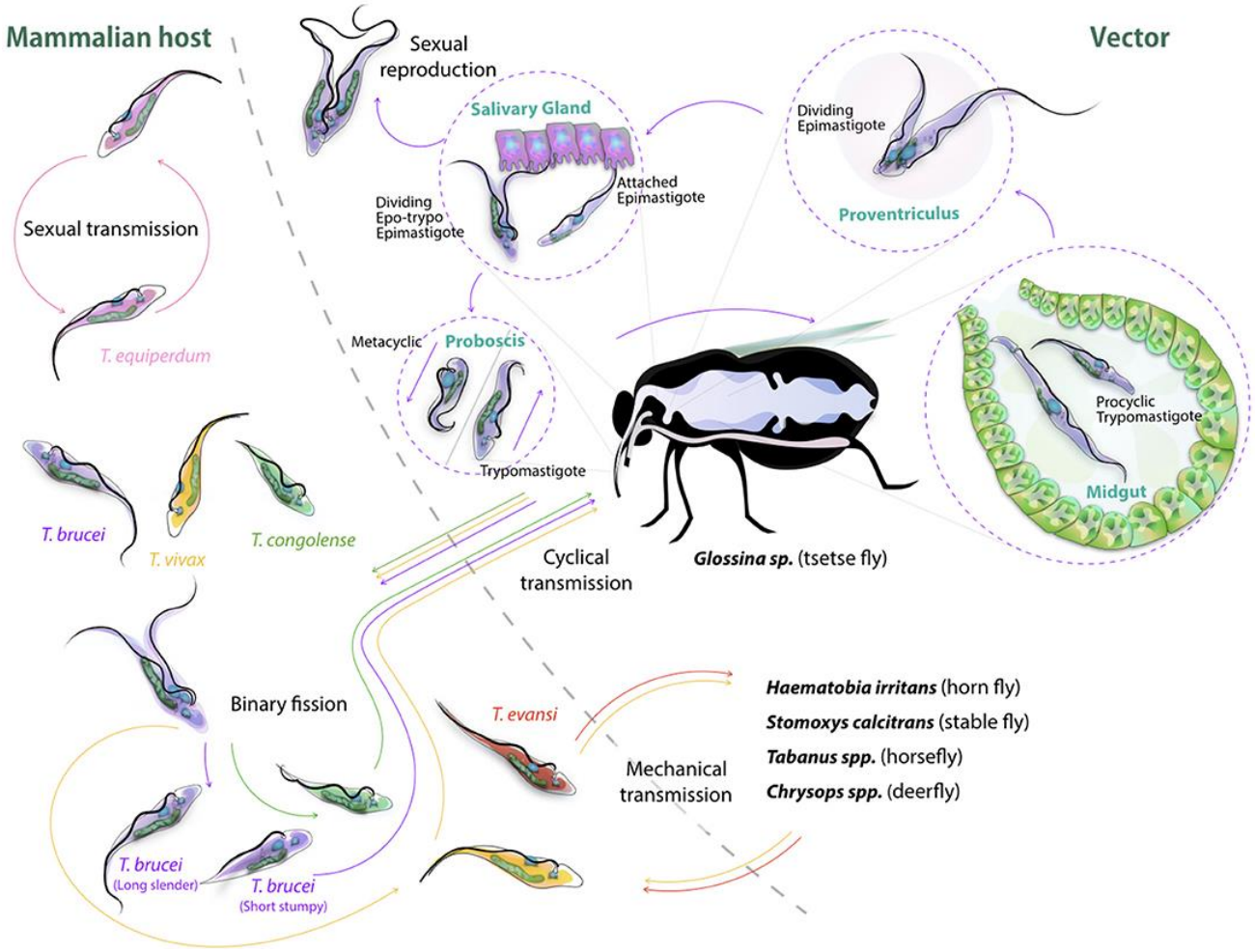


Figure 4. The life cycle of salivarian trypanosomes.

Tsetse and other biting flies facilitate the cyclic transmission of salivarian trypanosomes. Short stumpy forms of parasite are sucked by the flies into inside the body. Later, the parasites are re-injected to the new target via proboscis in the infective form of metacyclic trypomastigote. This transmission mode works in *T. brucei*, *T. congolense*, and *T. vivax*. *Trypanosoma vivax* and *T. evansi* spread through mechanical transmission, while *T. equiperdum* is transmitted through sexual transmission only (Radwanska *et al*., 2018).

1.4 Economic impact and zoonotic aspects of bovine trypanosomiasis

AAT is estimated to cause death toll of 3 million cattle annually and consumption of 35 million doses of expensive trypanocidal drugs (Kabayo, 2002). It is challenging to assess the actual impact of HAT because the disease affects the marginal community in the remote areas with the lack of health care facilities and infrastructures (Matemba *et al*., 2010). In 2008, HAT hit the ninth rank out of 25 deadliest human infectious and parasitic diseases in Africa (Fevre *et al*., 2008).

Estimating the economic losses due to *T. evansi* infection is complicated because the indirect costs have not been sufficiently determined. Most calculations were counted according to the mortality cost and spending on chemotherapeutic interventions. In Middle East countries, *T. evansi* mainly affects dromedary camels among domestic animal species (Aregawi *et al*., 2019). Several outbreaks of camel trypanosomiasis with high mortality and abortion were reported from this region (Aregawi *et al*., 2019). The prevalence and economic loss in cattle are largely unknown. Despite its significance, *T. evansi* infection in livestock, commonly known as surra, is considered as neglected parasitic disease and still not well studied (Figure 5).

Normally, humans are not susceptible to the AAT causing trypanosomes owing to the trypanolytic activity of apolipoprotein L-I (apoL-I) which presents in human blood (Vanhamme *et al*., 2003). It is considered to be the primary innate immune system against trypanosomes infection. However, 19 cases of "atypical" human trypanosomiasis (a-HAT) have been reported in the past. These a-HAT were caused by *T. b. brucei*, *T. evansi*, *T. congolense*, *T. vivax*, and *T. lewisi*, which are usually known as non-infective to humans (Truc *et al*., 2013). Although the risk to humans seems to be minimal, precautions are recommended when handling blood, tissues and animals infected with the agents of animal trypanosomiasis.

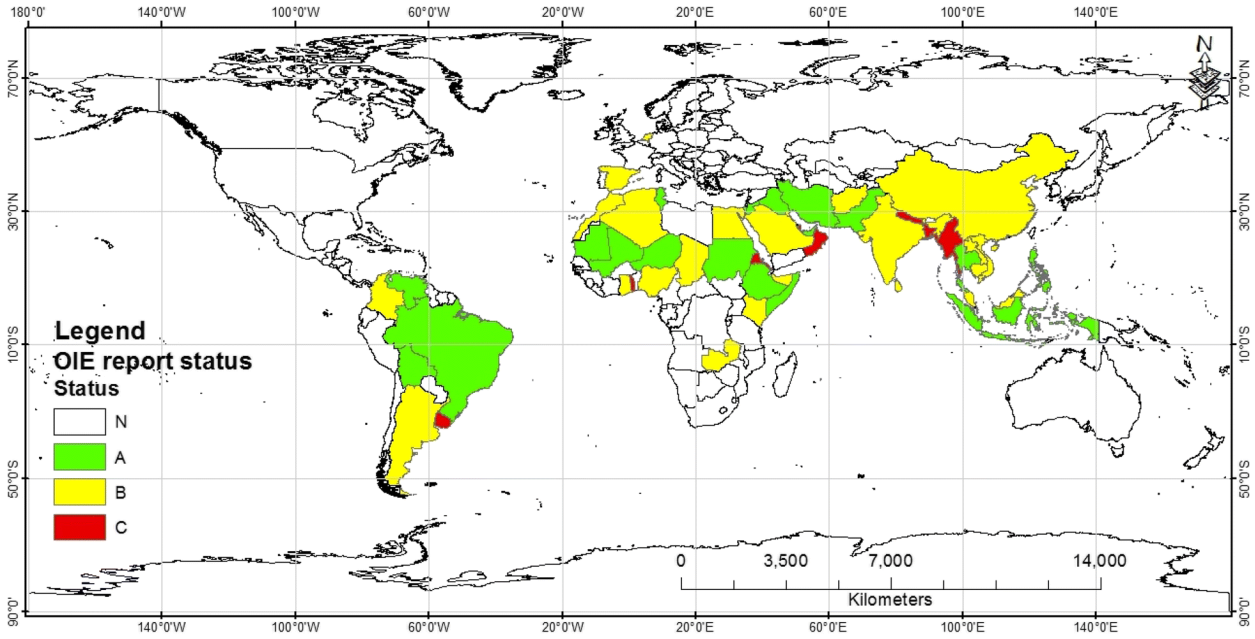


Figure 5. World map demonstrating disagreement between OIE case report of surra by countries and geographical distribution of surra according to published literature.

The colors show the OIE report status and scientific reports of surra in the world. Green: countries with published evidence on surra and reporting surra to OIE, yellow: countries with published evidence on surra and not reporting to OIE, red: countries with case report to OIE but no published evidence, and white: countries without published evidence or without report to OIE. From this map, it is speculated that the real distribution of *T. evansi* might differ from the official country reports.

Source: Aregawi *et al.*, 2019.

1.5 PCR systems for identification of animal African trypanosomes

Polymerase chain reaction (PCR) has been used to identify trypanosomes both in vectors and in host blood (Masiga *et al*., 1992; Wuyts *et al*., 1994; Majiwa *et al*., 1994; Lehane *et al*., 2000; Malele *et al*., 2003), for collecting epidemiological data (Solane *et al*., 1999), typing new trypanosomes (Gibson *et al*, 2001), diagnosis and treatment. As multiple infections of trypanosomes in both vector and host are expected, identifying several trypanosome species through a single PCR using only one pair of primers is ideal.

PCR targeting the internal transcribed spacer (ITS) 1 region of rDNA was developed as a universal single PCR to detect mixed infection of trypanosomes (McLaughlin *et al*., 1996; Desquesnes *et al*., 2001). ITS1 is a preferable target for a universal test because of its highly conserved flanking regions and size variability among trypanosomes species and subgroups. This locus has 100–200 copies and each transcribed unit is composed of 18S, 5.8S and 28S rRNA genes separated by two ITS regions (Desquesnes *et al*., 2002).

KIN primers amplify the ITS1 (situated between 18S and 5.8S rRNA genes) of the sequence ranged from 300 - 800 bp (Desquesnes *et al*., 2002) (Figure 6). Later, the ITS1 PCR system using CF and BR primers demonstrated higher diagnostic sensitivity than KIN primers (Njiru *et al*., 2005). The CF and BR primers detect more *T. vivax* samples than the KIN primers and do not amplify the nonpathogenic *T. lewisi* and *T. theileri.* Recently, Illumina sequence-based species identification method with modified primer sets was also established (Gaithuma *et al*., 2019). The application of next-generation sequencing (NGS) for ITS1 region can address the issue of mixing up of the *T. vivax* and the *T. godfreyi* due to the overlapping base-pair size (Gaithuma *et al*., 2019). NGS can also avoid the problem of inaccuracy due to misidentification of trypanosome species, caused by non-specific amplification (Gaithuma *et al*., 2019).

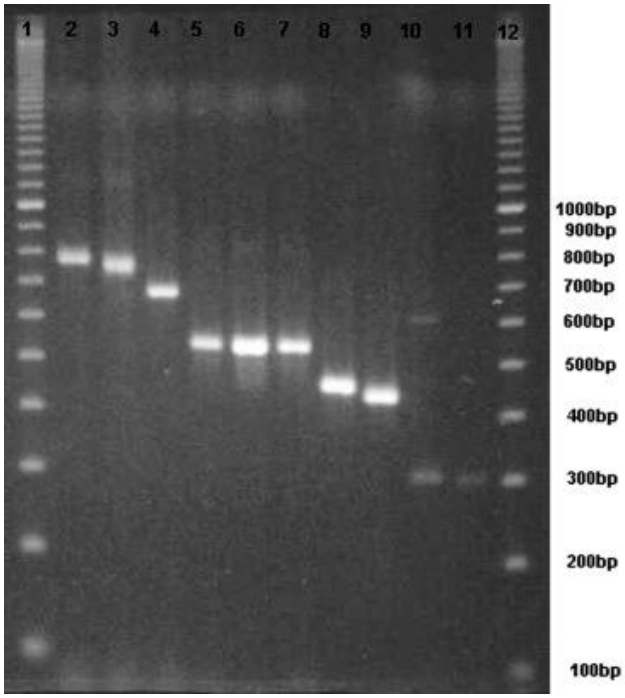


Figure 6. Electrophoresis gel of ITS1 amplicons photographed under ultraviolet light

This gel image shows the base-pair size of ITS1 amplicon of African trypanosomes: lane (1,12) 100 bp marker; (2–11) PCR products obtained with DNA of: (2) *T. congolense* forest; (3) *T. congolense* savannah; (4) *T. congolense* Kenya Coast; (5) *T. evansi*; (6) *T. equiperdum*; (7) *T. brucei*; (8) *T. theileri*; (9) *T. simiae*; (10) *T. vivax*; (11) *T. vivax*.

Source: Desquesnes *et al*. (2001)

1.6 Detection system for human African trypanosomes

Trypanosoma brucei gambiense and *T. b. rhodesiense* are responsible for HAT in West/Central and East Africa, respectively. Since it is almost impossible to correctly identify the *T. brucei* subspecies by only observing their morphology or geographical origin, the genetic marker genes are used for the accurate subspecies identification. Only *T. b. rhodesiense* possesses serum resistance-associated (*SRA*) gene, while majority of the *T. b. gambiense* (type-1) have the *T. b. gambiense*-specific glycoprotein (*TgsGP)* gene and therefore, those genes were used as a genetic marker to discriminate them from others in *Trypanozoon* (Welburn *et al*., 2001; Radwanska *et al*., 2002). Both *SRA* and *TgsGP* genes encode a protein which is involved in normal human serum resistance (Radwanska *et al*., 2002), the ability required to survive in the human blood. However, the *T. b. gambiense* which lacks TgsGP marker gene (type-2 *T. b. gambiense*) is also present. *SRA* is an expression site-associated gene in *T. b. rhodesiense* and is located upstream of the VSG in the active telomeric expression site (Xong *et al*., 1998). SRA has a key role in *T. b. rhodesiense* resistance to lysis by normal human serum (NHS) (Xong *et al*., 1998).

1.7 Purpose of study

It is widely believed that *Trypanozoon,* including *T. evansi*, originated in Africa and spread across the world (Desquesnes *et al*, 2013b). It has been speculated that *T. evansi* evolved from a *T. brucei* in camels that had temporarily entered the sub-Saharan tsetse belt, and adapted to mechanical transmission by biting flies (Hoare *et al*, 1972). However, a recent study suggested the independent origins of *T. evansi* from *T. brucei* strains, in which the ability of *T. evansi* to be transmitted mechanically occurred repeatedly (Kamidi *et al*, 2017). The parasite spread from North Africa toward the Middle East, Turkey, India, up to Russia, across all of Southeast Asia, down to Indonesia and the Philippines, and was also introduced by the conquistadores into Latin America (Hoare *et al*, 1972; Luckins, 1988; Reid, 2002).

Syria is one of the Middle Eastern countries situated in intersection of Africa, Asia and Europe. Thus, it can be speculated that Syria is geographically located in the "gate", where *T. evansi* started to spread worldwide from its origin in Africa. Therefore, Syria can be a key place to know the evolution of *T. evansi*, where the parasite moved out of its place of the origin and started to spread worldwide. It is suggested by Desquesnes *et al.* (2013) in their review that surra is present in Syria, but there is no case report to OIE, and no literature is found particularly on the molecular study of *T. evansi* in Syria (Aregawi *et al.*, 2019; Desquesnes *et al*., 2013).

Malawi is the endemic country of *T. b. rhodesiense* HAT and AAT in Africa. The prevalence of HAT and AAT in the country have reached epidemic proportions a decade ago (Davison, 1990; Chisi *et al*., 2011a; Simarro *et al*., 2012). Cattle are one of the most economically important livestock animals in Malawi (Schmidt, 1969; Chintsanya *et al*., 2004). Cattle are known to be the reservoir of *T. b. rhodesiense* in other African countries

(Kaare *et al*., 2007; Von Wissmann *et al*., 2011), but there is no report and lack of information about it in Malawi.

Understanding the genetic diversity of parasite and its epidemiology are the first step for the integrated effort to control the disease. It is necessary to closely monitor the parasite epidemiology and genetic diversity in order to follow their geographical distribution and to investigate their pathogenicity in potential new hosts (Weber *et al.*, 2019). This will be useful to actively prevent outbreaks of AAT and to identify any potential changes in life cycle and pathogenicity (Weber *et al.*, 2019). The information on genetic diversity is expected to support epidemiology study by providing a fine picture of *T. evansi* from the point of view of the sub-typing of trypanosomes, virulence pattern, and investigation on what adaptation involved in survivability of trypanosomes in different hosts. In particular, the information on genetic diversity of *T. evansi* will provide a fine picture of the information for the broader host adaptation and parasite migration. Therefore, study in genetic diversity is important for disease control.

Cattle have been used to study the control of AAT in trypanotolerant host animals (Morrison *et al*., 2010). It is believed that host genetic variation also involves in differences in the severity of the disease (Courtin *et al.*, 2007; MacLean *et al*., 2007). It is generally known that the severity of the bovine trypanosomiasis differs depend on the region. However, the potential impact of parasite genetic diversity on disease severity and progression has received less attention. Therefore, the aim of this study was to provide information on the distribution of bovine trypanosomes and genetic diversity in Syria and Malawi in order to provide better knowledge for the control of bovine trypanosomiasis and HAT in these countries.

2. CHAPTER 1

Molecular identification of *Trypanosoma evansi* from cattle in Syria

2.1 Summary

T. evansi, the "surra" disease-causing agent, is a blood protozoan parasite that infects a wide range of mammalian species an unlimited geographical region. It causes anemia, weight loss, and even death of the infected livestock that brings great losses in agriculture sector. However, the full epidemiological information of *T. evansi* is lacking, especially in developing countries, and the risk of the disease is largely underestimated. Syria can be a gate to learn about *T. evansi* evolution, where the parasite moved out of its place of origin in sub-Saharan Africa and started to spread worldwide. However, there is no molecular study of *T. evansi* in Syria. Therefore, this is the first epidemiological report of *T. evansi* in Syria using molecular biological methods. This study emphasizes the importance of controlling Surra in the country as a highly endemic region.

In this study, 207 samples of blood DNA collected from Holstein Friesian crossbred cattle in the central region of Syria in May 2010 were screened for *T. evansi*, aiming to determine the prevalence of the parasite. *Trypanosoma evansi* was screened by PCR targeting the internal transcribed spacer (ITS) 1 region, and 27 samples were found positive (13%), which is relatively high considering that no clinical symptoms were observed. The ITS1 amplicons were later subjected to *RoTat1.2*-PCR for the detection of *T. evansi* type A. For the characterization of genetic diversity of *T. evansi*, sixteen positives samples were obtained from *ESAG6* PCR, and these were successfully sequenced. Phylogenetic analysis based on the *ESAG6* revealed that Syrian sequences clustered together, with the majority in clade 10 (9 sequences) and clade 9 (4 sequences), while the remaining sequences were distributed in clade 1 (1 sequence) and clade 5 (2 sequences). Genetic diversities were revealed in the sequence analysis of *ESAG6* from the Syrian samples. The obtained genotype did not have association with regions, countries, or animal host. Further investigations in cattle and other domestic animals are necessary in Syria.

2.2 Introduction

T. evansi, the causative agent of "surra" disease, classified in the subgenus *Trypanozoon*, is a flagellated protozoan parasite (Donelson *et al*., 1998). It shares some characteristics with other *Trypanozoon* species, including *Trypanosoma brucei* and *Trypanosoma equiperdum*, in terms of morphology and genome sequences (Masiga *et al*., 1990; Carnes *et al*, 2015). However, while *T. brucei* undergoes a complex cycle of differentiation and multiplication in tsetse flies, *T. evansi* does not have a vector stage and is only transmitted mechanically. Unlike *T. brucei*, *T. evansi* has lost the maxicircles of kinetoplastid mitochondrial DNA, which are required to undergo the procyclic form in tsetse flies. This makes *T. evansi* unable to reproduce in tsetse flies (Lai *et al*, 2008). Instead, *T. evansi* is transmitted mechanically by a wide range of biting flies including tabanids and stomoxes, by vampire bats, and by ingestion of raw meat (Desquesnes, 2004; Desquesnes *et al*., 2013a). Since *T. evansi* is not restricted to the distribution of tsetse flies, it has the largest geographical distribution and host range among salivarian trypanosomes (Lun *et al*., 1995; Desquesnes *et al*., 2013a). *Trypanosoma evansi* infects a broad range of domestic animals and wildlife but is more pathogenic to camelids and equids (Donelson *et al*., 1998). It has been described that the susceptibility of *T. evansi* is highly variable depending on the host species and presumably on the region (Desquesnes *et al*., 2013a).

To differentiate *Trypanozoon* from other trypanosomes, polymerase chain reaction (PCR) targeting the internal transcribed spacer (ITS) 1 region of ribosomal RNA (rRNA) has been commonly used (Njiru *et al*, 2005; Gaithuma *et al*., 2019; Salim *et al*, 2011). Identification of each species in *Trypanozoon*, including *T. evansi* and *T. brucei*, is complicated because of their twinning characteristics (Masiga *et al*., 1990; Claes *et al*, 2004). Although *T. evansi* has no maxicircles, they still possess short minicircle DNA that encodes guide RNAs for RNA editing (Borst *et al*, 1979; Borst *et al*., 1987). Based on its minicircle restriction digestion profile, *T. evansi* is divided into types A and B (Borst *et al*., 1987; Njiru *et al*, 2006). *Trypanosoma evansi*

type A is the most common species found in Africa, South America, and Asia (Birhanu *et al*, 2016). In contrast, *T. evansi* type B is found in Eastern Africa. To date, the prevalence of *T. evansi* type B remains largely unknown, especially in Asia (Borst *et al*., 1987; Ngaira *et al*, 2005; Hagos *et al*, 2009; Salim *et al*, 2011; Birhanu *et al*, 2015).

The variant surface glycoprotein (*VSG*) of *RoTat1.2* is specifically present in *T. evansi*, particularly in type A, but not in *T. brucei* strains, and has been utilized to differentiate *T. evansi* from other *Trypanozoon* members (Claes *et al*, 2004; Urakawa *et al*, 2001; Verlo *et al*, 2001). Because some of the diagnostic strategies for *T. evansi* rely on the presence of the *RoTat1.2*, such as *RoTat1.2* loop-mediated isothermal amplification (LAMP) and the serological card agglutination test for *T. evansi* (CATT)/RoTat1.2, only *T. evansi* type A, but not type B, can be detected using these methods (Bajyana *et al*, 1988; Elsaid *et al*, 1998; Salim *et al*, 2018). Furthermore, it has not been studied whether the presence of the *Rode Trypanozoon antigen* type 1.2 (*RoTat1.2*) gene of *T. evansi* is related to pathogenesis or other biological phenotypes.

For specific diagnosis of *T. evansi* type B, several tests have been developed, including EVAB-PCR, targeting a type B-specific minicircle DNA sequence; a PCR; and LAMP targeting type B-specific *VSG JN 2118Hu* (Ngaira *et al*, 2005; Njiru *et al*, 2006; Njiru *et al*, 2010). However, recent investigation demonstrated that the *VSG JN 2118Hu* is considered to exist not only in *T. evansi* type B but also in the *T. b. brucei* and *T. b. gambiense* (Konnai *et al*, 2009). Latest study found 3-bp deletion in the C-terminal region of F1-ATP synthase γ subunit gene, A281del mutation, differentiates *T. evansi* type A, *T. evansi* type B, and *T. equiperdum* (Birhanu *et al*, 2015; Carnes *et al*, 2015). This mutation has been only found in *T. evansi* type A and *T. equiperdum* so far, while it is intact in the type B (Carnes *et al*, 2015). This is explained as an adaptation of the loss of functional kinetoplast DNA in *T. evansi/ T. equiperdum* (Birhanu *et al*., 2015; Lai *et al*., 2008). However, to rely solely on F1-ATPγ for characterization of *T. evansi* subtypes is a flawed approach because it includes only particular features of *T. evansi*

from Africa limiting its usage to differentiate the other *T. evansi* subtypes outside Africa (Kamidi *et al*., 2017). Considering the limitations on the detection of *T. evansi* type B, currently, the ITS1-PCR and *RoTat1.2*-PCR are the sensible and common method for subtyping among *T. evansi* (Konnai *et al*., 2009; Salim *et al*., 2011) (Table 1).

The transferrin receptors of trypanosomes are encoded by two *expression-site-associated-genes 6 (ESAG6)* and *ESAG7* (Kabiri *et al*., 2001; Steverding *et al*., 1996). The *ESAG6* and *7* genes are transcribed as the parts of the polycistronic VSG mRNA (Figure 7). The polycistronic VSG mRNA is transcribed from the active subtelomeric expression site (ES). There are 20 available ES in their genome but only one out of the 20 is active at a time. This mutually exclusive switching of the diversified transferrin receptors ensures a sufficient iron uptake escaping from anti-transferrin receptor antibodies (Bitter *et al*., 1998; Kabiri *et al*., 2001). In addition, the switch of *ESAG6* and *ESAG7* genes may affect trypanosome ability to take up transferrin molecules efficiently from different mammalian species. This made trypanosomes able to adapt and to survive in a wide range of hosts (Gerrits *et al*., 2002; Steverding *et al*., 2006). Considering the polymorphic nature of *ESAG6* gene, many studies have used it to estimate the genetic diversity in trypanosomes (Holland *et al*., 2001; Isobe *et al*., 2003; Mekata *et al*., 2009). Even though a series of studies had been conducted, it is not clear that the genetic diversity of *ESAG6/7* within *T. evansi* is related to the biology or infectivity of the trypanosome in mammalian host and whether it is related to countries/regions or host species (Sarkhel *et al*., 2017; Witola *et al*., 2005).

Collectively, the aim of this study was to determine the prevalence and characterize the genetic diversity of *T. evansi* in Syria. This epidemiological study and genetic diversity information of the parasites will help for improving the control measures in this region and understanding host tropism and adaptation of *T. evansi* in different animals and regions worldwide.

Table 1. PCR systems for identification and differentiation of *Trypanosoma evansi*.

Abbreviation of system	Target sequence	Purpose
ITS1	Internal Transcribed Spacer (ITS1) of rDNA	Screening for pathogenic trypanosomes including *Trypanozoon* strains. The ITS1 region of trypanosomes varies in size which can be utilized for identification each species in which for the *Trypanozoon* is approximately 480 bp.
RoTat1.2	Variable Surface Glycoprotein of trypanosomes (VSG) of Rode *Trypanozoon* Antigen (RoTat) type 1.2	Identification of *T. evansi* type-A. Any *Trypanozoon* including *T. evansi* type-B lack this gene.
ESAG6/7	Expression Site Associated Gene (*ESAG*) 6/7	Investigating genetic diversity among the parasite based on the polymorphisms in the *ESAG6* genes.
F1-ATP	F1-ATP γ subunit gene	Screening for *Trypanosoma evansi.* This mutation has been only found in *T. evansi* type A, while it is intact in the type B. However, the limitation is that currently it cannot cover all worldwide types of *T. evansi* and only includes the particular features of *T. evansi* from Africa. Hence, it cannot be used for *T. evansi* subtyping thoroughly.

JN 2118HU	VSG JN 2118HU	Screening for *T. evansi* type-B. However, recent study revealed that this gene is considered to exist not only in *T. evansi* type B but also in the *T. b. brucei* and *T. b. gambiense.* Hence, it cannot be used for *T. evansi* in Africa where the tsetse and non-tsetse transmitted *Trypanozoon* are overlapping.
EVAB	Type B minicircle	Screening for *Trypanosoma evansi* type-B. Any *Trypanozoon* including *T. evansi* type-A lack this gene.

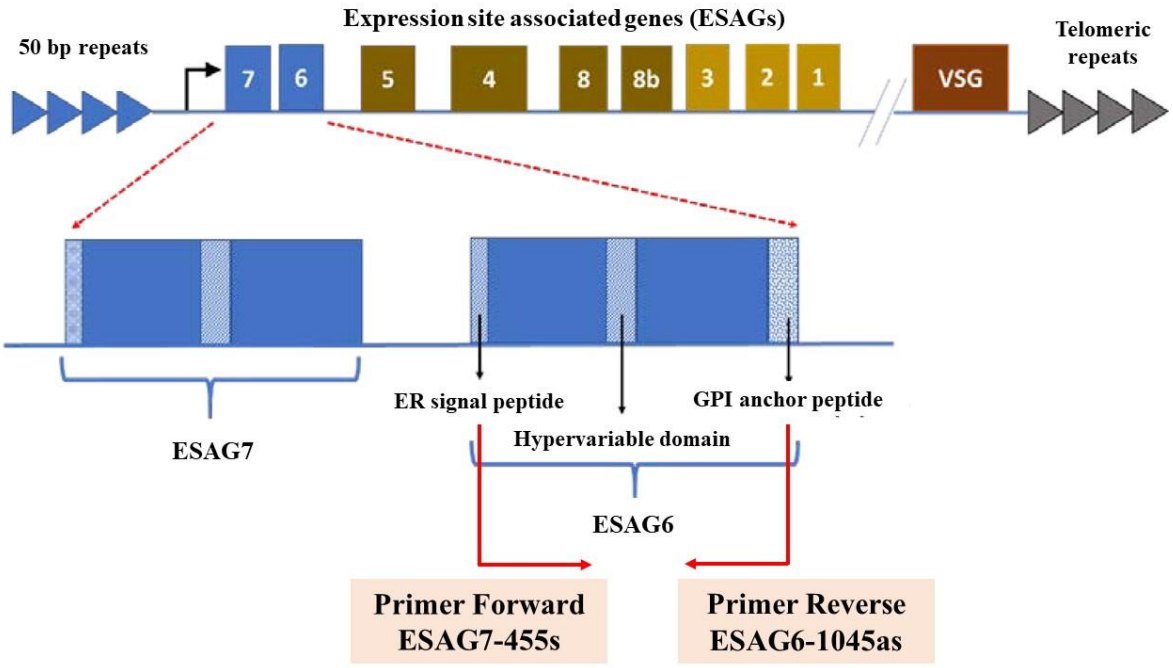


Figure 7. Schematic representation of the Variant Surface Gycoprotein (VSG) gene expression site including the specific location of Expression site-associated gene (*ESAG*) 6 and 7.

ESAGs 7 and 6 are located at the most proximal, while the VSG is at the most distal of the expression sites. The red arrows indicate the amplification site of *ESAG6* primers including Forward ESAG7-455s and Reverse ESAG6-1045as. ESAG6 and 7 sequences are highly homologous, but ESAG7 lacks the 3′ sequence encoding a signal for the glyco-phosphatidyl inositol (GPI) anchorage to the cell membrane. Hence, a reverse primer that would bind only to this GPI sequence was utilized for *ESAG6* amplification (Isobe *et al*., 2003).

Source: Kariuki *et al*., 2019

2.3 Materials and Methods

2.3.1 DNA Samples

A total of 207 blood DNA samples were used, which were collected from clinically healthy Holstein Friesian crossbred cattle in the central region of Syria in May 2010. The sampling sites were Huleh (*n* = 51), Qyser (*n* = 28), Hama (*n* = 73), Ghab (*n* = 32), and Salmia (*n* = 23) (Figure 8). The samples in Huleh, Qyser, Hama and Ghab were taken from extensive-farming raised animals, while the samples from Salmia were taken from indoor-kept animals. The collection of field samples was approved by the Syrian government through the Ministry of Agriculture and supported by veterinarians and staff working at the Society for Protection of Animals Abroad in Syria. Sample collection methods were followed as described previously (Terkawi *et al*., 2012). Readily prepared DNA samples were previously provided and used by Terkawi *et al*. (2012). In this study, microscopic parasite examination and serological tests for *T. evansi* diagnosis were not conducted.

2.3.2 PCR

PCR amplification of the ITS1 region of the rRNA was conducted to screen trypanosomes, including *Trypanozoon*. For the amplification of the ITS1 region, the primer set CF and BR was used (Njiru *et al*., 2005) (Table 2). Each reaction included 5 µl Ampdirect plus (Shimadzu, Japan), 0.05 µl BIOTAQ HS DNA Polymerase (5 U/µl) (Bioline, UK), 0.5 µl of each 10 mM primer, 2.95 µl RNase-free water, and 1 µl extracted DNA. The thermocycling profile started with an initial hold for 10 min at 95 °C, followed by 40 cycles at 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products 8.3 were electrophoresed on 1.2% agarose S (Nippongene, Japan) in Tris-acetate EDTA buffer pH and stained using GelRed (Biotium, USA) dye

before being visualized under UV light. The ITS1-positive samples were subjected to PCR specific for *T. evansi* (type A), which amplifies 151 bp of the *RoTat1.2 VSG* fragment by using the primer set TeRoTat920F and TeRoTat1070R (Konnai *et al*., 2009) (Table 2). The *RoTat1.2* amplicons were electrophoresed on a 2.2% agarose gel. PCR amplification of the 740 bp fragment of *ESAG6* was performed using *ESAG7* F455 and *ESAG6* R1045 primers as described by Isobe *et al*. (2003) (Table 2). *ESAG6* amplicons were electrophoresed on a 1.2% agarose gel. The reaction was performed for 35 cycles at 58 °C annealing temperature in *RoTat1.2* PCR and 40 cycles at 60 °C in *ESAG6* PCR; the other thermocycling conditions were as mentioned above. The master mix conditions were the same as those for ITS1 PCR. Increased sensitivity of *ESAG6* amplification was achieved using the newly designed nested PCR. The second round of PCR was performed using the inner primer set (Table 2). The reaction mixture was the same as that used for the initial *ESAG6* PCR. The thermal cycling conditions used were the same as that for the initial PCR except for the modification to 35 cycles.

2.3.3 Sequence and phylogenetic analysis of ESAG6

The *ESAG6* PCR products were purified by ExoSAP-IT (GE healthcare/USB, USA) following the manufacturer's instructions. Purified PCR products were sequenced using the Big-Dye Terminator v3.1 (Applied Biosystems, USA) on an automated capillary sequencer (Applied Biosystems 3130 Genetic Analyzer; Applied Biosystems Japan Ltd., Tokyo, Japan). DNA sequences were edited using ApE (Davis, 2012). The DNA sequence data were aligned against 47 sequences of *ESAG6* deposited in GenBank using the ClustalW program in the MEGA7 software (Kumar *et al*., 2016). A phylogenetic tree was constructed using neighbor-joining (NJ) algorithms. For the trees provided by NJ methods, bootstrap

branch supports were calculated from 1,000 pseudo-replicates following the rule of branch consistency.

2.3.4 Statistical analysis

Multiple logistic regression test was conducted to understand the association of infection rate in animals and ages, locations, anemia, and co-infection with *Babesia* spp. Statistical analysis was conducted to compare the infection rate of *T. evansi* in animals and co-infection with *Babesia* spp. using the data from Terkawi *et al*. (2012).

2.3.5 PCR sensitivity test

Sensitivity of the PCR systems for ITS1, *RoTat1.2, and ESAG6* was validated using primer sets of CF and BR, TeRoTat920F and TeRoTat1070R, and *ESAG7* F455 and *ESAG6* R1045, respectively. The detection limit was examined in four replicates using 10-fold serial dilutions of DNA extracted from *T. evansi* IL3354 isolate cultured *in vitro.*

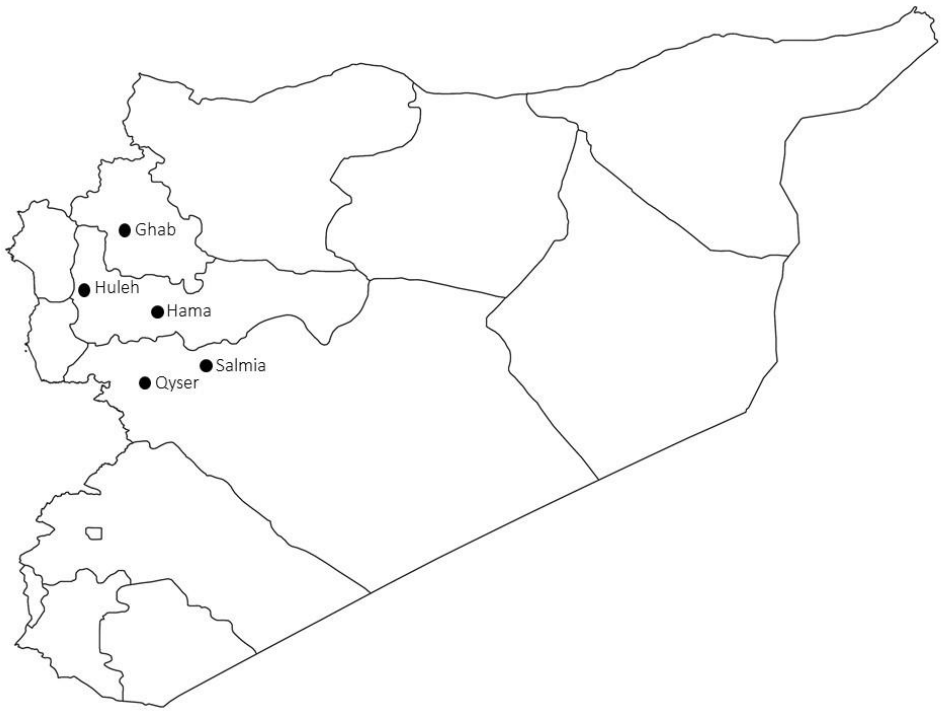


Figure 8. Map of Syria showing the five sampling locations

Sampling location were situated in the central region of Syria. The sampling sites were Huleh, Hama, Qyser, Ghab, and Salmia. Hama is located at more than 200 m above sea level.

Table 2. The list of primers used in this study.

Primers	Forward	Reverse
ITS1	CF 5′-CCGGAAGTTCACCGATATTG-3′	BR 5′-TGCTGCGTTCTTCAACGAA-3′
RoTat 1.2	TeRoTat920F 5′-CTGAAGAGGTTGGAAATGGAGAAG-3′	TeRoTat1070R 5′-GTTTCGGTGGTTCTGTTGTTGTTA-3′
ESAG 6/7	*ESAG7* F455 5′-CATTCCAGCAGGAGTTGGAGG-3′	*ESAG6* R1045 5′-TGTTCACTCACTCTCTTTGACAG-3′
Inner primer of *ESAG 6/7*	Forward 5′-GCAGGAGTTGGAGGAAATGA-3′	Reverse 5′-TGAGCTCAGCCTCTTTCTGC-3

2.4 Results

2.4.1 Molecular detection of T. evansi *by PCR*

DNA samples were subjected to PCR amplification of the ITS1 region and *RoTat1.2* for molecular identification of *T. evansi*. Sanger sequencing was carried out to validate the results of ITS1-PCR, and it was confirmed that all 27 ITS1-positive samples contained *T. evansi.* The present study showed a 13.0% prevalence of *Trypanozoon* infection in cattle by ITS1 PCR screening (Table 3 and Table 4). *RoTat1.2*-PCR amplified in 17 samples that were ITS1-positive, but it failed to amplify in the other 10 samples.

2.4.2 The infection rate of T. evansi *according to location, packed cell volume (PCV) value, age, and co-infection with* Babesia *spp.*

The highest prevalence of *T. evansi* was observed in Ghab (34.4%, n = 11), followed by Qyser (25.0%, n = 7). There was significant association between *T. evansi* infection and sampling regions ($P < 0.0001$, Table 4). In contrast, *T. evansi* infection in the sampled cattle did not have any association with anemia (Table 5), age (Table 6), and co-infection with *Babesia* spp. (Table 7). There was no significant association between anemia and the presence of *T. evansi* or *Babesia* spp.

2.4.3 Sequencing and phylogenetic analysis based on ESAG6

All ITS1-positive samples were subjected to PCR amplification of the *ESAG6* gene for the characterization of genetic diversity of *T. evansi*. Sanger sequencing was carried out to validate the results of ITS1-PCR. Sixteen samples were positive in *ESAG6* PCR, and these were successfully sequenced. Phylogenetic analysis based on the *ESAG6* revealed that Syrian sequences clustered together, with the majority in clades 10 (9 sequences) and 9 (4 sequences),

while the remaining sequences were distributed in clades 1 (1 sequence) and 5 (2 sequences) (Figure 9). This result indicated that some of the major genotypes were shared within the three sampling locations in Syria, and genetic diversity was observed in our samples.

2.4.4 Analytical sensitivity test of ITS1, RoTat1.2*, and* ESAG6-*PCR*

This experiment aimed to compare the sensitivity of PCRs using three different sets of primers. PCR sensitivity tests for ITS1, *RoTat1.2, and ESAG6* were conducted. It was repeated four times to confirm the reproducibility of the results. The minimum amount of DNA detectable by PCR was 1×10^{-4} ng for ITS1 PCR (two positives out of four experiments), 1×10^{-2} ng for *RoTat1.2* PCR (one positive out of four experiments), and 1×10^{-4} ng for *ESAG6* (one positive out of four experiments; Figure 10 and Table 8). ITS1 PCR and *ESAG6* PCR showed higher sensitivity than *RoTat1.2* PCR.

Table 3. PCR screening result for *T. evansi*

ITS1	*RoTat1.2*	*ESAG6*	Number of samples	
+	+	+	9	10
		-	1	
	-	+	7	17
		-	10	
-	-	-	180	

+ : PCR-positive, - : PCR-negative.

Table 4. Comparisons of the prevalence of *T. evansi* infections on the basis of sampling region.

City	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
Huleh	51	1	2.0%
Hama	73	6	8.2%
Qyser	28	7	25.0%
Ghab	32	11	34.4%
Salmia	23	2	8.7%
Total	207	27	13.0%

Table 5. Comparisons of the prevalence of *T. evansi* infections on the basis of PCV value.

PCV	Status	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
<24%	Anemic	9	2	22.2%
24-46%	Normal	198	25	12.6%
Total		207	27	13.0%

Table 6. Comparisons of the prevalence of *T. evansi* infections on the basis of age.

Age (years)	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
1-2	55	6	10.9%
3-5	99	14	14.1%
Above 5	53	7	13.2%
Total	207	27	13.0%

Table 7. Comparisons of the prevalence of *T. evansi* infections on the basis of coinfection with *Babesia* spp.

Category	Total number of samples	ITS1 positives with 480bp (putative *T. evansi*)	Positive rate
Absence of *Babesia* spp.	160	19	11.9%
Presence of *B. bovis*	16	1	6.2%
Presence of *B. bigemina*	31	7	22.6%
Total	207	27	13.0%

Figure 9. Phylogenetic tree constructed based on the nucleotide sequences of the *ESAG6* in *Trypanosoma brucei* and *Trypanosoma evansi.*

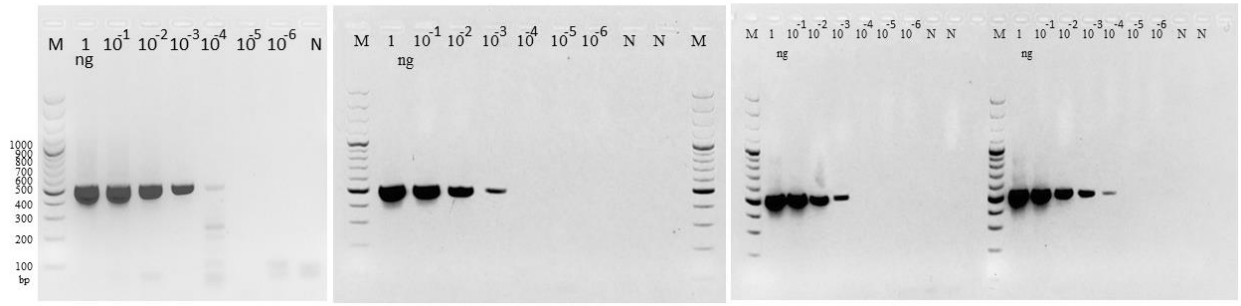
The entry data were represented according to their countries of origin—Peru, Philippine, Egypt, India, Thailand, Bolivia, and Syria; the hosts—cattle, camel, horse, donkey, buffalo, deer, and pig; and the GenBank accession numbers. The dotted entry names are the nucleotide sequences obtained from this study. The bootstrap confidence values at the nodes illustrate the percentage of times the group occurred out of 1,000 trees, and the bar depicts the genetic distance.

Table 8. The results of PCR sensitivity test in four replicates using different sets of primers: ITS1, *RoTat1.2*, and *ESAG6*.

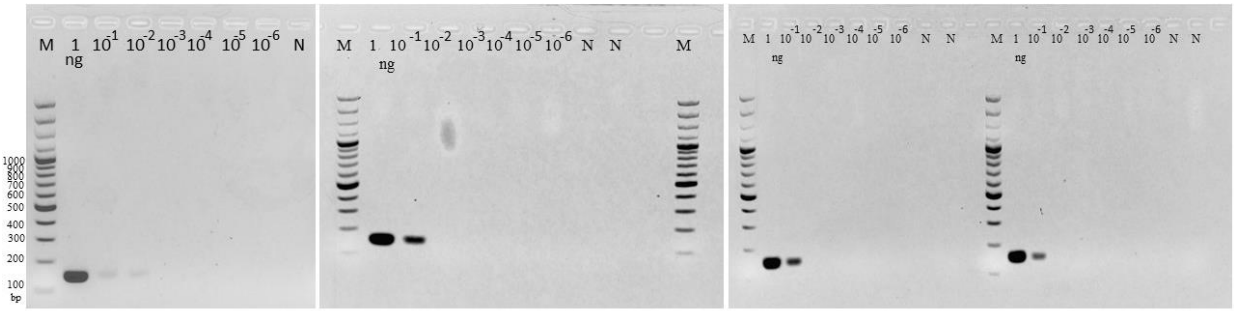
PCR	DNA concentration of positive control							N
	1 ng	10^{-1} ng	10^{-2} ng	10^{-3} ng	10^{-4} ng	10^{-5} ng	10^{-6} ng	
ITS1	4/4	4/4	4/4	4/4	2/4	0/4	0/4	0/4
RoTat1.2	4/4	4/4	1/4	0/4	0/4	0/4	0/4	0/4
ESAG6	4/4	4/4	4/4	3/4	1/4	0/4	0/4	0/4

The detection limit of PCR systems was examined using 10-fold serial dilutions of DNA extracted from purified parasites of *T. evansi* IL3354 isolate. The numbers in the first row and N indicate the concentration of DNA (ng) and the negative control, respectively. The numbers before slash (/) indicate the positive numbers in independent four times PCR experiments.

ITS1 PCR



RoTat1.2 PCR



ESAG6 PCR

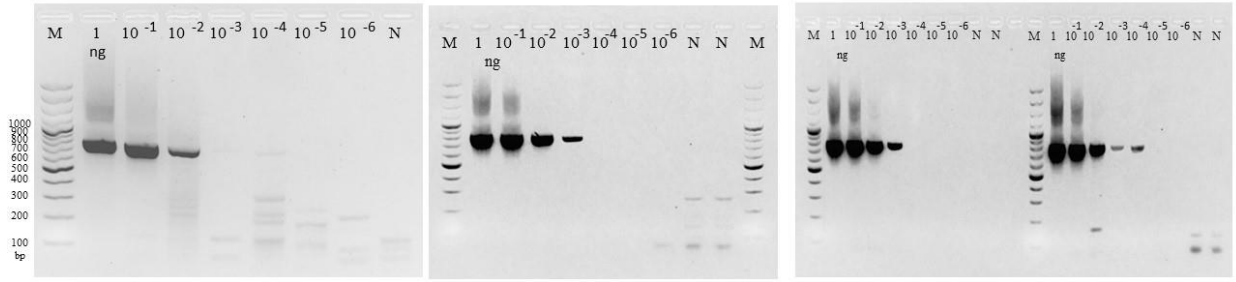


Figure 10. Evaluation of the PCR sensitivity using different sets of primers: ITS1, RoTat1.2, and *ESAG6*.

Numbers indicate the quantity of DNA used (ng). M and N indicate molecular weight markers (100 bp) and negative control, respectively. The detection limit was examined in four replicates using 10-fold serial dilutions of DNA extracted from *T. evansi* IL3354 isolate cultured *in vitro.*

2.5 Discussion

In the present study, I described the prevalence of *T. evansi* infection among cattle in Syria for the first time. ITS1 PCR was used to determine the presence of *T. evansi* in the studied samples because Syria is not a habitat of the tsetse flies, the *T. brucei* vectors, which only inhabit sub Saharan Africa (Vreysen *et al*., 2013). The positive rate determined by the ITS1 PCR screening was 13.0% and relatively high considering that no clinical symptoms were observed. I observed that all age groups of cattle were equally affected by the infection of *T. evansi*. In my analysis, 37% of the ITS1 PCR positive samples was negative for *RoTat1.2* PCR possibly owing to low parasitemia, presence of *T. evansi* type B, or the performance gap between ITS1 PCR and *RoTat1.2* PCR, in which *RoTat1.2* PCR had lower sensitivity compared to ITS1 PCR. To confirm the *RoTat1.2* negativity in ITS1 PCR positive samples, further analyses are required, including microscopic parasite examination, serology assay to detect RoTat1.2 antigen, and highly sensitive nucleic acid detection system.

Geographic factors played a role in the infection rate distribution in this study. Ghab, followed by Qyser, were the most prevalent areas of *T. evansi* infection in this study. Both of these regions were green areas dedicated to the agriculture sector in the country, unlike the other three areas. In Ghab and Qyser, the population of livestock was dense and animal movements were frequent. Poor farm hygiene and weather conditions in the areas provided suitable environment for horseflies (tabanus flies) to reproduce extensively. Farmers in Ghab and Qyser practiced a free-range management system where animals were released during the day to graze freely and returned home at dusk to sleep, which could increase the exposure to horseflies. These factors elevated the possibility of disease transmission in Ghab and Qyser. Given the significant differences in occurrence of *T. evansi* among the studied regions, it is necessary to investigate the geographical distribution of horseflies as parasite vectors in Syria. The higher altitude of Hama has possibly become a geographical barrier for wildlife trespasses,

which might explain the difference in the prevalence (Terkawi *et al*., 2012). In addition, the indoor management farm in Salmia contributed to lesser animal contact with fly vectors, which was one of the transmission factors.

In Asia, Holstein Friesian cattle are susceptible to *T. evansi* infection, and infected cattle frequently exhibit a significant decrease in PCV profiles and body weight as well as a negative effect on milk yield and fertility, including abortion (Payne *et al*., 1992; Payne *et al*., 1993; Kashiwazaki *et al*., 1998; Pholpark *et al*., 1999). In this study, the sampled cattle were a mixed breed of Syrian local and Holstein Friesian. Syrian local cattle are known to be resistant to trypanosome infection. This may explain the lack of association between infection in cattle and anemia. Considering the observed high prevalence and mild symptoms, these cattle might be one of the potential reservoirs of *T. evansi* in Syria.

The studied regions were endemic to other blood parasites of *Babesia* spp.; therefore, we also analyzed the correlation between *Babesia* spp. and *T. evansi* using the molecular analysis data of Terkawi *et al*. (2012) in the same samples collected. We did not find any significant association in co-infection of *Babesia* spp. and *T. evansi*. This is possibly because of limited interaction in terms of vector, lifecycle (one is an intracellular parasite and the other is extracellular), and immunogenicity (presumably acquired immunity does not cross-react with each other).

In this study, it was challenging to obtain sequence by *ESAG6* PCR due to its sensitivity problem in our samples. Hence, we designed inner primers for nested PCR, and they worked well to solve the problem. Considering the limitation of *ESAG6* PCR, we suggest that nested PCR is necessary to do for detection in sample with low parasite and mixed infection. The obtained sequences of *ESAG6* are available at GenBank under the following accession numbers: LC440031.1, LC519566.1, LC519567.1, LC519568.1, LC519569.1, LC519571.1, LC519572.1, LC519573.1, LC519574.1, LC519575.1, LC519576.1, LC519577.1,

LC519578.1, LC519579.1, and LC519580.1. Sequence analysis of the *ESAG6* of *T. evansi* in Syria showed certain diversity. The major genotypes were clades 10 and 5, and the genotypes were found in three sampling locations. An association of genotypes with countries, regions, or host species was not observed in this study. The diversity of transferrin receptors has been shown to be possibly related to the need for antigenic variation to escape from host immune responses (Trevor *et al*., 2019). This is the first report of molecular detection of *T. evansi* in Syria. Therefore, additional epidemiological study of the parasites is necessary. Further investigations in cattle and other livestock animals is also required to improve the control measures against *T. evansi* in Syria.

3. CHAPTER 2

Application of ITS1 PCR and nanopore sequencing as a broad detection system for African cattle trypanosomes in Malawi

3.1 Summary

Human African Trypanosomiasis caused by *T. b. rhodesiense* in Eastern/Southern Africa is a zoonosis for which the transmission between humans and animals, including livestock, is crucial. AAT is caused by other pathogenic trypanosomes, including *T. congolense*, *T. vivax*, and *T. brucei brucei*. Compared to neighboring in eastern and southern Africa where *T. b. rhodesiense*-type HAT is endemic, *T. b. rhodesiense* prevalence in tsetse flies is higher in Malawi. Cattle are one of the most economically important livestock animals in Malawi which have a potential as reservoir hosts for *T. b. rhodesiense* due to high exposure of humans to agriculture. This study aimed to identify trypanosome-infecting cattle in Malawi in order to understand the importance of cattle in the transmission dynamics of HAT and AAT. A total of 446 DNA samples from cattle blood from three regions of Malawi, Nkhotakota, Kasungu and Lilongwe, were screened for African trypanosomes by PCR targeting the internal transcribed spacer 1 region (ITS1). The obtained amplicons were sequenced using a nanopore type portable next-generation sequencer, MinION, for validation. Among the sequence-confirmed trypanosome species, *T. congolense* was the most common parasite found in this study (5.6%; 25 of 446), and it was most prevalent in Nkhotakota (10.8%; 20 of 185), followed by Kasungu (2.5%; 5 of 199). *Trypanosoma vivax* was found in Nkhotakota (6.5%; 12 of 185). *Trypanozoon* was found in Kasungu (1.5%; 3 of 199). No trypanosomes were detected in the Lilongwe samples. Notably, 6 out of the 9 positive signals in the ITS1-PCR amplicons in the gel analysis were found to be nonspecific amplifications after nanopore sequence analysis, indicating that variation by nanopore sequencing improve reliability for species identification. ITS1 PCR cannot differentiate species of *Trypanozoon*. Hence, further PCR screening targeting the human serum resistance-associated (SRA) gene was conducted to detect *T. b. rhodesiense*. T. *b. rhodesiense* was detected in samples from Kasungu (9.5%; 19 of 199) and Nkhotakota (2.7%; 5 of 185). In my study, most of the SRA-PCR-positive samples were negative for ITS1 PCR.

Thus, the SRA PCR test is still recommended for screening human-infective *T. b. rhodesiense* in a more sensitive and specific manner. I report the presence of *T. b. rhodesiense* and animal African trypanosomes from cattle at the human–livestock–wildlife interface for the first time in Malawi. This study confirmed that cattle are potential reservoirs for HAT in Malawi.

3.2 Introduction

AAT, also known as nagana, is caused by *T. congolense, T. vivax,* and *T. brucei brucei* (Spickler, 2018). Infection in domestic animals is usually severe, unlike in wildlife, in which it is usually nonpathogenic (Connor, 1994). AAT affects domestic animals, including cattle, goats, sheep, and pigs, and its pathogenicity differs according to the host species (Connor, 1994; Spickler, 2018). Clinical symptoms include fever, anemia, loss of weight and productivity, abortion, decreased fertility, edema, paralysis, and even death (Bekele, 2015). AAT remains a major threat to animal health and stock farming inside the tsetse belt (Connor, 1994).

Human African trypanosomiasis (HAT) or sleeping sickness occurs in two forms with different features, known as *T. b. gambiense* and *T. b. rhodesiense* infections (Büscher *et al*., 2017). HAT caused by *T. b. gambiense* is an anthroponotic disease that depends mostly on human-to-human transmission: humans act as the main reservoir with a minor role for animal reservoirs (Franco *et al*., 2014). Contrariwise, infection with *T. b. rhodesiense* is a zoonotic disease, affecting mainly animals (wildlife and livestock): humans are considered to be accidental hosts (Franco *et al*., 2014). The control of *T. b. rhodesiense*-type HAT is challenging because animals, both wild and domestic, act as reservoirs for disease transmission (WHO, 2013; Franco et al., 2014). Necessary control measures face obstacles because animal infections are difficult to monitor, unlike in humans, where they can be easily tracked. In addition, parasite-harbouring animals might not show any clinical symptoms, hence complicates the necessary measure to control the parasite infection (Brun et al., 2010). Nevertheless, parasite-harbouring animals can pass the parasite to the biting tsetse flies; maintaining the cycle of transmission of the parasite (Brun et al., 2010). Wildlife have long been known to be the major reservoir of *T. b. rhodesiense* (Ng'ayo et al., 2005). However, recent studies have revealed that livestock can also act as potent reservoir hosts for *T. b.*

rhodesiense owing to close contact of cattle with humans in agriculture activity, and thus, cattle can put humans in a higher risk of HAT infection (Waiswa *et al*., 2003). Cattle are one of the most economically important livestock animals in Malawi (Schmidt, 1969; Chintsanya *et al*., 2004). In Uganda, cattle are implicated to be the principal domestic reservoirs of *T. b. rhodesiense* (Welburn *et al*., 2001; Von Wissmann *et al*., 2014), and they were also documented as reservoirs of *T. b. rhodesiense* in Kenya and Tanzania (Kaare *et al*., 2007; Von Wissmann *et al*., 2011).

In Malawi, *T. b. rhodesiense*-caused HAT has been a burden for decades (Franco *et al*., 2017). Unlike typical *T. b. rhodesiense* infections, HAT in Malawi is characterized by the distinct clinical sign of chronic hemolymphatic stage infection without the formation of a chancre and this makes diagnosis difficult (MacLean *et al*., 2010; Chisi *et al*., 2011b). Endemic foci of HAT in the country are the Nkhotakota, Kasungu, and Rumphi districts (Madanitsa *et al*., 2009; Chisi *et al*., 2011a), where large national parks exist.

An epidemiological study of bovine trypanosomiasis caused by *T. congolense, T. vivax*, and *T. brucei* was conducted to clarify their distribution in Malawi using an indirect anti-trypanosomal antibody-detection ELISA (AbELISA) (Van den Bossche *et al*., 2000). The study proved that the AbELISA could be a useful tool in establishing the distribution of disease (Van den Bossche *et al*., 2000). However, the specificity of the IgG ELISA was questionable, and as in other serological assays, false positives may have been present (Hopkins *et al*., 1998; Van den Bossche *et al*., 2000). In contrast, PCR of the internal transcribed spacer (ITS) 1 region of ribosomal RNA (rRNA), which can distinguish species by product size, has been widely used to identify trypanosome species (Njiru *et al*., 2005; Gaithuma *et al*., 2019). However, ITS1 PCR cannot distinguish subspecies in *Trypanozoon* (*T. brucei brucei, T. brucei rhodesiense, T. brucei gambiense, T. evansi*, and *T. equiperdum*) (Njiru *et al*., 2005; Gaithuma *et al*., 2019). Hence, the detection of *T. b. rhodesiense* by PCR has been widely conducted by

targeting the human serum resistance-associated (SRA) gene which is unique in *T. b. rhodesiense* (Radwanska *et al*., 2002). Identification of species by ITS1 PCR is sometimes difficult due to ambiguous and nonspecific signals, which may result in false positive annotations because of subjective human decisions. In addition, it is impossible to differentiate between *T. godfreyi* and *T. vivax* because they share similar base-pair size of ITS1 PCR product.

MinION, developed by Oxford Nanopore Technologies, is a portable next-generation sequencer (NGS) that connects to a laptop computer through a USB cable (Loman *et al*., 2015; Mongan *et al*., 2020). MinION is unique among sequencing tools because it identifies nucleotides in a nanoscale ion channel (nanopore) by detecting specific changes in the electric current when DNA passes through the nanopore (Loman *et al*., 2015). DNA sequencing has a potential to be a definitive diagnostic method for detecting pathogenic species, and several studies have reported the application of MinION for pathogen identification (Ashton *et al*., 2014; Quick *et al*., 2014; Greninger *et al*., 2015; Sugano *et al*., 2015). Unlike conventional sequencers, MinION is economically affordable, allowing sequence analysis without preinstallation of expensive equipment, and not requiring a separate electric supply after connecting to a laptop computer (Sugano *et al*., 2015). Furthermore, operating MinION does not require sophisticated skills in biological research (Sugano *et al*., 2015). Given these features, genotyping of pathogens on-site with MinION is now feasible (Sugano *et al*., 2015).

The use of Illumina-type NGS for ITS1 amplicons gives higher specificity and resolution for species identification than PCR alone (Gaithuma *et al*., 2019). However, these instruments are not portable, affordable, or practical for field applications. In this study, I used MinION NGS to determine the prevalence of human and animal parasitic trypanosomes in cattle in Malawi.

3.3 Materials and methods

3.3.1 Ethics approval

The ethical clearance for animal sampling was obtained from the Ministry of Agriculture, Irrigation and Water Development in Malawi through the Department of Animal Health and Livestock Development with reference number 10/15/32/D.

3.3.2 Sample collection and DNA extraction

This is a descriptive, cross-sectional study of trypanosome infection in cattle, conducted by analyzing DNA material from 446 bovine blood samples. Samples were collected from cattle in three districts in Malawi: Kasungu, Nkhotakota, and Lilongwe, in February and March 2018 during the rainy season. Information on farmers and cattle populations was obtained from the District Agriculture Development Office (DADO) for each district through their District Animal Health and Livestock Development Officer (DAHLDO). The sampling map (Figure 11) was constructed using the free and open-source geographic information system QGIS (QGIS Development Team, 2020).

Villages located in the vicinity of Kasungu National Park and Nkhotakota Wildlife Reserve were deliberately selected as sampling sites because these areas are locations of human–livestock–wildlife interface and are endemic for tsetse flies carrying *T. b. rhodesiense*. Both clinically healthy animals and animals with clinical signs were randomly selected within farms. In Kasungu and Nkhotakota districts, a total of 199 and 185 cattle blood samples were collected from 29 and 26 farms, respectively. These farms were selected on purpose from a farmer registry list, since the selected areas are situated in the human–livestock–wildlife interface area in the vicinity of Kasungu National Park and Nkhotakota Wildlife Reserve (Figure 11). In Kasungu, 10,240 smallholder farms (92,677 cattle) exist. Chulu (12°49'3.912"S,

33°18'10.008"E), Lisasadzi (13°16'20.682"S, 33°8'11.446"E), and Chipala villages (13°7'2.761"S, 33°19'7.314"E) are located in this district, in which 47, 20, and 19 farmers live, respectively. I randomly selected 15, 7, and 7 farms from a farmer registry and collected 62, 72, and 65 blood samples, respectively. In Nkhotakota, 1,822 smallholder farms report 13,650 cattle. Mphonde **(**12°48'19.8"S, 34°11'27.2"E) and Linga (12°56'9.438"S, 34°13'36.438"E) villages are located in this district, in which 29 and 43 farmers live, respectively. We randomly selected 11 and 15 farms from a farmer registry list and collected 84 and 101 blood samples, respectively.

Both Kasungu National Park and Nkhotakota Wildlife Reserve are tsetse-infested areas and the major HAT/AAT foci in the country [12, 38, 57]. *Glossina morsitans morsitans* and *G. pallidipe*s are the dominant tsetse fly species in these areas, while *G. brevipalpis* is also known to exist to some extent [57]. The major cattle breed in these areas is Malawi Zebu, a local breed in the country also known as Angoni cattle (Small East African Zebu) in Eastern Zambia [39]. This breed is known to be susceptible to trypanosomiasis [58]. Cattle are kept in a free-range management system where animals are released during the day to graze freely and return home at dusk. Tsetse flies are often found at the sampling sites; thus, the frequencies of tsetse bite in the cattle in these areas are expected to be high. The other biting flies, tabanids, or *Stomoxys* spp. are also commonly found in these areas.

In Lilongwe district, there are only two commercial farms, and 62 blood samples were collected from one of these farms. Holstein Friesian cattle are maintained with a semi-intensive farm management system, where animals are kept in paddocks. Tsetse flies are not found in this area, and no HAT/AAT cases have been reported in the past from this area. However, the other hematophagous flies including tabanids and *Stomoxys* spp. are commonly found in Lilongwe, which might facilitate the mechanical transmission of trypanosome, especially *T. vivax*.

Before sampling, the venipuncture site was disinfected with a methylated spirit swab. Then, 5 mL of blood were drawn by venipuncture of the external jugular vein into vacutainer EDTA tubes. Packed cell volume (PCV) counting was conducted to determine anemia; animals with a PCV < 24% were considered anemic. For molecular detection purposes, approximately 200 µL of blood were subjected to DNA extraction using a KURABO QuickGene DNA whole blood kit (Kurabo Industries Ltd.), following the manufacturer's protocol.

3.3.3 Detection of trypanosomes by ITS1 PCR

DNA samples were subjected to PCR amplification of the rRNA ITS1 region to identify all pathogenic African trypanosome species/subspecies. ITS1 PCR was carried out using the primers by Gaithuma *et al*. (2019) (Table 9) considering its better sensitivity compared to the CF and BR primers which were used in the previous study as observed in the PCR sensitivity test (Figure 12). PCR was performed in the final volume of 25 µL, comprising 12.5 µL Ampdirect plus (Shimadzu, Japan), 0.125 µL BIOTAQ HS DNA Polymerase (5 U/µL) (Bioline, UK), 0.625 µL of each 10 mM primer, 0.5 µL of 2% DMSO, 8.625 µL RNase-free water, and 2 µL extracted DNA. The temperature and cycling profile included initial denaturation at 95°C for 10 min followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 90 s, extension at 72°C for 2 min, and final extension of 7 min at 72°C. PCR products were examined by electrophoresis in 1.5% agarose S (Nippongene, Japan) in Tris-acetate EDTA (TAE) buffer pH 8.3 and stained using GelRed (Biotium, USA) dye before being visualized under ultraviolet light in a transilluminator.

Signals on gels were judged by three investigators independently. They were classified as follows: positive (clear band), negative, and ambiguous (faint band or smearing). Trypanosome species were identified based on the size differences for members of the subgenus *Trypanozoon* (*T. b. brucei*, *T. evansi*, *T. b. rhodesiense*, and *T. b. gambiense*), a constant product of

approximately 415–431 bp, 560–705 bp for *T. congolense*, 331–343 bp for *T. simiae*, 220 bp for *T. godfreyi*, 269–350 bp for *T. theileri*, and 226–238 bp for *T. vivax* (Gaithuma *et al*., 2019) (Table 10). The plasmids that contain the TA-cloned ITS1 fragment of *T. congolense*, *T. brucei*, *T. vivax*, *T. godfreyi*, and *T. simiae* (Nambala *et al*., 2018) were combined and used as a positive control for PCR and gel analysis.

3.3.4 MinION library preparation for sequencing

To prepare MinION sequencing library, amplicons of the ITS1 PCR were used as template then indexes were added by additional PCR. The indexed amplicons were further ligated with adapter DNA provided by Oxford nanopore then sequence ready library was obtained. Positive, ambiguous samples, and a positive control from ITS1 PCR were sequenced as follows. For the indexing, reagents comprised 5 μL Ampdirect plus (Shimadzu, Japan), 0.05 μL BIOTAQ HS DNA Polymerase (5 U/μL) (Bioline, UK), 0.2 μL of each 10 mM ITS1-index primers (Table 9), 0.5 μL DMSO 2%, 2.55 μL RNase-free water, and 2 μL extracted DNA. PCR conditions were as follows: an initial hold at 95 °C for 10 min, followed by 10 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 min. Amplicon of the index PCR was prepared for MinION-compatible DNA libraries using the Ligation Sequencing Kit 1D SQK-LSK109 and Native Barcoding Kit EXP-NBD103 (Oxford Nanopore Technologies, UK) as per their instruction manuals.

Equal concentrations of each sample were pooled together to obtain 12 indexed amplicons for each pool, aiming total of 3,000 ng in 50 uL. Concentration of each sample is calculated by Qubit. After all samples were pooled in one PCR tube, the indexed amplicons were purified by AMPure XP (Beckman Coulter, USA). 90uL AMPure XP was added into the pooled sample, mixed by pipetting 10 times, and left in room temperature, not in the magnet. Tube was moved into magnet, kept for about 2 minutes until getting clear, and pipetted off the supernatant. Tube

was kept on magnet and the beads were washed with 200 µl of freshly prepared 75% ethanol without disturbing the pellet, the 75% ethanol were removed using a pipette and discarded, and this step was repeated. Tube was spinned down and placed back on the magnet, then any residual ethanol was pipetted off. Tube was allowed to dry for ~30 seconds without drying the pellet to the point of cracking. Tube was removed from the magnetic rack. Pellet was resuspended in 45 µl distilled water (DW) and incubated for two minutes at room temperature. 45 µl of eluate was removed and retained into a clean new PCR tube. 1 µl of pooled DNA was quantified by a Qubit fluorometer aiming 2,000 ng equal for each pooled sample.

End repair and dA-tailing were performed to the pooled, barcoded amplicons using the NEBNext UltraII End Repair/dA-Tailing module (New England Biolabs, UK) per the Oxford Nanopore 1D Native barcoding genomic DNA sequencing protocol. In PCR tube, following mixtures were combined together by gently flicking the tube then spindown, including: 45 µl 2000 ng pooled indexed DNA, 5 µl DW, 7 µl Ultra II End-prep reaction buffer, and 3 µl Ultra II End-prep enzyme mix. Mixture was incubated for 15 minutes at 20° C and 5 minutes at 65° C using the thermal cycler. 60 µl of resuspended AMPure XP beads were added to the end-prep reaction and mixed by pipetting. Mixture was incubated on a Hula mixer (rotator mixer) for 5 minutes at room temperature and spinned down. Tube was moved into magnet, kept for about 2 minutes until getting clear, and pipetted off the supernatant. Tube was kept on magnet and the beads were washed with 200 µl of freshly prepared 70% ethanol without disturbing the pellet, the 70% ethanol were removed using a pipette and discarded, and this step was repeated. Tube was spinned down and placed back on the magnet, then any residual ethanol was pipetted off. Tube was allowed to dry for ~30 seconds without drying the pellet to the point of cracking. Tube was removed from the magnetic rack. Pellet was resuspended in 25 µl DW and incubated for two minutes at room temperature. 25 µl of eluate was removed and retained into a clean

new PCR tube. 1 µl of end-prepped DNA was quantified by a Qubit fluorometer aiming 1,500 ng equal for each end-prepped sample.

Subsequently, the purified, end-prepped DNA was barcoded with the following materials: 22.5 µl equally 1,500 ng end-prepped DNA, 2.5 µl Native Barcode 1-12, and 25 µl Blunt/TA Ligase Master Mix. Reaction was incubated for 30 minutes at 23°C (room temperature) using thermal cycler. 50 µl of resuspended AMPure XP beads was added to the reaction and mixed by pipetting. Mixture was incubated on a Hula mixer (rotator mixer) for 5 minutes at room temperature and spinned down. Tube was moved into magnet, kept for about 2 minutes until getting clear, and pipetted off the supernatant. Tube was kept on magnet and the beads were washed with 200 µl of freshly prepared 70% ethanol without disturbing the pellet, the 70% ethanol were removed using a pipette and discarded, and this step was repeated. Tube was spinned down and placed back on the magnet, then any residual ethanol was pipetted off. Tube was allowed to dry for ~30 seconds without drying the pellet to the point of cracking. Tube was removed from the magnetic rack. Pellet was resuspended in 65 µl DW and incubated for two minutes at room temperature. 65 µl of eluate was removed and retained into a clean new PCR tube. 1 µl of barcoded DNA was quantified by a Qubit fluorometer aiming 1000 ng equal for each barcoded sample.

The purified, barcoded DNA was ligated with the Adapter Mix (AMX) using the NEBNext Quick T4 DNA Ligase (New England Biolabs, UK). In PCR tube, equimolar amounts of each barcoded sample were pooled together ensuring that sufficient sample is combined to produce a pooled sample of 1500 ng total. Following mixtures were combined together by gently flicking the tube then spindown, including: 65 µl 1500 ng pooled barcoded sample, 5 µl Adapter Mix II (AMII), 20 µl NEBNext Quick Ligation Reaction Buffer (5X), and 10 µl Quick T4 DNA Ligase. Reaction was incubated for 10 minutes at 23°C (room temperature) using thermal cycler. 50 µl of resuspended AMPure XP beads was added to the reaction and mixed

by pipetting. Mixture was incubated on a Hula mixer (rotator mixer) for 5 minutes at room temperature and spinned down. Tube was moved into magnet, kept for about 2 minutes until getting clear, and pipetted off the supernatant. Tube was kept on magnet and the beads were washed with 250 µl Short Fragment Buffer (SFB), flicked the beads to resuspend, spinned down, then returned the tube to the magnetic rack and allowed the beads to pellet. The supernatant was removed using a pipette and discarded, and this step was repeated. Tube was spinned down and placed back on the magnet, then any residual ethanol was pipetted off. Tube was allowed to dry for ~30 seconds without drying the pellet to the point of cracking. Tube was removed from the magnetic rack. Pellet was resuspended in 15 µl Elution Buffer (EB) and incubated for 10 minutes at room temperature. 15 µl of eluate was removed and retained into a clean new PCR tube. 1 µl of barcoded DNA was quantified by a Qubit fluorometer aiming 1,000 ng for the sequencing library.

The prepared library is used for loading into the flow cell and stored on ice until ready to load. The flowcell was primed for loading and sequencing of the library. The flow cell priming mix was prepared as follows: 30 µl of thawed and mixed Flush Tether (FLT) was added directly to the tube of thawed and mixed Flush Buffer (FLB), and it was mixed by pipetting up and down. 800 µl of the priming mix was loaded into the flow cell via the priming port, avoiding the introduction of air bubbles and left for 5 minutes. In a new tube, the library was prepared for loading as follows: 37.5 µl Sequencing Buffer (SQB), 12 µl DNA library, and 25.5 µl Loading Beads (LB).75 µl of sample was added to the flow cell via the SpotON sample port in a dropwise fashion. Each drop was confirmed to flow into the port before adding the next, then the sequencing was started.

3.3.5 Detection of trypanosome species using MinION sequencing

Base-calling and de-barcoding were conducted using Guppy (Oxford Nanopore Technologies). De-indexing was performed using custom scripts. In brief, indexed primer sequences were aligned on each MinION read using LAST (Kiełbasa *et al*., 2011), and the best hit indexes at both terminals were subsequently assigned. De-multiplexed reads were aligned with the nucleotide dataset using BLASTn (Altschul *et al*., 1990). I kept the best hit for each read and counted the species name that appeared in the output, i.e., *T. vivax*, *T. godfreyi*, *T. evansi*, *T. brucei*, *T. equiperdum*, *T. congolense*, *T. simiae*, and *T. theileri*. The counts for *T. brucei*, *T. evansi*, and *T. equiperdum* were summed up and regarded together as *T. brucei*. With the ITS1 PCR and sequencing, I cannot differentiate subgenus in *Trypanozoon*. However, *T. evansi* is known to spread out of the tsetse belt, not in Malawi. Therefore, the detected *Trypanozoon* was regarded as *T. brucei* in this study to emphasize the findings. Each taxon was programmatically assigned on the basis of the population of the read counts if it shared more than 20% of the total and more than 50 read counts. A schematic workflow was also provided (Figure 13). The prevalence of trypanosomes was determined according to the results of MinION sequencing.

3.3.6 Detection of T. b. rhodesiense by SRA PCR

SRA PCR was employed to identify *T. b. rhodesiense* using the primers by Radwanska *et al*. (2002) (Table 9). Reagents used for each reaction included 5 μL Ampdirect plus (Shimadzu, Japan), 0.05 μL BIOTAQ HS DNA Polymerase (5 U/μL) (Bioline, UK), 0.2 μL of each 10 mM primer, 2.55 μL RNase-free water, and 2 μL extracted DNA. The temperature and cycling profile included an initial hold for 10 min at 95°C, followed by 40 cycles at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final extension of 5 min at 72°C. PCR products were examined

by electrophoresis in 2% agarose S (Nippongene, Japan) in TAE buffer and stained using GelRed (Biotium, USA) dye before being visualized under ultraviolet light.

3.3.7 Statistical analysis

Multiple logistic regression test was conducted to understand the association of infection rate in animals and regions and anemic status (*P* value < 0.05).

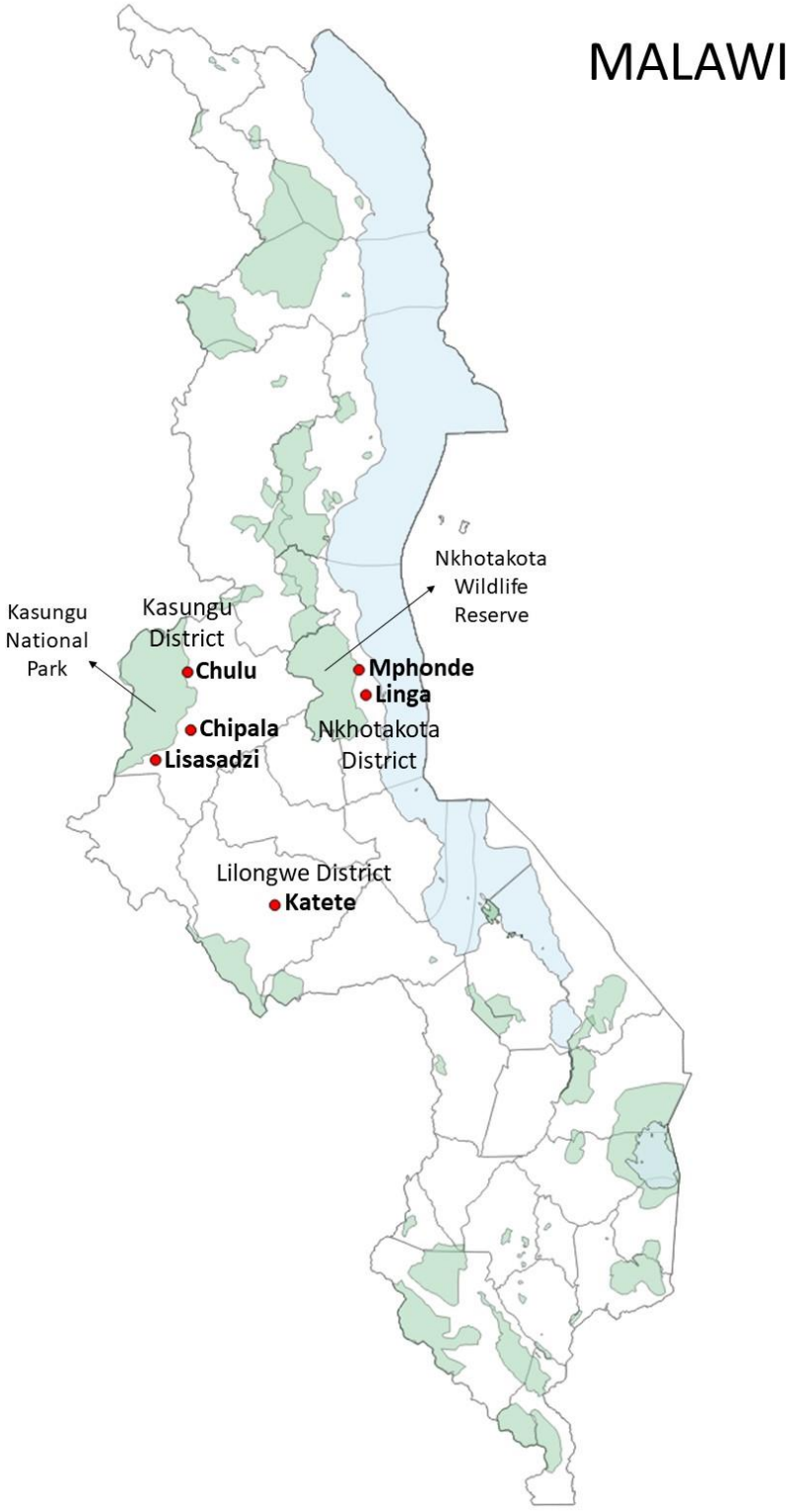


Figure 11. Map of Malawi showing sampling sites.

Black arrows point at Kasungu National Park and Nkhotakota Wildlife Reserve. Sampling points at Nkhotakota and Kasungu district were located outside the nature reserves in <1 km proximity.

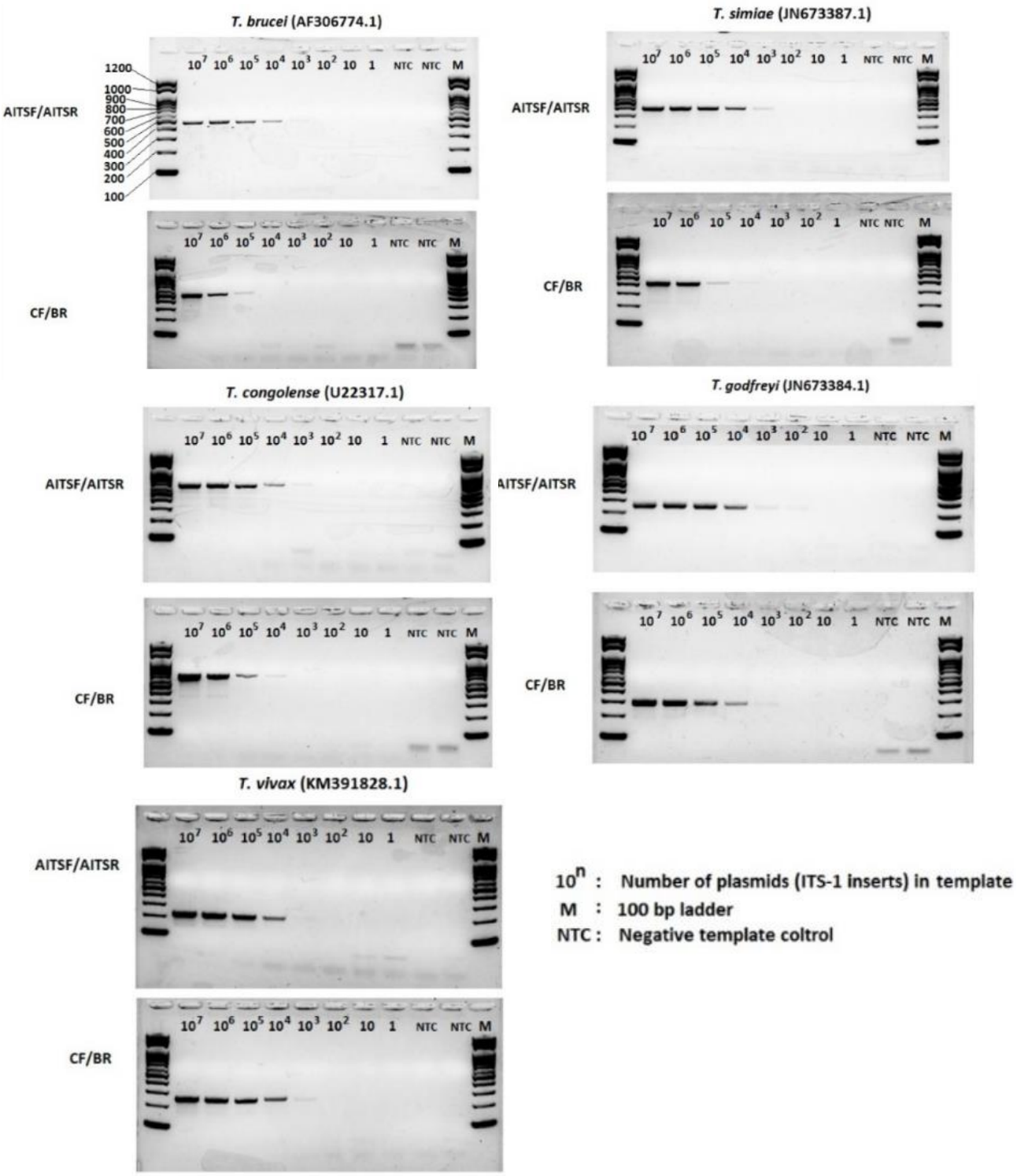


Figure 12. Comparison between the sensitivity test of the AITSF/AITSR primers and CF/BR primers.

The detection limit of each PCR was examined using 10-fold serial dilutions of Trypanosome ITS1 sequences inserts cloned in the pGEMT-easy vector (Gaithuma *et al*., 2019).

Table 9. List of primers and their sequence used in this study.

Primer name	Primer sequence 5` - 3`
Forward AITS1	[index 1-12]-CGGAAGTTCACCGATATTGC
Reverse AITS1	[index 1-12]-AGGAAGCCAAGTCATCCATC
index1	CTATACAGCATGAG
Index2	AGAGTCTAGCTAGC
Index3	TGCGACACATGTGA
Index4	GACTATGCAGTGCA
Index5	ACGCGTGCATCTAC
Index6	TCGAGTAGTCTCAG
Index7	GTATCATGTCAGCA
Index8	AGCTAGTAGCTACT
Index9	CGAGACGATACTCT
Index10	TAGATGCTCGCGAG
index11	GCTACGCTGAGTAG
index12	TCTCAGCGCAGTGA
Forward SRA	ATAGTGACAAGATGCGTACTCAACGC
Reverse SRA	AATGTGTTCGAGTACTTCGGTCACGCT

Table 10. Comparison between the amplicon sizes of AITSF/AITSR primers and CF/BR primers.

Trypanosoma species/ sub-species	AITSF/R Amplicon lengths (b.p)	CF/BR Amplicon lengths (b.p)	ITS1/ITS2 Amplicon lengths (b.p)
Trypanosoma congolense other isolate	633 - 705	686 - 717	600 - 674
Trypanosoma congolense riverine/ forest-type	660	713	-
Trypanosoma congolense Kilifi-type	560	613	529
Trypanosoma cruzi	544 – 570	-	512 - 518
Trypanosoma rangeli	525	-	455 - 494
Trypanosoma cf. varani	511	-	480
Trypanosoma kuseli	504 – 506	-	473
Trypanosoma otosspermophili	504	-	472
Trypanosoma lewisi	504	-	473 - 475
Trypanosoma grosi	424 – 435	-	401 - 404
Trypanosoma brucei/ evansi	415 – 431	468 - 484	384 - 394
Trypanosoma simiae	331 – 343	369 - 397	300 - 319
Trypanosoma cf. cervi	333 – 340	-	303 - 312
Trypanosoma avium	322	-	291
Trypanosoma congolense Tsavo-type	316	369	285
Trypanosoma theileri	269 – 350	-	238 - 265
Trypanosoma godfreyi	220	273	189
Trypanosoma vivax	226 - 238	248 - 264	164 - 187

Source: Gaithuma *et al.,* 2019

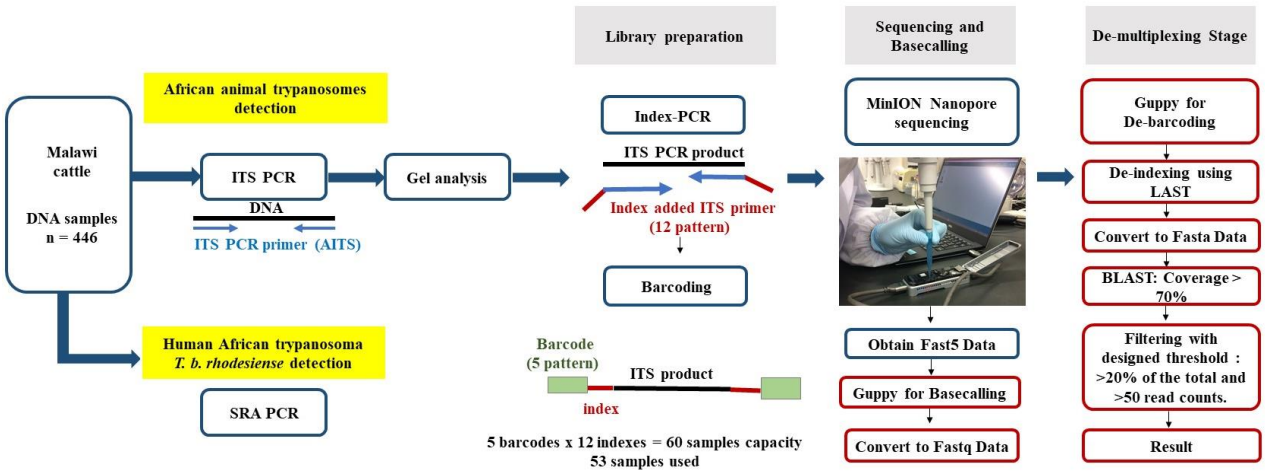


Figure 13. Workflow for molecular detection of African trypanosomes.

The workflow depicts structural steps for detection of African animal and human trypanosomes with serial arrows on the lateral side of cattle samples and a bottom arrow one, respectively. The section on the identification of trypanosome species by sequencing is divided into three steps: library preparation, sequencing and basecalling, and de-multiplexing. Bioinformatics analyses required in the experiments are framed in red borderline.

3.4 Results

3.4.1 ITS1 PCR gel analysis

Forty-four positive, 8 ambiguous, and 394 negative samples were obtained by gel analysis. The ambiguous samples were the samples with faint and smearing bands. For positive samples, I identified 21 amplicons with a single band size of 620–700 bp, indicating a single *T. congolense* infection; 9 amplicons with a single band size of 480 bp, indicating a single *Trypanozoon* infection; 9 amplicons with a single band size of 250 bp, indicating a single *T. vivax* or *T. godfreyi* infection; and 2 amplicons with a single band size of 350 bp, indicating a single *T. theileri* infection. Three amplicons showed clear multiple bands, indicating two mixed infections of *T. congolense* and *Trypanozoon* (A35 and A37; Figure 14, Table 11) and one mixed infection with *T. congolense* and *T. vivax* (D66; Figure 14, Table11).

3.4.2 MinION-based detection method for trypanosomes

In total, 503,039 reads were obtained for 53 multiplexed ITS1 amplicons, including one positive control. Taxonomic identity was determined for 369,673 reads using a BLAST homology search. For *T. congolense*, 24 samples (including single and mixed infections) were positive by gel analysis, and *T. congolense* sequences were detected in the same 24 samples and one additional sample, A17, by the MinION system (Figure 14, Table 11). For *T. vivax*, 10 samples were positive, and the same 10 samples were confirmed by sequencing, as well as two additional samples, D07 and E60. E60 was a mixed infection with *T. congolense* and *T. vivax* infection. For *T. theileri*, two samples were positive in both gel analysis and the MinION system.

In contrast, 11 samples positive for *Trypanozoon* were identified by gel analysis. However, using the MinION system, *Trypanozoon* sequences were detected in only three of these 11

products (Table 11). Most cases (B24, B25, B38, D20, D23, D42, D65, D76) with the exception of A37, produced reads for *Trypanozoon* that were much lower than my threshold (50 reads minimum). For A37, 172 reads for *Trypanozoon* out of 2,965 reads were obtained, but reads were below my criteria, with a minimum of 50 reads and more than 20% of total reads, and therefore, this sample was negative even though I could not eliminate the possibility of actual *Trypanozoon* infection.

Three confirmed cases of mixed infections were observed (A35, D66, and E60; Figure 14, Table 11). E60 showed a clear signal for *Trypanozoon* and a faint signal for *T. godfreyi* or *T. vivax*. However, the MinION system provided substantial numbers of sequences to show that the sample was a mixed infection with *T. congolense* and *T. vivax* (Table 11). Another case, A37, appeared to be a mixed infection with *T. congolense* and *Trypanozoon* by gel analysis, but MinION results did not support *Trypanozoon* infection as described above. Thus, in several cases, disagreement between ITS1 PCR gel analysis and ITS1-MinION sequence analysis was observed, likely due to nonspecific amplification and misjudgment of the ITS1-PCR gel analysis.

3.4.3 Prevalence and distribution of African trypanosomes determined by ITS1-MinION

The overall prevalence of trypanosomes calculated by ITS1-MinION was 8.3%, as determined by MinION sequencing data. *T. congolense* was the most prevalent trypanosome (5.6%), followed by *T. vivax* (2.7%), *Trypanozoon* (0.7%), and *T. theileri* (0.4%). I found three mixed infections: one *T. congolense* with *Trypanozoon*, and two *T. congolense* with *T. vivax* (Table 12). The parasites were detected in two districts, with the highest prevalence being in Nkhotakota (16.2%), followed by Kasungu (3.5%) (Table 12), while no parasites were detected in Lilongwe samples. There was a significant difference in prevalence between the study sites ($P < 0.05$).

3.4.4 Prevalence and distribution of human-infective trypanosomes determined by SRA PCR

T. b. rhodesiense was detected in cattle in both HAT-active foci by SRA PCR. A higher prevalence of *T. b. rhodesiense* was found in samples from Kasungu (9.5%; 19 of 199), followed by Nkhotakota (2.7%; 5 of 185) (Table. 11). No *T. b. rhodesiense* was identified in the Lilongwe samples. The detected number of *T. b. rhodesiense* was much higher than the detected number of *Trypanozoon* as indicated by the ITS1-MinION detection system. Out of 24 SRA PCR-positive samples, only 1 sample (B23) was positive when analyzed by the ITS1-MinION detection system, suggesting superior sensitivity of SRA PCR relative to ITS1 PCR (Figure 15).

3.4.5 Association between infection by African trypanosomes and the anemic status of cattle

There was a significant association in the anemic status (PCV ≤ 24%) between cattle with trypanosome infection and without infection ($P < 0.05$) (Table 13). Of 16 anemic animals, 11 tested were positive for trypanosome infections, indicating a 69% prevalence, but in nonanemic cattle, only 6% were infected with trypanosomes (26 of 430). In particular, infection with *T. congolense* showed a significant association in anemia status compared to other trypanosome species ($P < 0.0001$). The odds of anemia in the presence of *T. congolense* was 39 times than the odds in its absence. A significant difference in the anemic status of *T. congolense*-infected cattle was observed in both single-infected cattle and mixed-infected cattle (Table 13).

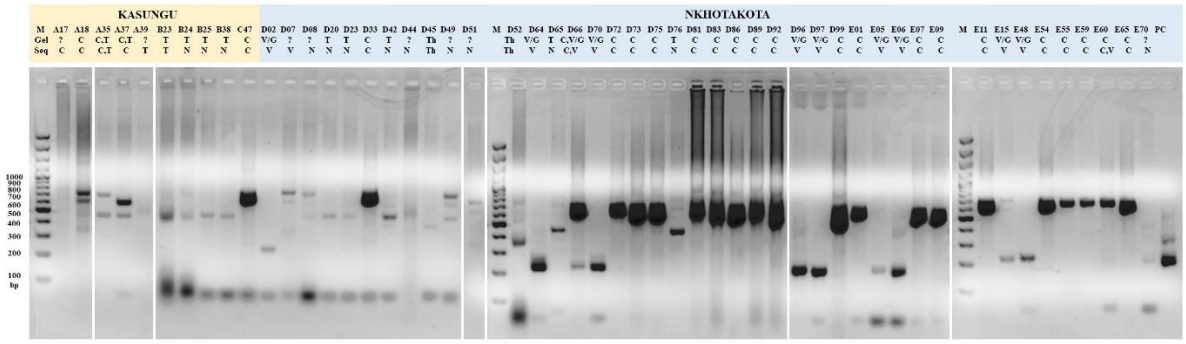


Figure 14. Gel images of the ITS1 PCR results for positive and ambiguous samples (N = 53, including one positive control).

The combination of number and letter referred to the sample identity. M and PC represent the abbreviation of 1 kb marker and positive control, respectively. The letter bellow explained the interpretation of the gel (Gel) and the analysis of MinION sequencing (Seq). C, *T. congolense*; T, *Trypanozoon*; V/G, *T. vivax* or *T. godfreyi*; S, *T. simiae*; Th, *T. theileri*; N, no result; and "?", ambiguous result."

Table 11. SRA PCR, ITS PCR, and MinION sequencing analysis for characterizations of trypanosome species.

No.	Sample ID	Region	SRA PCR	ITS1											
				Gel image result of PCR	MinION sequencing analysis	Number of hit reads by BLAST								No hit*	Fasta $
						T. vivax	*T. godfreyi*	*Trypanozoon*	*T. congolense*	*T. simiae*	*T. theileri*	*Trypanosoma sp.*	Others	Total	
1	A17	Kasungu		?	*T. congolense*	9	0	519	5605	0	25	346	1774	8278	3930	12208
2	A18	Kasungu		*T. congolense*	*T. congolense*	0	1	1	1188	0	54	17	160	1421	1053	2474
3	A20	Kasungu	*T. b. rhodesiense*													
4	A35	Kasungu		*T. congolense, Trypanozoon*	*T. congolense, Trypanozoon*	1	0	353	905	0	0	28	18	1305	216	1521
5	A37	Kasungu		*T. congolense, Trypanozoon*	*T. congolense*	1	1	172	2273	3	0	11	7	2468	497	2965
6	A39	Kasungu		?	*Trypanozoon*	3	0	1209	5	0	0	172	52	1441	1432	2873
7	A46	Kasungu	*T. b. rhodesiense*													
8	A51	Kasungu	*T. b. rhodesiense*													
9	A53	Kasungu	*T. b. rhodesiense*													
10	A54	Kasungu	*T. b. rhodesiense*													
11	B04	Kasungu	*T. b. rhodesiense*													
12	B06	Kasungu	*T. b. rhodesiense*													
13	B08	Kasungu	*T. b. rhodesiense*													
14	B13	Kasungu	*T. b. rhodesiense*													
15	B14	Kasungu	*T. b. rhodesiense*													
16	B17	Kasungu	*T. b. rhodesiense*													
17	B19	Kasungu	*T. b. rhodesiense*													
18	B23	Kasungu	*T. b. rhodesiense*	*Trypanozoon*	*Trypanozoon*	4	0	278	2	0	23	24	5	336	552	888
19	B24	Kasungu		*Trypanozoon*	Negative	1	0	0	6	0	0	0	58	65	451	516
20	B25	Kasungu		*Trypanozoon*	Negative	4	0	16	2	0	24	6	17	69	361	430
21	B38	Kasungu		*Trypanozoon*	Negative	111	0	21	2	0	0	61	32	227	1735	1962
22	B42	Kasungu	*T. b. rhodesiense*													
23	B53	Kasungu	*T. b. rhodesiense*													
24	B68	Kasungu	*T. b. rhodesiense*													
25	C21	Kasungu	*T. b. rhodesiense*													
26	C35	Kasungu	*T. b. rhodesiense*													
27	C47	Kasungu		*T. congolense*	*T. congolense*	59	327	0	1953	0	0	280	12	2631	971	3602
28	C50	Kasungu	*T. b. rhodesiense*													
29	D02	Nkhotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	4676	0	0	7	0	0	346	1	5030	3154	8184
30	D07	Nkhotakota		?	*T. vivax*	66	1	3	6	0	0	4	41	121	149	270
31	D08	Nhkotakota		?	Negative	13	0	1	8	0	0	0	197	219	2232	2451
32	D11	Nhkotakota	*T. b. rhodesiense*													
33	D15	Nhkotakota	*T. b. rhodesiense*													
34	D20	Nhkotakota		*Trypanozoon*	Negative	0	0	0	19	0	0	0	111	130	2210	2340
35	D23	Nhkotakota		*Trypanozoon*	Negative	1	0	0	26	0	1	0	26	54	447	501
36	D33	Nhkotakota		*T. congolense*	*T. congolense*	0	0	0	4167	0	0	6	2	4175	240	4415
37	D37	Nhkotakota	*T. b. rhodesiense*													
38	D42	Nhkotakota		*Trypanozoon*	Negative	1	0	0	30	0	7	0	7	45	501	546
39	D44	Nhkotakota		?	Negative	5	3	0	5	4	22	8	20	67	748	815
40	D45	Nhkotakota		*T. theileri*	*T. theileri*	58	0	0	2	0	457	52	159	728	448	1176
41	D49	Nhkotakota		?	Negative	162	0	0	14	0	47	26	981	1230	4515	5745
42	D51	Nhkotakota		?	Negative	61	1	0	4	0	11	11	2064	2152	1433	3585
43	D52	Nhkotakota		*T. theileri*	*T. theileri*	194	0	0	0	0	517	64	42	817	286	1103
44	D64	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	19991	10	2	14	11	3	2518	17	22566	16442	39008
45	D65	Nhkotakota		*Trypanozoon*	Negative	19	0	0	21	0	7	3	139	189	1197	1386
46	D66	Nhkotakota		*T. congolense* , *T. vivax* or *T. godfreyi*	*T. congolense* , *T. vivax*	6096	0	0	7422	0	0	332	2	13852	6010	19862
47	D70	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	1665	0	0	13	0	0	52	1	1731	1051	2782
48	D72	Nhkotakota		*T. congolense*	*T. congolense*	55	0	0	6752	0	0	12	19	6838	661	7499
49	D73	Nhkotakota		*T. congolense*	*T. congolense*	264	0	0	2242	3	0	56	5	2570	1451	4021
50	D75	Nhkotakota		*T. congolense*	*T. congolense*	4	0	0	21190	0	1	31	27	21253	2409	23662
51	D76	Nhkotakota		*Trypanozoon*	Negative	1	0	0	385	0	0	0	540	926	9635	10561
52	D81	Nhkotakota		*T. congolense*	*T. congolense*	0	0	0	49935	0	0	52	9	49996	4983	54979
53	D83	Nhkotakota		*T. congolense*	*T. congolense*	0	0	0	5607	0	0	8	3	5618	557	6175
54	D86	Nhkotakota		*T. congolense*	*T. congolense*	6	0	0	15852	0	0	16	25	15899	1448	17347
55	D89	Nhkotakota		*T. congolense*	*T. congolense*	9	5	2	2818	4	0	100	28	2966	1273	4239
56	D92	Nhkotakota		*T. congolense*	*T. congolense*	130	9	2	806	12	0	19	2	980	265	1245
57	D96	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	586	6	2	9	10	0	27	3	643	653	1296
58	D97	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	3183	0	0	11	0	0	187	5	3386	5869	9255
59	D99	Nhkotakota		*T. congolense*	*T. congolense*	27	0	0	12565	0	0	13	4	12609	1384	13993
60	E01	Nhkotakota		*T. congolense*	*T. congolense*	10	0	0	10592	0	0	9	7	10618	1119	11737
61	E05	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	3681	1	0	36	0	0	238	5	3961	1740	5701
62	E06	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	2922	0	0	23	25	0	157	2	3129	2868	5997
63	E07	Nhkotakota		*T. congolense*	*T. congolense*	20	0	0	9592	0	9	16	7	9644	837	10481
64	E09	Nhkotakota		*T. congolense*	*T. congolense*	4	0	0	14794	0	0	7	127	14932	1214	16146
65	E11	Nhkotakota		*T. congolense*	*T. congolense*	3	0	0	12758	0	0	13	7	12781	1116	13897
66	E15	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	14753	0	0	57	0	58	595	682	16145	13594	29739
67	E29	Nhkotakota	*T. b. rhodesiense*													
68	E32	Nhkotakota	*T. b. rhodesiense*													
69	E48	Nhkotakota		*T. vivax* or *T.godfreyi*	*T. vivax*	3061	0	0	121	0	1	180	7	3370	1380	4750
70	E54	Nhkotakota		*T. congolense*	*T. congolense*	57	0	0	10470	0	0	9	46	10582	637	11219
71	E55	Nhkotakota		*T. congolense*	*T. congolense*	145	0	0	5908	0	15	20	31	6119	527	6646
72	E59	Nhkotakota		*T. congolense*	*T. congolense*	16	1	1	15840	0	28	27	37	15950	2372	18322
73	E60	Nhkotakota		*T. congolense*	*T. congolense, T. vivax*	2529	192	0	2051	11	0	285	3	5071	1720	6791
74	E65	Nhkotakota		*T. congolense*	*T. congolense*	18	0	0	31134	0	0	46	357	31555	3999	35554
75	E70	Nhkotakota		?	Negative	1907	130	0	323	63	0	1560	20	4003	6607	10610
76	PC+			*T. vivax* or *T.godfreyi*, *T. simiae*, faint *Trypanozoon*	*T. vivax*	10318	4516	589	1610	5605	0	4144	0	26782	10787	37569

No hit* shows the number of reads that could not be identified by BLAST. FASTA $ describes the total number of obtained reads processed into BLAST. “PC+” and “?” refer to positive control and ambiguous results, respectively.

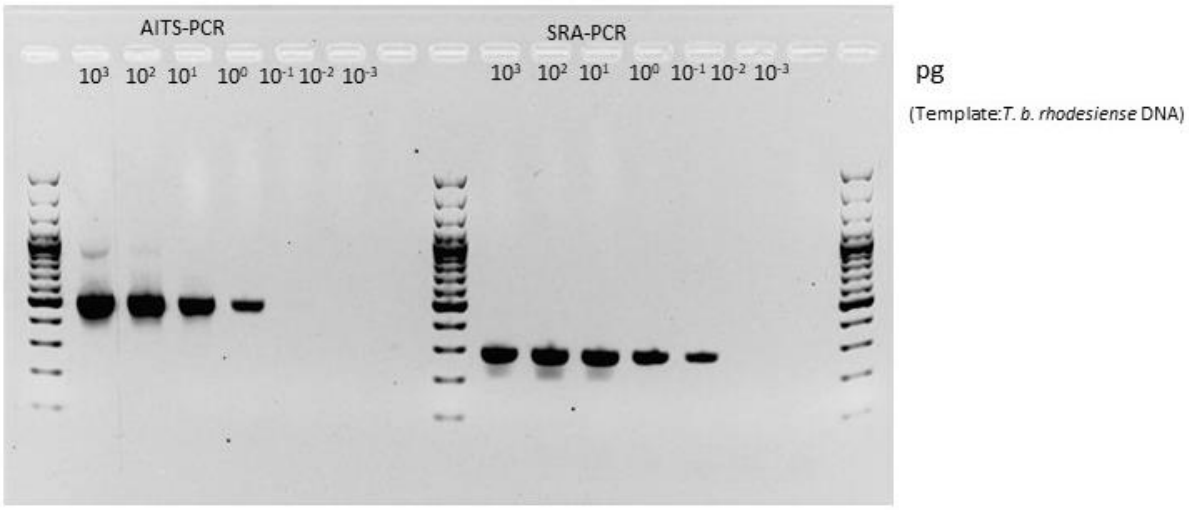
Samples which were negative in both the SRA PCR and the ITS1 PCR are not shown here.

Table 12. Detection of trypanosome species by analysis of MinION sequencing of ITS1 PCR products.

Sampling sites	Total number of samples	Number of positive samples			
		T. congolense	***T. vivax***	***Trypanozoon***	**Any trypanosome species**
Kasungu	199	5 [1] (2.5%)	0 (0%)	3 [1] (1.5%)	7 (3.5%)
Nkhotakota	185	20 [2] (10.8%)	12 [2] (6.5%)	0 (0%)	30 (16.2%) *
Lilongwe	62	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	446	25	12	3	37
Prevalence		5.6%	2.7%	0.7%	8.3%

Numbers in brackets [n] indicate samples with a coinfection involving multiple trypanosomes. Numbers in parentheses represent the prevalence of each trypanosome, or infected cattle per district. Asterisk (*) indicates that the prevalence in Nkhotakota is significantly higher than in Lilongwe and Kasungu ($P < 0.05$). Samples with *T. theileri* are counted as negative in this table.

Figure 15. Comparison of detection limit of SRA PCR and ITS1 PCR using *T. b. rhodesiense* IL1501 pure DNA.



This figure demonstrates the comparison of sensitivity test results between SRA PCR and ITS1 PCR. SRA PCR was observed to have higher sensitivity than ITS1 PCR.

Source: Squarre *et al*., 2020

Table 13. Anemia concerning trypanosome species analyzed by MinION sequencing of ITS1 PCR products.

PCV	Status	*T. congolense*	*T. vivax*	*Trypanozoon*	All trypanosomes	Total animals	No trypanosomes
<24%	Anemic	62.5% (10/16)*	12.5% (2/16)	6.2% (1/16)	68.7% (11/16)*	16	5
24-50%	Normal	3.5% (15/430)	2.3% (10/430)	0.5% (2/430)	6% (26/430)	430	404
Total		5.6%	2.7%	0.7%	8.3% (37/446)	446	409

The proportion in parentheses (n/n) represents the number of trypanosome-infected animals compared to the total number of animals. Asterisk (*) indicates that the anemic status in *T. congolense* infection is significantly higher than infection with *T. vivax* and *Trypanozoon* ($P < 0.05$).

3.5 Discussion

For molecular detection of trypanosomes in epidemiological studies, a popular method is PCR targeting the trypanosome conserved region of ITS1 rRNA (Njiru *et al*., 2005; Gaithuma *et al*., 2019). Most trypanosome species can be identified by the size of their amplicon (Njiru *et al*., 2005). This method is more sensitive and specific than ordinary microscopic observation and is relatively easy to perform (Njiru *et al*., 2005). In contrast, the annotation of species is occasionally difficult in the cases of ambiguous signals. Differentiation of *T. godfreyi* from *T. vivax* is tentative owing to their similar amplicon sizes. Amplicons from major infections may mask amplicons from minor infections in the cases of multiple infection. In this study, I applied next-generation sequencing (NGS) technologies, particularly MinION, a field-friendly, portable, rapid, and affordable NGS device. ITS1 amplicons, including ambiguous signals, were subjected to sequencing analysis. Identification of *Trypanosoma* species by gel electrophoresis and sequencing analysis was largely consistent for *T. congolense, T. theileri*, and *T. vivax*. This result suggests that MinION sequencing can detect and differentiate trypanosome species. In addition, sequencing methods appreciably remedied four downsides of gel observation: ambiguous signals, similar amplicon sizes, nonspecific signals, and multiple infections. I successfully detected a substantial number of reads from samples with ambiguous ITS1 PCR amplicons. *Trypanosoma godfreyi* and *T. vivax* were differentiated from each other. On the other hand, nonspecific amplification hampers correct identification and can lead to false positives. I observed 11 signals of approximately 450 bp corresponding to *Trypanozoon*, but most were not supported by sequencing. These could be annotated as false positives without sequencing validation. Apart from nonspecific signals, all samples definitively annotated by the gel assay had more sequence reads than the threshold of 50. Since I detected enough reads for other trypanosome species from gel analyses, the discrepancy presumably derived from

nonspecific PCR amplification of fragments of the same size as the *Trypanozoon* amplicon. Multiple infections were also successfully confirmed by sequencing. All these observations support the assertion that ITS1 PCR validated by sequencing analysis using MinION increases the reliability of parasite detection. Conversely, I obtained only tentative annotation in some samples, even though substantial sequence reads were acquired. Annotation depends on the threshold, and I applied a conservative value to eliminate false positives rather than allow false negatives. BLAST homology search and de-indexing steps were possible sources of error owing to the low accuracy of MinION sequencing. The effective range of ratios and absolute numbers of parasites in mixed infections should also be determined. These points should be clarified and optimized in future studies. Taken together, the combination of gel and sequencing analysis led to more reliable conclusions than either technique alone could have done. This combined approach has the potential to exclude false positives by nonspecific amplification and less-objective human decisions. Sequencing itself became cost-effective because of the multiplex sequencing system using MinION indexes and barcodes. The quality of field samples is occasionally poor and leads to unexpected nonspecific amplification, as observed in my cases. MinION provides sequence information that can exclude nonspecific amplification and thus improve specificity (Yamagishi *et al*., 2017). Therefore, the utilization of MinION-based sequencing in this study will help increase in the reliability of PCR-based epidemiology.

ITS1 PCR in combination with MinION sequencing provides sequence information to identify a broad range of trypanosome species in a more reliable manner compared to the conventional ITS1 PCR with gel analysis only (Gaithuma *et al*., 2019). However, this method has a limitation in characterizing *Trypanozoon* subspecies owing to the highly conserved sequence of the species (Cuypers *et al*., 2017). In addition, ITS1 PCR is known to have less sensitivity compared to other published primer sets targeting repeat sequences (Ahmed *et al*.,

2013; Masiga *et al*., 1992; Sloof *et al*., 1983), or SRA PCR (Squarre et al., 2020) (Figure 15). In my study, most of the SRA-PCR-positive samples were negative for ITS1 PCR, suggesting that ITS1 PCR alone is of low sensitivity and cannot used to investigate the prevalence of *Trypanozoon* especially for *T. b. rhodesiense* as reported before (Sloof *et al*., 1983). However, as species-specific primers cannot identify other trypanosomes, the ITS1 PCR system offers value for detecting the parasites broadly in the same reaction with less time and cost. To make the ITS1-MinION system more useful for epidemiological studies, future studies should be required to develop new primers targeting ITS1 or other regions, as a simultaneous sensitive diagnosis method. In the current ITS1-MinION system, the SRA PCR test is still recommended for screening human-infective *T. b. rhodesiense* in a more sensitive and specific manner.

In this study, the most common trypanosome detected was *T. congolense*. This is in agreement with reports from other southern African countries (Van den Bossche *et al*., 2000; Mamabolo *et al*., 2009). *Trypanosoma congolense* and *T. vivax* were more prevalent in Nkhotakota than in Kasungu, with no parasites seen in Lilongwe. Possible reasons for this trend may be due to the different methods applied for AAT control. Nkhotakota wildlife services have intensified blue cloth targets in the park to reduce the number of tsetse flies. In Kasungu, local veterinarians and farmers apply an anti-parasitic treatment, Berenil, to the cattle twice every year before and after the herding season. Berenil (diminazene aceturate) is a common therapeutic and preventive medicine for animal trypanosomiasis that has been used for >60 years (Kuriakose *et al*., 2019). Since samples were collected during the rainy season, I may expect a higher prevalence of parasites in the dry season when disease transmission peaks (Majekodunmi *et al*., 2013; Ngonyoka *et al*., 2017). In both Kasungu and Nkotakota areas, cattle are usually bitten when brought into close contact with flies at river crossings, village water holes, or other tsetse fly habitats in the field (Mulligan, 1970). On the other hand, semi-intensive farming was applied in Lilongwe, where the cattle were kept in paddocks, thus

preventing the mechanical transmission of *T. vivax* in cattle by biting flies. However, as *T. vivax* was detected in Nkhotakota, this emphasizes the necessity of sustained non-tsetse vector control for animal trypanosomiasis in the region. In addition, since I collected limited number of cattle samples only at three locations during short period, a broader countrywide survey is required to assess the overall AAT/HAT situation in the country.

The PCV value of infected cattle was significantly lower than that of non-infected cattle. Anemia has long been considered the main clinical sign of trypanosomiasis in both humans and animals (Fiennes *et al*., 1954; Fiennes *et al*., 1970). Anemia associated with infection has been previously associated with lower productivity of infected animals (Trail *et al*., 1990; Trail *et al*., 1993). Consistent with previous studies (Tasew *et al*., 2012; Mamoudou *et al*., 2015), my findings confirmed that cattle with *T. congolense* infection tended to be anemic, as compared to those infected with of other trypanosome species, suggesting the importance of controlling AAT, especially *T. congolense*.

Malawi is rich in ecosystems where humans, livestock, and wildlife populations exist close to one another. Compared to neighboring countries such as Uganda, Kenya, and Tanzania in eastern and southern Africa where *T. b. rhodesiense*-type HAT is endemic (Von Wissman *et al*., 2011; Auty *et al*., 2012; Welburn *et al*., 2001), *T. b. rhodesiense* prevalence in tsetse flies is higher in Malawi, according to xenomonitoring data (Alibu *et al*., 2015). As Kasungu and Nkhotakota are hotspots of HAT in Malawi after Rumphi (not analyzed here), the presence of *T. b. rhodesiense* in cattle samples has been debated (Chisi *et al*., 2011a; Auty *et al*., 2012). The Kasungu National Park and the Nkhotakota Game Reserve are the main foci of *Glossina morsitans morsitans*, the tsetse fly. Tsetse density in both areas is high, and the prevalence of HAT and AAT reached epidemic proportions a decade ago (Davison, 1990; Chisi *et al*., 2011a; Simarro *et al*., 2012).

Studying the human-livestock-wildlife interface is essential now, and even more so in the future because of human population expansion and agricultural development (Gondwe *et al*., 2009). This expansion induces farmers and their livestock to migrate closer to wildlife conservation areas, increasing their exposure to tsetse flies. Here, the surveyed areas were in close proximity, within <1 km, to the Kasungu and Nkhotakota national parks. The extensive farming system allowed cattle to interact with infected wildlife and tsetse flies during grazing activities at the human–livestock–wildlife interface, increasing the possibility of pathogen-sharing and disease transmission in the populations involved.

In this study, I identified both human- and animal-infective trypanosomes residing in cattle at human-livestock-wildlife interface areas in Malawi. A limited number of trypanosomiasis and tsetse control programs addressing both diseases have been conducted in the area (Meyer *et al*., 2016). AAT and HAT control activities are interdependent since both diseases share the same transmission vector and host. Control programs targeting flies and animal populations are necessary to achieve HAT control (Jali, 2019). This study contributes to improving knowledge regarding the status of trypanosomiasis in Malawi. My findings emphasize the need for sustainable integration between AAT and HAT control measures and collaborative human and animal health care services under the One Health concept, which are indispensable in tackling HAT.

This is the first study that showed the prevalence of animal and human infective trypanosomes in cattle in Malawi. The use of MinION sequencing in combination with ITS1 PCR increased the reliability of PCR-based trypanosome detection. However, for more sensitive and specific detection of human infective *T. b. rhodesiense*, SRA PCR is recommended to combined with the ITS1-MinION analysis. Trypanosome-susceptible cattle harbor both human and animal infective trypanosomes, implying its role as a potential reservoir

of *T. b. rhodesiense*. This study emphasizes the urgent need for sustainable control measures within the context of One Health approach for AAT and HAT in livestock.

4. GENERAL CONCLUSIONS

Bovine trypanosomiasis has long been known to hamper agriculture and farming productions in many countries. Despite its significance, as typical for the parasitic disease, trypanosomiasis is largely neglected compared to other viral or bacterial diseases in livestock. To control the disease and to suppress its impact, a thorough intervention should be taken. Studies in the genetic diversity of trypanosomes and its epidemiology provide relevant information to guide and give a clear direction for the control measures of the disease.

The present study was intended to provide information on the prevalence and genetic diversity of *T. evansi* in Syria and African trypanosomes in Malawi. The summary of this study can be described into several points as follows;

1. The report on molecular detection of *T. evansi* in Syria is the first epidemiological study of *T. evansi* in the country. This study emphasizes the necessity for further investigations of the parasite in cattle and other livestock in Syria to improve control measures against *T. evansi* in the country.
2. Sequence analysis of the *ESAG6* of *T. evansi* in Syria showed genetic diversity. Several genotypes were observed in Syrian cattle, and the genotype did not have an association with regions, countries, or animal hosts.
3. The combination of ITS1 PCR with MinION sequencing provides sequence information to detect a broad range of trypanosome species in a more reliable manner compared to the conventional ITS1 PCR with gel analysis alone. MinION sequencing improved the specificity of the detection and could solve the common problems which usually observed in gel analysis, including ambiguous signals, similar amplicon sizes, nonspecific signals, and multiple infections, and thus, it could avoid false positives

results. Hence, the utilization of MinION-based sequencing in this study will help increase the reliability of PCR-based epidemiology.

4. This is the first study to assess the prevalence of animal and human infective trypanosomes in cattle at the human–livestock–wildlife interface in HAT foci in Malawi. This emphasizes the urgent need for interventions in the transmission dynamics between humans and livestock since the high possibility of frequent exposure between them might lead to pathogen sharing. It is necessary to apply integrated control measures within the context of One Health approach for *T. b. rhodesiense*-type HAT in livestock at human–livestock–wildlife interfaces as well as to promote a sustainable non-tsetse vector control for animal trypanosomiasis in Malawi currently neglected.
5. Cattle play an important role in trypanosomes epidemiology as a host animal. The trypanosomes transmission starts when the vector flies take up the bloodmeal from cattle as a food resource. Since cattle have close contact with humans in agriculture activity; thus, cattle can put humans in a higher risk of HAT infection. In my study, trypanosome-susceptible cattle from local breed might harbor human infective trypanosomes, indicating its role as a potential reservoir of *T. b. rhodesiense,* the causative agent of HAT in Malawi. Understanding the significance of cattle or other potential reservoir diseases in livestock could help to decide the feasible control measures of trypanosomiasis in the region.

The trypanosomes detection system used in this study has the potential to be applied in the future research for genetic diversity of parasite, and characterization of the trypanosomes up to sub-species. The findings in this dissertation will serve relevant contributions to the epidemiology of pathogenic trypanosomes by identification of trypanosomes species, understanding the genetic diversity, and determining the potential animal reservoirs to improve the control measures of bovine trypanosomiasis in the specific region.

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