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WEST AFRICAN CENTRE FOR CELL BIOLOGY OF INFECTIOUS PATHOGENS

THE INTERPLAY OF ANIMAL AFRICAN TRYPANOSOMIASIS PREVALENCE, IRON HOMEOSTASIS, AND TRANSFERRIN RECEPTOR VARIABILITY IN LIVESTOCK OF THE GREATER ACCRA REGION, GHANA

BY
FERNANDE AMANDINE MBAMBA MAGRA
(10704380)

THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY DEGREE IN MOLECULAR AND CELL BIOLOGY OF INFECTIOUS PATHOGENS

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DECLARATION

I, Fernande Amandine Mbamba Magra, declare that the entire work described in this thesis is my own, carried out under the supervision of Prof. Theresa Manful Gwira, Dr. Calvin Tiengwe, and Dr. Kwabena Sarpong. I would like to indicate that references to other works were duly acknowledged. I certify that this thesis or part of it has never been submitted for a degree or any other qualification elsewhere.

STUDENT: Fernande Amandine Mbamba Magra (10704380)

Signature : Date : 25/04/2023

SUPERVISORS

Prof. Theresa Manful Gwira (Principal supervisor)
West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology (BCMB)

Signature: Date: 25/04/2023

Dr. Calvin Tiengwe (Co-supervisor)
Imperial College London

Signature: Calvin Tiengwe Date: 25/04/2023

Dr. Kwabena Sarpong (Co-supervisor)
West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology (BCMB)

Signature: Date: 25/04/2023

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LIST OF ABBREVIATIONS

HAT: Human African Trypanosomiasis

AAT: Animal African Trypanosomiasis

Tf: Transferrin

TfR: Transferrin receptor

ESAG: Expression site associated gene

VSG : Variant surface glycoprotein

PCR : Polymerase chain reaction

T : *Trypanosoma*

b: *brucei*

TIBC: Total iron binding capacity

TSI: Total serum iron

TS: Transferrin saturation

HCT: Haematocrit

WHO: World health organisation

NSSCPs: National Sleeping Sickness Control Programs

ELISA: Enzyme-linked immunosorbent assay

IFAT: Indirect fluorescent antibody technique

CATT: Card agglutination test

LAMP: Loop-mediated isothermal amplification

LFA: Low-flow assay

M1: Macrophage type1

B-cell : Lymphocyte-B cells

INF-y : Interferon-y

IL : Interleukin

RBC: Red blood cells

TNF: Tumour necrosis factor

MIF: Macrophage migration inhibitory factor

OIE: World Organization for Animal Health

ITS: Internal transcribed spacer

IM: Intramuscular

IV: Intravascular

SC: Subcutaneous

BBB: Blood brain barrier

FP: Flagellar pocket

ISG: Invariant surface glycoprotein

SRA: Serum resistance-associated

BSF: Blood stream form

CP: Cysteine proteinase

Ig: Immunoglobulin

TSE: Trans-sialidase

IFX: invariant flagellum from *T. vivax*

QS: Quorum sensing

SIF: Stumpy induction factor

PTU: Polycistronic transcriptional unit

SL: Spliced leader

mRNA: Messenger RNA

TSS: Transcription start site

UTR: Untranslated region

RBP: RNA binding protein

NTD: N-terminal domain

CTD: C-terminal domain

BES: Bloodstream expression site

IRP: Iron regulatory protein

IRE: Iron responsive element

CSIR: Council for Scientific and Industrial Research

GA: Greater Accra

LIPREC: Livestock and Poultry Research Centre

WASH: West African Shorthorn

EDTA: Ethylenediaminetetraacetic acid

CBC: Cell blood count

Hb: Haemoglobin

OD: Optical density

FBS: Foetal bovine serum

HMI-9: Hirumi's modified Iscove's medium

MW: Molecular weight

AA: Amino acid

FACS: Fluorescence-activated cell sorting

HBSG: HEPES-buffered saline with glucose

TL: Tomato lectin

PBS: Phosphate-buffered saline

PBST: Phosphate-buffered saline

HRP: Horseradish peroxidase

GPI: Glycosylphosphatidylinositol

ABSTRACT

Background

African trypanosomiasis is a parasitic disease that can infect both humans and animals. In humans, the disease is known as human African trypanosomiasis (HAT), while the animal form is called animal African trypanosomiasis (AAT). Although the burden of HAT has significantly decreased over the years, AAT remains a significant constraint to livestock production in several sub-Saharan African countries, including Ghana. Several species of African trypanosomes can infect humans, domestic and/or wild animals. As majority of parasite, trypanosomes rely on host-derived iron for survival and disease establishment. Consequently, the parasites express a divergent transferrin receptor (TfR) that facilitates host transferrin uptake to satisfy their iron needs.

Previous studies reported that TfR diversification enables trypanosomes to expand their mammalian host reservoirs, but these studies were primarily performed using *in-vitro* systems. It remains unknown whether this phenomenon occurs during natural infections. Additionally, TfR is co-transcribed from the same polycistronic transcription unit as Variant Surface Glycoproteins (VSGs), which are critical for immune evasion. There are ~10 million densely packed VSG molecules per trypanosome cell that shield invariant surface proteins from host-derived antibodies. VSGs can exist either as homodimers or trimers, but whether VSG oligomerisation influences the uptake of essential ligands such as transferrin remains unknown. Numerous studies have identified iron deficiency as the primary cause of anaemia during AAT, yet only a limited number have described changes in iron status in naturally-infected livestock.

In light of these knowledge gaps, this study sought to examine the prevalence of AAT in cattle, sheep, goats, and pigs in the Greater Accra Region, Ghana, assess changes in the iron status of livestock during trypanosome infection, determine whether TfR variability facilitates trypanosome colonisation of livestock during natural infections, and finally, examine whether VSG oligomerisation influences the uptake of host ligands.

Methods

Blood samples were collected from 456 animals (155 cattle, 138 sheep, 132 goats, and 31 pigs) from six farms in the Greater Accra region. DNA was extracted from the blood collected, and nested diagnostic polymerase chain reactions (PCRs) were performed using *Trypanosoma* species-specific primers amplifying tubulin gene. Subsequently, PCR amplicons were subjected to Sanger sequencing for trypanosome species validation. ELISA and colourimetric assays were performed using the serum to determine ferritin concentration, total serum iron (TSI), transferrin saturation (TS), and total iron-binding capacity (TIBC) as indicators of iron status during infections. Additionally, the haematocrit (HCT) levels were compared between infected and non-infected livestock. To determine the variable region of TfR, RNA was extracted from the blood of livestock infected with *T. b. brucei*. Following this, cDNA was synthesised and used to perform conventional PCR. Finally, cell lines expressing different VSGs belonging to the main classes (dimers and trimers) were used to measure transferrin uptake.

Results

Of the 456 animals screened, 73 (16%) were infected, of which 50 (11%) were infected with *T. brucei*, 16 (3.5%) with *T. theileri*, and 5 (1.1%) with *T. congolense*. Mixed infections with *T. congolense* and *T. theileri* were observed only in two animals at two study sites. Overall, sheep were the most infected, with an infection of rate 29.7% (41/138), compared to 16.8% (26/155) in cattle, 3.8% (5/132) in goats, and 3.2% (1/31) in pigs. An increase in ferritin concentration was observed in infected cattle (Mean = 394 ng/mL) compared to non-infected controls (Mean = 283 ng/mL); the difference observed was statistically significant (p-value = 0.047). However, the

ferritin concentration in infected and non-infected sheep was low (Mean = 39 ng/mL). TIBC was very high in infected cattle and sheep (p-value = 0.0001) compared to non-infected animals, while TSI and TS showed a decrease in all the infected livestock compared to non-infected controls. Moreover, a significant reduction in HCT levels in goats was observed compared to cattle and sheep (p-value = 0.0001); however, the difference between infected and non-infected animals was not significant. TfR sequences from sheep, cattle, and goats were compared, a huge diversity was observed between and within the different livestock species in the variable region of ESAG7 that was analysed. Finally, a significant reduction in transferrin uptake was observed in trimeric VSG-expressing cell lines compared to dimeric VSG cell lines (p-value = 0.001).

Conclusion

The prevalence of AAT in this study was low; the predominant circulating trypanosome species was *T. brucei*, and sheep were the most infected animals. This is the first study to report a high prevalence of AAT in sheep in the Greater Accra Region. Therefore, as seen in cattle, equal attention should be given to other livestock when implementing control measures for AAT elimination. Furthermore, the data showed that during AAT, there are significant changes in the iron status of infected livestock. Also, for the first time, this study reported diversity in TfR sequences in trypanosomes from naturally infected livestock. Therefore, this suggests that parasites express a unique TfR in different mammal hosts. Finally, this study provided evidence that the VSG structure may influence nutrient uptake.

CHAPTER ONE

1.1 Introduction

Trypanosomiasis is a severe disease caused by protozoan parasites found in sub-Saharan Africa both humans and animals can be infected. Trypanosomes are etiologic agents of the disease and members of the genus *Trypanosoma*. Trypanosomes can proliferate in the blood, lymphatic and nervous systems (Muhanguzi *et al*., 2017). Only *T. brucei rhodesiense* and *T. brucei gambiense* can cause human infection, sleeping sickness commonly called human African trypanosomiasis (HAT). Meanwhile, *T. congolense*, *T. vivax*, *T. theileri*, and *T. brucei brucei* cause animal African trypanosomiasis (AAT) also called nagana (Auty *et al*., 2016; Ofori *et al*., 2022; Ekloh *et al*., 2023). Other trypanosome species such as *T. godfreyi*, *T. simae*, *T. evansi*, *T. dionisii*, *T. elephantis*, *T. vegrandis*, *T. gilleti* can also infect many domestic and wild animals, but the susceptibility of mammal hosts to specific strains of trypanosomes varies (Anderson *et al*., 2011; Kasozi *et al*., 2021). Although the incidence of HAT has drastically reduced, and the disease is likely to be eliminated by 2030 (Franco *et al*., 2022), the challenge persists with AAT which is recognised as a severe infectious disease limiting the livestock expansion in SSA (Auty *et al*., 2016; Dieng *et al*., 2022). In Africa, reports have shown that about 55 million cattle are at risk of being infected by various trypanosome species; additionally, about 3 million of these cattle are thought to die yearly (Dieng *et al*., 2022; Giordani *et al*., 2016). A systematic review published in 2022 reported the prevalence of AAT in several African countries located in various regions (East, Central, Southern and West Africa). Two countries recorded the highest AAT prevalence: Zimbabwe and Ghana, 53% and 61%, respectively (Dieng *et al*., 2022). The high prevalence of AAT reported, therefore, calls for concern. Also, several studies have clearly shown that animals are now reservoirs for the human species, *T. brucei gambiense* (Njiokou *et al*., 2010) and *T. brucei rhodesiense* (Hamill *et al*., 2013). The presence of these reservoirs has been suggested to be the major factor causing a resurgence of the disease after the implementation of specific protective measures (Njiokou *et al*., 2010; Hamill *et al*., 2013). Trypanosomes are mostly present in the blood of hosts. Also, studies have demonstrated that these parasites can be found sequestered in adipose tissue (Trindade *et al*., 2016) and under the skin (Capewell *et al*., 2016). Furthermore, trypanosomes can also be found in the brain during the late phase of the infection (Smith *et al*., 2017). Infected tsetse flies (*Glossina sp*.) are responsible for transmitting trypanosomes to a different host by a bite during the blood meal.

In the livestock population, an essential feature of trypanosome pathogenesis is the hyper-activation of macrophages, causing massive destruction of erythrocytes by phagocytosis resulting in anaemia (Stijlemans *et al*., 2018). Anaemia experienced during AAT results from various factors; however, the most important among them is iron-homeostasis dysregulation. Like in other chronic diseases, during trypanosomiasis, signs of anaemia are associated with an imbalance between erythrophagocytosis and erythropoiesis (Stijlemans *et al*., 2018). This directly impacts iron homeostasis by affecting the recycling process and sequestration performed by macrophages. All these events induce iron depletion, causing a severe challenge for extra-cellular parasites to establish and maintain the infection. Moreover, the host's immune defence plays a critical role in iron availability through mechanisms requiring cytokines, cellular proteins, peptides, and hormones (Ebanga *et al*., 2020). Hepcidin, secreted by hepatocytes, is essential to create low-iron conditions mainly by down-regulating ferroportin; this, therefore, affects the cellular export of iron to the bloodstream. Evidences suggest that during trypanosomiasis, several changes occur in the host affecting iron homeostasis; however, this has not yet been studied in naturally infected livestock.

African trypanosomes require iron, just like every other living organism for their survival in the host. Iron is an essential and crucial cofactor for several biological processes, like cell proliferation, gene expression, regulation, and synthesis of DNA, RNA as well as proteins (Basu *et al*., 2016). The most important source of iron for these parasites is transferrin (Tf), an iron-binding protein (Taylor & Kelly, 2010). During *T. brucei*'s life cycle, the parasite passes from the vector to the mammalian host. Throughout this cycle, the parasite takes several forms to adapt to various environmental changes and ensure its survival. *T. brucei*, precisely the bloodstream form, expresses a unique receptor that binds to the host's Tf. The receptor is the *T. brucei* transferrin receptor (TfR), which is critical for iron uptake through endocytosis of the host's Tf (Kariuki *et al*., 2019; Benz *et al*., 2018). Expression site-associated genes (ESAG) 6 and 7 encode for TfR, which is a heterodimeric protein complex. The association of ESAG6 and ESAG7 is needed to interact with the Tf protein (Steverding *et al*., 1995). ESAGs are always co-transcribed with the variant surface glycoprotein (VSG) gene, which constitutes the major component of the compact surface coat that gives the parasite the ability to elude the host's defence system, essentially both arms of the immune system (Didier Salmon *et al*., 1994). In the past, VSGs were described as homodimers, but recently researchers have reported two main classes: dimers and trimers (Sara *et al*., 2023). The expression of both VSG and TfR is essential for the parasites to establish the infection. The genome of trypanosomes contains about 15 specific expression sites; ESAGs are multi-copy genes and are present in each expression site; only one is active at a specific time (Hertz-Fowler *et al*., 2008). Several expression sites encode TfR that might be structurally related but differ in function. Therefore, few studies reported little diversity between TfR among different mammalian hosts, which can significantly impact the binding affinity for Tf, as well as the ability of trypanosomes to survive in the sera of these mammalian hosts (Bitter *et al*., 1998).

1.2 Rationale

In Ghana, like in several African countries, AAT is a major constraint to livestock production. This is confirmed by the high prevalence recently reported (Dieng *et al*., 2022). Moreover, in Ghana, livestock production has considerable economic and socio-cultural benefits. Especially to rural households, via the provision of nutrition, as an employment avenue as well as capital accumulation for owners. Generally, livestock production in SSA accounts for 40% of the total household income and in Ghana, these statistics are similar; the range varies between 44.1% and 51.5% (Agriculture Ghana report, 2020). When there is a reduction in production, this might significantly impact the livelihoods of most rural dwellers (Abro *et al*., 2021). The dependence of farmers on livestock is undeniable; therefore, appropriate measures must be put in place to improve the management of AAT in the country. Although the prevalence of AAT seems to be high in the country, most of the data obtained were from the Northern region, and only cattle were sampled (Bakari *et al*., 2017; Dieng *et al*., 2022; Ofori *et al*., 2022). Limited studies describe the dynamics and the burden of AAT in livestock in other regions of Ghana, such as the Greater Accra region. Therefore, the prevalence reported is not representative of the whole country, and the dynamics of the disease still need to be described in many regions where several private and public farms are also found. Furthermore, few studies have been conducted to determine the impact of the disease on other primary livestock, such as sheep, goats, and pigs. Also, the pathogenesis of AAT in infected livestock has not been fully described. Anaemia is the most important symptom experienced during trypanosomiasis and is used to determine the severity of the disease (Degneh et *al*., 2017; Meharenet & Tsegaye, 2020). Studies suggest that iron deficiency is the leading cause of anaemia during AAT. However, no study describes changes in the iron status of naturally infected animals. Also, it remains unclear if there is a correlation between those changes and disease pathogenesis. Though during the infection, iron levels in the hosts are depleted, *T. brucei* is still able to fulfil its iron needs through the uptake of the host Tf available in the serum via a unique receptor TfR. The hypothesis is that *T. brucei* expresses a specific TfR depending on the final host to increase the binding affinity with the Tf molecule. TfR is essential for Tf uptake, but it is unknown if the sequence polymorphisms correlate with infections in different livestock (Trevor *et al*., 2019). The type of TfR expressed might influence Tf uptake, just as the type of VSG (dimeric or trimeric). This is because of the genomic location of ESAG6 and ESAG7 as well as VSG genes and the fact that these genes are always co-transcribed together. However, no study has been conducted so far to determine the possible influence of the VSG expressed on Tf uptake.

1.3 Hypotheses and aims

1.3.1 Hypotheses

1. Important changes occur in the iron status of infected livestock, and those changes vary among different hosts.

2. TfR sequence polymorphism allow trypanosome adaptation and transferrin uptake in different hosts.

3. Transferrin uptake is influenced by TfR sequence polymorphism and the type of VSG expressed.

1.3.2 The aims of the study

Aim 1: Determine the prevalence of AAT and describe the iron status of trypanosome-infected livestock.

Aim 2: Identify transferrin receptor sequence diversity *in vivo* in different livestock and determine the correlation between the type of VSG expressed and transferrin uptake.

1.3.3 Specific objectives

1. Determine AAT prevalence and trypanosome species circulating in livestock (cattle, sheep, goats, and pigs).
2. Describe changes in the iron status of trypanosomes-infected livestock.
3. Identify TfR sequence polymorphisms in trypanosome-infected livestock.
4. Determine the influence of the type of VSG expressed on the transferrin uptake.

1.4 Significance of the study

The data generated from this study will describe the real burden of the animal African trypanosomiasis in the primary livestock in the Greater Accra region. The main trypanosome species in circulation and places with high disease incidence will also be identified. Also, the key iron parameters that are significantly modified in the infected livestock will be determined. These findings are relevant because they will provide meaningful information required to design appropriate strategies for better disease management. Finally, this study will discover if *T. brucei* expresses a unique transferrin receptor in various animal hosts and if the structure of the VSG expressed influence transferrin uptake.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 African trypanosomiasis

African trypanosomiasis is a severe parasitic infection endemic in sub-Saharan Africa (SSA), affecting humans and animals. Some blood-feeding flies and tsetse flies transmit trypanosomes; these insects are primarily found in humid and semi-humid parts of Africa. The animal form of the disease, animal African trypanosomiasis (AAT), affects cattle and other livestock. AAT is predominantly caused by *Trypanosoma congolense* (*T. congolense*), *Trypanosoma vivax* (*T. vivax*), and *Trypanosoma brucei spp* (*T. brucei spp*) (Haag *et al*., 1998; Ekloh *et al*., 2023). AAT is found within a mainland of approximately 10 million km^2, including 36 counties in Africa where exposure to the main vector (tsetse fly) and trypanosome infection is high (Meyer *et al*., 2016). AAT causes important reduction in the farming sector affecting the quality of the meat and milk delivered to the market and impacting the global economy. AAT is the topmost constraint on SSA's food production and economic growth. Also, the human form of the disease exists, known as human African trypanosomiasis (HAT). Two subspecies of *T. brucei* cause HAT. Although AAT and HAT are found in similar geographical locations in Africa, *rhodesiense* HAT is predominant in East and South Africa. In contrast, *gambiense* HAT is dominant in Central and West Africa. However, the incidence of HAT has reduced drastically, and the disease has been targeted for elimination (Jose *et al*., 2022).

2.2 Human African trypanosomiasis

2.2.1 Epidemiology and distribution

T. b. gambiense and *T. b. rhodesiense*, two subspecies of the flagellated unicellular protozoan parasite *T. brucei*, cause sleeping sickness or HAT (Büscher *et al*., 2017). Infected tsetse flies (*Glossina*) transmit the disease within specific foci of endemicity in SSA (Jose *et al*., 2022; Simarro *et al*., 2012; Simarro *et al*., 2010).

Studies have reported that HAT is not only detected in SSA countries but also in Europe, America, and Asia among travellers, tourists, and migrants (Simarro *et al*., 2012). HAT caused by *T. b. gambiense* is considered anthroponotic (Büscher *et al*., 2018) and represents the majority of reported disease cases (Jose *et al*., 2020). However, HAT caused by *T. b. rhodesiense* is a zoonotic disease; livestock and wildlife can serve as reservoirs of the parasites (Fèvre *et al*., 2008; Franco *et al*., 2014; Matovu *et al*., 2020; WHO, 2013). Furthermore, the disease progression is different for these two parasites; *gambiense* HAT produces a chronic form of the disease, while *rhodesiense* HAT results in the acute form (Jose *et al*., 2014; Kennedy, 2013). If HAT is wrongly diagnosed and untreated, the outcome is death (Jamonneau *et al*., 2012).

HAT mainly affects people living in poor communities in SSA, which is why the disease is considered a neglected tropical disease (WHO, 2012). HAT caused several disastrous epidemics in the past; therefore, the disease was tagged as a “colonial disease” (Headrick, 2014; Wylie and Lyons, 1994). The most recent escalation in infection cases was in the 1990s, when per year, more than 25,000 cases were reported (Hide, 1999; Smith *et al*., 1998). In 1997 and 1998, about 35,000 cases were reported (WHO, 2013), and over 300,000 people were at risk of the disease. As a result, volunteers and stakeholders formed the National Sleeping Sickness Control Programs (NSSCPs) in various endemic countries for better disease management. NSSCPs were coordinated by the

WHO and supported many non-governmental organisations, pharmaceutical companies, several research institutions, and charitable foundations (Simarro *et al*., 2011; Simarro *et al*., 2008). Due to the spectacular achievement of these collective efforts, within a decade, the incidence of HAT drastically reduced, and the disease has been under effective control for a long time.

HAT was targeted for elimination as a public health problem by 2020; however, due to the COVID-19 pandemic and other challenges, this could not be achieved, although most known foci were reported disease-free. Therefore, WHO's new goal is to eliminate HAT caused by *T. b. gambiense* by 2030 (Compaoré *et al*., 2022; WHO, 2022).

2.2.2 Clinical Manifestations

Clinical symptoms of HAT, whether caused by *T. b. gambiense* or *T. b. rhodesiense,* are similar. Sleeping sickness symptoms and signs are divided into two main stages. The first stage during which trypanosomes are found in the lymphatic systems and the bloodstream exclusively, is called the hemolymphatic phase. Generally, during this phase, patients experience non-specific symptoms such as joint pain, headaches, and fever; these are mostly misdiagnosed, leading to poor disease management. The second stage is the meningoencephalitic phase, where trypanosomes invade the central nervous system. Specifically, trypanosomes bypass the blood-brain barrier (BBB), modify the sleep cycle, and cause mental disorders and paralysis. These symptoms can lead to patient death if treatment is not administered. The sleeping pattern modification during the infection results in “sleeping sickness”. The two phases described above during *gambiense* HAT last almost three years, whereas for *rhodesiense* HAT progression to the second stage requires a few weeks, and death results within months (Odiit *et al*., 1997). The unique way of differentiating the two infections is the possible appearance of the trypanosomal chancres after the tsetse fly bite.

For *gambiense* HAT, the appearance of the chancre is rare, but patients infected with *T. b. rhodesiense* mostly experienced trypanosomal chancre (Boatin *et al*., 1986; Blum *et al*., 2012; MacLean *et al*., 2010). Local erythema characterises the chancre and is described as the lesion that appears at the bite site. Other signs can also be associated with the chancre, such as heat, tenderness, and oedema. When the trypanosomal chancre is present, this can be useful for clinical diagnosis (Boatin *et al*., 1986; Blum *et al*., 2012; MacLean *et al*., 2010).

2.2.3 Diagnosis of HAT

Diagnosis of HAT has improved considerably in terms of sensitivity, specificity, and cost. Also, the number of equipped health facilities has expanded, reinforcing the capacity for passive detection and increasing epidemiological understanding of the disease (WHO, 2022). HAT is distinguished by the discontinuous presence of parasites in the patient's blood and irregular fever. The diagnosis aims to establish that a person is infected using parasitological, serological, and molecular methods. Various samples such as serum, blood, urine, and cerebrospinal fluid can be used to achieve this. During the first stage of the disease, parasitological techniques are the most indicated (Magona *et al*., 2003). The presence of motile parasites can be detected by examining wet blood film, albeit lacking sensitivity. Moreover, centrifugation can be performed to concentrate the parasites, and the buffy coat can be examined; these techniques are relatively sensitive (Kasozi *et al*., 2021). When parasitaemia is low, phase contrast and dark field microscopy improve sensitivity.

Serological and molecular methods are more sensitive and specific than parasitological techniques. Serological tests frequently used are enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody technique (IFAT), and the card agglutination test for trypanosomiasis

(CATT) to diagnose gambiense HAT. For the molecular tests, the most used are PCR, LOOP/LAMP and low-flow assay (LFA) (Omowumi *et al*., 2020).

2.2.4 Chemotherapy

Human African trypanosomiasis is generally treated using few drugs; prescription is done considering the stage of the disease and the infecting *T. brucei* subspecies. These drugs are eflornithine, suramin, melarsoprol, pentamidine, and nifurtimox. *T. b. gambiense* infections are treated during the first stage with pentamidine administrated intravenously for a week. Some of the adverse reactions associated with this treatment are discomfort due to the introduction of the needle to administer the drug, hypotension, and also hypoglycaemia (Santos *et al*., 2014). For the second stage, the drug of choice is melarsoprol for *gambiense* HAT. Studies indicated that this drug is not well tolerated because of adverse reactions such as peripheral neuropathy, skin rash, vein sclerosis, and encephalopathic syndrome. In some endemic regions, resistance to melarsoprol was reported (Fairlamb & Horn, 2018). Therefore, this drug was replaced by eflornithine for the second-line treatment. Whereas for *rhodesiense* HAT, the drug used is suramin for about 30 days during the first stage of the disease; for the second stage, melarsoprol is used. Previous studies showed that *T. b. rhodesiense* is resistant to eflornithine (Santos *et al*., 2014). Recently, in 2019 an oral drug, fexinidazole, was recommended by the WHO for treating *gambiense* HAT. Fexinidazole is currently used to treat stage I and non-severe stage II infections. It reduces treatment logistics as an oral treatment taken once daily for ten days, but patients must stick to the therapy to prevent relapse (Dickie *et al*., 2020). Using fexinidazole as an oral medication has made it simple to treat HAT and demonstrated that producing safer and simpler drugs was possible.

2.3 Animal African Trypanosomiasis (AAT)

2.3.1 Geographic distribution

The main pathogenic *Trypanosoma* species causing AAT are *T. vivax*, *T. congolense,* and *T. b. brucei*, belonging to the salivary group (Haag *et al*., 1998). These have been named due to their mode of transmission to the mammalian host, which occurs through the saliva of an infected tsetse fly. Domestic animals such as cattle, pigs, sheep, goats, and many others are prone to nagana with one or multiple species of *Trypanosoma* (mixed infection, frequently observed). Animals can experience acute or chronic forms of the disease, resulting in colossal mortality, morbidity and even infertility if no treatment is administered (Leach & Roberts, 1981; Connor, 1992). AAT causes a significant reduction in farming and agricultural production, negatively impacting the economy in SSA. Trypanosome species transmitted by tsetse flies are endemic in SSA, known as the tsetse fly belt occurring roughly between latitudes 10 degrees North and 20-30 degrees South. In addition, some tsetse fly species are found in the southwestern parts of the Arabian Peninsula. Due to animal importation and mechanical transmission, *T. vivax* can spread above the tsetse fly belt, favouring the establishment of *T. vivax* in the Caribbean, Central and South America regions, typically tsetse flies free. Some trypanosomes causing AAT and their preferred hosts are summarised in Table 2.1.

Table 2.1: Some trypanosome species and their hosts

Host species	Trypanosome species	References
Horses, pigs, cattle, goats, sheep, donkey	*T. vivax*	(Nimpaye *et al*., 2003, Birhanu *et al*., 2015; Bakari *et al*., 2017; Fetene *et al*., 2021)
Sheep, goats, pigs, cattle, horses, dogs	*T. congolense*	(Nimpaye *et al*., 2003; Nakayima *et al*., 2012)
Cattle	*T. theileri*	(Bakari *et al*., 2017; Matovu *et al*., 2020; Ofori *et al*., 2022, Ngomtcho *et al*., 2017)
Monkey, pigs, cattle	*T. simiae*	(Nimpaye *et al*., 2003), (Nakayima *et al*., 2012), (N'Djetchi *et al*., 2017)
Cattle, horses, dogs, cats, camels, sheep, goats, and pigs	*T. b. brucei*	(Cordon-Obras *et al*., 2010; Jirků *et al*., 2015; Ngomtcho *et al*., 2017)
Camel, horse, goat, sheep, donkey, pig	*T. evansi*	(Jirků *et al*., 2015)
Crocodiles	*T. grayi*	(Fermino *et al*., 2013)
Horse, donkeys	*T. equiperdum*	(Claes *et al*., 2005; Davaasuren *et al*., 2019)
Pigs	*T. suis*	(Rodrigues *et al*., 2020)
Humans, pigs, sheep, cattle,	*T. b gambiense*	(Cordon-Obras *et al*., 2010; N'Djetchi *et al*., 2017; Njiokou *et al*., 2010)
Humans, pigs	*T. b rhodesiense*	(Hamill *et al*., 2013)
Pigs	*T. godfreyi*	(Hamill *et al*., 2013)

2.3.2 Transmission

So far, various types of transmission have been described: cyclical and mechanical. Natural biological vectors for trypanosomes are *Glossina ssp*, commonly called tsetse flies, responsible for the cyclical transmission of these parasites to mammalian hosts (Uilenberg, 1998). The development of trypanosomes in the vector requires one or more weeks to become fully infective (Santos *et al*., 2014). Also, African trypanosomes can be transmitted mechanically through other bloodsucking insects (Masiga *et al*., 1996). Mechanical transmission might be most relevant for *T. vivax* (because of the short life cycle); this was observed in South America. Furthermore, in that region, *Stomoxys spp* and *Tabanidae* are suggested to be the primary vectors, and additionally, venereal and placental transmission have also been described (Santos *et al*., 2014). Once infected with trypanosomes, animals become chronic carriers, and any stress can reactivate the silent infection. The survival of trypanosomes outside the host like in the environment is drastically reduced.

2.3.3 Clinical signs

Clinical manifestations of AAT in species such as goats, cattle, pigs and sheep have been well-documented compared to other animal species, though these signs are often rare (Santos *et al*., 2014). Many cases are chronic, but an acute form of the disease can be observed, leading to death within weeks. Generally, a localised swelling is observed at the fly's bite, but the chancre is barely noticed. During the infection, primary symptoms are anaemia, lymphadenopathy, fever, and weight loss often associated with reduced meat and milk production. Furthermore, a loss of appetite and diarrhoea can be experienced by the animals, followed by the development of oedema, cardiac lesions, corneal opacity, and several neurological signs. Early abortions and cases of infertility have also been described (Silva *et al.*, 2013). Immunosuppression was described during infection with African trypanosomes, resulting in secondary infections (Onyilagha & Uzonna, 2019). If untreated, animals presenting chronic signs can die, and also, during the recovery period, if animals are stressed, they can recidivate. Acute, chronic and sub-clinical forms of AAT were documented in dogs, and the symptoms are the same as in ruminants (Desquesnes *et al.*, 2022).

2.3.3.1 Immune response to trypanosomes infections and anaemia

African trypanosomes interact with their mammalian hosts, which triggers the activation of innate and adaptive immune responses. These parasites are extracellular and continuously exposed to the host's immune system. Due to co-evolution, described as a balanced growth regulation system, trypanosome parasites survive longer without causing the host's death to complete their life cycle and ensure transmission (Paulnock *et al.*, 2010). This uncommon balance has two essential components, an effective type 1 cellular/pro-inflammatory immune response and a strong humoral antiparasite B-cell response during the first peak of parasitaemia resulting in parasite control and temporal host resistance (Magez *et al.*, 2008; Stijlemans *et al.*, 2012). Significantly, trypanosomes have developed several immune mechanisms such as antigenic variation, immunosuppression and B-cell depletion/loss of B-cell memory to avoid elimination and, therefore, ensure the disease establishment (Stijlemans *et al.*, 2018). If unchecked, the pro-inflammatory immune response mediated by the activated myeloid phagocyte system can cause severe damage to the host. Therefore, the degree of the inflammatory response triggered and the ability of the host to control this response determine whether immunopathology (anaemia and tissue damage) develops and helps differentiate between trypanosusceptible and trypanotolerant livestock.

In vivo models using mice where specific genes were knocked were helpful in revealing mechanisms involved in trypanosomiasis-associated pathogenicity and anaemia (Stijlemans *et al.*, 2018). Generally, during African trypanosomiasis, anaemia occurs at all stages of the disease and can be divided into separate phases. First, there is a massive erythrophagocytosis during the acute stage following the parasite clearance. Then, a recovery phase is observed, and finally, the chronic phase coincides with the development of progressive anaemia. Facts stated previously suggest the critical role of myeloid cells in anaemia development. In addition, pro and anti-inflammatory cytokines were reported to play a role in anaemia onset and progression (Musaya *et al.*, 2015).

2.3.3.2 Host and parasite-derived factors that contribute to acute anaemia

Anaemia is defined as a condition where the number of red blood cells in circulation is low, and the mechanisms underlying African trypanosomes-associated-anaemia are multifactorial. Several molecules were shown to influence the development of anaemia at various disease stages. Available data suggest that acute anaemia results as part of the innate immune response in response to the infection, where parasite-derived factors (extracellular vesicles) and host-derived factors (M1 type cells) trigger the production of pro-inflammatory molecules to control the disease. Specifically, during the acute phase, INF- γ produced is the main factor causing acute anaemia (Naessens, 2006). Additionally, the parasites' extracellular vesicles can fuse with the red blood cells (RBC), causing erythrophagocytosis and potentially enhancing anaemia (Naessens, 2006). A partial recovery phase mediated through erythropoiesis and production of anti-inflammatory (IL-10) helps moderate the pathogenic effects of M1. Importantly, depending on the severity of the infection and the host’s ability to mount and maintain IL-10 production, anaemia is either moderate (trypanotolerant animals) or severe (trypanosusceptible). Another important host-derived factor regulating the progression of anaemia is the macrophage migration inhibitory factor (MIF) which promotes continuous activation of the pro-inflammatory immune response and inhibits the production of RBC. Moreover, IFN- γ, TNF-α, and MIF are molecules that favour the massive destruction of RBC and inhibit erythropoiesis (Stijlemans *et al.*, 2018). On the contrary, the production of IL-10 promotes erythropoiesis by reducing the effects of pro-inflammatory cytokines (Stijlemans *et al.*, 2018). The degree of anaemia during the infection is determined by the balance between pro and anti-inflammatory cytokines (Musaya *et al.*, 2015). Reports suggest that chronic anaemia is caused by a disproportional immune response (Naessens, 2006). Briefly, the mechanisms behind the development of the chronic anaemia experienced by the animals during AAT seem to differ based on the trypanosome species. Therefore, studies suggest that anaemia during infections with *T. b. brucei* and *T. congolense* is caused by two different mechanisms (Stijlemans *et al.*, 2018). Anaemia development caused by *T. b. brucei* infections are due to the persistence of M1 promoting massive destruction of RBC as well as iron retention and reduced erythropoiesis due to low iron condition and the presence of pro-inflammatory cytokines that inhibits maturation of RBC. However, anaemia developed during *T. congolense* infections is suggested to be caused by iron accumulation in the spleen, followed by an increased release of bilirubin in the blood (Stijlemans *et al.*, 2018). Furthermore, hyperbilirubinemia could trigger the externalisation of phosphatidylserine on RBC and then promote the destruction of RBC. Specifically, during *T. congolense* infections, an increased ferritin expression causing low iron conditions, thrombocytopenia and splenomegaly results in hemodilution, the primary proposed mechanism underlying anaemia development (Tilg *et al.*, 2002).

2.3.4 Diagnosis

Animals infected with African trypanosomes present barely any clinical symptoms; therefore, the clinical examination does not help to establish a proper diagnosis (CABI, 2019). The diagnosis requires identifying trypanosomes in animals' blood using parasitological methods (CABI, 2019). Parasites are likely to be isolated in the blood at the early stages of the infection. Trypanosomes are not expected to be identified in animals with chronic disease and are hardly detected in asymptomatic carriers. Animal blood freshly collected is used to perform blood films and examined microscopically to identify motile parasites. The motility of trypanosomes is determined using phase-contrast illumination or a brightfield background. Thick and thin blood films are prepared to be examined after staining with agents such as methylene blue, Giemsa and May-Grunwald-Giemsa (Uilenberg, 1998). Moreover, other techniques such as haematocrit centrifugation, quantitative buffy coat technique, mini anion-exchange chromatography and the phase contrast buffy coat technique/dark field are useful to diagnose trypanosome infections. Mixed infections are reported when animals are infected with more than one trypanosome species.

2.3.4.1 Serological techniques

Serodiagnosis of AAT requires various serological techniques to detect specific antibodies produced or circulating trypanosomal antigens. The specificity and sensitivity of these tests are variable (Bengaly *et al*., 2002). For instance, the diagnosis of *T. evansi* involved the use of ELISA and card agglutination test for trypanosomiasis (CAAT), which rely on detecting antigen-antibody reactions (Reid & Copeman, 2003). In the field, the sensitivity of ELISA is 81.7%, but the specificity is 15% which is very low (Desquesnes *et al*., 1999). Rapid diagnostic tests are available and are fast and straightforward to use; therefore, they can be integrated routinely for the diagnosis of livestock. Some of these tests are: CAAT and indirect detection card agglutination tests (LATEX) (Verloo *et al*., 2000), the specificity of these tests is 95% and 82%, respectively. During latent infections, parasitological techniques are not helpful; therefore indirect latex agglutination test (Suratex), which can detect circulating trypanosomal antigens, is used (Nantulya & Diall, 1998). CAAT can specifically detect immunoglobulin (Ig)M produced at the early stage of the disease; this test can detect AAT early. Once the disease has been established, IgM produced can be detected by ELISA. Many studies have demonstrated that ELISA is specific and sensitive for diagnosing trypanosomes (Singh *et al*., 1993; CABI, 2019). However, a survey by Desquesnes et al. (2011) has shown that the sensitivity of CAAT in cattle was only 12%. Still, in horses and camels, the sensitivity was high (Desquesnes *et al*., 2011). Moreover, specific parasite antigens circulating in the blood can be detected using antibodies directed against them. This usually confirms the presence of the parasite within the host. However, antibodies being detected indicate trypanosomal infection, but whether it is an active or passive infection is challenging to prove. After treating animals with recommended drugs, though trypanosomes are cleared, antibodies are still detectable. This is one of the disadvantages associated with the use of serological tests. Studies have shown that trypanosomal antigens circulating in the system are rapidly cleared once parasites are killed; therefore, the presence of these antigens in the animals indicates that the parasites are alive (Uilenberg, 1998).

Serology is indicated mainly for a presumptive diagnosis because past infections cannot be distinguished from active infections; cross-reactions with other trypanosome species are frequent. Finally, using serological tests in case of low parasitaemia is not recommended.

2.3.4.2 Molecular techniques

The most popular molecular technique, PCR, is used to identify trypanosomes precisely at the genus, species or subspecies level in tissue samples or blood. Currently, the World Organization for Animal Health (OIE) is prone to using PCR assays to identify trypanosome species. Most available primers allow the detection of *T. b. brucei*, *T. theileri*, *T. congolense* and *T. vivax* and many other species; however, identifying some specific species remains challenging. To amplify many trypanosome species, internal transcribed spacer-1 (ITS-1) (Bakari *et al*., 2017; Cox *et al*., 2010) or tubulin genes can be targeted using the PCR technique, and gel electrophoresis is performed to differentiate these organisms according to fragment size (Ofori *et al*., 2022).

Aside from PCR, another technique called Loop-mediated isothermal amplification (LAMP) can detect trypanosomes in animals. LAMP is extremely sensitive and do not require thermal cycling steps. The assay is performed at the same temperature (isothermal reaction); this reduces the reaction's time allowing the amplification of the parasite DNA in the sample (Kuboki *et al*., 2003).

2.3.4.3 Treatment and control of AAT

The incidence of AAT in endemic areas is high; therefore, achieving effective treatment and proper control of the disease is challenging. Nevertheless, over the last decades, many improvements have been made concerning the treatment and management of AAT. Few chemical compounds are available for treating of AAT, but diminazene aceturate and isometamidium chloride are the two drugs primarily used because of their confirmed efficacy (Singh *et al*., 1995; Jithendran *et al*., 1997; Jain *et al*., 2000; Singh & Choudhri, 2002). Furthermore, drugs such as suramin (specific to *T. evansi*), quinapyramine chloride (used to prevent AAT in livestock), quinapyramine sulphate, cymelarsan (appropriate for camel), and many others (Table 2.2), are used to treat AAT. OIE recommends administering diminazene aceturate intramuscularly to treat sheep, goats, pigs, and cattle. In certain parts of the world, trypanosomes have developed resistance against diminazene aceturate (Giordani *et al.*, 2016). Therefore, either isometamidium chloride or melarsomine hydrochloride is administrated intramuscularly when resistance against the drug of choice diminazene aceturate is observed (Desquesnes, 2004). To avoid trypanosome resistance, alternating diminazene aceturate and isometamidium chloride is recommended (Desquesnes, 2004).

Table 2.2: Some drugs available for the treatment of AAT

Drug	Proposed mechanism	Action/Species/host	Route of administration	Limitations Side effects	References
Homidium Bromide Homidium chloride	Inhibits topoisomerase- II during DNA biosynthesis	(Prophylaxis and treatment)/ *T.vivax*, *T. congolense* (less active *T. b brucei*, *T. evansi*) Cattle, sheep, goats and dog	IM (deep cattle), IV (sheep, goats, pigs)	Toxic, drug resistance	
Diminazene aceturate	Inhibits kinetoplastid DNA biosynthesis	(Treatment)/ *T. congolense*, *T. vivax* (less effective *T.b. brucei*, *T. evansi*) Cattle, sheep, goats and dogs	IM or SC	Highly polar, poor penetration BBB, toxic	
Quinapyramine sulphate	Trypanostatic, Inhibits Kinetoplastid DNA biosynthesis, loss of ribosomal function	(Treatment)/ *T. congolense*, *T. vivax*, *T. b brucei* and *T. evansi*, *T. simiae*, *T. equiperdum*/ Camels, horses, pigs, dogs, cattle	SC	Toxic at high doses, fast resistance acquisition	
Isometamidium chloride	Inhibits topoisomerase-II during DNA biosynthesis	(Prophylaxis and treatment)/ *T. congolense*, *T. vivax* (less effective *T. brucei*, *T. evansi*)/ cattle, sheep, goats, horses, camels	IM (deep)	Toxic, highly irritant	(Giordani et al., 2016; Kasozi *et al.*, 2022)
Suramin sodium	Non-specifically binds to L-α-glycerophosphate oxidase	(Treatment and prophylaxis)/ *T. evansi*, *T. b. brucei*, *T. equiperdum*/ Camels, horses	IV	May be toxic for horses, IM can cause necrosis	
Melarsomine dihydrochloride	Inhibition of trypanothione reductase	(Treatment)/ *T. evansi*, *T. b. brucei*, *T. equiperdum*/	SC or IM	Metabolise fast in the plasma	

2.3.5 Current status of vaccine development

Progress in developing a vaccine against African trypanosomes has been promising, although hampered by several considerable challenges such as antigenic variation, the complexity of the parasite's life cycle, lack of success in identifying non-variable immunogenic proteins and moderate immune response during the infection. Therefore, until today no functional vaccine is available against African trypanosomiasis, mainly because of the abovementioned challenges. Vaccines against trypanosomiasis initially targeted the VSG parasite surface coat. Ideally, VSG would have been a promising vaccine candidate if not for the antigenic variation. The first attempts to develop a vaccine used VSG molecules as targets (Cornelissen *et al*., 1985); however, with time, researchers in the field realised that using that approach was challenging due to antigenic variation. In the past decades, three main strategies have been explored to develop an anti-trypanosome vaccine, non-variable antigen vaccine, transmission-blocking and anti-disease.

Using invariant trypanosome proteins or a mixture of such targets is the rationale behind several attempts to develop an anti-trypanosome vaccine. The flagellar pocket (FP) plays a central role in nutrient uptake. FP is composed of several surface receptors and invariant surface molecules like haptoglobin-haemoglobin and low-density lipoprotein receptors, invariant surface glycoprotein (ISG) ISG65 and ISG75, and serum resistance-associated (SRA) (Field and Carrington, 2009). FP was targeted using two independent approaches, mainly due to this organelle's high number of molecules and receptors. In an initial study, an antigen derived from *T. b. rhodesiense* FP was inoculated into cattle; then, those animals were released to their natural habitat to be exposed and challenged by infected tsetse flies (Mkunza *et al*., 1995). The experiment was conducted for 15 months, corresponding to three rainy seasons; results showed a decrease in the disease prevalence among immunised cattle compared to untreated controls. In the second study, a mouse model was used instead of cattle allowing researchers to have better control over some parameters. Immunisation was performed using *T. brucei* purified material. When mice were challenged with 500 parasites (BSF of *T. brucei*) for 100 days, about 60% did not develop parasitaemia (Radwanska *et al*., 2000). However, no protection was observed when the number of parasites used to challenge mice was increased (1000 parasites or more).

When ISG65 and ISG75 were discovered, these molecules are invariant trypanosome surface proteins, and they were thought to be good vaccine candidates. However, although it was reported that ISG75 triggers the production of antibodies, no protection was observed when recombinant proteins were administered as a vaccine (Ziegelbauer & Overath, 1993). In another attempt, mice were immunised once without any boost, then 175 days later, challenged with 500 *T. brucei* GVR35 parasites. Results were similar to those observed when mice were immunised with FP-derived antigens and challenged with fewer parasites. Briefly, partial protection was observed for 60 days (Lança *et al*., 2011); the protection observed was strongly associated with IgG2a antibody response, indicating an IFN-γ cytokine environment. Also, Ca^{2+} ATPase TBCA2 antigen formulated in a *Vibrio cholera* ghost-based vaccine was used to immunise BALB/c mice; results obtained were once again similar to those previously reported with FP-derived antigens and ISG75 DNA vaccine (Ramey *et al*., 2009).

Trypanosome tubulin and actin proteins were used as vaccine candidates in many studies (Kateete *et al*., 2012; Kurup & Tewari, 2012; San Qiang Li *et al*., 2009; Lubega *et al*., 2002; Tewari *et al*., 2015). These proteins constitute the intracellular cytoskeleton structure and are involved in motility and intra-cellular organelle transport. However, they are reachable by the host immune defence, making them attractive as vaccine candidates. In almost all the studies where those proteins were used, the resting period separating the final boost and the challenge with parasites was relatively short of characterising the vaccine-induced memory. However, the proposed mechanism of immunisation with either tubulin or actin proteins was the activation of the innate immune response due to an improved inflammatory environment resulting in better control of parasitaemia during trypanosome infection.

Almost all studied anti-trypanosome vaccines were performed in mice because relevant hosts are difficult to handle in the laboratory environment. However, this approach has been questioned by many researchers, suggesting that relevant hosts should be used to study the effectiveness of potential vaccine candidates. So far, only a few vaccine candidates have been tested in cattle; one of them is *T. congolense* cysteine proteinase (CP) congopain; however, after vaccination, only trypanotolerant N'Dama cattle were able to mount a proper IgG1 response while Boran cattle which are trypanosusceptible did not (Greca & Magez, 2011; Magez *et al*., 2020). Fully vaccinated cattle presented high titres of anti-CP serum and anti-VSG antibodies, indicating better parasitaemia control (Authié *et al*., 2001). To improve immunogenicity, CP was coupled with the catalytic domain of the α2-macroglobulin enzyme (Huson *et al*., 2009); different adjuvants were used. However, Quil A™ was the adjuvant selected for future studies (Kateregga *et al*., 2012). The main concept of this approach is to modulate the trypanosome-induced pathology and reduce the inflammatory response caused by the disease; here, the parasite is not directly targeted. However, the complete molecular basis or the mechanism underlying this approach is poorly understood.

Some *T. brucei* enzymes have also been used as vaccine targets, especially trans-sialidase (TSE) was targeted. Mice were immunised with a single dose of a TSE encoding plasmid DNA, then challenged with 500 *T. brucei* GVR35 parasites after 175 days. Partial protection was observed for a minimum of 60 days (Coustou *et al*., 2012). Unfortunately, no molecular mechanism explaining this protection was provided. However, in another attempt to develop a vaccine against *T. congolense*, using Tco, TSE protection levels observed were around 15-40% (Silva *et al*., 2009). Once again, the vaccine-induced protection mechanism was not addressed, mainly due to the short period between the final boost and the parasite's challenge (fewer days).

The last approach re-explored by the Magez group is the anti-pathology vaccination; unlike other anti-trypanosome vaccination, the idea is to stop the disease progression. This was further explored after discovering the VSG-GPI anchor, the critical protein driving inflammatory pathology and macrophage-derived tumour necrosis factor (TNF) production (Magez *et al*.,1998). This vaccination approach was applied for *T. congolense*, *T. b. brucei* and *T. evansi* using a liposome-based GPI vaccine. Mice received many doses of the GPI liposome vaccine; then, the parasite challenge was performed, and the severity of the infection was reduced, increasing the lifespan of immunised mice (Stijlemans *et al*., 2007). Various experiments concluded that the vaccine could cause modulation of the inflammatory response explaining the protection observed (Stefan Magez *et al*., 2020).

More recently, systematic genome-led vaccinology was applied using a mouse model to search for potential vaccine candidates against AAT caused by *T. vivax*. Briefly, immunisation was done with a unique recombinant protein containing the extracellular region of a conserved cell surface protein located to the flagellum membrane, named invariant flagellum from *T. vivax'* (IFX) (Autheman *et al*., 2021). Vaccination with IFX significantly reduced the parasite multiplication rate, and robust, long-lasting protection was elicited beyond at least 170 days. Also, it was shown that immune serum was passively transferred, and monoclonal antibodies produced could induce sterile protection. Furthermore, immunised mice were dissected many months after parasite inoculation; interestingly, no viable parasite was detected. However, one limitation was that sera from infected cattle found in AAT endemic areas were not immunoreactive to IFX, raising questions about the suitability of this vaccine candidate in naturally infected hosts. The various vaccine candidates identified are summarised in Table 2.2.

Table 2.3: Summary of some available vaccine candidates

Type of vaccine	Antigen	Antigen preparation	Boosts/host	Time gap boost-challenge	Parasite load	Outcome	References
Intra-muscular	*T. b. rhodesiense* FP	Parasite isolated	3/cattle	14 days or more	Natural exposure	Partial protection	(Mkunza *et al.*, 1995)
I.p.	*T. b. brucei* AnTaR FP	Parasite isolated	3/mouse (Balb/c)	3 weeks	500–10^3	Partial/no protection	(Radwanska *et al.*, 2000)
I.p.	*T. b. brucei* MITat ISG65, ISG75	Recombinant protein	3/mouse (C57bl/6)	11 days	10^4	No protection	(Ziegelbauer & Overath, 1993)
I.p.	*T. b. brucei* ISG75	Plasmid DNA	1/mouse (Balb/c)	175 days	500	Partial protection	(Lança *et al.*, 2011)
I.p.	*T. b. brucei* GuTat10.0 Ca^{2+} ATPase TBCA2	Recombinant protein	3/mouse (Balb/c)	6 weeks	10^6	No protection	(Ramey *et al.*, 2009)
Sub-cutaneous	*T. brucei* (UTRO010291B) Tubulin rich fraction	Parasite isolated	3/mouse (no strain indication)	Not indicated	10^3-10^5	Partial (cross-species) protection	(Ramey *et al.*, 2009)
Sub-cutaneous	*T. evansi* (STIB806) β-Tubulin	Recombinant protein	3/mouse (Balb/c)	6 days	10^3	Partial (cross-species) protection	(San Qiang Li., 2007)
Intra-muscular	*T. evansi* (EU483116) β-Tubulin	Plasmid DNA	2/mouse (Swiss albino)	35 days	10^3	No protection	(Kurup & Tewari, 2012)
Sub-cutaneous	*T. evansi* (EU483116) β-Tubulin	Recombinant protein	2/mouse (Swiss albino)	14 days	10^3	No protection	(Tewari *et al.*, 2015)
Sub-cutaneous	*T. evansi* (STIB806) Actin	Recombinant protein	3/mouse (Balb/c)	6 days	10^3	Partial (cross-species) protection	(San Qiang Li *et al.*, 2009)
Sub-cutaneous	*T. congolense* CP1 & CP2	Recombinant protein	4/cattle (Boran)	1 month	Tsetse bite	Improved recovery	(Authié *et al.*, 2001)
Intra-muscular	*T. b. brucei* Sialidase	Plasmid DNA	1/mouse (Balb/c)	175 days	500	Partial protection	(Coustou *et al.*, 2012)
I.p.	*T. congolense* Sialidase	Recombinant protein	4/mouse (Balb/c)	10–14 days	10^4	Partial protection	(Benoît Stijlemans *et al.*, 2007)
I.p.	*T. b. brucei* AnTat GPI	GPI-Liposomes	2/mice (C57bl/6 and KOs)	3 weeks	5×10^3	Cross-species anti-pathology	(Perdomo *et al.*, 2016)

Notes: I.p. intraperitoneal; FP, Flagellar Pocket; ISG, Invariant Surface Glycoprotein; GPI, glycosylphosphatidylinositol; CP, Cysteine protease., IFX; Invariant flagellum antigen from T. vivax'.

2.4 Trypanosome parasites

2.4.1 Introduction

Trypanosomes are unicellular parasites belonging to the Kinetoplastea class and the Trypanosomatida order. Kinetoplastea are organisms that have a nucleus, mitochondrion, and flagellum. These parasites exhibit different forms, from long, thin, motile spindle shapes to spherical organisms with a tiny flagellum. A unique feature of kinetoplatids is the architecture of their mitochondrial DNA, known as the kinetoplast, visible by light microscopy after appropriate staining like DAPI. Trypanosomes present remarkable biological features and have been used as a reference model for several aspects of eukaryotic molecular cell biology (Walochnik & Duchêne, 2016).

2.4.2 The life cycle of *Trypanosoma brucei*

T. brucei has a complex lifecycle, with five crucial developmental stages occurring in the mammalian host and the tsetse fly. These are the forms the parasite can take: slender metacyclic trypomastigotes, stumpy, metacyclic trypomastigotes, epimastigotes, and procyclic forms. An infected tsetse fly during a blood meal injects metacyclic forms of the parasites causing infections in humans or animals. Trypanosomes enter the lymphatic system and colonise the bloodstream. Once in the host, they differentiate into slender forms, which are proliferative. These parasites can also migrate to other parts, such as the adipose tissues, the central nervous system, and the skin. Long slender forms differentiate into short stumpy non-replicative forms. Then, stumpy forms are picked by tsetse flies during a blood meal. Trypanosomes multiply via binary fission; their life cycle within the hosts is exclusively extracellular. They transform into procyclic form in the tsetse fly midgut, then move to the salivary gland and differentiate into epimastigotes. When these parasites reach the salivary glands, they continue their proliferation and differentiate into non-proliferative metacyclic, infective forms. The development of the parasites within the vector takes 20 to 30 days to complete their maturation (Aksoy *et al*., 2003; Fenn & Matthews, 2007) (Figure 2.1).

Stumpy and slender forms exhibit biological variations essential for the *T. brucei* life cycle. One main variation occurs in the proteins expressed on the parasite's surface. Unlike the stumpy forms, slender forms express the variant surface glycoprotein (VSG), allowing the bloodstream forms (BSF) to change their surface coat constantly. Antigenic variation is a crucial feature that allows the parasite to evade the host immune system (Santos *et al*., 2014)

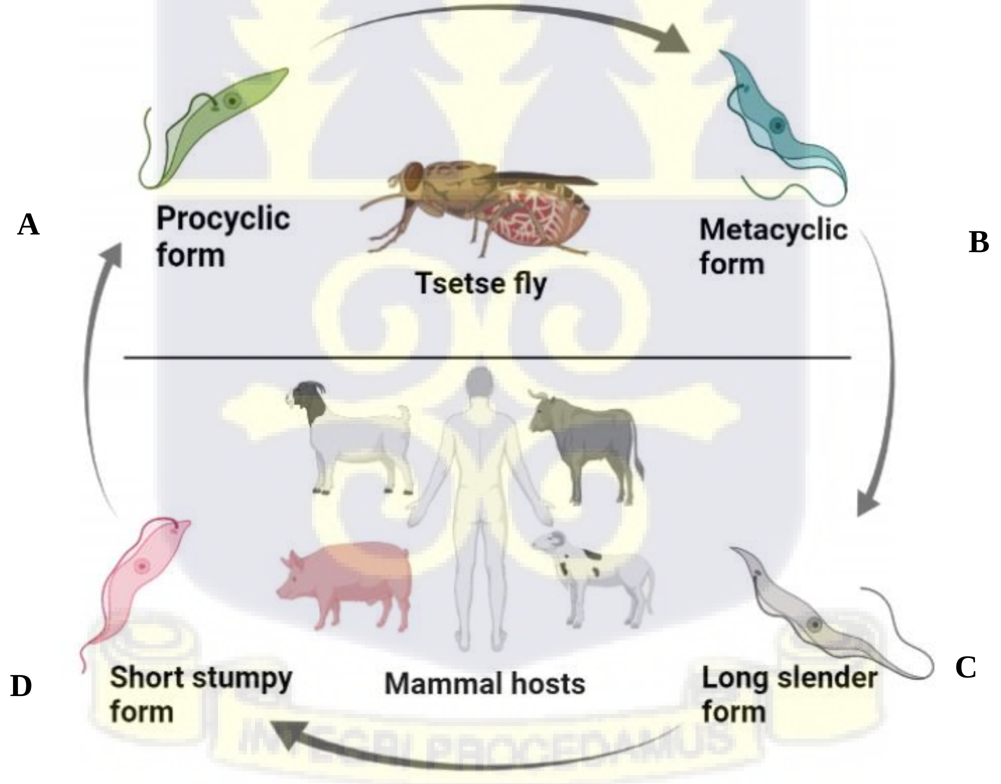


Figure 2.1: The lifecycle of *Trypanosoma brucei*. (A) Procyclic forms are present in an infected tsetse fly. However, after approximately14 days' trypanosomes colonise the salivary glands to be transformed into free-swimming parasites **(B)** metacyclic trypanosomes are the infective forms transmitted to mammal hosts. Once in the host, metacyclic forms are transformed into **(C)** long slender forms and later into (D) short stumpy forms, which can be picked by another tsetse fly during a blood meal, and the life cycle starts again. ***Source: Biorender***

2.4.3 Modifications during the life cycle

The life cycle of *T. brucei* and other trypanosome species shows some variations. For all trypanosome species, controlling the cell cycle requires tight regulation of gene expression to respond to various extracellular changes. According to many studies, the only well-documented stage is the differentiation of BSF of *T. brucei* to stumpy forms. It was shown that the transition from slender to stumpy forms is regulated by quorum sensing (QS). When the density of the long slender forms is optimal, this leads to the secretion of a specific molecule, the "stumpy induction factor" (SIF). Stumpy forms are formed once the concentration of SIF reaches a certain threshold. QS has been studied extensively; briefly, the primary function of this process is to prolong the parasite's survival within the host and promote transmission because stumpy forms cannot multiply in the blood; they survive better and colonise the midgut of the tsetse fly.

Recently, a group of researchers showed that slender forms could survive in the fly's midgut and differentiate directly into the procyclic stage, improving the success of tsetse flies infection (Schuster *et al*., 2021). Also, they discovered that highly motile long slender forms could arrive at the entry of proventriculus; this migration event occurs before the parasite moves to the next stage of the life cycle (Schuster *et al*., 2021).

2.4.4 African trypanosomes genetics

Trypanosomes have a complex genome with specific features not found in other eukaryotic genomes. The genome is organised in a polycistronic transcriptional unit (PTUs); therefore, to produce mature mRNAs, trans-splicing and polyadenylation must occur. Trans-splicing requires the addition of a spliced leader (SL), a sequence of 39 nucleotides derived from the SL-RNA to the 5'end of the mRNA (Martínez-Calvillo *et al*., 2010). SL contains a 7-methylguanosine cap and 4 methylated nucleotides at the sequence start to generate mature mRNA (Günzl *et al*., 2015).

PTUs are also conserved; the degree of synteny between different species is high. Previously, scientists thought that trypanosomatids lack introns. However, two introns were found after re-examining the *T. brucei* genome; *T. brucei PAP* gene encoding poly (A) polymerase and *T. brucei* (Tb927.8.1510) encoding a putative RNA helicase (Günzl, 2010). Therefore, these mRNAs are processed by cis and trans splicing mechanisms. Protein-coding genes are in large PTUs containing 10 to 100 genes oriented in the same direction. Several reports have shown that among trypanosomatids, the gene order within PTUs is relatively conserved (Martínez-Calvillo *et al*., 2010). Within the PTUs, genes do not have the same function and can therefore be expressed at different stages. A single transcriptional start site (TSS) can transcribe only one PTU; this drastically reduces the role of several gene expression regulators. For instance, in *T. brucei*, most genes down-regulated are closer to the TSS, whereas up-regulated genes are distant. Furthermore, the location of genes within the PTUs influences the process of gene expression during different stages of the cycle (Siegel *et al*., 2011). Surprisingly, the position of many genes does not depend on their transcriptional regulation (Campbell *et al*., 2003; Martínez-Calvillo *et al*., 2010; Kelly *et al*., 2012).

In trypanosomes, processing and controlling mRNA stability are two essential mechanisms. Specific elements in the 3' untranslated region (UTRs) regulate the stability of the mRNA. The half-lives of fully mature mRNA are variable among trypanosome species and depend on the life cycle stage. Also, both stability of the mRNA and the rates of degradation of the precursor mRNA are essential for the half-life of the mRNA. If polyadenylation occurs late, the chances for the mRNA to be degraded increase (Clayton, 2014; Jackson *et al*., 2015).

RNA-binding proteins (RBPs) have been described to bind regulatory elements in the 3' UTR of their target genes to achieve their primary function, regulating gene expression. RBPs are more abundant than mRNAs, the abundance of RBPs influences the stability of mRNA and, thus, protein translation (Clayton, 2014; Clayton, 2013). Furthermore, protein-coding genes can be regulated during the translation process.

2.4.4.1 RNA Polymerase I transcription

Expression Site Associated Genes (ESAGs) and VSG are organised in PTUs with a unique promoter found 67 bp upstream of the TSS (Günzl *et al.*, 2015). Bloodstream expression sites (BESs) in the PTUs are specific to trypanosomes and are only transcribed by RNA polymerase I (RNA pol I). However, RNA pol I transcribe ribosomal RNA (rRNA) in eukaryotes (Günzl *et al.*, 2015). RNA pol I in *T. brucei* can transcribe 45S rRNA and procyclin and BESs genes found outside the nucleolus (Das *et al.*, 2008). Studies suggest that *T. brucei* uses RNA pol I at the BES to allow the high-rate transcription occurring at the active expression site to generate VSG mRNA (Alsford *et al.*, 2012).

2.4.5 Surface molecules

2.4.5.1 Variant surface glycoprotein (VSG)

The BSF of *T. brucei* is protected by a thick coat of VSG, described as homodimers for many years. However, recently another class has been characterised by concentration-dependent trimers (Sara *et al.*, 2023). VSGs represent about 5-10% of the cell proteins, and the molecular weights of the VSG molecules vary between 50-65 kDa. All mature VSG proteins are attached to the surface of the parasite by GPI-anchor and have two regions, an N-terminal domain (NTD), which is large (300-400 amino acids), and a C-terminal domain (CTD), smaller with only about 80-120 amino acids. The NTD is the region that harbours the GPI anchor (Aresta-Branco *et al.*, 2019). On the other hand, the CTD is less immunogenic, and the sequence is highly conserved compared to NTD (Sara *et al*., 2023). However, NTD is thought to be highly immunogenic and only 10-30% identity is observed in the sequence from variant to variant (Berriman *et al*., 2005). This implies that the NTD of VSGs are essential for antigenic variation in African trypanosomes.

The BSF of *T. congolense* and *T. brucei* have another essential protein, the transferrin receptor, encoded by two genes, ESAG 6 and 7. These genes are located upstream of VSG genes within the expression site and are co-expressed. The parasite expresses both the VSG and the transferrin receptor to ensure its survival within the host.

2.4.6 Antigenic variation and monoallelic exclusion

Antigenic variation is a mechanism used by African trypanosomes to evade the immune response mounted by the host by constantly changing their surface coat (Vanhamme *et al*., 2000). Approximately 10 million, monoallelelically-expressed molecules of VSGs coat undergo repeated cycles of switching critical for the parasite's survival within the host (Bangs, 2019; Horn, 2019; Manna *et al*., 2014). This allows a cyclic process of rapid parasite growth, increasing the parasitemia, immune response and then the prevalent VSG variant is cleared. Subsequently, there is a noticeable growth of immune-escape parasites expressing different VSGs (Sara *et al*., 2023). This contributes to maintaining the infection state in the host, causing long-term morbidity and mortality and the only means to clear the infection is by using appropriate drugs (Mugnier *et al*., 2016). The key factors essential for antigenic variation are the thousands of VSG genes and pseudogenes in *T. brucei* genome serving as the parasite's extensive antigen repertoire (Sara *et al*., 2023). One VSG gene is expressed at each point in time; although the parasites have more than 2000 VSG genes and pseudogenes (Alsford *et al*., 2012).

Before transcription occurs, VSG genes must be placed either in a BES or a metacyclic expression site (MES), which are telomeric expression sites (Jackson *et al*., 2013). This depends on where the activation occurred, either within the mammalian host or the tsetse fly salivary glands. BESs have been studied extensively due to their abundance in the genome. Also, they contain large PTUs and carry many other genes ESAGs, and most have been described as cell surface proteins (Jackson *et al*., 2013).

BES has been described as the main site where antigenic variation occurs, as they contain the unique active VSG locus in the host. At each point in time, one BES is active, and only one VSG gene is transcribed. The genome of *T. brucei* harbours 15 BESs expressing similar but not identical copies of VSG and ESAGs (Hertz-Fowler *et al*., 2008). In *T. brucei* Lister 427, all the BESs are highly conserved in the overall structure and the order of the genes (Hertz-Fowler *et al*., 2008). However, one specific expression site in *T. b. rhodesiense* is described as a resistant expression site because it carries the serum-resistance associated (SRA) gene conferring resistance to the lysis by human serum (Bart *et al*., 2015). African trypanosomes achieve antigenic variation using four defined mechanisms: *in-situ* switch, gene conversion, telomere exchange and segmental gene conversion (Vanhamme *et al*., 2000). Activation of a new BES is described as *in-situ* switch and when in the active expression site homologous recombination of the VGS occurs, this mechanism is telomere exchange. The two mechanisms described above, trigger limited variation, and occur mainly during the early stage of the infection. However, segmental gene conversion and gene conversion mechanisms require gene recombination resulting in a new VSG sequence (Alsford *et al*., 2012; Vanhamme *et al*., 2001). The two last mechanisms described have a higher potential to generate diversity and enable the parasite to prolong the infection.

2.5 Importance of iron in trypanosomes

Iron is one of the essential nutrients in both prokaryotes and eukaryotes (Basu *et al*., 2016). The ability to catalyse several electron transfer reactions makes iron indispensable for many pathways (Rouault & Klausner, 1997). Iron generally exists in two main forms, Fe^{2+} and Fe^{3+}, but other forms have also been described. Due to its toxicity, cells have evolved systems to regulate iron uptake. Besides solving iron supply challenges, cells must also regulate free iron levels. In excess, iron can catalyse the "Fenton reaction", and the products of this reaction cause severe damage to the cell (Stadman, 1993) and other essential molecules such as DNA, proteins, and lipids (Gudjoncik *et al*., 2014; Imlay & Linn, 1988; Kaplan & Ward, 2013; Krewulak & Vogel, 2008; Waldvogel-Abramowski *et al*., 2014). Fenton reaction is toxic, explaining the need for specific proteins to maintain iron homeostasis in many living organisms. Iron homeostasis checks iron absorption and sequestration in eukaryotic cells to maintain reasonable free iron levels. Furthermore, iron can be stored in either soluble or insoluble form, corresponding to ferritin and hemosiderin, respectively.

Iron plays multiple functions in almost all living organisms, including *T. brucei* and most of these functions are shared by the parasite and the host. Some of these functions are cytochrome-based oxidative respiration and reduction of ribonucleotides for DNA synthesis (Weinberg, 2010). The quantity of iron absorbed by a mammal host daily after a regular meal is about 1.5 mg. Furthermore, enterocytes perform iron absorption via complex mechanisms in the upper part of the gut, the duodenum, and the proximal jejunum. Iron is absorbed in two forms: haem-iron (from haemoproteins) or non-haem-iron (from vegetables and grain), and several import proteins are involved in the absorption of the two primary ionic forms, Fe^{2+} and Fe^{3+} (Reyes-López *et al*., 2015).

Serotransferrin (Tf) is the unique transporter of Fe^{3+} in the blood serum of mammals. Tf is a bi-lobe glycoprotein with a molecular weight of 80 kDa; each lobe has two domains with Fe^{3+} binding sites. Depending on the physiological pH of the serum, Tf can bind either mono-or di-ferric iron atoms, transforming an Apo-Tf (iron-free) into holo-Tf (iron-laden) (Aisen & Listowsky, 1980; Brummett *et al*., 2017). The binding affinity of the two lobes differs; reportedly, the C-terminal lobe binds Fe^{3+} more tightly, and then iron molecules are slowly released (Reyes-López *et al*., 2015). Tf is expressed in the spleen, kidneys, liver, and CNS of most mammals (Macedo & de Sousa, 2008).

Like all pathogens, African trypanosomes successfully established infection by colonising the host. However, this process requires the presence of nutrients and growth factors, such as iron. The interaction between parasites and their hosts is complex in that; the hosts obtain iron from the diet to satisfy their iron demands; they must simultaneously sequester this nutrient from various invaders (Reyes-López *et al*., 2015). This mechanism of defence is called nutritional immunity; several hosts use this process to protect their essential nutrients, and this results in the killing of these invading pathogens put in a vulnerable state. Tf is an iron-carrier protein, but neither transferrin nor its homolog exists in many protozoan parasites, specifically in *T. brucei*. Therefore, to fulfil their iron needs these parasites must obtain iron from the host Tf through a receptor called *T. brucei* transferrin receptor (TfR). Moreover, several parasites such as *Plasmodium spp* (Haldar *et al*., 1986; Rodriguez & Jungery, 1986), *Trichomonas* (Sutak *et al*., 2008), *Leishmania spp* (Voyiatzaki & Soteriadou, 1992), *Trypanosoma cruzi* (Corrêa *et al*., 2008), and *Entamoeba histolitica* (Reyes-López *et al*., 2001), express TfR that recognises the same transferrin molecule of mammalian cells.

2.6 Transferrin receptor genomic location, context, and structure

The TfR is essential for iron uptake through receptor-mediated endocytosis (Benz *et al.*, 2018; Kariuki *et al.*, 2019). ESAG 6 and 7 encode for TfR, which is a heterodimeric protein complex. ESAG6 has been described as a heterogeneously glycosylated protein containing a GPI anchor, but ESAG7 is a glycoprotein with no GPI. The association of ESAG6 and ESAG7 is needed to interact with Tf molecule (Steverding *et al.*, 1995). ESAGs are generally co-transcribed with VSG genes (Figure 2.2), constituting the major component of the compact surface coat that gives trypanosomes the ability to elude the host's defence system (Didier-Salmon *et al.*, 1994). Expression of VSG and TfR is essential for the disease establishment by *T. brucei* parasite (Tiengwe *et al.*, 2017). Several expression sites encode multiple TfR structurally related but with different binding affinities to various Tf. Reportedly, differences between TfRs are observed among various mammalian hosts, which influence the binding affinity for Tf, and therefore the ability of trypanosomes to survive in the sera of these hosts (Bitter *et al.*, 1998; Trevor *et al.*, 2019).

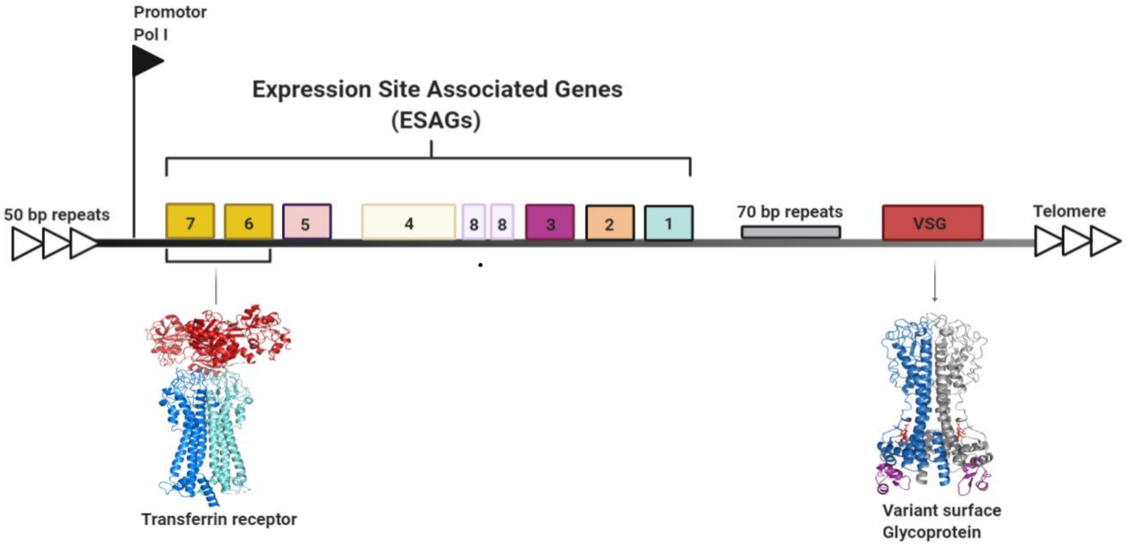


Figure 2.2: Expression of both Trypanosoma brucei transferrin receptor and variant surface glycoprotein. Two expression site-associated genes (ESAG6 and 7) encode for the transferrin receptor, a heterodimeric protein. These two genes are co-transcribed with VSG genes encoding the variant surface glycoprotein, constituting the trypanosome's primary surface coat. Multiple expression sites exist; however, a unique polycistronic VSG mRNA is transcribed with the help of the RNA pol I from the active expression site. ***Source: Biorender adapted from Stevending et al., 2003.***

2.6.1 TfR localisation

T. brucei lives extracellularly in the host’s bloodstream and the TfR is only expressed by the BSF to mediate Tf endocytosis. However, this receptor is structurally and evolutionary distinct from the host TfR and is found within the parasite's flagellar pocket (Maier & Steverding, 1996; Steverding *et al*., 2012; Steverding *et al*., 1995). Two essential proteins constitute TfR and, are encoded by ESAG6 and ESAG7. For the binding of host Tf, an association of ESAG6 and ESAG7 proteins is required (Hertz-Fowler *et al*., 2008; Steverding *et al*., 2000). Trypanosomatids have developed an organelle-like compartment, the flagellar pocket, where uptake of various nutrients occurs. This justifies the presence of the TfR within the flagellar pocket, which is the only known site of exocytosis and endocytosis (Landfear & Ignatushchenko, 2001; Perdomo *et al*., 2016). Interestingly, Tf is internalised by trypanosome parasites by a known molecular mechanism different from that observed in mammals.

Briefly, the GPI anchor found on the cell membrane ensure binding of TfR to Tf resulting in Tf internalisation by clathrin-coated pits. Due to the low pH in the endosome, iron is released from the Tf molecule resulting in the low affinity of Tf for the TfR (Maier & Steverding, 1996). Therefore, apo-Tf (iron-free molecule) is released, then transported to lysosomes, and degraded by cysteine-protease and cathepsins-specific enzymes found in *T. brucei* (Steverding *et al*., 2012; Steverding *et al*., 1995). TfR is then recycled to the flagellar pocket to bind a new holo-Tf (Mehlert *et al*., 2012; Steverding *et al*., 2012). The entire mechanism is described in (Figure 2.3). Unlike *T. brucei*, in mammalian cells, Tf is attached to the receptor and transported to the extracellular milieu to bind new iron (Steverding *et al*., 2012).

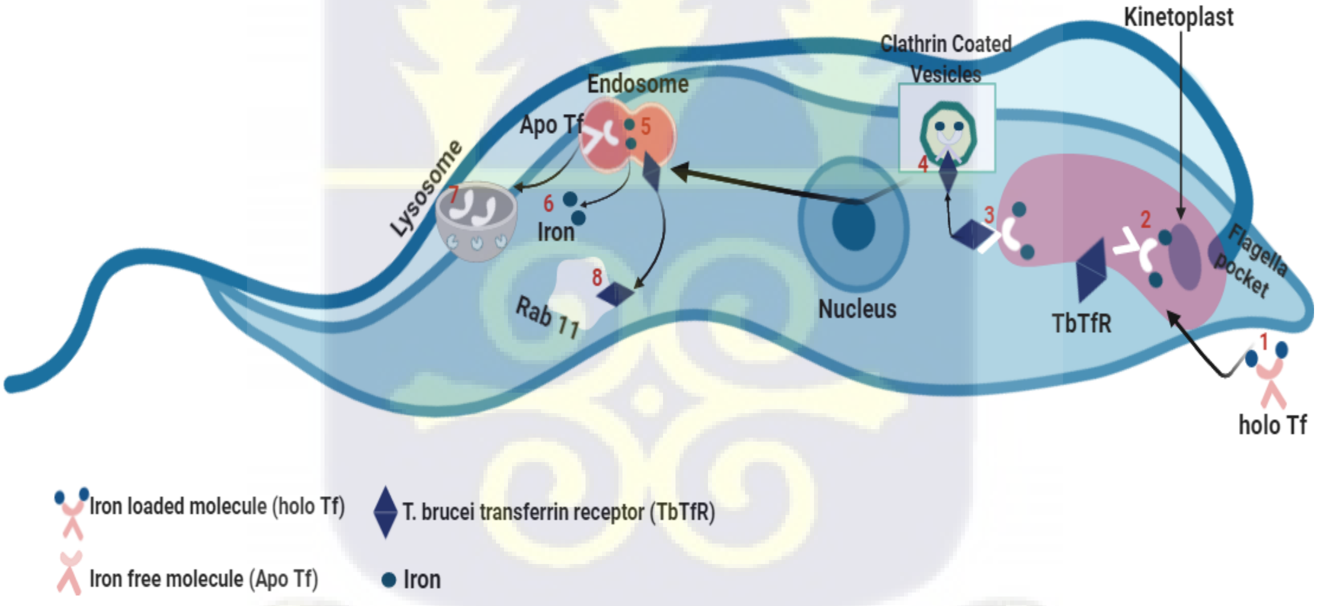


Figure 2. 3: Iron acquisition in *Trypanosoma brucei* via transferrin-mediated endocytosis. The delivery of iron into the bloodstream forms of *T. brucei* is mediated by host transferrin that is taken up via a unique receptor (transferrin receptor) expressed by the parasite. The parasite internalises host transferrin by receptor-mediated endocytosis. Holo transferrin (iron-loaded molecule) is bound by the *T. brucei* transferrin receptor (*TfR*), which is located at the flagellar pocket; clathrin-coated pits, therefore, internalise the complex (transferrin-TfR). After acidifying the pH within the endosomes, iron is released and transported to the cytoplasm. A transferrin-free molecule or apo-transferrin is cleaved by specific enzymes in the lysosomes. Finally, the *TfR* is recycled by Rab11-positive vesicles and returned to the membrane of the flagellar pocket to be used again. ***Source: Biorender adapted from Reyes-Lopez et al., 2012***

2.6.2 Structure of *T. brucei* transferrin receptor (TfR)

TfR subunits share an identical fold with VSGs, the trypanosome surface coat (Salmon *et al*., 1997; Didier-Salmon *et al*., 1994; Steverding, 1997). Furthermore, TfR recognises their ligand only when VSG is densely packed; endocytosis occurs in vesicles that emerge from the parasite flagellar pocket (Steverding *et al*., 1995). Antibodies have access to the flagellar pocket and the cell surface of *T. brucei*. This, therefore, raises an important question about how TfR avoids detection. No research has been conducted to answer this question confidently.

Using the *T. brucei* Lister 427 genome, 14 TfRs families were identified, and the structure of TfR was solved (Trevor *et al*., 2019). Trevor and colleagues observed that ESAG6 and ESAG7 are elongated heterodimers containing three long alpha-helices. The N-terminal presents two helices; this corresponds to the long helices of VSGs, and the third helix strengthens the fold. Each subunit of the membrane-proximal side contains a short alpha-helix linking the two subunits of the heterodimer. Data further confirmed that ESAG6 and ESAG7 share similar folds; the sequence identity is 80%. The membrane-distal loops adopt multiple conformations to allow asymmetric binding of both subunits to one Tf molecule (Trevor *et al*., 2019).

2.6.3 Transferrin receptor polymorphism

Different expression sites (ES) encode TfRs that are not identical but similar (Bitter *et al*., 1998; Steverding *et al*., 1995) which results in the polymorphisms observed in the TfR. Bitter and colleagues previously established that these subtle variabilities in the TfR affect the binding affinity to transferrin from different hosts and, subsequently, this might affect the ability of *T. brucei* to survive in the bloodstream of these hosts. To prove this, an *in-vitro* system was used where the BSF of *T. brucei* was exposed to transferrin from many mammals. Data confirmed that the binding affinity varied, influencing the iron uptake rate. *T. brucei* infects a wide range of animals, such as cattle, pigs, goats, sheep, and many other animals; each of these animals' transferrin varies, therefore requiring a unique TfR.

Previous studies suggest that depending on the host where *T. brucei* is found, a unique TfR having high affinity with that transferrin molecule is expressed. This is only possible because the parasite can switch between different expression sites. Several experiments were performed to confirm this; details of experimental procedures and results are found in the paper published by Bitter et al. (1998). Furthermore, the study results by Mussmann and colleagues also reported the existence of polymorphisms in the TfR (Mussmann *et al*., 2004; Trevor *et al*., 2019). The variant 221a (lab-strain) producing TfR with low affinity for canine Tf was cultured and transferred to a dog serum-based medium. This triggered the selection of clones that have switched to at least three different ES compared to the wild type (Hirumi & Hirumi, 1989; Trevor *et al*., 2019).

However, all these findings were recently challenged by new the data Trevor and colleagues published, indicating that a single TfR binds transferrin from various mammalian hosts. Previously, most studies suggested that polymorphism in TfR is required to cope with different transferrin sequences, allowing this parasite to infect various mammals (Bitter *et al*., 1998; Fast *et al*., 1999). To refute this old thought, when BES1 and BES17 receptors were used, the binding affinity to Tf from eight different hosts was analysed, and surface plasmon resonance was used to measure the binding of Tf variants. Interestingly, results showed that BES17 bound all eight Tf variants but with different binding affinities. This suggests that a unique receptor BES17 can ensure appropriate Tf uptake under favourable physiological conditions. Also, the results indicate that TfR saturation ensures that Tf covers the surface of the receptor most exposed. This inhibits antibody recognition and decreases the risk of the host immune system detecting and clearing the parasite (Trevor *et al*., 2019). The second receptor tested BES1 was shown to bind weakly to some Tf variants, and for four of these variants, no binding was observed (Trevor *et al*., 2019). Binding affinities of BES17 to the eight Tf were more significant than the BES1. Therefore, the new hypothesis is that trypanosomes express several receptors to avoid clearance by immunoglobulins produced to target Tf binding site, ensuring continuous Tf uptake (Trevor *et al*., 2019). The mechanism of receptor switching to a novel antigenic variant might reset the system to limit possible competition. The diversity of TfR allows antigenic variation because polymorphism is not focused on residues in contact with Tf but rather across the third membrane-distal of the receptor, which is predominantly exposed.

2.7 The regulation of iron and trypanosome transferrin receptor

Iron plays a central role in many pathways, but this nutrient's uptake and concentration in the cell must be controlled (Stijlemans *et al*., 2015). Therefore, the expression level of TfR is regulated to control the uptake of iron in the parasite and to prevent the generation of reactive free radicals causing damage in the cell in the physiological presence of oxygen (Taylor & Kelly, 2010). The mechanism of iron regulation in mammalian cells is performed by iron regulatory proteins (IRP) binding iron-responsive elements (IRE) (Fast *et al*., 1999; Manta *et al*., 2000), but how this task is achieved in *T. brucei* remains unclear.

2.7.1 Iron regulation in mammals vs trypanosomes

In mammalian cells, two different aconitases were identified: mitochondrial and cytosolic aconitases. Several studies described cytosolic aconitase as iron regulatory protein-1 (IRP1) (Gruer et al., 1997; Fast *et al*., 1999; Manta *et al*., 2000). Aside from being involved in regulating iron in mammalian cells, this enzyme plays a vital role in the citric acid cycle. Moreover, another iron-regulatory protein was described (IRP-2), but the function of this protein has not been studied extensively (Pantopoulos, 2004). However, some studies have been conducted to understand how IRP-1 or cytosolic aconitase works (Gruer *et al*., 1997; Pantopoulos, 2004). Due to evolution, cytosolic aconitase has gained the ability to bind RNAs harbouring iron-responsive elements (IRE) (Gruer *et al*., 1997). *T. brucei* contains one copy of aconitase gene encoding isozymes active in both the cytosol and the mitochondria. In comparison, multicellular eukaryotes contain different genes encoding mitochondrial and cytosolic aconitases (Müllner *et al*., 1989). In mammalian cells, IRP-1 can switch between aconitase to RNA binding activities, but IRP-2 has only RNA-binding protein activity (Pantopoulos, 2004).

Regulation of transferrin receptor and iron storage through ferritin protein is essential to achieve iron homeostasis. Studies revealed that mRNAs of TfR and ferritin carry IREs within their UTRs (Menotti *et al*., 1997; Erlitzki *et al*., 2002; Paul, Piccinelli & Tore Samuelsson, 2007). Under low iron conditions, IRP-1 and IRP-2 bind to IREs, stabilising the TfR mRNA and inhibiting ferritin mRNA's translation. This increases TfR-dependent uptake while reducing the storage of iron into ferritin. If the intracellular iron level is high, the interaction between IRP/IRE decreases, and TfR mRNA is degraded. In contrast, the ferritin mRNA is translated, resulting in iron storage (Pantopoulos, 2004) (Figure 2.4). IRP/IRE system described above is required for iron homeostasis in mammalian cells, but this system is not active in trypanosomes. However, under low iron conditions without aconitase, TfR is up-regulated, suggesting that aconitase is not the IRP in the parasites (Fast *et al*., 1999). Therefore, this model of iron regulation is questioned which suggests that *T. brucei* might have developed another mechanism to achieve iron homeostasis.

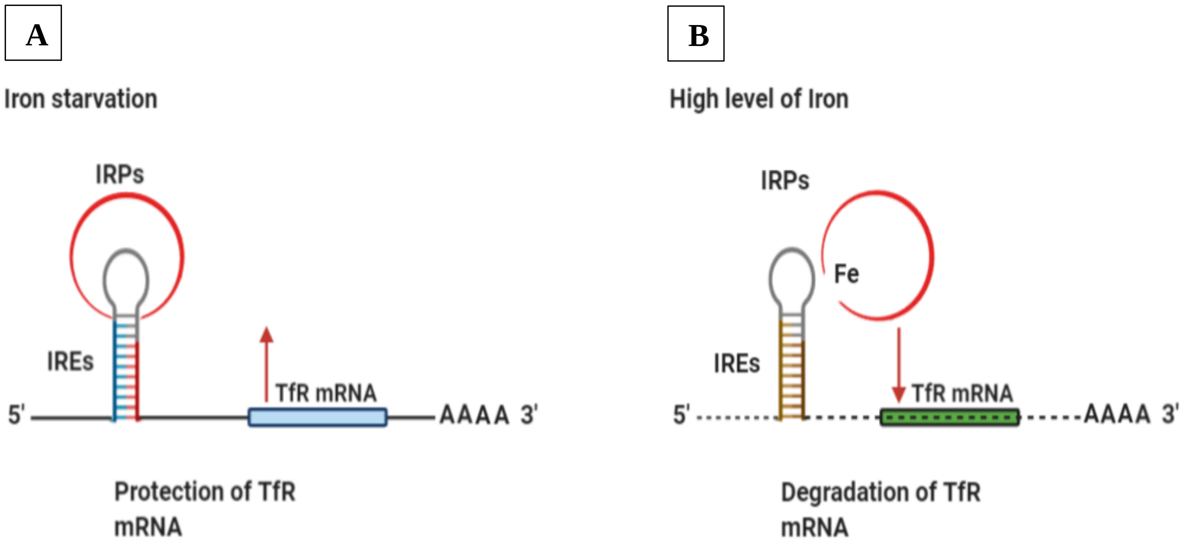


Figure 2.4: Iron regulation via the control of transferrin receptor (TfR) mRNA in mammals. (A) Iron starvation. Under low iron conditions, iron-regulatory proteins (IRPs) bind to iron-responsive elements (IREs); this leads to the protection of the TfR mRNA and induces an increase in the levels of TfR. **(B)** High level of iron. However, under excess iron conditions, IRPs bind to ferritin (Fe), inducing the degradation of the TfR mRNA, and this causes a significant decrease in the TfR levels. ***Source: Biorender adapted from Anderson et al., 2012***

2.7.2 Regulation of *T. brucei* transferrin receptor

Fast and colleagues were the first to report that aconitase is not the IRP in *T. brucei* and that expression of TfR is regulated by the level of iron available in the host (Fast *et al*., 1999). After inducing iron starvation in the BSF of *T. brucei* using deferoxamine, an increase of TfR expression was observed (a 3-fold change), and subsequently, transferrin uptake rate. This confirms that the availability of iron in the environment modulates the expression of the parasite TfR. Also, the amount of ESAG6 mRNA, one of the genes encoding TfR, was fivefold regulated by a post-transcriptional modification (PTM), suggesting that PTMs are essential for iron regulation in *T. brucei* (Fast *et al*., 1999). To understand the function of the cytoplasmic aconitase in *T. brucei*, aconitase gene was deleted, and results showed that aconitase was not essential for iron regulation in *T. brucei* (Fast *et al*., 1999).

T. brucei, acquires iron exclusively from the host, which is done using the TfR as mentioned previously. Excess or low levels of iron conditions lead to the parasite's death, and the necessity to find the right balance is undeniable. Therefore, the only way for the parasite to achieve iron homeostasis is by controlling the expression of the TfR according to the need. This explains why this receptor has been studied extensively over the years.

2.8 New perspective of AAT in Ghana and direction for future work

The farming sector is an essential part of agriculture in Ghana and contributes to economic and social development. Specifically, livestock and crop production are critical in generating food and revenue farming a significant part of the agricultural GPD (Adams *et al.*, 2021). AAT is a major constraint in livestock production in Ghana. Several efforts have been put in place to improve disease management in the country. However, to achieve this, it is essential to understand the dynamics of the disease. In Ghana, some studies have reported the prevalence of AAT and trypanosome species in circulation (Bakari *et al.*, 2017; Ekloh *et al.*, 2023; Nakajima *et al.*, 2012; Ofori *et al.*, 2022). However, for most of these studies, the animals sampled were only cattle; also, the sample size was not representative and finally, few areas were visited. Therefore, in some parts of the country, the dynamics of AAT is still poorly understood, making it difficult to evaluate the real burden of the disease, and this impairs the disease management in the country. Studies must be conducted determining the incidence of AAT in many parts of the country and so identify trypanosome species in circulation to determine the severity of the infection in the major livestock. Iron is an essential nutrient for the survival of African trypanosomes within the host. Several studies showed that inducing iron starvation in trypanosomes results in death; hence exploring that field can be interesting to gain more knowledge about the parasite’s biology and possibly identify new drug targets. To fulfil their iron needs, the parasites use TfR for transferrin uptake an iron-loaded molecule found in the host. The structure of TfR was recently described (Trevor *et al*., 2019), boosting studies in that field. Moreover, polymorphisms in this receptor were reported but whether this is observed in field parasites collected from various animals remains unknown.

Moreover, transferrin uptake can be influenced by polymorphism in the TfR, this was reported many years ago. However, another key factor that might influence transferrin uptake is the structure (dimer or trimer new classification) of the VSG expressed (unpublished data). Therefore, studies must be conducted to answer all these relevant questions and to add to the current knowledge.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Ethics statement

Ethical clearance was sought from the Council for Scientific and Industrial Research (CSIR) (RPN012/CSIR-ACUC/2020). Furthermore, approval was obtained from the respective farms where blood samples were collected. Farm managers and heads of units were asked to sign an informed consent form containing the study's purpose, procedures, voluntariness, risks, and benefits. Experienced animal handlers restrained animals humanely before a well-trained veterinarian collected the blood.

3.2 Study sites

For this study, six farms located in four districts in the Greater Accra region (GA) (Figure 3.1) were selected. Those study sites were: The University of Ghana Livestock and Poultry Research Centre (LIPREC) located at Ashaley Botwe, Prampram (Potters city), the Council for Scientific and Industrial Research (CSIR) at Katamanso and Pokuase, and two private farms located at Apollonia and Oyibi. The farms in these geographical areas were selected based on the prevalence of the disease (AAT) reported previously (Ofori *et al.*, 2022; Ekloh *et al.*, 2023), the vegetation surrounding the farm, the breed of the selected animals, and the density of tsetse flies. At almost all the study sites the vegetation observed is the savannah-type but at the Pokuase site the vegetation is semi-forest. More details about the breed of the livestock sampled at each study site are found in Table 3.1.

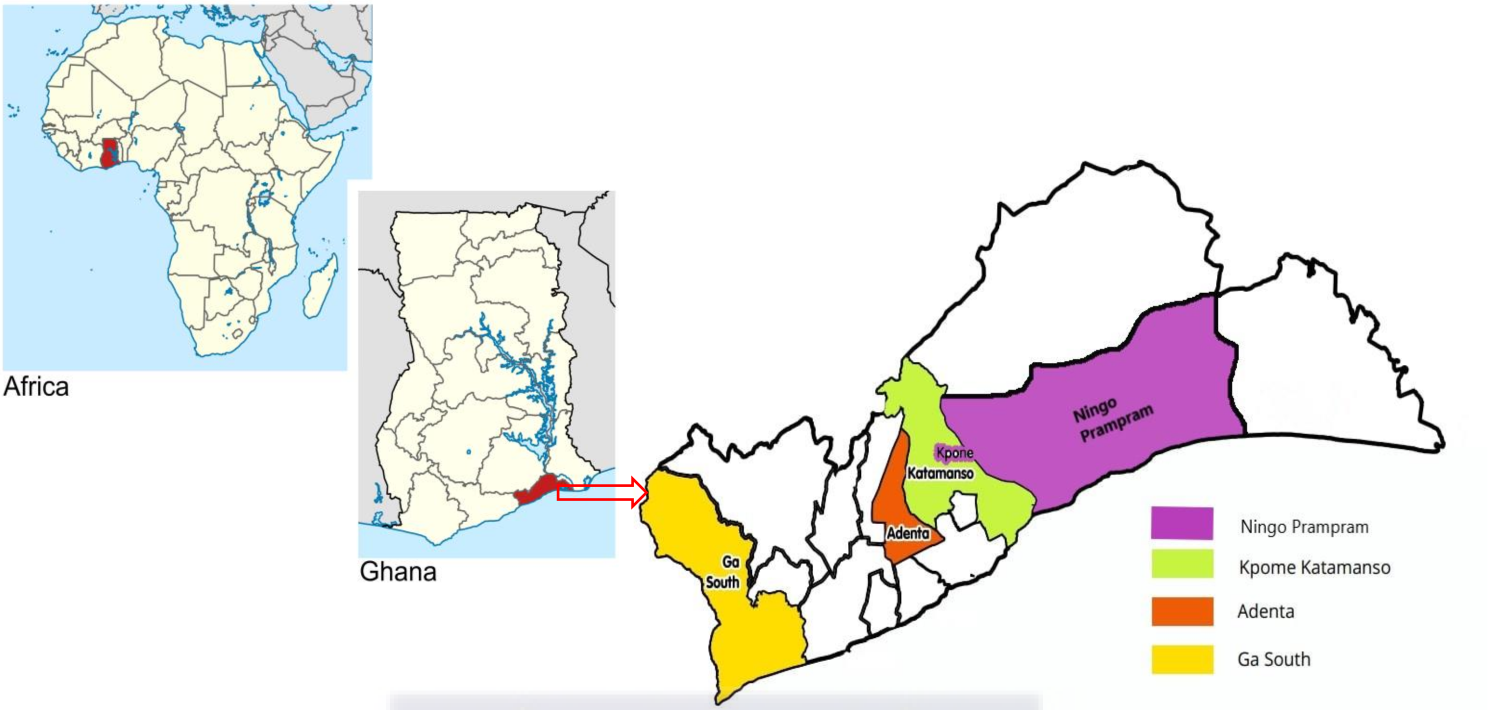


Figure 3.1: Greater Accra map showing the four main districts where sample collection was performed. Kpome Katamanso (Katamanso), Adenta (Ashaley Botwe, Appolonia and Oyibi), Greater Accra South (GA South) Pokuase Ningo Prampram (Potters city). ***Map adapted from Aninagyei et al., 2020), Get Map was used to draw the map.***

Table 3.1: Animal species and breed at the different study sites

Study sites	Animal species	Breed
Ashaley-Botwe (LIPREC)[a]	Cow	Sanga, Sanga cross and WASH
	Sheep	Nungua type, Sahelian and Forest type
	Goat	Short-legged type and Long-legged type
	Pig	NA
Katamanso (CSIR)[b]	Cow	N'DAMA, WASH, Sanga, and Sanga cross
	Sheep	Crossbreed and Sahelian type
	Goat	Crossbreed and Short-legged
	Pig	NA
Pokuase (CSIR)	Cow	N'DAMA,
	Sheep	Crossbreed
Prampram	Cow	Sokoto
Appolonia and Oyibi	Sheep	Nungua type and Crossbreed
	Goat	Short-legged and Crossbreed

WASH: *West African Shorthorn a) Livestock and Poultry Research Centre; b) The Council for Scientific and Industrial Research*

3.3 Sample collection

A cross-sectional study was carried out; samples were collected on two separate occasions, the first round from January 2020 to August 2020 and the second round from January 2022 to April 2022.

During the first sample collection, the livestock selected were cattle, sheep, goats, and pigs (Figure 3.2) aged between 1-9 years and randomly sampled regardless of breed or sex. Almost all the livestock species were sampled for the second round except pigs. In total, 456 animals were screened for the detection of trypanosome infections, 132 goats, 155 cattle, 138 sheep and 31 pigs. The number of animals sampled per site was 175 at Ashaley Botwe, 118 at Katamanso, 80 at Pokuase, 45 at Prampram, and 20 and 18 at Appolonia and, Oyibi, respectively.

Table 3.1 summarises all the livestock sampled at the various study sites for the first round; details about the animals sampled during the second occasion are found in the appendices (appendix C1, C2 and C3). Animals sampled for this study did not receive any treatment against AAT for at least a year before blood samples were collected. Each animal was bled from the jugular vein, and sometimes the tail and 10 mL of blood was taken, 2 mL was transferred in EDTA tubes (Sarstedt AG & Co, Nümbrecht, Germany) and 3 mL in Tempus RNA tube (Invitrogen, Applied Biosystems^{TM}, USA) and 5 mL in a tube without anticoagulant. Tubes containing blood (EDTA tubes) were placed on ice and immediately transported to the Small Animal Hospital to perform a cell blood count (CBC). The remaining samples were taken to the Molecular Biology Laboratory at the Biochemistry, Cell and Molecular Biology Department, University of Ghana. RNA, DNA, and serum were extracted using appropriate molecular techniques and kept at the recommended temperature for further use.

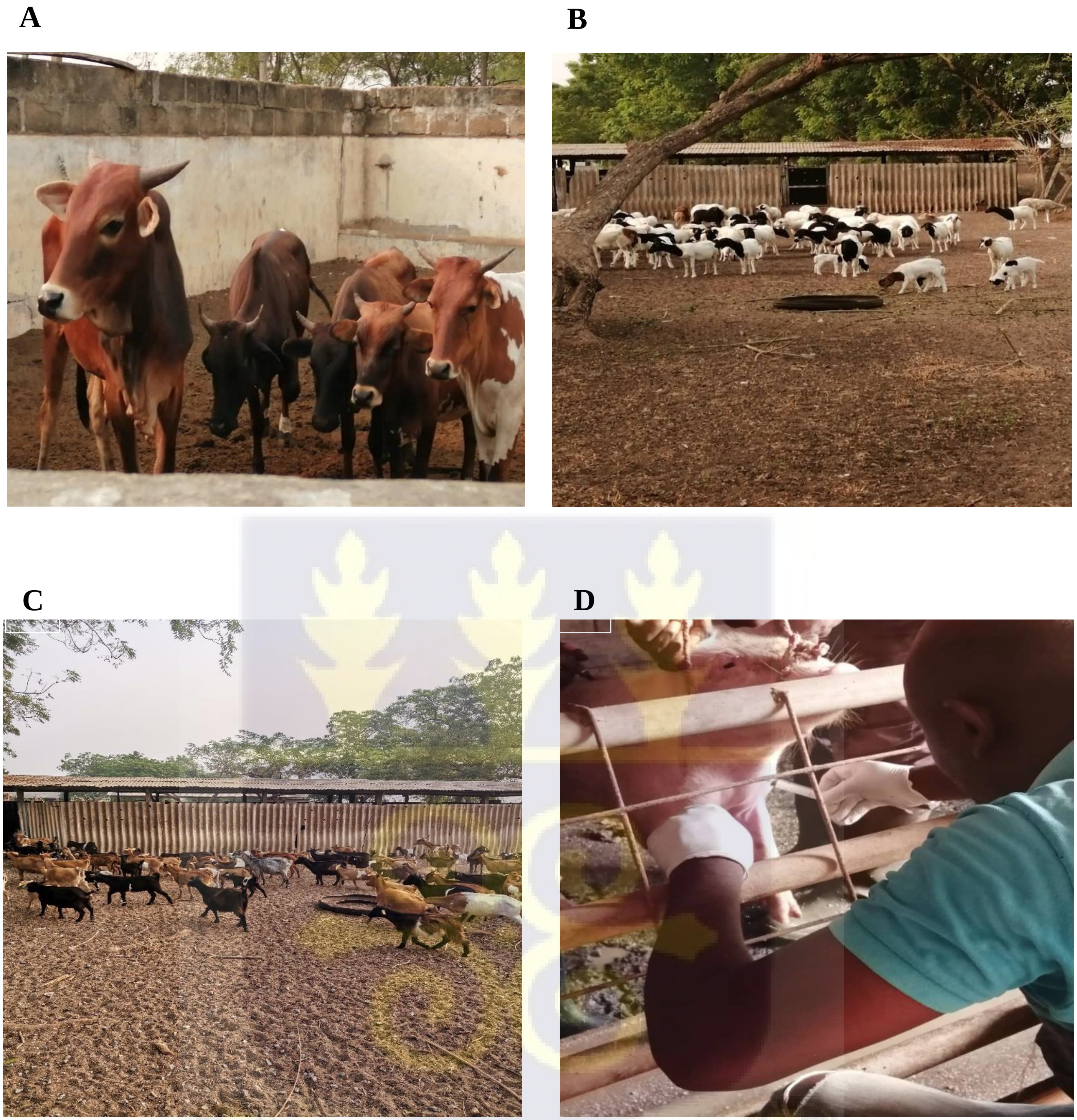


Figure 3.2: **Four livestock species sampled. (A)** cattle showing signs of weight loss; **(B)** sheep breed Nungua black head and the Sahelian breed; **(C)** goats cross breed; **(D)** collection of blood by a veterinarian from the jugular vein of a pig.

3.4 Experimental procedures

3.4.1 Microscopy for the identification of motile parasites in the blood of livestock

Thin films were prepared from a drop of whole blood collected into EDTA tubes, using a Pasteur pipette. These were fixed with 3 drops of absolute methanol and air-dried. After drying, the slides were stained with 30 % Giemsa solution for 20 minutes. The stained slides were washed under running water for 5-10 seconds and placed in a vertical position to air dry. When dried, the slides were viewed under light microscopy at 100X magnification with a single drop of immersion oil.

3.4.2 DNA extraction from the blood of animals

DNA was extracted from the cattle, goats, sheep, and pigs' blood using Qiagen QIAmp DNA blood maxi kit (spin protocol) (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. Briefly, the blood collected in the EDTA tube was used for the DNA extraction. Out of the 3 mL, only 200 µL of the blood was transferred into a 1.5 mL microcentrifuge tube containing 20 µL QIAGEN Protease and mixed. The lysis Buffer AL (200µL) was added, and the mixture was vigorously shacked and incubated at 56℃ for 10 minutes. Then, 70% ethanol was added and mixed thoroughly, and the mixture was transferred onto the QIAamp Mini spin column and centrifuged at 8000 rpm for 1 minute. The flow-through and the collection tube were discarded. The QIAamp Mini spin column was placed in a new 2 mL collection tube, and 500 µL of the Buffer AW1 was added and centrifuged at 8000 rpm for 1 minute. The flow-through and the collection were discarded, the QIA amp Mini spin column was placed into a new 2 mL collection tube, and 500 µL of Buffer AW2 was added, then centrifuged at full speed for 3 minutes. The flow-through and the collection tube were discarded. Finally, for the elution step, the QIA amp spin column was placed in a new 1.5 mL microcentrifuge, 200 µL Buffer AE was added and incubated at room temperature for 15 minutes and centrifuged at 8000 rpm for 1 minute to elute the DNA.

3.4.3 RNA extraction

RNA was extracted using the PAXgene Blood RNA kit (PreAnalytiX by QIAGEN) according to the manufacturer's recommendations with few modifications. Blood from various animal species (cattle, goats, sheep, and pigs) were collected in Tempus tubes. These tubes contain 6 mL of an RNA stabilising solution which lyses the cells considerably; then 3 mL of the animal blood was directly transferred into the tube and vigorously mixed for 10 seconds. Briefly, the content of the Tempus tube was transferred into a 50 mL falcon tube and 3 mL of PBS was added, bringing the final volume in the falcon tube to 12 mL. Then the falcon tube was vortexed at the maximum speed for 30 seconds, followed by a centrifugation step at 4℃ at 3000g for 30 minutes. After centrifugation, the supernatant was carefully removed, and the tube was left inverted for 2 minutes. To resuspend the RNA pellet, 350 µL of resuspension buffer was added into the tube and briefly vortexed. The sample was then transferred into a 1.5 microcentrifuge tube, and 300 µL of binding buffer and 40 µL of proteinase K were added and mixed for 5 seconds and incubated at 55℃ using a shaker incubator for 10 minutes. The lysate obtained from the previous step was directly pipetted into a PAXgene Shredder Spin column and centrifuged at full speed for 3 minutes. The supernatant was transferred into a new 1.5 microcentrifuge tube, and 350 µL of absolute ethanol was added and vortexed, then moved into a new PAXgene RNA Spin column and centrifuged. Then 350 µL of the wash buffer1 was added into the column and centrifuged; next, 80 µL of DNase I was added onto the column and incubated a room temperature for 15 minutes. Then, 350 µL of the wash buffer1 was pipetted into the column and centrifuged. Next, 500 µL of the wash buffer2 was added into the column and spun. This process was repeated twice. Lastly, 80 µL of the elution buffer was added to the column, incubated for 5 minutes at room temperature, and centrifuged. The pure RNA obtained in a fresh Eppendorf tube was incubated at 65°C for 5 minutes, then immediately stored at -80°C for further use.

3.4.4 Serum separation

Blood samples collected in tubes without anti-coagulant were placed on the bench at room temperature to clot for 4 hours, and serum was separated by centrifugation at 2500 rpm for 15 minutes. The sera obtained were aliquoted into three 1.5 mL Eppendorf tubes for each sample and kept at -80°C until further use.

3.4.5 Conventional PCR primers

This study used different sets of primers for trypanosome identification, RNA trapping, cDNA preparation and amplification of *T. brucei* transferrin receptor (TfR). The first set of primers was nested multiplex primers described by Ofori *et al*., 2022. These primers specifically targeted part of the tubulin gene cluster (Figure 3.3). They were designed using available databases to identify known and unknown trypanosome species by nested PCR (Table 3.2, Table 3.3). These primers are specific, sensitive and cost-effective (Ofori *et al*., 2022). The second set of primers was carefully designed to capture trypanosome RNA mixed with host RNA. A specific trypanosome parasite feature explored was the SL, a sequence of 39 nucleotides ligated to the mature messenger RNA. Therefore, SL primers were designed and synthesised (Eurofins Genomics, company). Also, primers such as oligodT and TfR (ESAG6&7) were used respectively for the cDNA synthesis and to amplify TfR (Table 3.4).

Table 3.2: Primers used for the first round of the PCR reaction

Primer	Primer sequence (5’ to 3’)
Outer forward	GGTGAGTTCTCCGAGGCCCGTG
Outer reverse 1	CCGTGCTCGTCGCTGATCAC
Outer reverse 2	CCGTGCTCATCGCTGATCAC
Outer reverse 3	CCGTGCTCGTCACTGATCAC

Table 3.3: Primers used for the second round of the PCR reaction

Primers	Primer sequences (5’ to 3’)
AD 00523 (Inner Forward)	TTAA CTGATCACYTCCCAGAACTT
TP1 (Inner Reverse)	TCGA GAGGAGGAYGTKGGAGGAGTACTA
TP2 (Inner Reverse)	TCAG GAGGAGGAYGTKGGAGGAGTACTA

Table 3.4: Primers used for cDNA synthesis and to confirm the expression of TfR

Primer	Primer sequence
CT573	GATCTACAGTTTCTGTACTAT
CT739	AACTAACGCTATTATTAGAA
CT865	CCCTTTACAAAATTGAGGATTC
CT866	CATCACTGCATTTTTTGCTTC

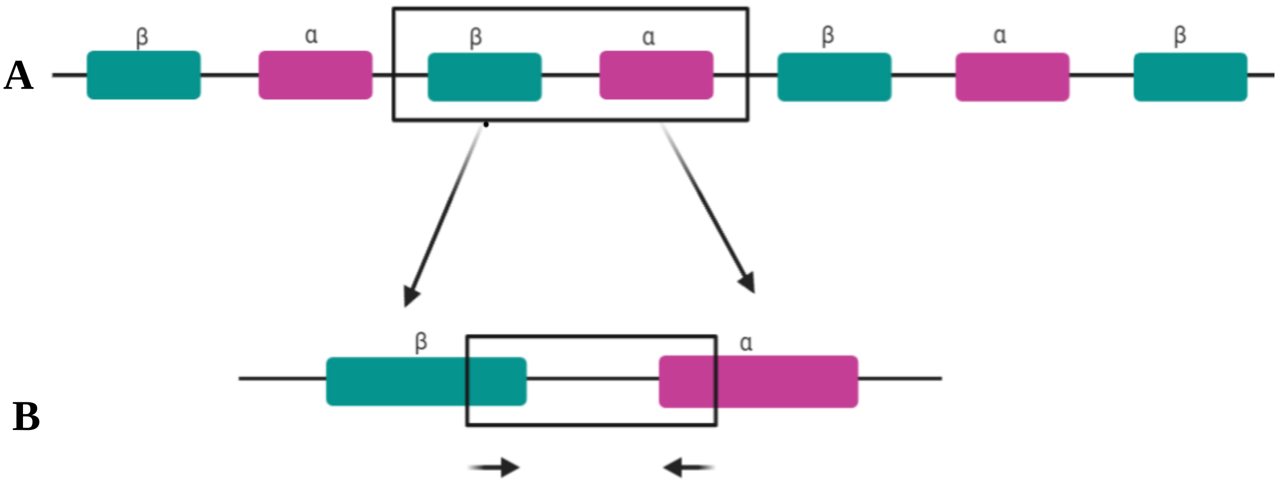


Figure 3.3: Cluster of tubulin genes in *Trypanosoma brucei*. (A) Tubulin genes are organised in tandem repeat; an alternation of alpha and beta genes is observed roughly in the genome. There are about 20 copies. Specifically, one alpha beta repeat is 3800 base pairs. ***(B)*** Segment of the gene amplified by nested PCR. The primers designed simultaneously amplify both parts of alpha and beta tubulin gene from trypanosome species possibly present in various whole blood DNA to be screened. Amplicon size is between 400 – 600 bp, depending on the trypanosome species.

3.4.6 Molecular identification of trypanosome species

Nested PCR was performed to identify trypanosome species. These primers were used to target the alpha and beta subunits of tubulin gene. The PCR master mix was prepared for each experiment, and the final volume was 50 µL. Specifically, 5 µL of unpurified whole blood DNA from various animal species (cattle, goats, sheep, and pigs) was used, and 45 µL of the master reaction was added. The master mixture of the reaction was made using 5X Mango *Taq* non-coloured reaction buffer with 1.5 mM $MgCl_2$, 1mM dNTPs, 1µM outer primers (both forward and reverse), and 1U polymerase Mango *Taq* (Bioline Reagents Ltd, UK). The PCR conditions were the initial denaturation step for 4 minutes at 94°C, subsequently, 30 cycles. For each cycle, 94°C for 40 seconds, 61°C for 40 seconds for the annealing step, 72°C for 45 seconds, the final extension at 72°C for 5 minutes and the cooling at 4°C. For the second round of PCR, the master mix of 50 µL was prepared, and the components were 5 µL of the PCR product of the first reaction and the set of outer primers targeting alpha and beta intergenic segments of tubulin gene were changed and replaced by inner primers (Table 3.6). The PCR conditions used for the second round were not changed. Then, 10 µL of the PCR product from the second round obtained after amplification was run on 1.5 % agarose gel; the voltage was 100 V for 60 minutes. Ethidium bromide was used as the intercalating agent to visualise using ultraviolet (UV) light using GE Healthcare Amersham Imager gel dock (Little Chalfont, UK); identifying trypanosomes was based on the band size of the PCR product. For trypanosome species confirmation, gel extraction was performed, and samples were sent for Sanger sequencing.

3.4.7 Gel extraction

Samples previously identified as trypanosomes positive by conventional PCR were selected for gel extraction. However, this method relied on the band size to determine the trypanosome-infecting species. The primers can identify four trypanosome species (*T. brucei*, *T. congolense*, *T. vivax* and *T. theileri*). Briefly, PCR products of the second round of the PCR of all the positive samples were used for this experiment. A 2% agarose gel was prepared, and ethidium bromide was added as the intercalating agent, then gently mixed and poured into a gel tray. Combs were used to create wells deep enough to load about 37 µL of PCR product for each positive sample, adding 3 µL of the loading dye. The samples were run for 60 minutes, and UV light was used to visualise and facilitate the excision of the bands. A sterile scalpel blade was used to excise the individual band from the agarose gel carefully and transferred directly to a sterile Eppendorf tube. Then, a pipette tip was used to break the gel into small pieces, and 300 µL of Buffer QG was added and vortexed and placed in a water bath set at 50°C for 30 minutes incubation. However, in between, vortexing steps were done after 10 minutes and 20 minutes and the final vortexing was done before adding 100 µL isopropanol, then vortexed again. The mixture was applied to a column and centrifuged for 1 minute at 3000 rpm, then 500 µL of Buffer QC was applied to the column once again and centrifuged for 1 minute at 3000 rpm. Then 750 µL of Buffer PE was pipetted into the column and allowed to rest at room temperature for 5 minutes, then centrifuged for 1 minute at the same speed (3000rmp). Finally, the column was transferred into a fresh Eppendorf sterile tube, and 30 µL of water (appropriate in case samples are intended to be sent for sanger sequencing) was added and incubated for 15 minutes before centrifugation; this step was repeated. Pure DNA was quantified immediately using Nanodrop and kept at -20°C before sequencing.

3.4.8 Sanger sequencing for species confirmation

Nested PCR was performed in order to select samples that were trypanosomes positive. Primers used for the two rounds of PCR targeted the outer and inner parts of the tubulin gene cluster. Relying on PCR alone is not enough to confirm the trypanosome species, although the primers are specific. Therefore, performing sequencing is a critical step, purified DNA obtained after gel extraction was used for Sanger sequencing. The samples were prepared following the instructions of the sequencing facility. Briefly, 10 µL of the DNA sample was mixed with 10 µL of the forward inner tubulin primer (AD00523). The concentration required for the PCR fragment and the primer was 20ng and 10 pmol/µL, respectively. The samples were shipped and sent to the University of Cambridge, where Sanger sequencing was performed. The nucleotide sequences obtained after sequencing were evaluated using BioEdit software (version 7.2), and based on the chromatograph quality, the sequence ends were trimmed before performing the Basic Local Alignment Search Tool (BLAST) using TriTrypDB (https://tritrypdb.org/tritrypdb/app) and NCBI database Nucleotide Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) databases.

3.4.9 Enrichment of *T. brucei* RNA and amplification of the *T. brucei* transferrin receptor

Limited protocols describe how *T. brucei* RNA can be selectively enriched from field samples. There was a need to adapt a working protocol only to enrich trypanosome parasites' RNA. Before processing the field samples, the protocol was optimised using *T. brucei* cells adapted lab strain (GUTat 3.1) added to the blood collected from six uninfected calves. Briefly, 3 mL of blood from each calf collected into Tempus tubes containing 6 mL stabilising reagent was spiked with 1 x 10^5 to 10^0 cells/mL of *T. brucei* cells to mimic natural infections. Then, after adding a known *T.* brucei cell density, RNA was extracted from each tube using the PAXgene Blood RNA kit (PreAnalytiX by QIAGEN), following the manufacturer's recommendations. Next, cDNA was prepared from the various RNA extracted using SuperScript™ III First-Strand Synthesis System (Invitrogen™).

3.4.10 Synthesis of the first strand cDNA (single-stranded cDNA: ss-cDNA)

SuperScript III was used for the cDNA synthesis, the instructions given by the manufacturer were followed with few modifications. Briefly, for the first step, 6 µL of total RNA was mixed with 1 µL of Oligo $d(T)_{23}$, random primer-mix and RNAse-out the mixture was incubated at 65℃ for 10 minutes and spun briefly then snapped on ice for 5 minutes. Next, to the mixture were added 4 µL of RT buffer, 2 µL of $MgCl_2$, DTT, SuperScript III RT and 1 µL of dNTPs and incubated at 50℃ for 50 minutes, then at 85℃ for 5 minutes and complete denaturation of the template RNA was done by adding 1µL RNase H (2U) and incubated at 37℃ for 20 minutes. The cDNA obtained was purified using Zymo DNA clean and concentrator described by the manufacturer. However, a slight modification was done for the final step; for the elution, 12 µL of nuclease-free water was used instead of the elution buffer provided to obtain a pure cDNA.

3.4.11 Synthesis of the double-stranded cDNA (ds cDNA)

The purified cDNA was used to synthesise a double-stranded cDNA, and the parasite's mRNA enrichment was performed at this stage. The cDNA obtained in the previous step was still a mixture of host and trypanosome parasites cDNA. Trypanosome-specific RNA was enriched by PCR using SL primers. Previous studies (Cuypers *et al.*, 2017; Mulindwa *et al.*, 2014) have shown that using SL to enrich trypanosome mRNA can be useful. The set-up for PCR was the following, 12 µL of pure cDNA mix and 38 µL of the master mix containing (5X Mango *Taq* noncolored reaction buffer, 1.5 mM $MgCl_2$, 1mM dNTPs, 1µM SL forward primer and 1U polymerase Mango *Taq* (Bioline Reagents Ltd, UK). The thermo-cycler was programmed with the following conditions: 98°C for 20 seconds, 95°C for 1 minute, 50°C for 60 minutes and 72°C for 60 minutes. Next, the ds cDNA was amplified briefly; to the second strand cDNA synthesis mix, 1 µL of each component was added, SL reverse primer, MangoTaq polymerase and SL forward primer. The following program was used for 10 cycles 95°C for 30 seconds, 39°C for 30 seconds and 68°C for 2 minutes, with a final extension at 68°C for 10 minutes. The ds cDNA was purified using a QIAquick PCR purification kit (Qiagen); then elution was done in 30 µL of EB buffer.

3.4.12 Amplification of the variable region of *T. brucei* transferrin receptor and next generation sequencing (NGS)

A study by Trevor and colleagues suggested that trypanosome parasites express a single transferrin receptor (TfR) to bind a variety of host transferrin (Trevor *et al.*, 2019). However, the study was conducted using *in vitro* systems. For this current work, only the variable regions of TfR were amplified from confirmed trypanosome-positive field samples. Briefly, pure *T. brucei* ds cDNA obtained in the previous step was used to amplify TfR; the primers used in this study targeted a portion of ESAG6/7 genes. The PCR components were 5 µL of pure ds cDNA used as a template and 45 µL of the master mix containing (5X Mango *Taq* noncolored reaction buffer, 1.5 mM $MgCl_2$, 1mM dNTPs, 1µM outer primers (CT865 and CT866), and 1 U polymerase Mango *Taq* (Bioline Reagents Ltd, UK). The PCR conditions were as follows: initial denaturation at 95°C for 4 minutes, and subsequent 25 cycles. For each cycle, denaturation was done at 95 °C for 1 minute, the annealing step at 58°C for 1 minute, then the extension was done at 72°C for 2 minutes, followed by a final extension at 72°C for 10 minutes, and the cooling was done at 4°C. Subsequently, 10 µL of the amplicon was resolved on 1.5 % agarose gel at 100 V for 60 minutes. Ethidium bromide was used as the intercalating agent to enable the visualisation by ultraviolet (UV) light using GE Healthcare Amersham Imager gel dock (Little Chalfont, UK); to confirm that TfR was successfully amplified. Then, the next step was to load the rest of the 40 µL, the band was cut out, and gel extraction was performed. Then, the products obtained were sequenced on the Illumina HiSeq platform by BGI technologies (Beijing, China).

3.4.13 Analyses of NGS data

The paired-end reads generated were aligned to the *T. brucei* clone 427_2018 reference genome v46 downloaded from TriTrypDB, using Bowtie2 as previously described by Langmead an Salzberg 2012, with the 'very-sensitive-local', MAPQ≥10 and pre-set alignment option. The alignments were converted to BAM format, reference sorted and indexed with Samtools as previously described by Li *et al*., 2009. Fragment counts were determined from the BAM files using featureCounts (Liao, Smyth, & Shi, 2014) with the following setting: -p (pair-end) -B (both ends successfully aligned) -C (skip fragments that have their two ends aligned to different chromosome) -t transcript (count level) -g gene_id (summarization level). Counts were normalized by dividing the number of counts in each sample by the total number of reads, and genes with a count of less than 5 were excluded. The data were then normalized using the maximum value and clustered by rows and columns using the seaborn Python package (Waskom, 2021). Furthermore, to quantify the count frequency of the first variable region of ESAG7 that binds the TfR (Cordon-Obras *et al*., 2013). "TTGGGTGCGGAAAGTATT(. *?)TGTAACCTAATGC" a specific regular expression was used in order to differentiate ESAG7 and ESAG6. The relevant reads were extracted from the RNA-seq FASTQ files using a custom Python script and counts were performed. To normalize the counts, the number of counts in each sample was divided by the total number of reads, and target sequences with a count of less than 2000 were excluded. Data were then normalized by raw using the z-score and clustered by rows and columns using the seaborn Python package (Waskom, 2021). The DNA sequences were converted to amino acid sequences using the Biopython python package (Cock *et al*., 2009).

3.4.14 Cell blood count, haematocrit determination and measurement of key parameters to determine the iron status of trypanosome-infected animals

3.4.14.1 Complete cell blood count and haematocrit determination

For this part of the project, only blood collected during the second round of sample collection was used. Briefly, blood was collected in EDTA tubes and mixed gently to run a complete cell blood count (CBC) using the URIT-5240Vet machine. The results were printed-out and analysed by a well-trained technician, and reports obtained were used to determine parameters such as (haemoglobin haematocrit, number of red blood cells and others).

3.4.14.2 Determination of the total iron binding capacity (TIBC) in infected and non-infected livestock

TIBC, the capacity of transferrin to bind iron, was measured in the trypanosome-infected and non-infected animals' serum. The kit used to perform this assay was Total Iron-Binding Capacity (TIBC) and Serum Iron Assay Kit (Colorimetric) (Sigma-Aldrich). TIBC was determined by a colorimetric method, and the absorbance was measured at 570 nm for standards and samples according to the manufacturer's recommendations. The following equation was used to determine the actual concentration of TIBC in each sample:

$$\text{TIBC } (\mu\text{mol iron/L}) = \frac{X}{V} \times \text{D} \times 10^3$$

where:

X = TIBC iron amount from a standard curve (nmol)

D = Sample dilution factor (if applicable; D = 1 for undiluted samples)

10^3 = Conversion factor mL to L

V = Volume of Serum Sample (μL)

3.4.14.3 Measurement of total serum iron (TSI) in infected and non-infected livestock

To determine the effect of trypanosome parasites on the circulating iron in infected animals, the TSI in the serum of some animals was measured using the same kit used to measure TIBC. TSI was measured by a spectrophotometric assay and calculated with the following equation:

$$\text{Total serum iron } (\mu\text{mol iron/L}) = \frac{Y}{V} \times \text{D} \times 10^3$$

where:

Y = Serum Iron amount from the standard curve (nmol)

D = Sample dilution factor (if applicable; D = 1 for undiluted samples)

10^3 = Conversion factor mL to L

V = Volume of Serum Sample (μL)

3.4.14.4 Determination of transferrin saturation (TS) in infected and non-infected livestock

Transferrin saturation (TS), which measures how much iron in the blood is bound to transferrin, was expressed as a percentage and determined with the formula below:

$$\% \text{ Transferrin Saturation} = \frac{\text{Total serum iron } (\mu\text{mol iron/L})}{\text{Total iron binding capacity } (\mu\text{mol iron/L})} \text{ x } 100$$

3.4.14.5 Determination of the serum ferritin concentration in infected and non-infected livestock

Ferritin concentration was measured in cattle, goats, and sheep serum by competitive and sandwich ELISA. Competitive ELISA was used for goats and cattle using Goat H-ferritin (FTH) and Bovine Ferritin (FE) ELISA Kits, respectively. However, ferritin was measured in sheep serum by two-site sandwich ELISA using Sheep Ferritin heavy chain (FTH1). The ELISA Kits were purchased from MyBioSource, and assays were performed according to the manufacturer's instructions. To quantify ferritin in goats and cattle, 100 µL of standards and samples (each sample was run in duplicate) were added to each well of a microtiter plate coated with anti-ferritin antibodies PBS (pH 7.0-7.2) was used at the blank control well; 50 µL of the conjugate was dispensed to each well except the blank control well and mixed gently before incubation at 37°C for 1 hour Then the microtiter plate was washed with the 1x washing solution and inverted and blot dried by hitting the plate onto absorbent paper. Subsequently, 50 µL of substrate A and 50 µL of substrate B were added to each well, including the blank control well; the microtiter plate was covered and incubated at 37°C for 20 minutes. Next, 50 µL of stop solution was added to all the wells and mixed several times. The optical density (OD) was immediately determined at 450 nm using a microplate reader

(Thermo Scientific Varioskan LUX). Results obtained from the standards were used to plot a standard curve to calculate ferritin concentration in the samples tested.

For sheep samples, the procedure was a little different; the principle of the FTH1 ELISA Kit is sandwich ELISA as previously indicated. Briefly, 100 µL of standards and samples (in duplicate) was added to the wells covered with adhesive films and incubated at 37°C for 2 hours. Each well was aspirated and washed. The process was repeated 3 times; the plate was inverted and blotted against a clean paper. Then, 100 µL of biotin-conjugate (1x) was added to each well, covered and incubated at 37°C for 1 hour. The plate was washed and dried, 100 µL of streptavidin-HRP (1x) was added to each well, and the microtiter was covered with the adhesive films and incubated at 37°C for 1 hour. Subsequently, washing was performed, the plate was dried, then 100 µL of the substrate solution was added to all the wells and incubated at 37°C for 20 minutes. Finally, 50 µL of the stop solution was added to each well, the OD was determined within 5 minutes using a microplate reader set at 450 nm. Standards were used to plot the standard curve, and ferritin concentration in sheep serum was measured.

3.4.15 Determination of the influence of the VSG structure on nutrient uptake

3.4.15.1 Determination of the growth rate of dimeric and trimeric VSG cell lines

T. brucei brucei Lister 427 (dimeric and trimeric VSGs) cell lines were grown at 37°C with 5% CO_2 in HMI-9 medium supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). All the different characteristics of the cell lines used are summarized in Table 3.5. The following drugs were added to the cultured cell lines for selection: 5 µg/mL G418 with 10 µg/mL blasticidin (VSG2, VSG3 and VSG615) or with 2.5 µg/mL phleomycin, 0.2 µg/mL puromycin and 5 µg/ml hygromycin (VSG1.8, VSG11 and VSG224 or

VSG3$^{\mathbf{parental}}$). Only 5 µg/mL hygromycin and 2.5 µg/mL phleomycin were used to select metacyclic VSG1954 (mVSG1954) and finally, 5 µg/mL G418 with 10 µg/mL blasticidin and 0.2 µg/mL puromycin for E7 GPI clones. For most experiments performed, cells were harvested during the exponential phase (~0.5-1.5 x 10^6 cells/mL). To determine the growth rate of the various cell lines and draw growth curves, cells were diluted to 1 x 10^5 cells/mL, counted every 24 hours and diluted back to 1 x 10^5 cells/mL for 4 days.

Table 3. 5: Characteristics of the different cell lines

MITat	VSG	Class	Local names	Copies	BES	MW (kDa)	NTD	AA
1.2	VSG2	Dimer	VSG221	1	1	51.0	N2	476
1.3	VSG3	Trimeric	VSG224	2	7	53.8	N4	509
1.11	VSG 11	Trimeric	VSGbR2	2-3	15	70.0	N4	518
1.8	VSG 1.8	Trimeric	VSGOD1	2	12	56.5	N4	466
M1.615	VSG615	Trimeric	NA	NA	NA	51.2	N4	480
M1.1954	VSG1954	Trimeric	NA	NA	NA	53.9	N4	521

MITat: Monomorphic strain; VSG: variant surface glycoprotein; NTD: N-terminal domain; AA: Amino acid; MW: Molecular weight; BES: Bloodstream expression site, NA: Not available.

3.4.15.2 Determination of the receptor-mediated endocytosis and transferrin uptake by fluorescence-activated cell sorting (FACS)

Cells were harvested (1 x 10^6) for all the cell lines described above and washed 3 times with HMI-9 medium containing 0.5 mg/mL bovine serum albumin (BSA). Following that, cells were resuspended in HMI-9/BSA and incubated at 37°C for 40 minutes. Following, 10 µg/mL of Alexa6467 conjugated Tf (Tf-467) from Human Serum (Invitrogen$^{\text{TM}}$) or 20 µg/mL Dylight 594-conjugated tomato lectin (TL-467) (Invitrogen$^{\text{TM}}$) were added, and incubation performed at 37°C for 45 minutes. Finally, cells were washed once using 1 x HEPES-buffered saline with glucose (HBSG), resuspended in 1 mL 1 x HBSG, transferred in a sterile tube and analysed with BD Fortessa II with excitation wavelength 650 nm and emission detection at 665 nm. Data analysis was performed using FlowJo v10.8.1 Software (BD Lifesciences).

3.4.15.3 Generation of parasites having TfR with two GPI anchors

The plasmid construct for ESAG7-GPI (E7$^{\text{GPI}}$, making an E7 expressing a GPI-anchored fusion of E6 C-terminus to C-terminus of E7) is described here (Tiengwe *et al*., 2017). Linearization was done using ClaI and FeSI with CutSmart as the buffer; then ethanol precipitation was performed. Briefly, for the ethanol precipitation, 295 µL of the linearized plasmid was used and 3 volumes of absolute ethanol added, the mixture was frozen for 60 minutes at -20°C. Centrifugation was done at 4°C for 25 minutes, and the supernatant was gently removed, and 1 mL of 70% ethanol added and mixed by inversion and centrifuged at 4°C for 5 minutes. Finally, the supernatant was removed, and the plasmid-DNA was air-dried for 15 minutes and resuspended in 30 µL of sterile water.

The cells used for the transfection were in total 5 x 10^7; after harvesting the cells, they were resuspended in 100 µL of transfection buffer (Roditi Tb-BSF). Then, 20 µg of the linearized plasmid was added to the mixture and transferred gently into the transfection cuvette, and electroporation was done using Amaxa Nucleofector™ 2b (Lonza) set to the program X00-1. Cells were gently removed and transferred into a culture flask containing 25 mL of warmed HMI-9 medium supplemented with 10% FBS without antibiotics; cells were allowed to recover for 6 hours, at 37°C with 5% CO_2. After 6 hours the appropriate drugs were added for clone selection and then plated into 24 well plates with 1 mL media per well and grown at 37°C, 5% CO_2 for 7 days before clone selection. The screening for transfected clones started on day 7 post-transfection, wells with noticeable cell growth were marked and checked closely till the density was considerably high. Then, the clones were transferred into 6 well plates, containing 5 mL of HMI-9 medium per well with the selected drugs. The obtained clones were grown, and then regularly diluted to be used for further experiments after appropriate validation.

3.4.15.4 Western blotting (anti-VSG and anti-TfR)

Cells were harvested (5 x 10^6) and washed once with 1 x phosphate-buffered saline (PBS). Then the same volume of SDS loading buffer and PBS were added to the cells and heated using a heating block set at 100°C for 10 minutes. Protein separation was performed by SDS-PAGE and Trans-Blot Turbo Transfer system (Bio-Rad) was used for the transfer onto PVDF membranes. Skimmed milk (5%) was prepared using PBST to block the membranes; this was done at 4°C overnight. The following day, membranes were incubated at room temperature for 60 minutes with the primary antibody. These were the antibodies used: anti-VSG221 (rabbit, 1:10000), and anti-TfR (rabbit, 1:2000). The various antibodies were diluted in 5% skimmed milk in PBST. After incubation, the membranes were washed with PBST 3 times for 1 minute, 2 times for 5 minutes, and 1 time for 10 minutes. Then, the membranes were incubated with the secondary antibody, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:5000) for 1 hour. Then, the washing step was carried out as described previously; however, the only difference was that for the last step, the time of the final washing was doubled. The SuperSignal West Pico Plus chemiluminescent substrate (Thermo Fisher Scientific) was prepared and immediately added to the top of the membranes just before visualization. Imaging was performed with the ChemicDoc XRS+ Imaging system (Bio-Rad) and the images were saved in a specific folder. Finally, the membranes were incubated with Ponceau S staining solution (Thermo Fisher Scientific) for 15 minutes for protein quantification and normalisation. The membranes were immediately imaged, using the same equipment and the program used was the Coomassie Fluor Orange.

3.4.15.5 Relative quantifications of TfR and VSG transcripts by Real-Time PCR (qRT-PCR)

Cells were harvested (1 x 10^7) from the various cell lines and RNA was extracted using the RNeasy Plus MiniKit (Qiagen). RNA concentration was determined with the Nanodrop, then diluted before performing qRT-PCR using the Luna Universal One-Step RT-qPCR Kit (New England Biolabs). The experiments were conducted following the manufacturer's recommendations. The various primers used are found in Table 3.6. All the various reactions were performed in triplicate, with technical and biological replicates. The comparative C_T ($\Delta\Delta C_T$) method was used to determine the fold change with ZFP3 as the endogenous gene for control and normalization.

Table 3.6: Primers used for qPCR

Primer	**Sequence (5’ to 3’)**
TbZFP3 F	CAGGGGAAACGCAAAACTAA
TbZFP3 R	TGTCACCCCAACTGCATTCT
ESAG6 F	AACAGATACTGACGGTGTATTGG
ESAG6 R	CCACCCTCAACGTACATACTTC
ESAG7 F	GACGGAGGTTTGCTGAAAGATA
ESAG7 R	GTGAGAACTGACATCACCGTATT
VSG2 F	CGCTGAAAGCCAACAACAAG
VSG2 R	CCGCATCGTTATGCCATTTG
VSG3 F	TTTCAATCGGACCCTACTTGG
VSG3 R	CCCTGTTTCTGAGTTCCTCTG

3.4.15.6 Amplification of TfR, sanger sequencing and data analysis

RNA previously extracted from the different cell lines was also used for cDNA synthesis. Briefly, cDNA was synthesized from 50 ng of RNA using iScript cDNA Synthesis Kit (Bio-Rad). The cDNA newly synthesized was used to perform conventional PCR to amplify TfR (ESAG6 and ESAG7). PCR products were run in 1.5 % agarose gel at 100 V for 45 minutes to confirm that TfR was successfully amplified. Then, gel extraction was performed using QIAquick Gel Extraction Kit (Qiagen) following the manufacturer’s instructions. The purified PCR products obtained were sent for sanger sequencing (Eurofins Genomics, UK). Samples were premixed with the forward and reverse primer in separate tubes; the final volume was 17 µL and the concentration was 20 ng.

The AB1 files obtained from the sequencing facility were used for sequence analysis. Benchling and FinchTV chromatogram viewer were used to view and check the sequence quality. Furthermore, FinchTV was also used to edit and clean the sequences before base calling. TriTrypDB was used to run a blastn to confirm that sanger sequences were indeed for TfR genes and to download the reference gene (Tb427.BES40.2). Then, Ugene-Unipro and Benchling were used to perform multiple sequence alignments using both the forward and reverse sequences of each sample.

3.5 Data analysis

GraphPad® Prism version 8.1 (GraphPad® Software Inc., California, USA) and RStudio version 4.13 software were used to analyse the data and to generate the various figures. Comparison between groups was done using either a t-test or Wilcoxon test, depending on the distribution of the data. To compare more than two groups, Anova or Kruskal Wallis was used, followed by Dunn test or Bonferroni as post-hoc tests. *P-value* < 0.05 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Molecular identification of trypanosome in livestock

To identify trypanosome species infecting the livestock, blood samples were collected from 456 animals (155 cattle, 138 sheep, 132 goats, and 31 pigs) at six study sites in the Greater Accra, a region previously reported to have low tsetse flies' density. Throughout the time that different livestock were screened, AAT outbreak was observed once at the Ashaley-Botwe. However, at one of the study sites (Katamanso), farmers reported symptoms observed in a specific group of sheep like those experienced during the acute form of AAT; unfortunately, this was not verified.

The approach was to bleed the livestock listed above; purified DNA was obtained from the blood and used to identify infecting trypanosome species by performing nested PCR targeting the intergenic region between the alpha and beta tubulin genes (Ofori *et al*., 2022). The representative agarose gels showing the identification of trypanosomes by PCR in sheep and cattle are shown in Figure 4.1 and Figure 4.2, respectively.

Trypanosomes were identified based on the band sizes of the products: *T. brucei* (424 bp), *T. congolense* (456 bp), *T. vivax* (586 bp) and *T. theileri* (646 bp).

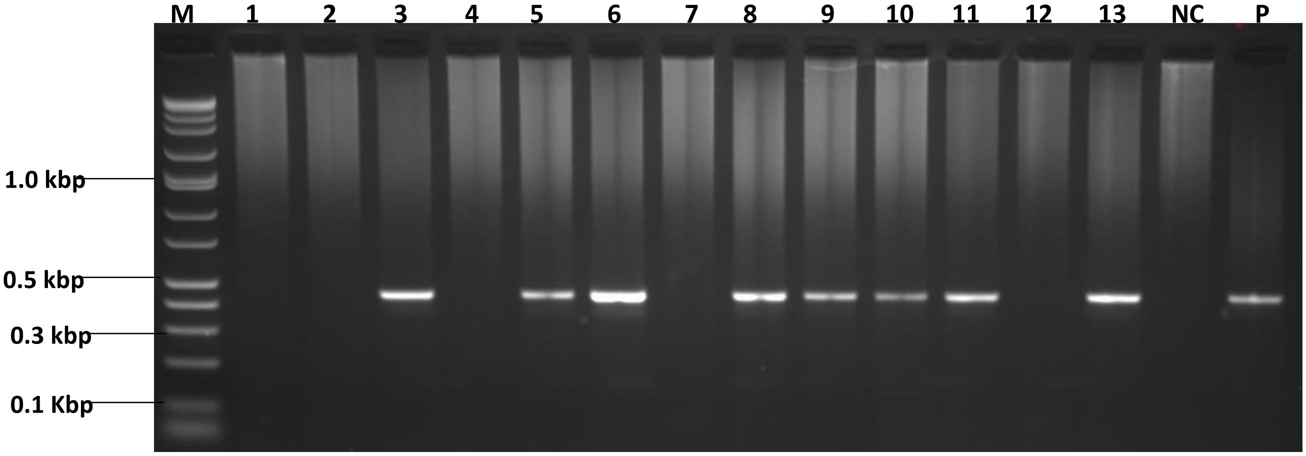


Figure 4.1: Identification of trypanosomes in the blood sheep livestock. An agarose gel picture shows the bands used to identify the *trypanosome spp* in each sheep. M: marker, P: positive control NC-: PCR H2O and 1-13: sheep IDs. Expected band sizes by nested PCR were *T. brucei*: 424 bp, *T. congolense*: 456 bp and *T. vivax*: 586 bp.

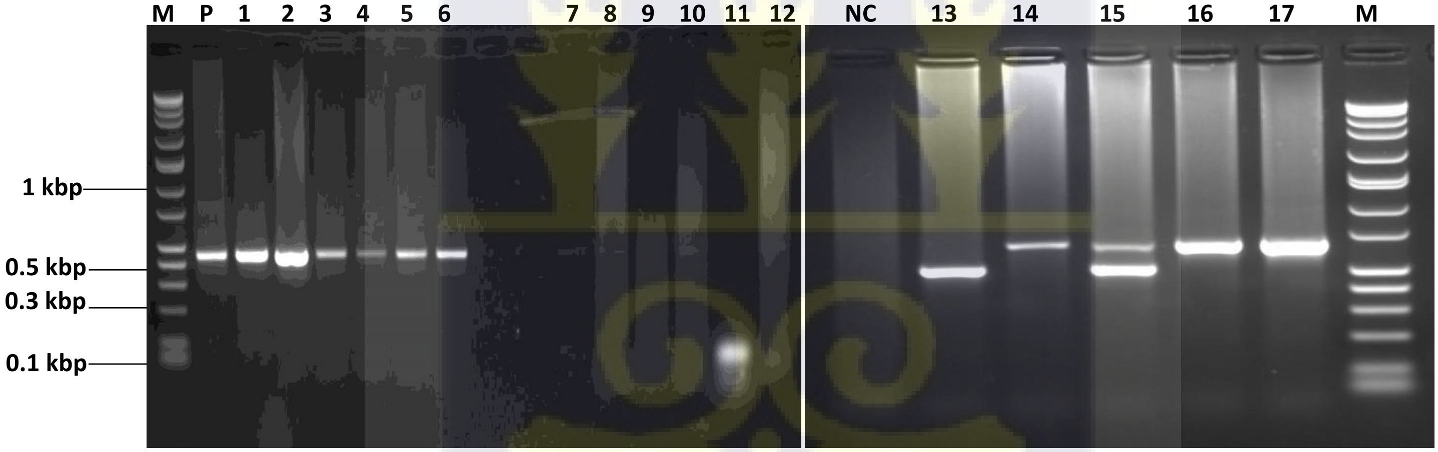


Figure 4.2: Identification of trypanosomes in cattle. An agarose gel picture shows the bands used to identify the *trypanosome spp* in each cow. M: marker, P: positive control NC-: PCR H2O and 1-17: cattle IDs. Expected band sizes by nested PCR were *T. brucei*: 424 bp, *T. congolense*: 456 bp and *T. vivax*: 586 bp.

4.2 The prevalence of trypanosome infections in livestock

The prevalence of AAT in livestock in GA and the proportions of single and mixed infections are shown in Figures 4.3A and 4.3B, respectively. The prevalence of trypanosome infections was 73/456 (16%), and most animals were infected with a single trypanosome species 71/73 (97%). 2/73 (3%) animals were infected with more than one trypanosome species or mixed infections. The proportion of infections in female animals was 54/326 (16.6%) and in males 19/130 (14.6%), the difference observed was not statistically significant (*p-value* > 0.05). Trypanosomes were detected in all four livestock; the proportion of the infection in each livestock species and the species identified are described in Table 4.1. Sheep were the most infected livestock, with 41/138 (29.7%), followed by cattle with 26/155 (16.8%). The lowest infection proportions were observed in goats and pigs, with 5/132 (3.8%) and 1/31 (3.2%), respectively.

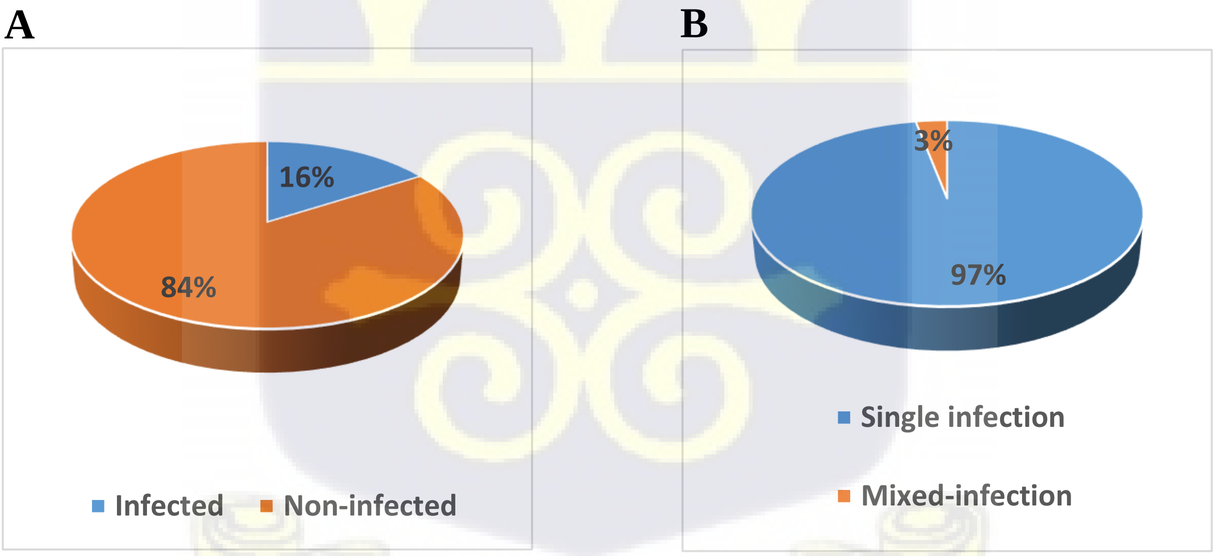


Figure 4.3: Trypanosome infections in livestock. (A) Prevalence of trypanosome infections in livestock. **(B)** Proportions of single and mixed infections. Sample size (n=456).

Table 4.1: Trypanosome species identified in livestock (percentages are shown in brackets)

Livestock	*T. brucei*	*T. theileri*	*T. congolense*	Mixed infection	Total infection
Cattle	21(80.7)	3(11.5)	1(3.9)	1(3.9)	26(16.8)
Sheep	26(63.4)	13(31.7)	2(4.9)	0	41(29.7)
Goats	3(60)	0	1(20)	1(20)	5(3.8)
Pigs	0	0	1(100)	0	1(3.2)
Total	50(68.5)	16(21.9)	5(6.8)	2(2.8)	73(16)

* Mixed infection = *T. congolense* + *T. brucei*

4.2.1 Trypanosomes identified in sheep

For sheep, all 138 (30 males and 108 females) animals sampled during the two rounds of sample collection were screened, and trypanosome species were identified. The analysis of the various gel electrophoresis coupled with Sanger sequencing showed that sheep were infected with the three most common pathogenic trypanosomes (*T. brucei*, *T. theileri* and *T. congolense*). Out of the 138 sheep, 41 (29.7%) were trypanosome positive. Of the infected sheep, *T. brucei* accounted for 73% (26/41), *T. theileri* for 32% (13/41) and *T. congolense* for 5% (2/41). Of the 108 female animals, 29 (27%) were infected, while out of the 30 males 12 (40%) were infected. However, the difference observed was not statistically significant (*p-value* = 0.18). Female sheep were infected with all three trypanosomes (*T. brucei* = 17, *T. theileri* = 10 and *T. congolense* = 2). However, male animals were only infected with two trypanosome species (*T. brucei* = 9 and *T. theileri* = 3). Of the 93 blackhead sheep 26 (28%) were infected, and for the crossbreed 15 out 45 (33%) were infected, the difference observed was not statistically significant (*p-value* = 0.4). The summary of trypanosomes identified and other details about the infected sheep are shown in Figures 4.4A – D.

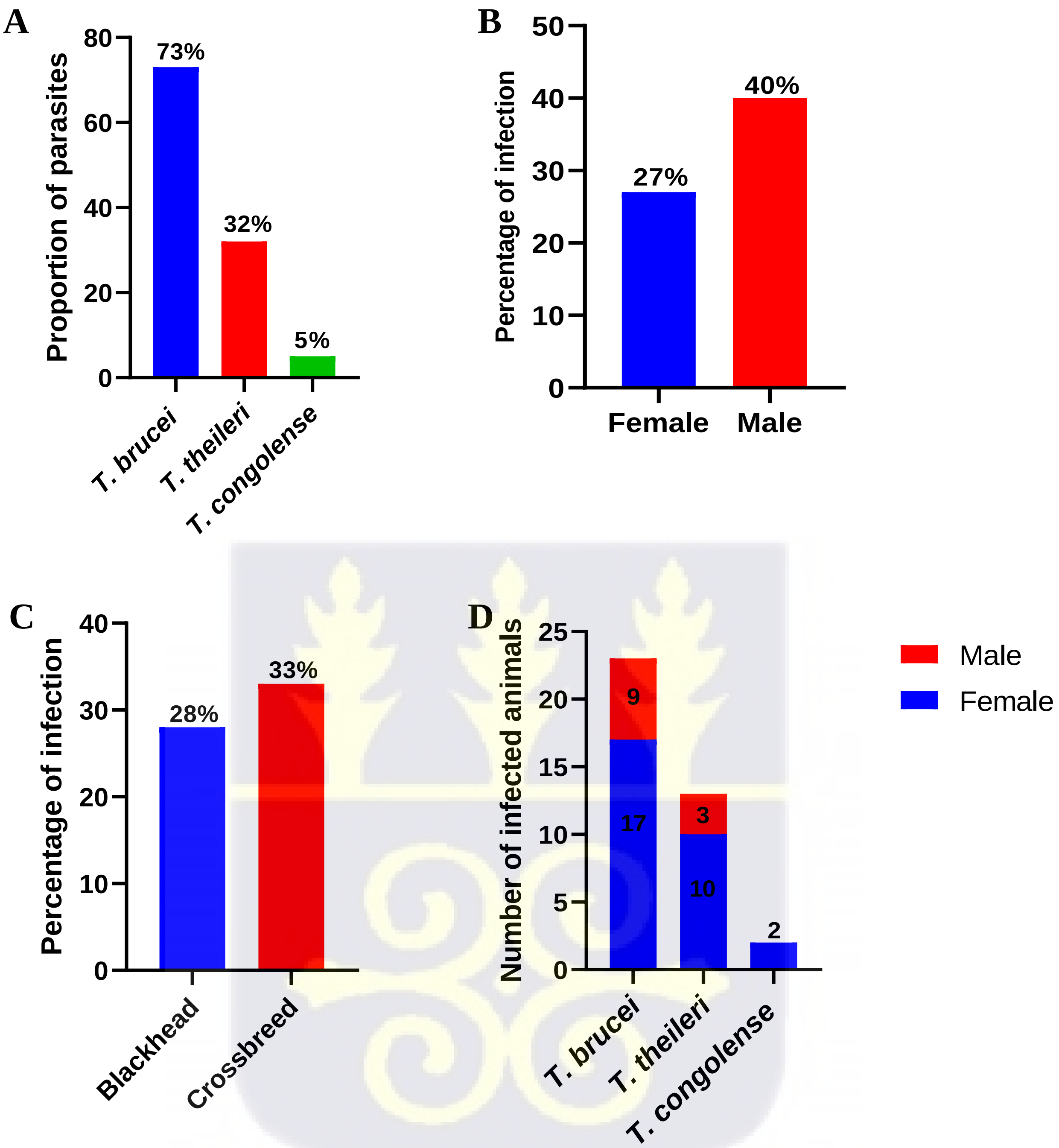


Figure4.4: Description of trypanosome infections in sheep. (A) Trypanosome species identified in sheep livestock. (B) Trypanosome infections per sex. **(C)** Distribution of trypanosome infections per the breed. **(D)** Distribution of trypanosome species per the sex of infected sheep. Sample size (n=138).

4.2.2 Trypanosomes identified in cattle

In total, 155 (40 males and 115 females) cattle were sampled, and screened to identify trypanosomes. Representative gel electrophoresis showing trypanosome species was previously shown in Figure 4.2. The three main trypanosome species infecting sheep were also the same as those infecting cattle. Out of the 155 cattle, 26 (16.8 %) were trypanosome positive and *T. brucei* accounted for 81% (21/26), *T. theileri* for 11% (3/26), *T. congolense* for 4% (1/26) and mixed infection of *T. congolense* and *T. brucei* for (4%) 1/26. Of the 40 males, 6 (15%) were infected and of the 115 female animals, 20 (17.4 %) were infected but the difference observed was not significant (*p-value* = 0.72). Female cattle were infected with all three trypanosomes identified, and the mixed infection was observed in a female animal (*T. brucei* = 15, *T. theileri* = 3, *T. congolense* = 1 and mixed infection of *T. congolense* and *T. brucei* = 1). However, male animals were only infected with one trypanosome species (*T. brucei* = 6). Of the infected cattle, 40% (12/30) were N'Dama breed, 22.5% (9/40) were Sanga breed and Sokoto breed 11% (5/45). The difference observed between the various breed infected was not statistically different. The summary of trypanosomes identified and other details about the infected cattle are shown in Figures 4.5A – D.

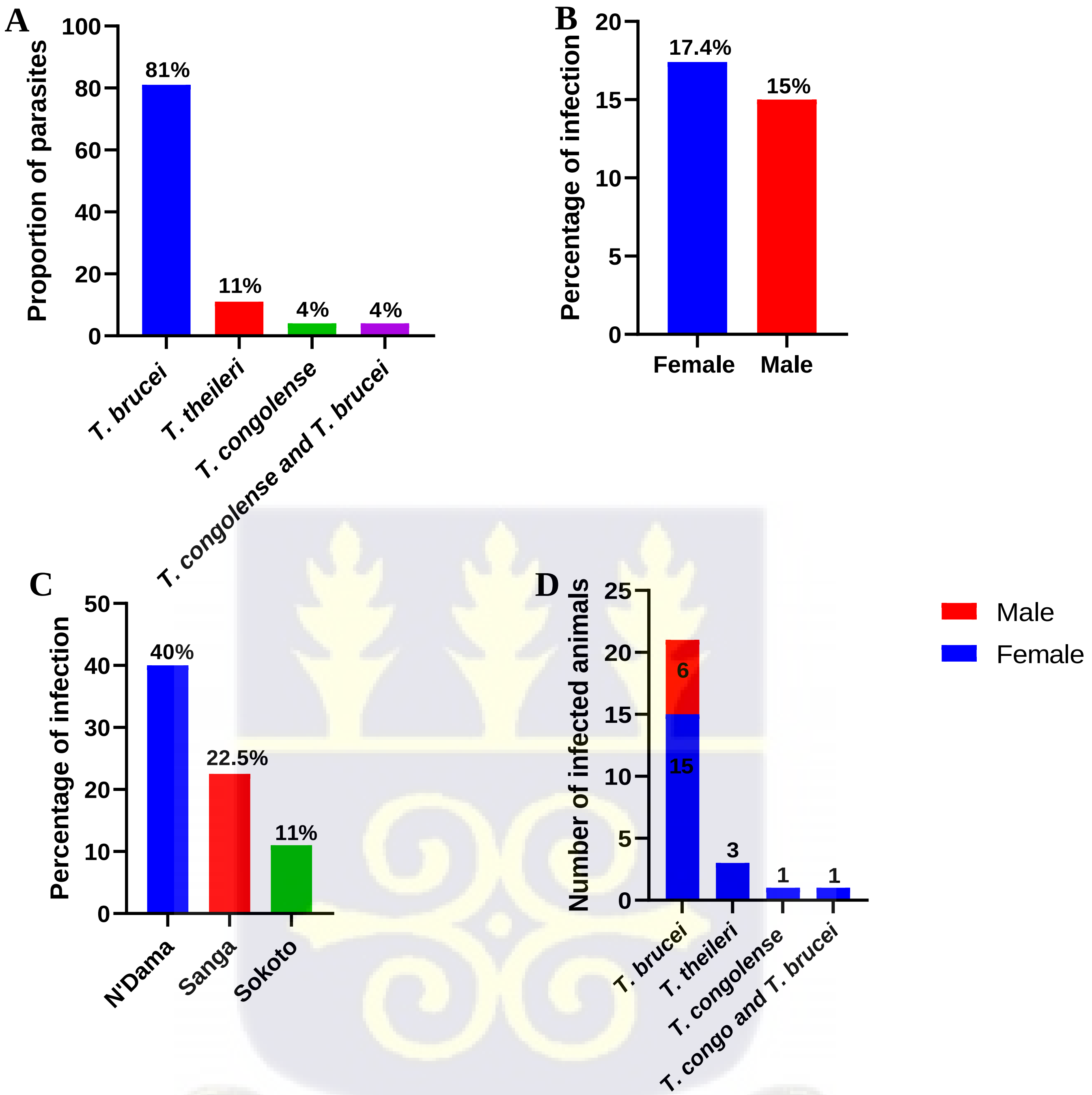


Figure 4.5: Description of trypanosome infections in cattle. (A) Trypanosome species identified in cattle livestock. **(B)** Trypanosome infections per gender. **(C)** Distribution of trypanosome infections per the breed. **(D)** Distribution of trypanosome species per the sex of infected cattle. Sample size (n=155).

4.2.3 Trypanosomes identified in goats

In total, 132 (46 males and 86 females) goats were sampled, and screened to identify trypanosomes. Only two trypanosome species were identified in goats. Of the 132 goats, 5 (3.8%) were trypanosome positive. Of the infected, *T. brucei* encounted for 60% (3/5), *T. congolense* for 20% (1/5) and mixed infection of *T. congolense* and *T. brucei* for 20% (1/5) (Figure 4.6A). Of the goats infected, 2% (1/46) was infected while 5% (4/86) females were found to test positive for trypanosomes. Female goats were infected with *T. brucei* and *T. congolense*. Mixed infections with *T. congolense* and *T. brucei* was observed in only male goats infected (Figure 4.6B). The infection observed in the different breeds were, 3% (2/66) short-legged, 10% (2/20) long-legged and 4.3% (1/46) crossbreed (Figure 4.6C). The summary of trypanosomes identified and other details about the infected goats are shown in Figures 4.6A – D.

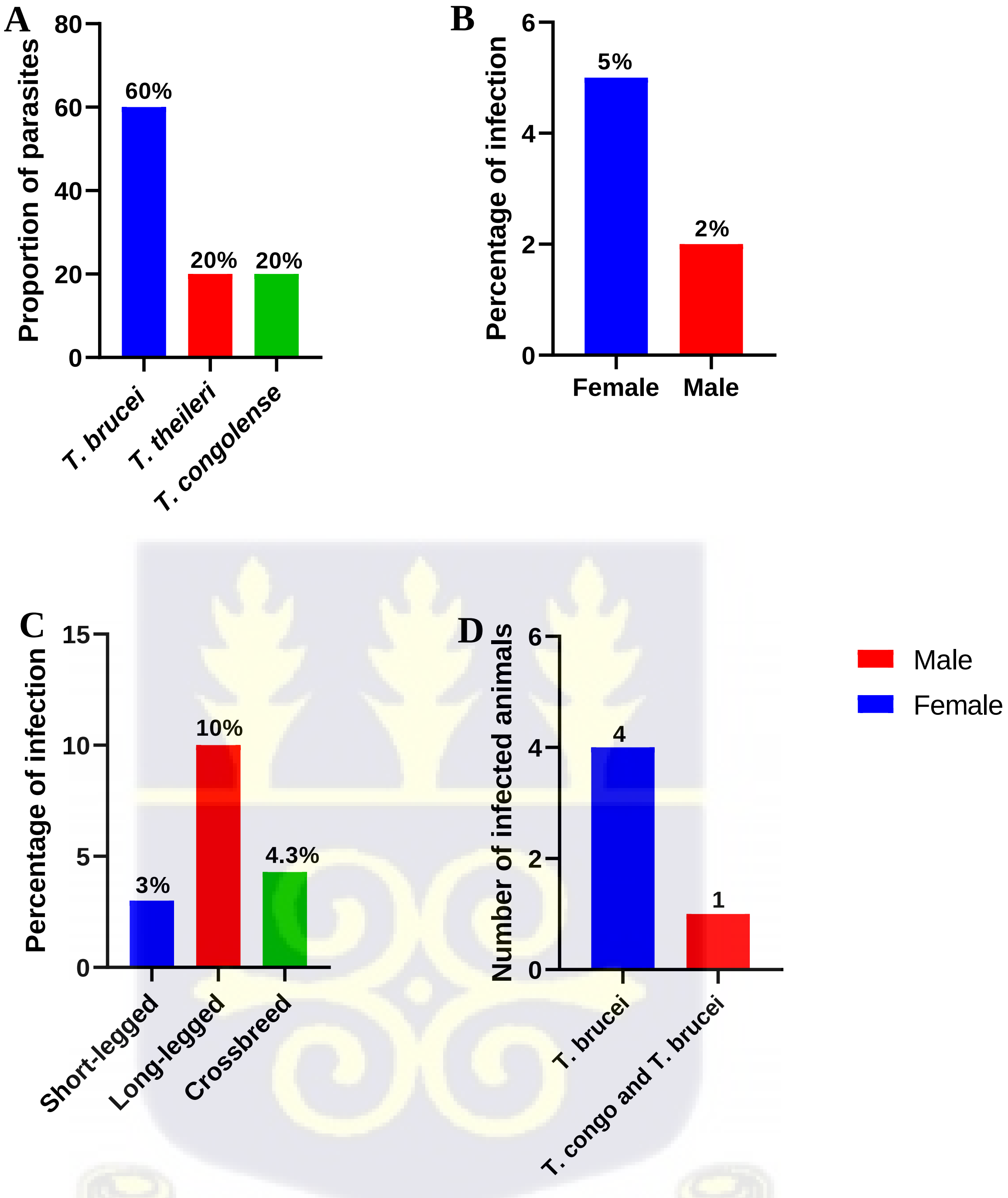


Figure 4.6: Description of trypanosome infections in goats. (A) Trypanosome species identified in goats. **(B)** Trypanosome infections per gender. **(C)** Distribution of trypanosome infections per the breed. **(D)** Distribution of trypanosome species per the sex of infected goats. Sample size (n=132).

4.2.3 Trypanosomes identified in pigs

A total of 31 (14 males and 17 females) pigs were screened to detect trypanosome infections by performing nested PCR. Of the 31 pigs, 1 female (3.2 %) was trypanosome positive. Information about the breed of the pigs sampled for this study was not recorded. Figure 4.7 shows the electropherogram of trypanosome identified in pigs. Based on the band size, the pig was infected with *T. congolense*.

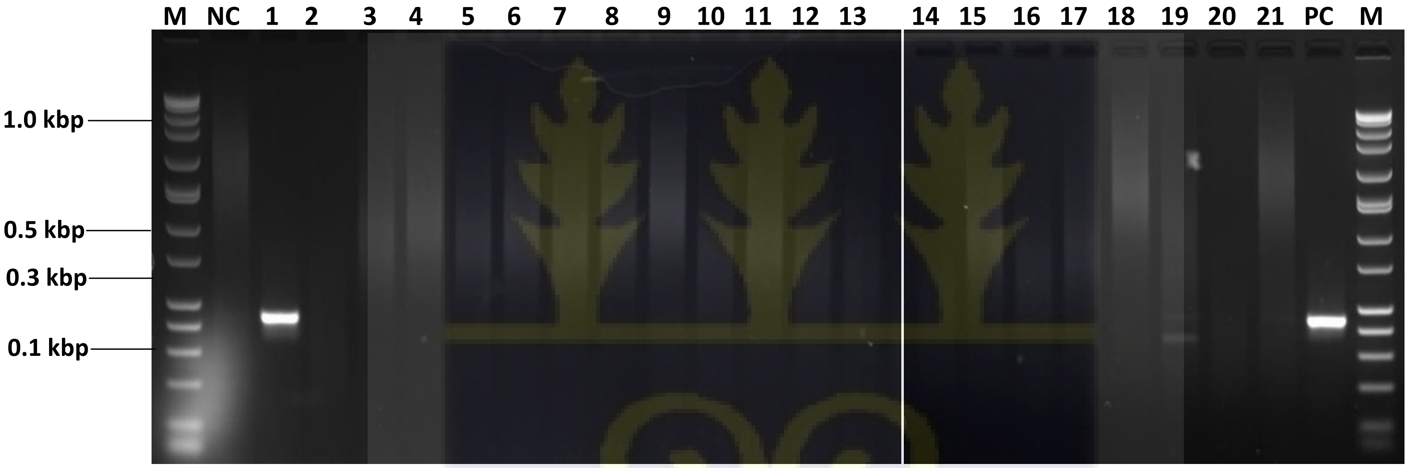


Figure 4.7: Identification of trypanosomes in pigs. An agarose gel picture shows the bands used to identify the t*rypanosome spp* in each pig. M: marker, P: positive control NC-: PCR H2O and 1-21: Pigs IDs. Expected band sizes by nested PCR were *T. brucei*: 424 bp, *T. congolense*: 456 bp and *T. vivax*: 586 bp.

4.3 Predominant trypanosome species infecting various livestock

Trypanosome species identified in the livestock screened in this study were compared to determine the most prevalent trypanosome species. Out of 73 animals that were trypanosome positive, 50 (68.5 %) were infected with *T. brucei*, 16 (21.9 %) with *T. theileri*, 5 (6.8 %) and mixed infections with *T. congolense* and *T. brucei* was observed in two animals. *T. brucei* was the dominant species in almost all livestock except pigs. However, *T. theileri* was the second most dominant species and was only identified in sheep and cattle. Of the 16 animals infected with *T. theileri*, 13 (81.2 %) were found in sheep, and 3 (18.8 %) were cattle. Statistically, the trypanosome infection rate in sheep was significantly higher (p-value = 0.006) than in other livestock. Although cattle were the second most infected livestock species, the observed difference (p-value = 0.4) compared to goats and pigs was not statistically significant, even though a lower prevalence was recorded. All the details about trypanosomes and the infection proportions in the various livestock are summarised in Table 4.2.

Table 4.2: Summary of trypanosome species identified in livestock (percentages are shown in brackets)

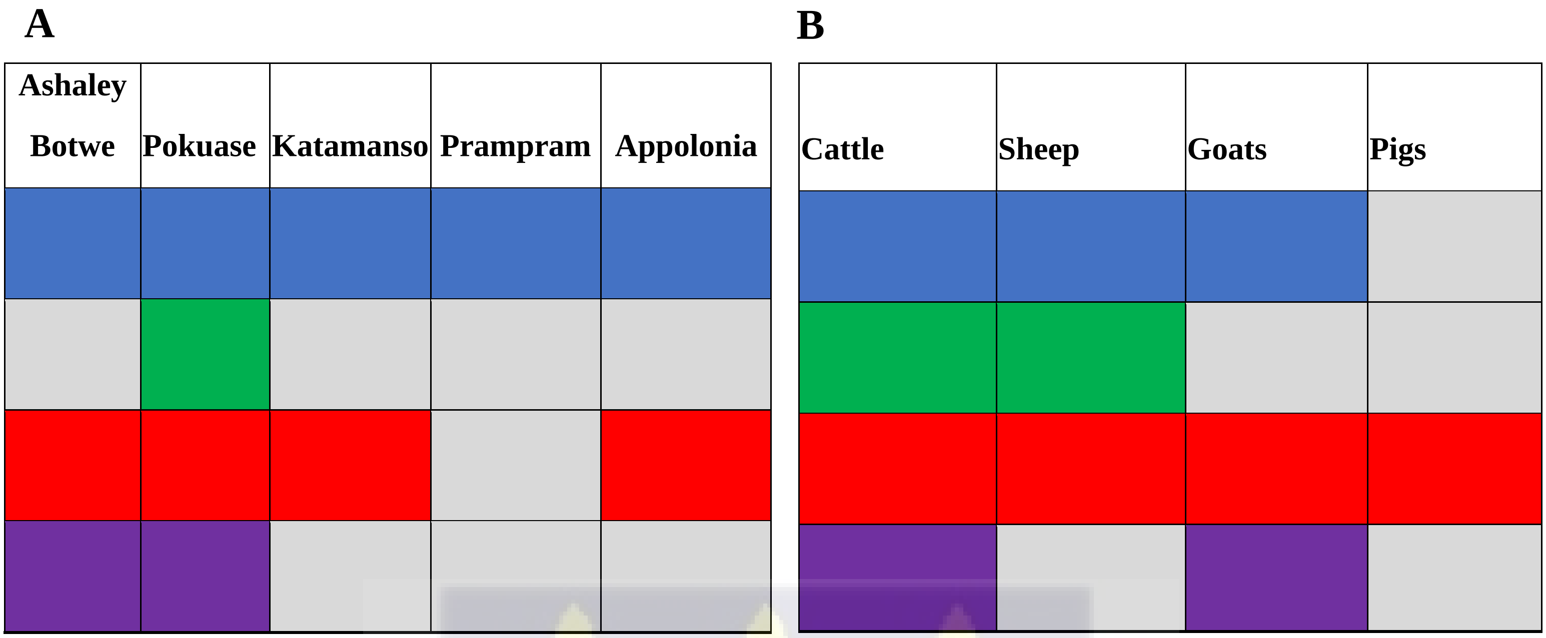
Livestock	*T. brucei*	*T. theileri*	*T. congolense*	Mixed infection	Total infection	χ2 value	P-value
Cattle	21(80.7)	3(11.5)	1(3.9)	1(3.9)	26(16.8)	0.02	0.87
Sheep	26(63.4)	13(31.7)	2(4.9)	0	41(29.7)	7.42	0.006*
Goats	3(60)	0	1(20)	1(20)	5(3.8)	3.31	0.09
Pigs	0	0	1(100)	0	1(3.2)	NA	1.19
Total	50(68.5)	16(21.9)	5(6.8)	2(2.8)	73(16)		

**Statistically significant, p-value < 0.05, mixed infection = T. congolense + T. brucei, Fisher's exact test was performed for pigs.*

4.4 Trypanosome species identified at the various study sites and livestock affected

Livestock animals from six study sites (private and non-private farms) were sampled for this study. Those study sites were Ashaley Botwe (Liprec farms), CSIR farms located at Katamanso and Pokuase, the private zoo at Prampram and farms located at Appolonia and Oyibi. Samples collected from all the sites were processed to identify trypanosome infections. Trypanosomes were identified at almost all the study sites except Oyibi, where no animal was found to be infected. The infection rate varied from one study site to another; however, *T. brucei* was the only trypanosome species identified at all the five study sites. Pokuase was the only study site where all three trypanosomes (*T. brucei*, *T. congolense* and *T. theileri*) were in circulation simultaneously. Mixed infections were observed at two specific sites: Pokuase and Ashaley Botwe. Pokuase was the only site where *T. theileri* was identified; however, *T. congolense* was identified at four study sites (Ashaley Botwe, Katamanso, Pokuase and Appolonia). Prampram was the only study site where only one trypanosome species (*T. brucei*) was circulating (Figure 4.8A).

Infecting species were analysed to confirm the preference of certain trypanosome species for specific livestock. Cattle and sheep were infected with the three trypanosomes identified in this study; goats were only infected with two species (*T. brucei* and *T. congolense*). The only pig that was found to be trypanosome positive was infected with *T. congolense*. Infections with two trypanosome species (mixed infections) were observed in cattle and goats. Finally, *T. congolense* was the only species detected in all four livestock screened (Figure 4.8B).



A

Ashaley Botwe	Pokuase	Katamanso	Prampram	Appolonia
🟦	🟦	🟦	🟦	🟦
⬜	🟩	⬜	⬜	⬜
🟥	🟥	🟥	⬜	🟥
🟪	🟪	⬜	⬜	⬜

B

Cattle	Sheep	Goats	Pigs
🟦	🟦	🟦	⬜
🟩	🟩	⬜	⬜
🟥	🟥	🟥	🟥
🟪	⬜	🟪	⬜

Figure 4.8: Trypanosomes identified per study site and livestock species. **(A)** Study sites and **(B)** Livestock species. **NA***:* Trypanosomes DNA not detected, **Mixed infection**: *T. congolense* + *T. brucei*

⬜ **NA**
🟦 ***T. brucei***
🟩 ***T. theileri***
🟥 ***T. congo***
🟪 **Mixed-Infection**

4.5 Trypanosome infections in livestock in the Greater Accra region

Trypanosome infection rates varied across the various study sites. Of the 175 livestock sampled at Ashaley Botwe, trypanosomes DNA was detected in 21 (12 %) animals. At Katamanso, 118 animals were screened, and 16 (13.6 %) were infected, while at Pokuase, 29 (36.3 %) animals were infected. Of the 45 animals sampled at Prampram, 5 (11 %) were trypanosome positive. Only two animals were infected at Appolonia of the 20 animals screened. No animal was found to have trypanosomes DNA at Oyibi (Table 4.3).

Table 4.3: Prevalence of Animal Trypanosomiasis in the Greater Accra Region

Study sites	**Number screened**	**Number infected**	**Prevalence (%)**	**$\chi2$ value**	***P-value***
Ashaley Botwe (LIPREC)[a]	175	21	12	1.16	0.28
Katamanso (CSIR)[b]	118	16	13.6	0.30	0.58
Pokuase (CSIR)	80	29	36.3	8.26	0.004
Oyibi	18	0	*NA*	*NA*	*NA*
Appolonia	20	2	10	0.22	0.63
Prampram	45	5	11.1	0.38	0.53

a) Livestock and Poultry Research Centre
b) The Council for Scientific and Industrial Research

4.6 Determination the haematocrit level to describe the of degree of anaemia the livestock

Anaemia is a condition in which the number of red blood cells or the haemoglobin concentration within them is lower than normal. Anaemia can be determined by the haematocrit, haemoglobin levels and number of red blood cells. Low haematocrit, haemoglobin levels and number of red blood cells indicate anaemia. To evaluate the degree of anaemia during trypanosome infections in selected livestock, haematocrit was measured and compared between infected and non-infected animals. Of the total 216 livestock screened for anaemia, (95 cattle, 80 sheep, and 41 goats); only 54 (25%) animals were anaemic. All goats sampled at the two study sites (Pokuase and Katamanso) were anaemic, representing 75% of all the livestock presenting anaemia, and 37.5% of the anaemic animals were infected with at least one trypanosome species (Table 4.4). Sheep were the most infected animals presenting anaemia. There was no statistical difference between infected and non-infected anaemic animals (p-value = 0.62). Age and sex of the livestock screened was not considered when comparing the haematocrit levels between trypanosome infected and non-infected. Similarly, the potential effect of the livestock species was assessed by comparing the respective proportions of each livestock per study site; the difference was not significant.

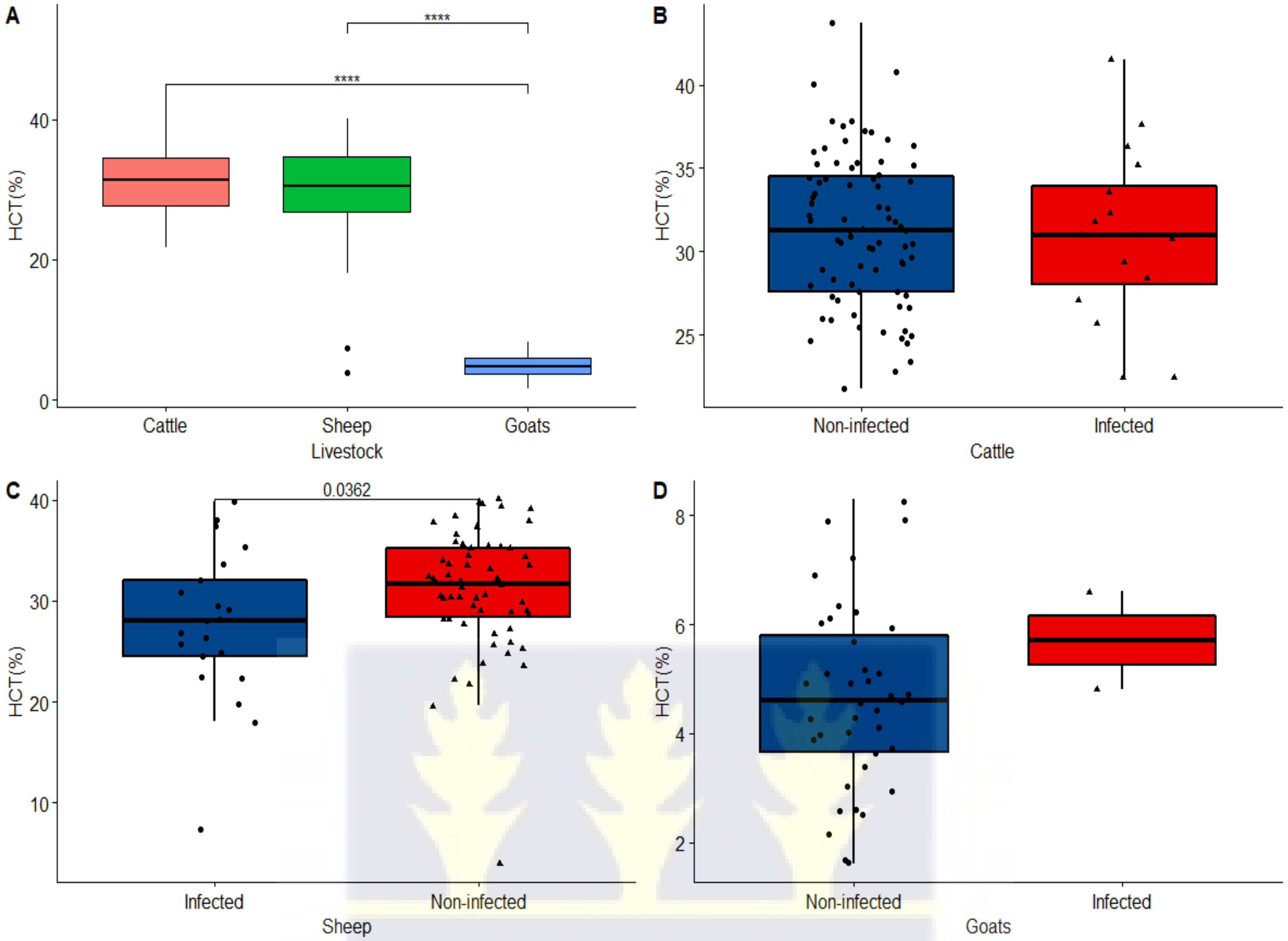
Table 4.4: Haematocrit and trypanosome infections

HCT	**Nbr screened**	**Proportion (%)**	**Infected**	**Non-infected**	**$\chi 2$**	***P-value***
Anaemic	54	25	15(37.5)	39(22.2)	1.84	0.62
Non-anaemic	162	75	25(62.5)	137(77.8)		
Total	216	100	40(100)	176(100)		

HCT: Hematocrit, Nbr = number

4.6.1 Comparison of the haematocrit levels in trypanosomes infected livestock

Haematocrit levels of infected and non-infected livestock were compared. This was done for the three primary livestock: cattle, sheep and goats. Among all the livestock, goats had the lowest hematocrit levels compared to cattle and sheep. However, in cattle and sheep, haematocrit levels were not different. The comparion between infected and non-infected livestock was performed. For cattle and goats, haematocrit levels in infected and non-infected animals were not stastistically different. However, in sheep, haematocrit level in infected animals was lower compared to the non-infected animals and the difference was statistically significant (p-value = 0.036). More details are found in Figure 4.9.



p=0.00012

p=0.0001

Figure 4.9: Haematocrit levels in infected and non-infected livestock. (A) Comparison of the haematocrit levels in the various livestock species: cattle, sheep, and goats. **(B)** Comparison of the haematocrit levels in infected vs. non-infected cattle. **(C)** Comparison of the haematocrit levels in infected vs. non-infected sheep. **(D)** Comparison of the haematocrit levels in infected vs. non-infected goats. Data from all the livestock collected at the various study sites were used in the analyses. The horizontal bar represents the Mean ± SEM. Statistical significance was tested using the Kruskal-Wallis test followed by the Dunn test, also Wilcoxon test and student-test were used where required to test the difference between infected and non-infected. *p-value* < 0.05 and ******p-value* < 0.01 were considered as statistically significant. Total sample size (n= 216), cattle (n=95), sheep (n=80) and goats (n=41).

4.6.2 Iron homeostasis in trypanosomes infected and non-infected livestock

To investigate the iron status of livestock during trypanosome infections, specific serum biochemical parameters such as serum ferritin, total iron binding capacity (TIBC), serum iron (TSI), and transferrin saturation (TS) were measured and compared between infected and non-infected livestock. Attributable to the dynamics of the disease, comparisons were made considering the host (sheep, cattle, and goats), the trypanosome infecting species, and the study site. Other factors, such as sex, breed, and age were not considered.

4.6.2.1 Ferritin concentration in livestock

Ferritin concentration was measured in 120 animals (59 cattle, 40 sheep and 21 goats) these included both infected and non-infected. The results showed that serum ferritin concentration varied from host to host; sheep had the lowest concentration compared to cattle and goats. There was an increase in the serum ferritin concentration observed in infected cattle compared to non-infected animals. The difference observed was statistically significant (p-value = 0.046). However, for sheep, there was no significant difference observed between infected and non-infected animals (p-value = 0.89). Finally, an increase was observed in ferritin concentration in infected goats compared to non-infected goats Figure 4.10.

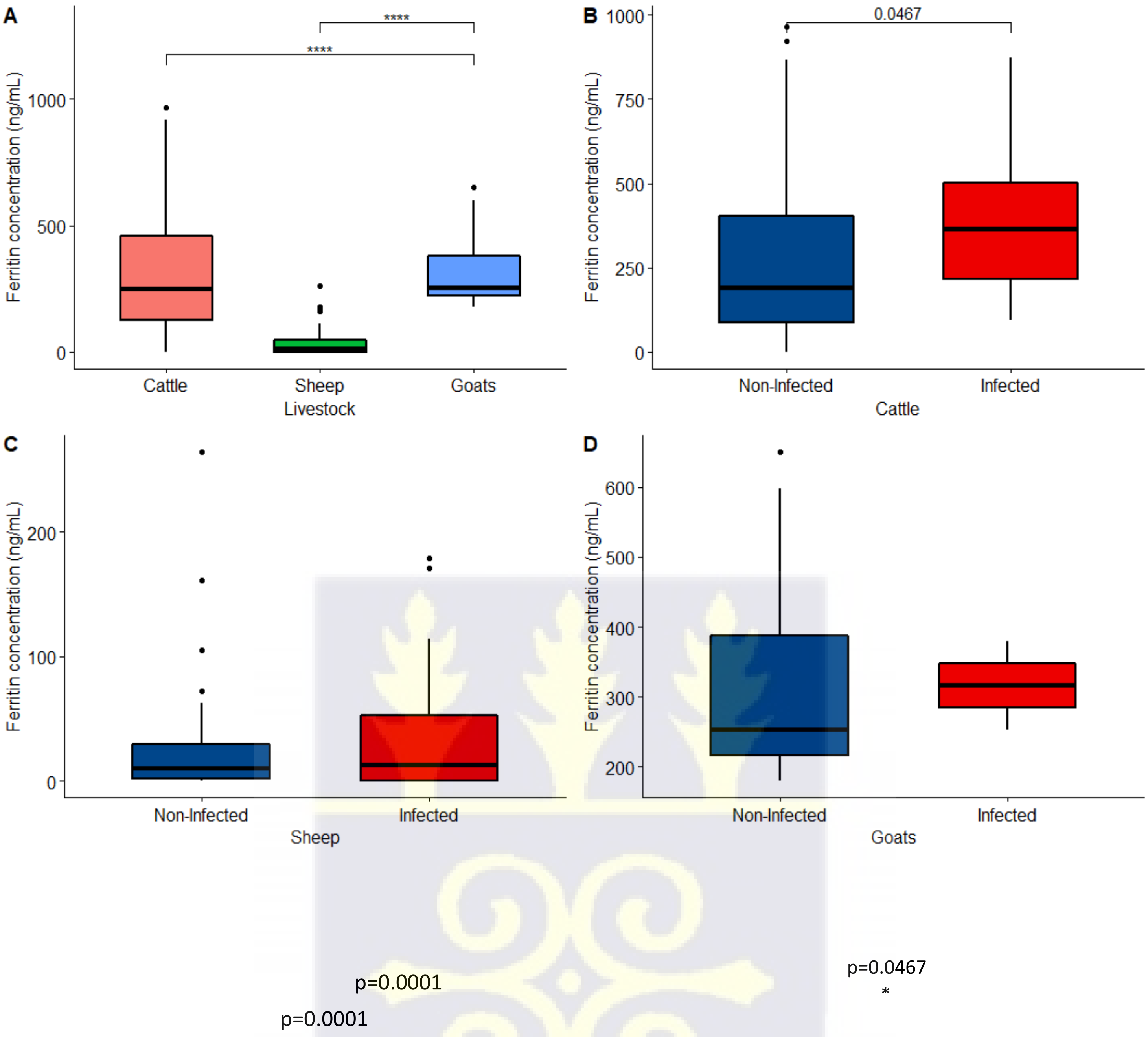


Figure 4.10 : Ferritin concentration in trypanosomes-infected and non-infected livestock. Comparison of **(A)** ferritin concentration in cattle, sheep, and goats. **(B)** Ferritin concentration in infected vs. non-infected cattle. **(C)** Ferritin concentration in infected vs. non-infected sheep. **(D)** Ferritin concentration in infected vs. non-infected goats. Data from all the livestock collected at the various study sites were used in the analyses. The horizontal bar represents the Mean ± SEM. Statistical significance was tested using the Kruskal-Wallis test followed by the Dunn test and Wilcoxon test was used to test the difference between infected and non-infected. *p-value* < 0.05 and ******p-value*** < 0.01 were considered as statistically significant. Total sample size (n= 120), cattle (n=59), sheep (n=40) and goats (n=21).

4.6.2.2 Determination of the total iron binding capacity, the total serum iron, and the transferrin saturation during trypanosome infections

To measure total iron binding capacity, total serum iron and determine the transferrin saturation sera from the animals were analysed using colorimetric assays. Two groups of animals were used, infected animals confirmed to be anaemic (cattle=10, sheep=10 and goats=2), and the control group was non-infected and non-anaemic animals from the same species (cattle=10, sheep=10 and goats=18). In general, sheep recorded the highest TIBC concentration compared to the other livestock. TIBC was more increased in infected sheep and cattle compared to non-infected animals. The difference observed was statistically significant (p-value = 0.0001). In goats, TIBC in non-infected animals was higher than in infected animals (Figure 4.11). TSI was significantly decreased in trypanosomes-infected cattle and sheep, but an exception was observed in goats (p-value = 0.04) (Figure 4.12). Also, transferrin saturation was low in trypanosomes-infected and anaemic cattle and sheep compared to non-infected animals (p-value = 0.0001). However, TS in infected goats was higher than in non-infected animals (p-value = 0.04) (Figure 4.13).

These different iron parameters were also compared between infected and non-infected livestock according to the study sites where samples were taken (Pokuase, Katamanso, Ashaley-Botwe, and Prampram), and there was no difference. Likewise, the infecting trypanosome species, *T. brucei*, *T. theileri,* and *T. congolense,* were considered to perform various comparisons. Then again, results showed no significant difference in the iron status of the multiple herds.

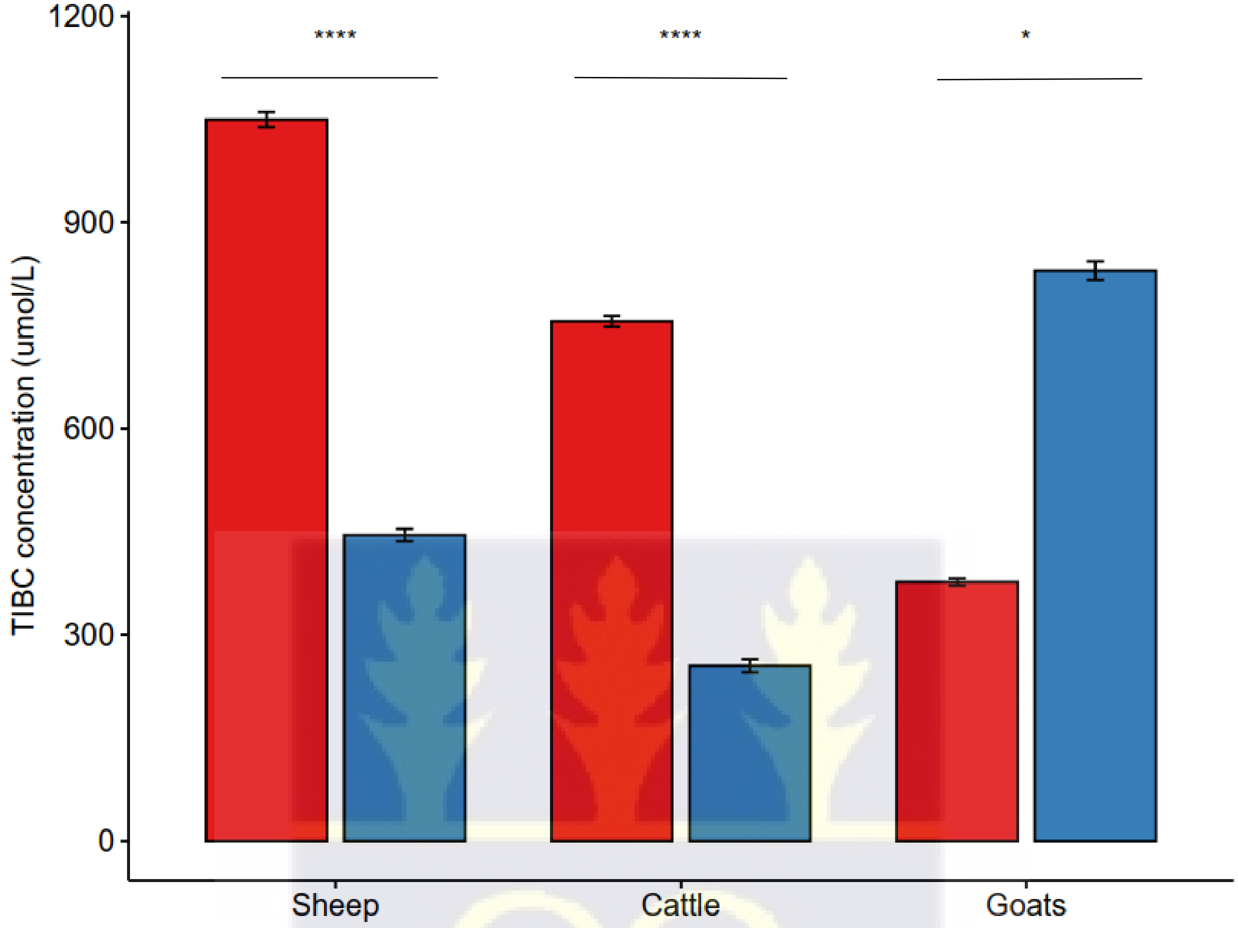


Figure 4.11: Effect of trypanosome infection on the total iron binding capacity (TIBC) in sheep, cattle, and goats. Mean±SE. **P-value* <0.05, *****P-value* < 0.01 were considered statistically significant. Animal status: red (infected) and blue (non-infected).

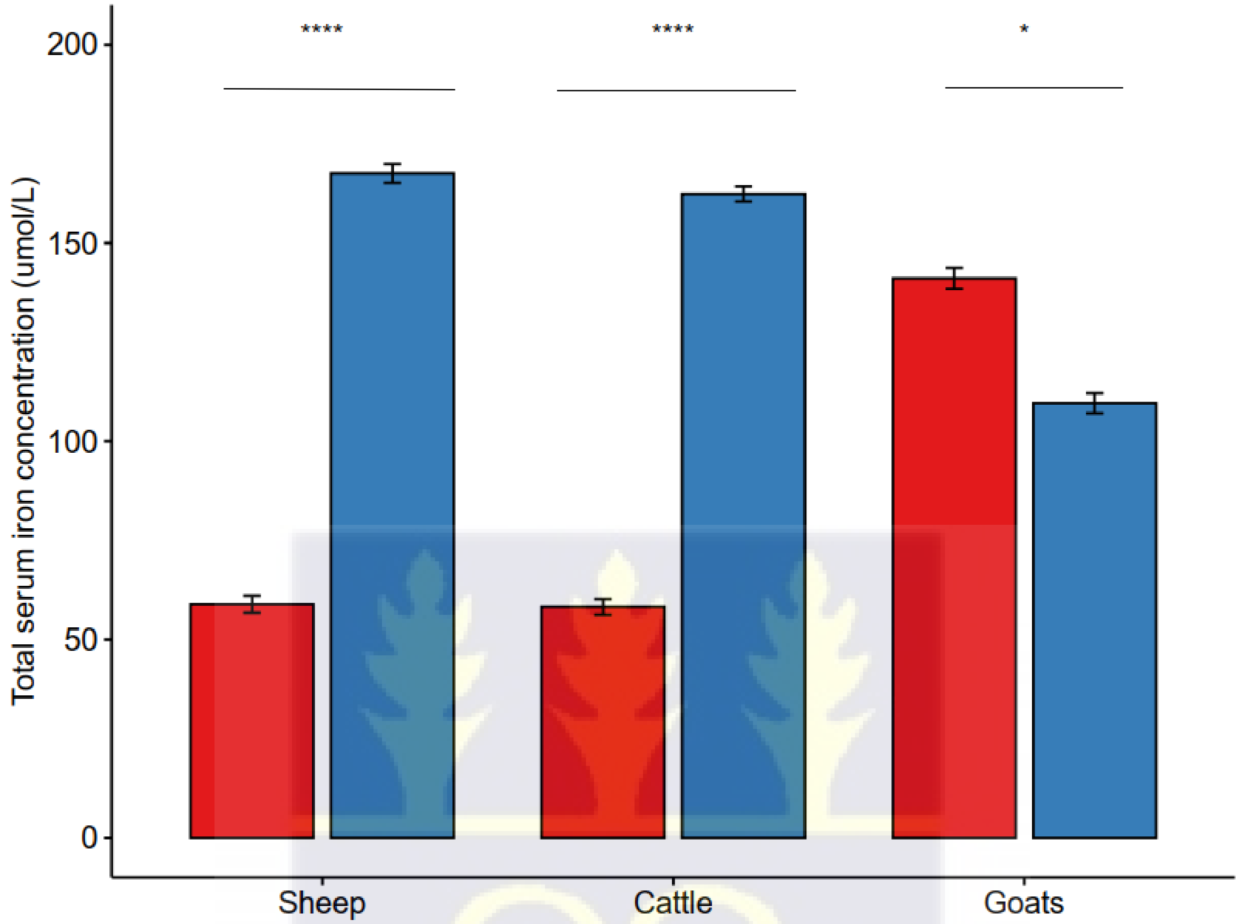


Figure 4.12: Effect of trypanosome infection on the total serum iron (TSI) in sheep, cattle, and goats. Mean±SE. **P-value* <0.05, ****P-value* < 0.01 were considered statistically significant. Animal status: red (infected) and blue (non-infected).

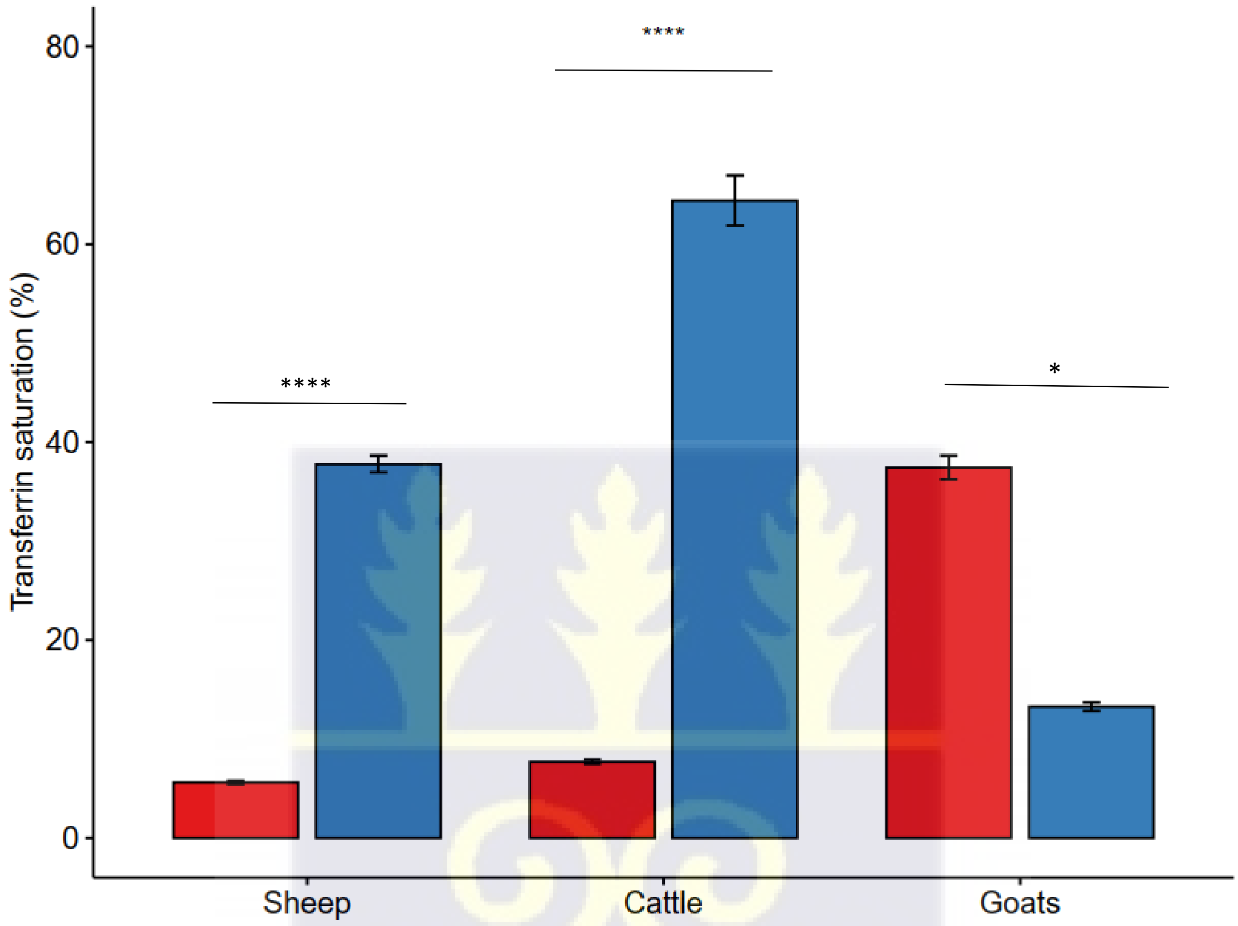


Figure 4.13: Effect of trypanosome infections on transferrin saturation (TS) sheep, cattle, and goats. Mean±SE. **P-value* <0.05, *****P-value* < 0.01 were considered statistically significant. Animal status: red (infected) and blue (non-infected).

4.7 Identification of transferrin receptor sequence diversity in naturally infected animals

To examine TfR sequence diversity in natural infections in livestock (cattle, sheep, goats), blood was collected, and the presence of trypanosome species confirmed. Then, RNA was extracted for only *T. brucei* positive animals and cDNA was synthesized and enrichment of trypanosome specific material was performed. The double stranded cDNA obtained was used to perform conventional PCR to amplify the variable region of TfR (ESAG6 and ESAG7), Figure 4.14 shows the variable region amplified and the consensus sequence analysed in the different livestock. The PCR amplicon was gel purified and sent for Illumina sequencing. The TfR sequences were compared from 10 infections: Cattle (n = 4), sheep (n = 5) and goat (n =2) and wild type GUTat 3.1 (wild-type) as a reference. The reads were mapped to the reference genome; in this case, Lister 427 was used.

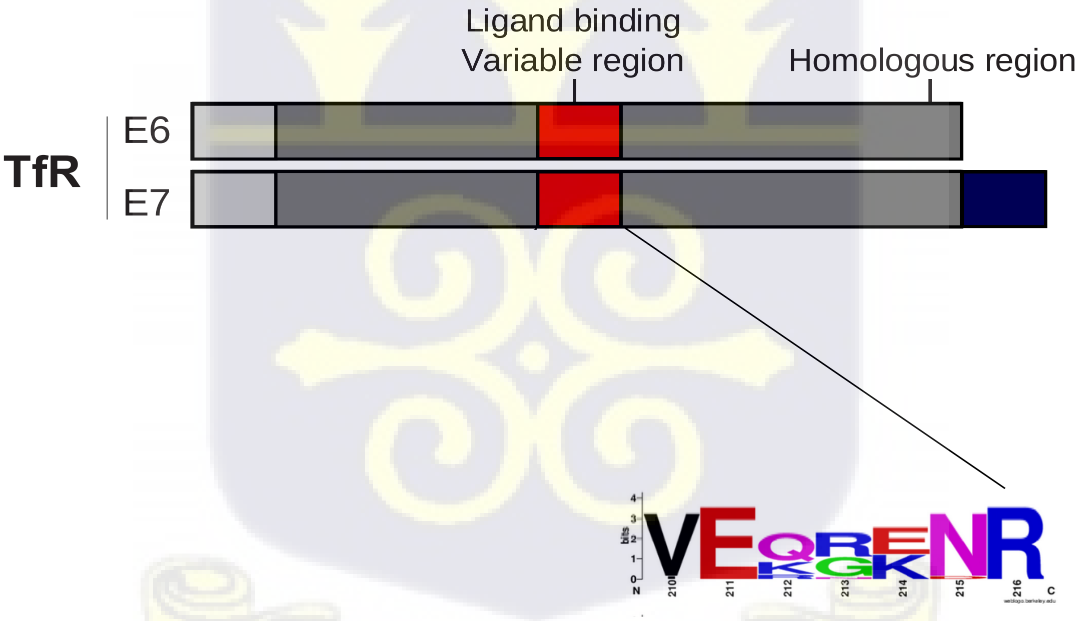


Figure 4.14: The transferrin ligand binding region (variable region) of TfR amplified for sequencing and the consensus sequence of amino acids analysed.

4.7.1: Mapping to chromosomes and expression sites, and analysis of sequence diversities

The reads were mapped to the various chromosomes and also to the expression sites, taking into consideration the 15 expression sites exist (Hertz-Fowler *et al*., 2008).

The reads were from the following samples: sheep (SH1-5), Cattle (CP1-3) and goat (GT1-2) and wild-type (GUTat 3.1); the dataset corresponds to read counts of a specific TfR gene in the *T. brucei* genome.

The heatmap was therefore generated (Figure 4.15), the colors show relative "levels of expression" (frequency distribution of reads) mapped to each expression site. High levels of expression (blue) and low levels of expression (white). For the samples, GT2-CP3, reads mapped to few expression sites mostly BES3, BES12. However, the reads for SH4 and CP4 mapped to at least 10 different BES and the wild-type used as a control mapped to almost all the 15 BES. The read counts in BES4, BES5 and BES7 were significantly low.

Next, the variable region was examined, specifically the VEQ/K sequence from the variable region of ESAG7. The consensus sequence in BES1 is VEQRKNRG but the consensus sequence from the animal data is VEQGENRG matching 19 out of 34 sequences. The results showed a significant difference in the livestock sequences analysed and those differences are observed between and within animal species, as shown in Figure: 4.16.

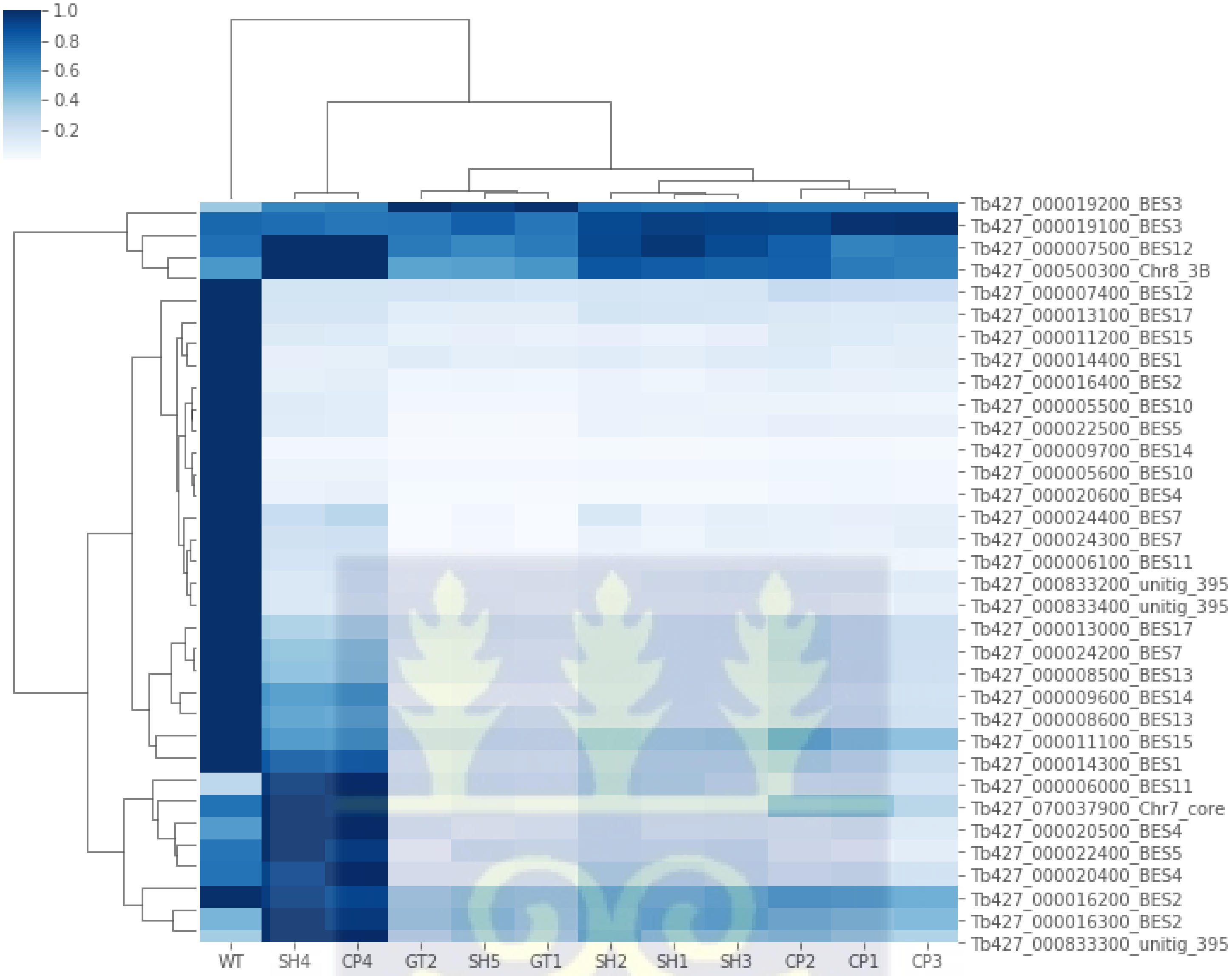


Figure 4.15: RNA-seq clustering. Mapped reads to chromosomes and expression sites (rows) and the samples as columns in the heatmap. The colours show relative "levels of expression" (frequency distribution of reads) mapped to each expression site. High levels of expression (blue) and low levels of expression (white).

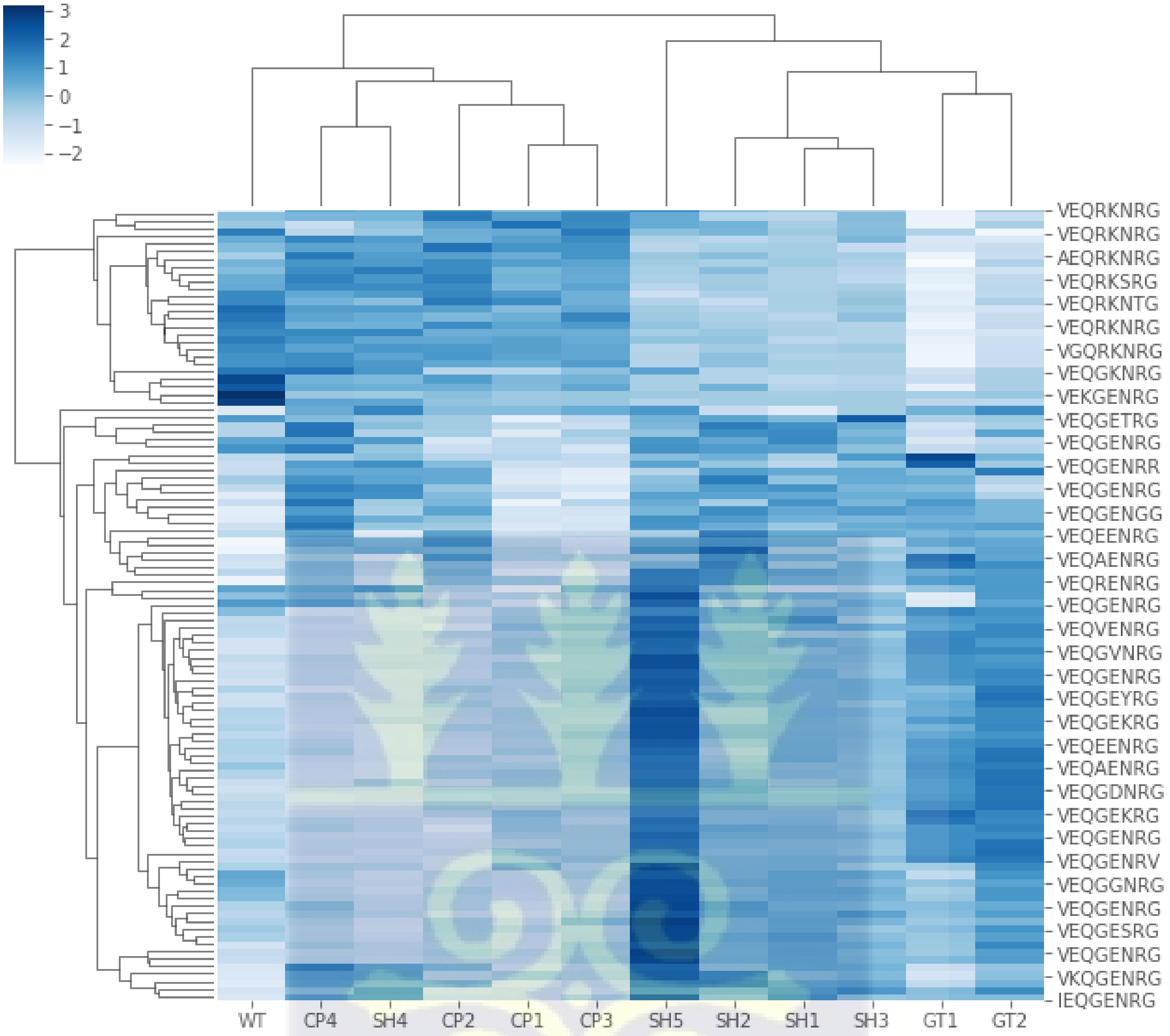


Figure 4.16: Analysis of the consensus sequence in TfR sequences of trypanosomes isolated from sheep, cattle, and goats. The figure clusters the samples (columns) and sequence counts (rows) of the first variable region in TfR that binds transferrin. Based on protein sequence similarity, 2 clusters of genetically diverse TfRs were observed. Trypanosomes in Cattle and 1 sheep clustered with the WT and those in Goats and most of the sheep clustered together.

4.8 Determination of the influence of the type of VSG expressed by *T. brucei* on the transferrin uptake

4.8.1 Cell lines characterisation and comparison of the growth rate

There are two main classes of VSG: dimers and trimers (Sara *et al*., 2023). The main goal was to determine whether the structure of the VSG expressed influences transferrin uptake. To answer this question, *T. brucei* various cell lines (Lister 427 strain) expressing the two main VSGs described were used. Briefly, the cell lines used were dimeric-VSG (VSG2) and trimeric-VSG (VSG3, VSG11, VSG615 and VSG1954). These cells were from the same and unique bloodstream expression site (BES1). Specifically, some of these cells were engineered via gene conversion such that the expression site 221 was replaced with VSG11 and VSG1.8.

First, the doubling time was determined over 72 hours for all the cell lines. Generally, the doubling time for most of the cells was approximately 8 hours. The data showed that there was not a significant difference in the doubling time of both dimeric and trimeric-VSG cell lines. However, among the trimeric-VSG, the doubling time of VSG1954 was approximately 20 hours and that for VSG615 was approximately 26 hours, these two cell lines are described as metacyclic VSGs. The difference observed was statistically significant (p-value = 0.041). The growth curves of all the cells used are summarized in Figure 4.17.

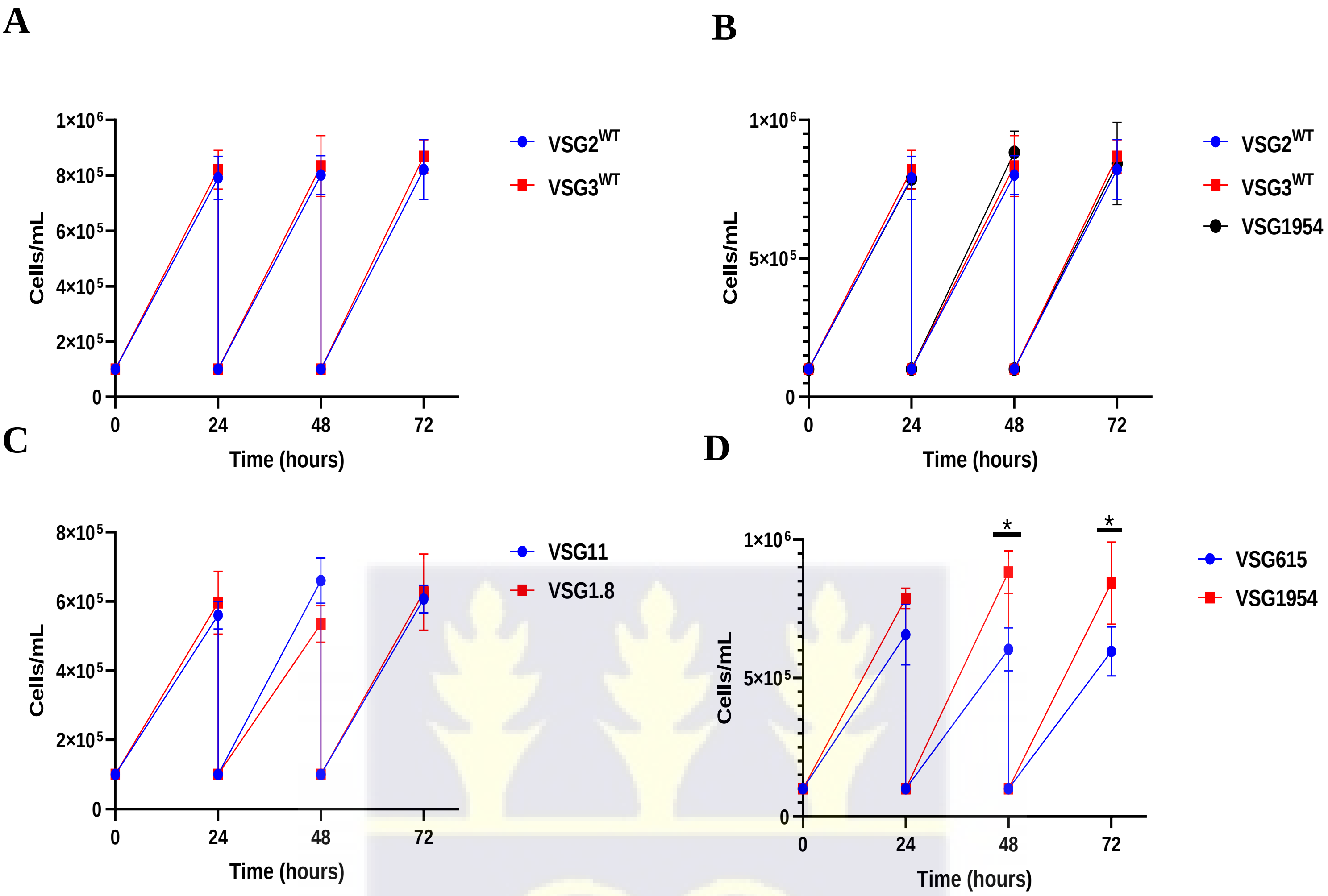


Figure 4.17: Growth rates of dimeric and trimeric VSG cell lines. Growth curve of **(A)** VSG2 and VSG3. **(B)**VSG2, VSG3 and VSG1954. **(C)**VSG11 and VSG1.8. **(D)**VSG615 and VSG1954. Cells were counted every 24 hours and diluted back to 1 x 10^5 cells/mL. Data presented as mean ± SD. * *p-value* < 0.05 by unpaired t-test. n = 3 biological replicates.

4.8.2: Determination of the influence of the VSG-structure on transferrin uptake

To investigate whether the class of the VSG expressed influences nutrient uptake, specifically transferrin (Tf) uptake flow cytometry was performed to measure the uptake of Tf-467 in the different cell lines previously described. Cells were pre-incubated in HMI-9 media serum-free this to trigger TfR turnover, therefore replacing ligated TfR on the surface of the parasites with un-ligated TfR freshly synthesized as described here (Tiengwe *et al*., 2017). However, normal receptor-mediated endocytosis was measured using a specific marker, tomato lectin (TL-467). TL-467 can bind poly-LacNAc repeats and the ManGlcNAc$_2$ core of paucimannose N-glycans on the flagellar pocket, this favours endocytosis and delivery to the lysosome (Fujimoto *et al*., 2008; Schwartz *et al*., 2013). There was no difference in TL-467 uptake between dimeric and trimeric cell lines (Figure 4.18A), this is an indication that general endocytosis was the same. Although there was a slight difference between VSG2 and VSG3, the difference was not statistically significant (p-value = 0.49). However, Tf uptake was reduced in VSG3 which is a trimeric VSG compared to VSG2 which is a dimer, the difference observed was statistically significant (p-value = 0.002). Likewise, Tf uptake was significantly reduced in VSG1.8 and VSG11 compared to VSG2 (p-value = 0.0071 and p-value = 0.001). In general, there was an important reduction of approximately 75% in Tf uptake in some trimeric VSG cell lines compared to the dimeric VSG. Furthermore, there was a reduction of more than 50% in Tf uptake observed among the different trimeric VSGs (VSG3, VSG11 and VSG1.8) and the metacyclic VSGs (VSG615 and VSG1954). The Tf uptake was significantly higher approximately 3-fold increase in VSG615 compared to VSG3 (p-value = 0.0001). Similarly, a significant Tf uptake increase was observed in VSG1954 compared to VSG11 (p-value = 0.0006) (Figure 4.18B).

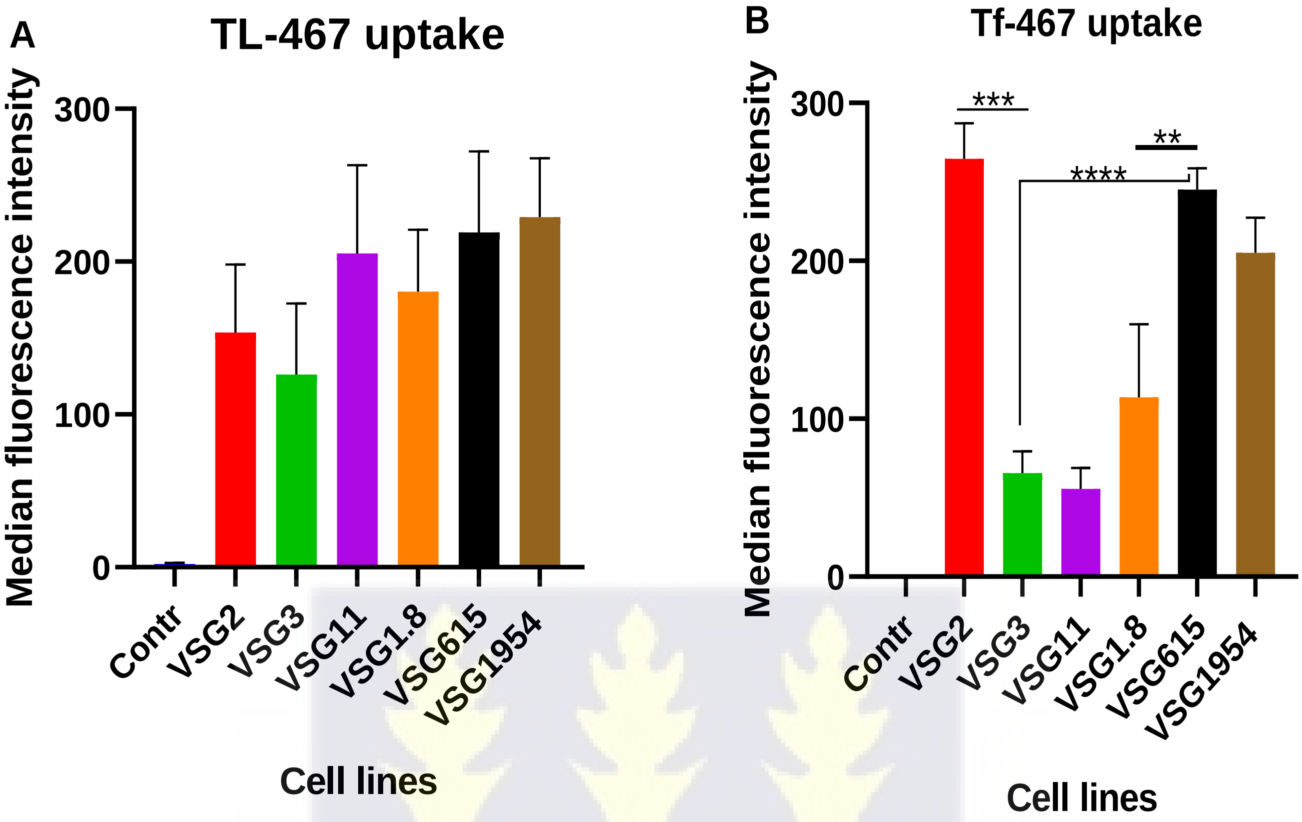


Figure 4.18: Transferrin uptake in dimeric and trimeric cell lines. (A) TL-467 and **(B)** Tf-467 uptake in the various cell lines. Median fluorescent intensity (arbitrary units) of n = 3 biological replicates. Dimeric VSG: VSG2; trimeric VSG: VSG3, VSG1.8, VSG11 and Metacyclic VSGs (VSG615 and VSG1954). Data presented as mean ± SD. ***p-value* < 0.01, ****p-value* <0.001, *****p-value* < 0.0001 by unpaired t-test.

4.8.3 Quantification of TfR levels in the different VSG cell lines

The results described above showed 75% reduction of Tf uptake in some trimeric VSG cell lines (VSG3, VSG11 and VSG1.8). TfR is essential for Tf uptake; therefore, it was necessary to determine whether the reduction observed in Tf uptake was due to the low expression of TfR by examining the ESAG6 and ESAG7 genes. To do this, mRNA was extracted from the different cell lines and qRT-PCR was performed to quantify the expression levels of the TfR genes. The primers used targeted ESAG6 and ESAG7 specifically, and to normalize, the endogenous gene *T. brucei* zinc finger protein 3 (ZFP3) was used. Results showed a reduction of approximately 50% and 60% of ESAG6 and ESAG7 mRNA levels in some trimeric VSGs (VSG11 and VSG1.8) compared to VSG2 and VSG3, respectively. However, there was no significant difference between VSG2 and VSG3 (p-value = 0.08). Likewise, no difference was observed when comparing the mRNA levels of ESAG6 and ESAG7 in VSG2 and the metacyclic VSGs; VSG615 (p-value = 0.22) and VSG1954 (p-value = 0.09) (Figures 4.19A-B). Next, a Western blot was performed to check whether the reduction in ESAG6 and ESAG7 in some cell lines resulted in a reduced TfR protein. TfR protein levels was not reduced in trimeric VSG expressing cells, this indicates that the reduced Tf uptake is not due to low TfR (Figure 4.19 C-D).

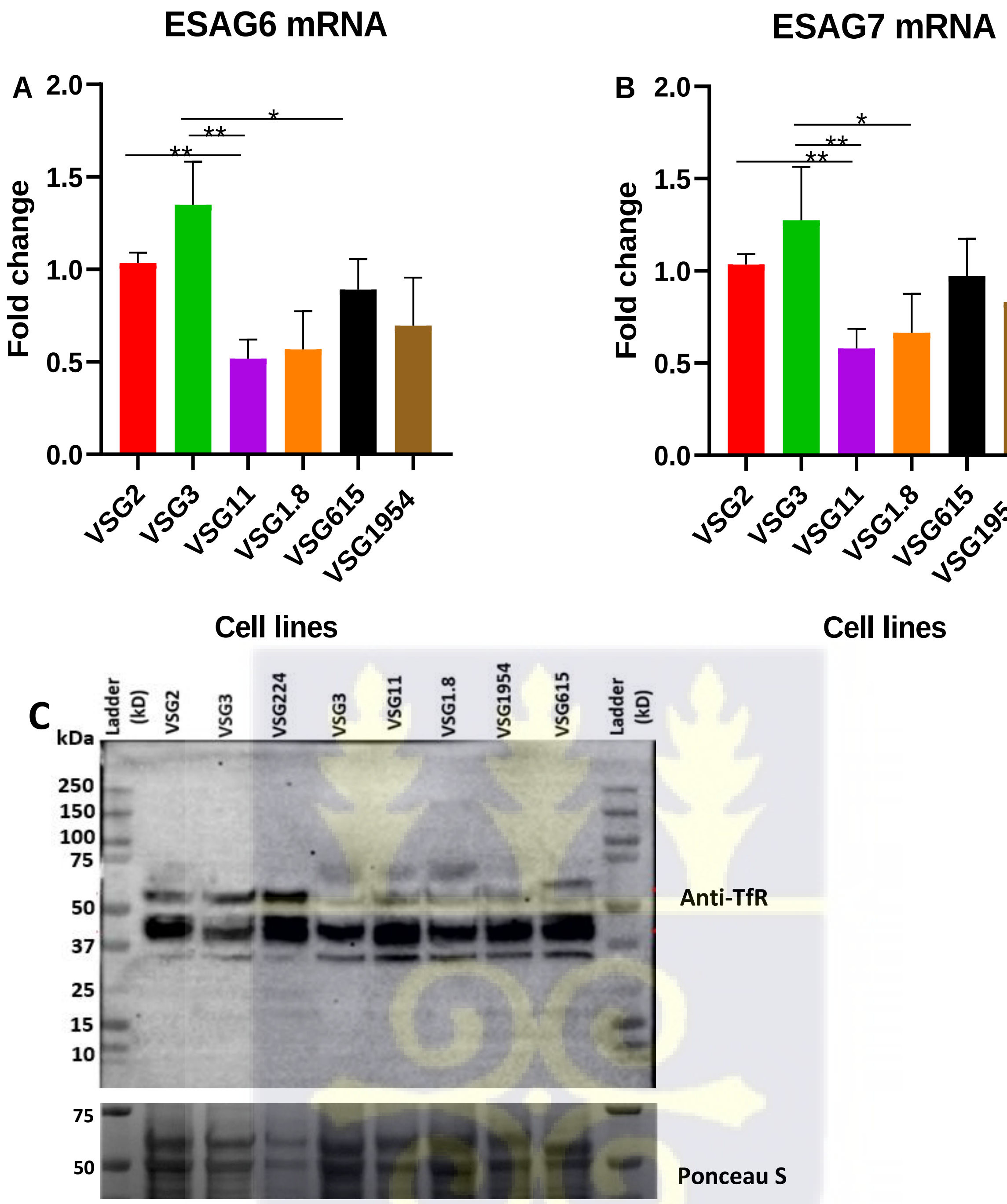


Figure 4.19: Quantification of TfR mRNA and protein levels in dimeric and trimeric cells. (A) qRT-PCR analysis of ESAG6 transcripts and **B)** ESAG7 transcripts presented as a fold change. ZFP was used as an endogenous control for normalization and data presented as mean ± SD. n = 3 biological repeats, each in technical triplicate. **(C)** Anti-TfR western blot and protein quantification. Ponceau stain included as a loading control for normalisation. E6: 50-60 KDa and E7: 42 KDa. **Dimeric VSG**: VSG2 (used to normalise other cells); **trimeric VSG**: VSG3, VSG224, VSG1.8, VSG11 and **Metacyclic VSGs** (VSG615 and VSG1954).

4.8.4: Characterisation of TfR expressed in the different cell lines

To investigate whether the reduction in Tf uptake observed in some cell lines was caused by the difference in the TfR sequence, the full-length TfR was amplified and sequenced. To do that, both dimeric and trimeric cell lines previously described were used. However, all these cells are from the same and unique bloodstream expression site (BES1). Therefore, technically the TfR expressed should be the same for all the cell lines. To verify this, mRNA was extracted, and cDNA synthesised, then amplification of the TfR was done by conventional PCR (Figure 4.20A), and the PCR products were gel purified and Sanger-sequenced. After getting the various sequences, BLAST was performed to validate that those sequences corresponded to *T. brucei* TfR. Following that, multiple sequence alignment was done, and the results showed that all the TfR sequences analysed were highly similar; the sequence identity varied between 97% and 98%. Therefore, there was no significant difference in the TfR expressed in both dimeric and trimeric VSG cell lines as expected. However, a single nucleotide change was observed from A to G, which is a transition in only the metacyclic VSGs (VSG615 and VSG1954); more details are found in Figure 4.20B.

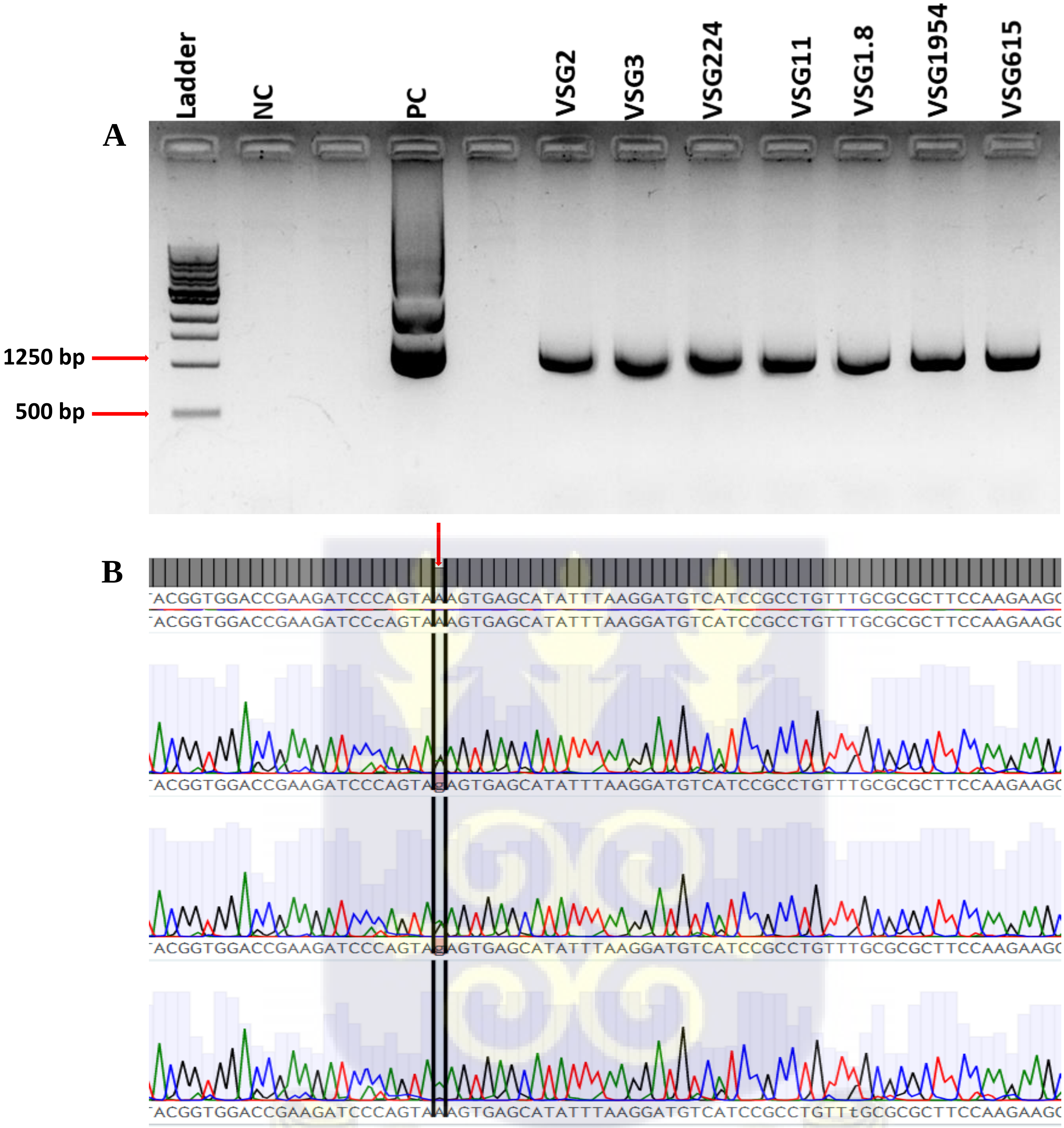


Figure 4.20: Comparison of TfR sequence in dimeric and trimeric cell lines. (A) Agarose gel pictures showing the amplification of TfR. M: marker, NC: negative controle and PC:positive controle and the samples are the different cell lines. Expected band size: 1250 bp **(B)** Chromatogram for a single the single nucleotide variant, indicated with the red arrow.

4.8.5 Generation of an ESAG7-GPI (E7^{GPI}) anchor expressing cell line

TfR is formed by two proteins (ESAG6 and ESAG7), only ESAG6 has a GPI anchor. Previously, it was reported that the GPI-valence in the bloodstream stage of *T. brucei* influences intracellular trafficking precisely transferrin uptake (Tiengwe *et al*., 2017). Therefore, the next step was to investigate whether making a TfR protein with two GPI anchors will rescue Tf uptake. VSG3$^{\mathbf{WT}}$ cell lines were used to transfect with the plasmid (E7$^{\mathbf{GPI}}$) (the plasmid construct is found in the appendix). Then, the uptake of Tf-467 was measured in VSG3$^{\mathbf{WT}}$ and the new E7$^{\mathbf{GPI}}$ cell lines by FACS as previously described. The same marker which is tomato lectin (TL-467) was used to control for general receptor-mediated endocytosis. There was an increase in the general endocytosis, the difference between TL-467 uptake in VSG3$^{\mathbf{WT}}$ and E7^{GPI} was statistically significant (p-value = 0.0436) (Figure 4.21A). However, a considerable reduction of 70% was observed in Tf-467 uptake in E7^{GPI} compared to the wild type (p-value = 0.0002) (Figure 4.21B).

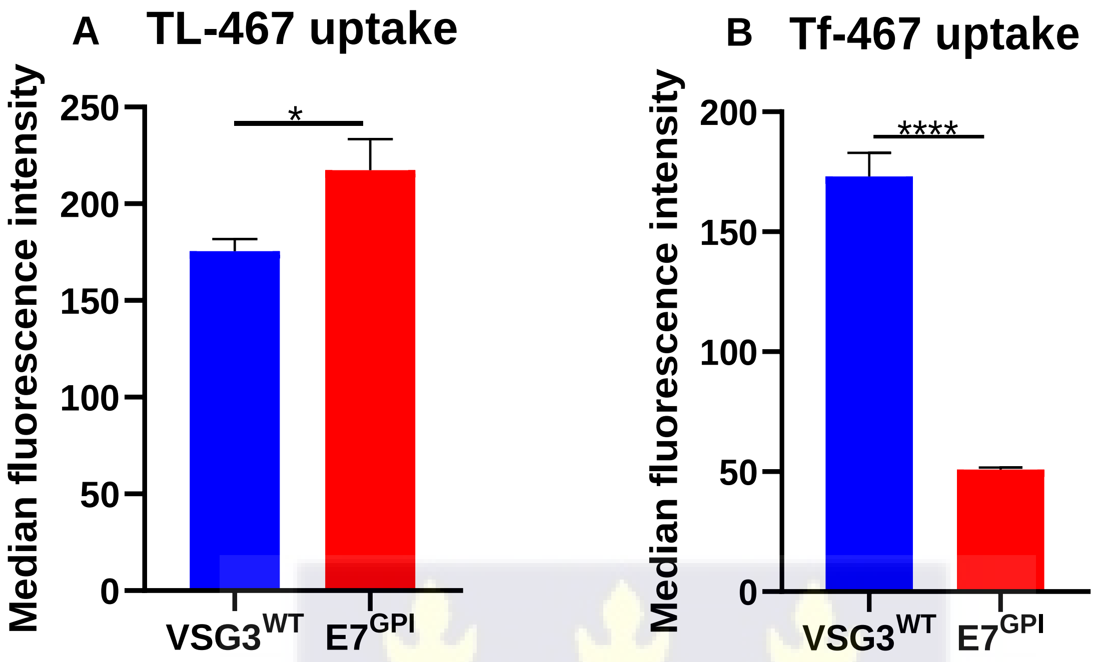


Figure 4.21: Transferrin uptake in E7^{GPI} expressing cell lines. (A) TL-467 and **(B)** Tf-467 uptake in VSG3^{WT} and E7^{GPI} cell lines. Median fluorescent intensity (arbitrary units) of n = 2 biological replicates. Data presented as mean ± SD. **p-value* < 0.05, *****p-value* <0.001 by unpaired t-test.

CHAPTER FIVE

5.0 DISCUSSION, LIMITATIONS, CONCLUSION, AND RECOMMENDATIONS

5.1 Discussion

This study was conducted to provide the current burden of animal trypanosomiasis in the primary livestock (cattle, sheep, goats, and pigs) in the Greater Accra Region and to understand the dynamics of the disease, provide information about anaemia levels and the iron status of trypanosome-infected animals, identify polymorphism in transferrin receptor in naturally infected livestock and determine how the VSG structures influence transferrin uptake.

The approach was to first detect circulating trypanosome DNA by a sensitive nested PCR (Ofori *et al*., 2022) confirmed by sequencing amplicons and to determine haematocrit levels, serum ferritin, total iron binding capacity, total serum iron, and transferrin saturation by performing colourimetric and ELISA assays. Further, RNA was extracted from the blood of trypanosome-infected animals, and cDNA was synthesised, and the variable region of TfR amplified and sequenced. Finally, specific cell lines from the two main VSG classes were used to measure transferrin uptake using FACS.

Trypanosome infections in livestock in Greater Accra

For this study, trypanosome infection could not be detected by microscopy, which could be firstly due to the low sensitivity of the parasitological diagnostic technique. Secondly, low parasitaemia is often observed in naturally infected animals. Therefore, molecular characterization through PCR remains the method of choice to identify *Trypanosoma* species due to its sensitivity and specificity. The sensitivity of PCR techniques has been shown in various epidemiological studies of trypanosomiasis in livestock and other wild animals (Ereqat *et al*., 2020; Fetene *et al*., 2021; Habeeb *et al*., 2021; Ofori *et al*., 2022) .

The overall prevalence of AAT in the Greater Accra Region is 16% and seems relatively low compared to other studies (Dieng *et al*., 2022; Ereqat *et al*., 2020; Muhanguzi *et al*., 2017). Specifically, in the Northern Region of Ghana, the prevalence of AAT was reported to be (61%) which was extremely high (Dieng *et al*., 2022). This could be explained by the importance of the farming sector in that region of the country. Many farms are found there, and the vegetation is very dense, helping to maintain a high tsetse fly challenge. Therefore, the low prevalence recorded in Greater Accra might be explained by factors such as, low tsetse fly challenge observed in the region in general and at the different study sites. Another important factor is the season during the sampling. Previously, some studies showed that depending on when the samples are collected, either during the rainy or the dry season, this can influence the prevalence of trypanosome infections. More infections are usually observed during the dry season compared to the rainy season (Majekodunmi *et al*., 2013). For this present study considerable number of animals were sampled during the rainy season. Also, the overall number of animals screened for this study could explain the result obtained, in the sense that studies that reported a high prevalence sampled a considerable number of animals. However, some studies conducted in other countries have recorded a lower prevalence than what is reported in this current study (Ebanga-Echi *et al*., 2020; Idehen *et al*., 2018).

The livestock were sampled on two separate occasions; for the first and second occasions, the prevalence of trypanosome infections was 13.8% and 18.5%, respectively. The difference observed, although not significant, might be due to the study sites for the two occasions of sample collection not the same farms were visited. However, all the samples were collected during the same season even though it was not the same year.

Trypanosome infections greatly varied across the different study sites. Pokuase (CSIR-farm) recorded the highest infection rate, mainly in sheep. The second study site with a high number of infected animals (sheep and cattle) was Katamanso (CSIR-farm), followed by Ashaley-Botwe (LIPREC). The difference in the infection rates observed might be due to variations in the number of animals sampled per study site and the vegetation surrounding the different places. Another critical parameter that could have influenced the infection rate is the handling of these animals by the farm managers and owners. Some farmers administer some drugs, including diminazene aceturate, to the livestock to prevent various infections, although this usually depends on the health state of these animals.

Trypanosomes were identified in all four livestock species sampled, even though the infection rates varied among these animals. The primary livestock sampled were (cattle, sheep, goats, and pigs) at different sites. Sheep were the livestock mostly infected; this result is contrary to observations that cattle are the most infected livestock (Ereqat *et al*., 2020; Muhanguzi *et al*., 2017; Tweneboah *et al*., 2021). A study conducted in Nigeria also reported a high infection rate in sheep compared to cattle and other livestock (Idehen *et al*., 2018). However, the infection rate reported in sheep by Idehen and colleagues was 6.9% significantly lower than what is reported in the present study (29.7%). The low infection rate (16.8%) recorded in cattle in this study might be due to the small sample size; only 155 animals were screened, as other studies reporting high disease prevalence in cattle sampled huge numbers (Habeeb *et al*., 2021; Muhanguzi *et al*., 2017; Tweneboah *et al*., ;2021). However, the infection rate presently recorded (16.8%) is higher than what some studies reported (Ebanga-Echi *et al*., 2020; Idehen *et al*., 2018; Tweneboah *et al*., 2021).

The infection rate in goats was significantly low (3.8%) compared to a previous study conducted in Gabon, where 140 goats were screened, and 7.8% were found to be infected with trypanosomes. However, the present data corroborate the findings of another study conducted in Nigeria, where 1054 goats were sampled and the prevalence reported was 4% (Behnke *et al*., 2011; Gutierrez *et al*., 2006). Surprisingly, goats and sheep are always kept in the same vicinity at the different study sites. However, different infection rates are reported; this could be due to the duration of the infection, the host tropism, or some specific genetic factors. Also, the breed of goats (short-legged) sampled for this study seems not susceptible to trypanosome infections compared to the other breed (long-legged), but this should be confirmed by conducting more extensive studies. Moreover, previously goats were reported to be resistant to trypanosome parasites (Gutierrez *et al*., 2006*)*. The lowest infection rate among all the livestock was reported in pigs. This could be due to the small sample size or the fact that primers used for the molecular diagnosis could not detect *T. simae,* which mostly infect pigs. A study conducted by Tweneboah and colleagues also reported low trypanosome infections in pigs (Tweneboah *et al*., 2021).

Trypanosome species identified in livestock and study sites

Trypanosome species identified in the livestock were: *T. brucei brucei*, *Trypanosoma theileri*, and *T. congolense*, similar to other studies (Ekloh *et al*., 2023; Simwango *et al*., 2017). Among these three species identified in this study, two (*T. brucei brucei* and *T. congolense*) are known to cause AAT in livestock (Masumu *et al*., 2009; N'Djetchi *et al*., 2017). However, of all the species identified, *T. brucei brucei*. was the most dominant (68.5%) across all the study sites. This finding is relatively uncommon because *T. vivax* and *T. congolense* have been reported by several studies as the prevalent species (Idehen *et al*., 2018; Muhanguzi *et al*., 2017; Tweneboah *et al*., 2021).

Also, previous studies conducted in our laboratory (Bakari *et al*., 2017; Ofori *et al*., 2022) reported *T. vivax* as the most dominant species. Meanwhile some of this study's samples were collected at the same study sites and seasons. This, therefore, indicates that the prevalence of trypanosome infections keeps changing. Most of the animals were infected with only one species of trypanosomes, but co-infections of *T. congolense* with *T. theileri* were seen in two animals (cow and goat). This is contrary to what was observed by Ofori and colleagues, where high number of co-infections were reported.

Specific trypanosome species were identified in different study sites and livestock. Pokuase was the only site where all three main trypanosome species were found simultaneously. This could be explained by the very dense vegetation surrounding the farm, and the high tsetse fly challenge observed at the site. However, at Ashaley-Botwe, Katamanso and Appolonia, only two species (*T. brucei* and *T. congolense*); this can be explained by the fact the vegetation observed at these sites was not dense; therefore, the tsetse flies challenge was moderate. *T. brucei* was identified at almost all the study sites, this suggests that majority of the flies found at the different study sites were infected with that specific trypanosome species. The few cases of co-infections observed in the present study were recorded at two specific sites, Ashaley-Botwe and Pokuase. This suggests that flies found at these farms were infected with more than one trypanosome species, therefore during the blood meal the flies transmitted the two species simultaneously.

Effect of sex and breed on trypanosome infections in livestock

Some host factors, such as sex and age, influence the susceptibility to trypanosome infections (Bezie *et al*., 2014). Previous studies have reported the impact of sex and breed of the livestock species on trypanosome infections (Ngongolo *et al*., 2020; Simwango *et al*., 2017). Specifically, female animals were reported to be more infected with trypanosome species than males (Ereqat *et al*., 2020; Simwango *et al*., 2017). The data from the present study also showed that among the infected livestock they were more females, and this was observed for most animal species. However, the difference observed was not statistically significant when comparing trypanosome infections in females (16.6%) and males (14.6%). This suggests that sex did not likely have any significant effect on trypanosome infections in the different livestock, corroborating what was previously reported by Ekloh et al. 2023 (Ekloh *et al*., 2023). However, the high number of trypanosome infections observed in females might be simply explained by the fact that more females were sampled. This is because in all the farms visited, female animals were always more than males. The reason is that most farmers prefer to keep female animals and have few males for mating.

The breed of the animal can also influence the susceptibility to trypanosome infections; for this study many breeds of different animal species were sampled. For cattle, five breeds (Sanga, Sokoto, WASH, N'Dama and Sanga cross) were sampled and trypanosome infections were found in three breeds, those were N'Dama, Sanga and Sokoto and the infection rates were 40%, 22.5% and 11% respectively. However, the difference observed was not significant suggesting that, the breed did not have a major effect on trypanosome infections in cattle. No trypanosome infection was found in two breed WASH and Sanger cross; this might be due to the limited number of animals sampled. For sheep four breeds (Nungua type or blackhead, Sahelian type, Forest type and Crossbreed) were sampled for this study. Trypanosome infections were found in only two breed, Nungua type or blackhead and crossbreed the infection rates were 28% and 33% respectively. No infection was observed in the other breeds, this because few sheep from these breeds were sampled. Moreover, Sahelian-type and Forest-type sheep are mostly found in the northern region of the country. Therefore, it was not a surprise that the two breeds of sheep found to be infected were the most represented because present in most of the farms that were visited. Finally, the different breeds of goats sampled were crossbreed, short-legged and long-legged. In general, few goats were found to be infected with trypanosome species. These findings suggest that goats might be less susceptible to trypanosomes, this is contrary to what was reported by Amisigo and colleagues.

Trypanosome infections and degree of anaemia in livestock

Haematocrit level was significantly lower in goats compared to cattle and sheep. There was no significant difference in haematocrit levels in sheep and cattle. Contrary to what other studies reported, severe anaemia was not observed in infected cattle (Degneh *et al*., 2017; Meharenet & Tsegaye, 2020). However, infected sheep and goats were anaemic, this was confirmed with low haematocrit levels. There was a significant difference in the haematocrit levels of infected and non-infected sheep although the number of infected animals was not that considerable. However, goats were found to be anaemic, but only two animals were infected out of the 41 animals sampled. Anaemia in goats was severe, but surprisingly the infection rate in these animals was very low. Therefore, this makes it very difficult to draw a proper conclusion while comparing infected versus non-infected animals. Results from this study showed that majority of animals classified as anaemic had no detectable trypanosomes. This suggests that other factors such as bacterial or parasitic infections or poor nutrition can cause anaemia (Degneh *et al*., 2017). Also, anaemic animal found to be trypanosome-negative might not be true negative; there is mounting evidence indicating that blood might not be appropriate for diagnosing trypanosomiasis due to tissue tropisms. Studies showed that trypanosome parasites prefer other tissues (adipose tissues, skin, and others) to blood; these parasites hide to avoid immune response and blood flow (Ekloh *et al*., 2023; Trindade *et al*., 2016). For this study, no association was found between the trypanosome species identified and the haematocrit level, indicating the degree of the anaemia.

Anaemia and changes in iron status during animal trypanosomiasis

Anaemia is a crucial immunopathological feature associated with trypanosomiasis, and the degree of the anaemia is a vital indicator of the severity of the infection. During trypanosomiasis, the immune response is characterized by a robust pro-inflammatory M1-type activation of the myeloid phagocyte system (MYPS), resulting in a decrease in iron. Over activation of M1-type, MYPS triggers the development of iron-deficiency anaemia as the most prominent pathological parameter in the host due to increased erythrophagocytosis (Stijlemans *et al*., 2015). During infection, the host sequestered iron to starve the parasite and therefore limit the growth of the parasite, this phenomenon is known as nutritional immunity. All these facts indicate that there are several modifications in the iron status of the host. The first parameter that was determined was ferritin, which is an iron-binding protein. Ferritin was quantified in the different livestock, both infected and non-infected animals. The normal value of ferritin varies in different hosts, results showed that compared to cattle and goats, sheep had the lowest ferritin concentration. The result agrees with a previous study where the normal range of ferritin concentration in sheep was found to be between 6.7 – 32 ng/mL (Badawi, 2014). There was a significant increase in ferritin concentration in infected cattle and goats compared to non-infected animals. This is in contradiction with what was observed in camels infected with trypanosomes, where ferritin concentration was rather low (Al-Rubaie *et al*., 2020). The observation made in this present study might be the result of trypanosome infections. However, there was no difference in ferritin concentration between infected and non-infected sheep.

TIBC is the capacity of transferrin to bind iron; therefore, when there is an increase of this parameter, this indicates low iron levels and vice versa. TIBC was significantly high in infected sheep and cattle compared to non-infected animals, similar results were observed in trypanosome infected camels (Al-Rubaie *et al*., 2020). These data, therefore, suggest that in infected cattle and sheep, the levels of free iron available are low. However, the inverse was observed in goats where TIBC was higher in non-infected than in infected animals. TSI was measured, and the results obtained showed that iron concentrations were significantly low in infected sheep and cattle. This was expected looking at the high TIBC recorded in these two specific livestock. However, although a slight difference was observed in infected and non-infected goats, iron levels were not significantly low in general. Normally, in case serum-iron levels are low, TS also decreases; the results from the present study confirmed those observations. A significant reduction was observed in TS in infected sheep and cattle; this is due to low serum iron levels observed in these livestock. Transferrin (holo-transferrin) is an iron-loaded molecule; therefore, if the levels of iron decrease the consequence is that more apo-transferrin (iron-free) molecules are found in circulation in the serum of the host, explaining the low TS. The changes observed in the TIBC, TSI and TS are considerable in trypanosomes-infected animals compared to non-infected animals. This suggests that trypanosomes cause significant changes in the iron status of infected animals. Similar findings were reported by Al-Rubaie and colleagues, where the iron status of trypanosomes-infected camels was significantly modified compared to non-infected camels (Al-Rubaie *et al*., 2020). The iron parameters measured in this current study can be good indicators of infection with trypanosomes, specifically for animals tested negative after diagnosis using techniques such as PCR or microscopy.

Polymorphisms in the transferrin receptor in naturally infected hosts

Iron is one of the most essential nutrients required for trypanosomes to survive in their hosts and to establish the disease. However, the only source of iron for trypanosomes in the host is through the uptake of transferrin (holo-transferrin). Therefore, *T. brucei* express a unique receptor TfR to bind the transferrin of the different hosts. The question was to know whether *T. brucei* expresses specific TfR depending on the host, to facilitate ligand binding in different hosts. This is because mammalian evolution triggered transferrins to diverge considerably; a typical example is that human and bovine transferrins differ by 30% in amino-acid sequence (Bitter *et al*., 1998). Therefore, TfR sequence diversity in natural infections in livestock (sheep, cattle, and goats) were examined and compared. These animals were selected because they are domestic and economically important and known to be the hosts of trypanosome parasites.

It was expected that TfR reads should map to a few expression sites (ideally one ES) indicating high levels of expression while most ES will show low levels of expression. This was the case for most animal samples but not for the wild type and two animals (SH4 and CP4) which shows high read counts for most expression sites. This is consistent with published data showing that minor variants observed at the population level derived from silent ES significantly contribute to the total available TfR (Gerrits *et al*., 2002). Data from the present study showed that BES3 and BES12 seem to be the most preferred in naturally infected livestock; however, BES1 is known to be the most used expression site for majority of the cell lines used for *in vitro* studies. Previous studies have identified specific variable regions in TfR, but for this work, only one region from ESAG7 was examined and compared among the different livestock. The results showed that the region examined was different in all the sequences analysed. Therefore, these data suggest that there is diversity in TfR expression observed within or between the different livestock species. This contradicts findings from another study (Trevor *et al*., 2019), which reported that *T. brucei* expresses only one TfR that can bind transferrin from various mammalian hosts. However, for the study conducted by Trevor et al. (2019) cell lines (lab-adapted parasites) were exposed to transferrin from four hosts (Cow, horse, rabbit, and pig). This does not mimic in anyways natural infections, therefore what was observed *in vitro* in the previous studies might not precisely reflect what is happening *in vivo*. The present study gives a better overview of what happens in a host infected with trypanosomes.

Effect of the VSG-structure on the growth rate and transferrin uptake

The growth rates of cell lines expressing dimeric and trimeric VSG were compared and the data showed no significant difference. This means the VSG structure does not directly affect the growth of trypanosome parasites. However, transferrin uptake in trimeric VSG cell lines was significantly low compared to the dimeric VSG. But the general receptor-mediated endocytosis was normal for all the different cell lines. This suggests that the low transferrin uptake observed in these cells is not the result of reduced endocytosis. TfR is essential for transferrin uptake, therefore reduction in the expression of this protein can significantly affect the uptake of this nutrient (Bitter *et al*., 1998; Gerrits *et al*., 2002). However, the data did not show a reduction in TfR protein levels in the trimeric VSG cell lines. This also suggests that the decrease in transferrin uptake is not due to low or absence of TfR in these cells. Moreover, TfR sequences from the various cell lines were analysed and no significant difference was observed. This result was expected because the cell lines although expressing different VSG were from the same bloodstream expression site (BES1), therefore TfR expressed should be the same (Hertz-Fowler *et al*., 2008). However, only a point mutation which was a transition, was observed in only the metacyclic VSGs (trimeric).

Tiengwe et al. (2017) showed that the GPI-valence influences intracellular trafficking when a TfR with GPI-anchors was made. Here, the TfR is found at the FP, but with the additional GPI-anchor added, this increases the quantity of TfR at the cell surface. Having more TfR at the surface of the parasite should increase transferrin uptake. However, results showed only an increase in the general receptor-mediated endocytosis, but transferrin uptake was not rescued but decreased. Overall, these data suggest that nutrient uptake might be influenced by the VSG structure. The hypothesis therefore is that trimeric VSGs have a complex structure, and probably due to overcrowding at the cell surface, uptake of some nutrients such as transferrin is affected.

5.2 Potential pitfalls and alternative approaches

1. In some study sites the number of animals sampled was very small and the livestock species were different. This makes it difficult to compare trypanosome infections across the various study sites and livestock.

2. To identify diversity in TfR, only the variable region was amplified and sequenced in different livestock, and a very limited number of livestock were used per animal species.

3. To determine the changes in iron status during animal trypanosomiasis, it would have been important to use the same number of animals for the two groups infected and non-infected. For some livestock species, the number of infected animals was low. Further studies should consider confounding factors such as infection with other parasitic or bacterial diseases.

5.3 Conclusion

The overall prevalence of AAT in Greater Accra was low, however, Pokuase area recorded a high infection rate. Trypanosome species identified in circulation in the region are *T. brucei*, *T. congolense*, and *T. theileri*, with *T. brucei* being the most dominant species. Trypanosome infections were detected in all the livestock screened (cattle, sheep, goats, and pigs) and at almost all the study sites; however, trypanosome infections significantly varied across the different livestock and study sites. This study showed that of all the livestock screened, sheep were the most infected. This is the first study to report sheep as the most trypanosome-infected livestock in Greater Accra. Furthermore, the results of this study brought evidence that several changes occur in the iron status of trypanosome-infected livestock, suggesting that iron-deficiency anaemia is the primary type of anaemia during animal trypanosomiasis. These findings indicate that iron parameters might be good indicators of trypanosome infections in livestock. However, more studies with larger sample size are needed to confirm this. Furthermore, for the first time, this study showed that in naturally infected hosts, *T. brucei* expresses a specific and unique TfR. Finally, this study demonstrated that the structure of the VSG expressed significantly affects uptake of some specific nutrients.

5.4 Recommendations

1. Extensive sampling of primary livestock (sheep, cattle, goats, and pigs) in other regions of the country should be done to determine the real burden of animal trypanosomiasis.

2. The same attention should be given to sheep and cattle when implementing control measures to eliminate AAT.

3. Iron parameters such as ferritin concentration, total iron binding capacity, total serum iron and transferrin saturation should be used to reinforce the diagnosis of trypanosome infections in livestock.

4. Full TfRs, not only the variable regions from a large number of infected livestock, must be amplified and sequenced for a better understanding of the importance of the receptor in natural trypanosome infections.

REFERENCES

Abro, Z., Kassie, M., Muriithi, B., Okal, M., Masiga, D., Wanda, G., … Chilongo, K. (2021). The potential economic benefits of controlling trypanosomiasis using waterbuck repellent blend in sub-Saharan Africa. *PLoS ONE*, *16*(7 July), 1–15. https://doi.org/10.1371/journal.pone.0254558

Adams, F., Ohene-Yankyera, K., Aidoo, R., & Wongnaa, C. A. (2021). Economic benefits of livestock management in Ghana. *Agricultural and Food Economics*, *9*(1). https://doi.org/10.1186/s40100-021-00191-7

Aisen, P., & Listowsky, I. (1980). Iron Transport and Storage Proteins. *Annual Review of Biochemistry*, *49*(1), 357–393. https://doi.org/10.1146/annurev.bi.49.070180.002041

Aksoy, S., Gibson, W. C., & Lehane, M. J. (2003). Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. *Advances in Parasitology*, *53*, 1–83. https://doi.org/10.1016/S0065-308X(03)53002-0

Al-Rubaie, L. S. G., Al-Qayim, M. A. J., Al-Rubaie, H. M. A., & Y., A. A. (2020). Study Iron Homeostasis of Infected and Non-Infected Iraqi Camels with Trypanosomiasis. *The Iraqi Journal of Veterinary Medicine*, *44*(2), 35–44. https://doi.org/10.30539/ijvm.v44i2.975

Alsford, S., Eckert, S., Baker, N., Glover, L., Sanchez-Flores, A., Leung, K. F., … Horn, D. (2012). High-throughput decoding of antitrypanosomal drug efficacy and resistance. *Nature*, *482*(7384), 232–236. https://doi.org/10.1038/nature10771

Anderson, N. E., Mubanga, J., Fevre, E. M., Picozzi, K., Eisler, M. C., Thomas, R., & Welburn, S. C. (2011). Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. *PLoS Neglected Tropical Diseases*, *5*(6). https://doi.org/10.1371/journal.pntd.0001211

Aresta-Branco, F., Erben, E., Papavasiliou, F. N., & Stebbins, C. E. (2019). Mechanistic Similarities between Antigenic Variation and Antibody Diversification during Trypanosoma brucei Infection. *Trends in Parasitology*, *35*(4), 302–315. https://doi.org/10.1016/j.pt.2019.01.011

Autheman, D., Crosnier, C., Clare, S., Goulding, D. A., Brandt, C., Harcourt, K., … Wright, G. J. (2021). An invariant Trypanosoma vivax vaccine antigen induces protective immunity. *Nature*, *595*(7865), 96–100. https://doi.org/10.1038/s41586-021-03597-x

Authié, E., Boulangé, A., Muteti, D., Lalmanach, G., Gauthier, F., & Musoke, A. J. (2001). Immunisation of cattle with cysteine proteinases of Trypanosoma congolense: Targetting the disease rather than the parasite. *International Journal for Parasitology*, *31*(13), 1429–1433. https://doi.org/10.1016/S0020-7519(01)00266-1

Auty, H., Morrison, L. J., Torr, S. J., & Lord, J. (2016). Transmission Dynamics of Rhodesian Sleeping Sickness at the Interface of Wildlife and Livestock Areas. *Trends in Parasitology*, *32*(8), 608–621. https://doi.org/10.1016/j.pt.2016.05.003

Badawi, N. M. (2014). Evaluation of some hematological values and ferritin concentrationin normal and emaciated Iraqi sheep. □□□□□□□ □□□□□□□ □□□□□□□ □□□□ □□□□□□□□□ | *Kufa Journal For Veterinary Medical Sciences*, *5*(2).

Bakari, S. M., Ofori, J. A., Kusi, K. A., Aning, G. K., Awandare, G. A., Carrington, M., & Gwira, T. M. (2017). Serum biochemical parameters and cytokine profiles associated with natural African trypanosome infections in cattle. *Parasites and Vectors*, *10*(1). https://doi.org/10.1186/s13071-017-2255-9

Bangs, J. D. (2019). *HHS Public Access*. *40*(12), 1–15. https://doi.org/10.1002/bies.201800181.Evolution

Bart, J., Cordon-obras, C., Vidal, I., Reed, J., Cuevas, L., Field, M. C., … Navarro, M. (2015). *Europe PMC Funders Group Localisation of Serum Resistance-Associated protein in Trypanosoma brucei rhodesiense and transgenic T . b . brucei*. *17*(10), 1523–1535. https://doi.org/10.1111/cmi.12454.Localisation

Basu, S., Horáková, E., & Lukeš, J. (2016). Iron-associated biology of Trypanosoma brucei. *Biochimica et Biophysica Acta - General Subjects*, *1860*(2), 363–370. https://doi.org/10.1016/j.bbagen.2015.10.027

Behnke, J. M., Chiejina, S. N., Musongong, G. A., Nnadi, P. A., Ngongeh, L. A., Abonyi, F. O., & Fakae, B. B. (2011). Resistance and resilience of traditionally managed West African Dwarf goats from the savanna zone of northern Nigeria to naturally acquired trypanosome and gastrointestinal nematode infections. *Journal of Helminthology*, *85*(1), 80–91. https://doi.org/10.1017/S0022149X10000295

Bengaly, Z., Sidibe, I., Ganaba, R., Desquesnes, M., Boly, H., & Sawadogo, L. (2002). Comparative pathogenicity of three genetically distinct types of Trypanosoma congolense in cattle: Clinical observations and haematological changes. *Veterinary Parasitology*, *108*(1), 1–19. https://doi.org/10.1016/S0304-4017(02)00164-4

Benz, C., Lo, W., Fathallah, N., Connor-Guscott, A., Benns, H. J., & Urbaniak, M. D. (2018). Dynamic regulation of the Trypanosoma brucei transferrin receptor in response to iron starvation is mediated via the 3’UTR. *PLoS ONE*, *13*(12), 1–12. https://doi.org/10.1371/journal.pone.0206332

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D. C., … El-Sayed, N. M. (2005). The genome of the African trypanosome Trypanosoma brucei. *Science*, *309*(5733), 416–422. https://doi.org/10.1126/science.1112642

Bezie, M. (2014). African Trypanosomes: Virulence Factors, Pathogenicity And Host Responses. *Journal of Veterinary Advances*, *4*(11), 732. https://doi.org/10.5455/jva.20141129012406

Birhanu, H., Fikru, R., Said, M., Kidane, W., Gebrehiwot, T., Hagos, A., … Büscher, P. (2015). Epidemiology of Trypanosoma evansi and Trypanosoma vivax in domestic animals from selected districts of Tigray and Afar regions, Northern Ethiopia. *Parasites and Vectors*, *8*(1), 1–11. https://doi.org/10.1186/s13071-015-0818-1

Bitter, W., Gerrits, H., Kieft, R., & Borst, P. (1998). The role of transferrin-receptor variation in the host range of Trypanosoma brucei. *Nature*, *391*(6666), 499–502. https://doi.org/10.1038/35166

Blum, J. A., Neumayr, A. L., & Hatz, C. F. (2012). Human African trypanosomiasis in endemic populations and travellers. *European Journal of Clinical Microbiology and Infectious Diseases*, *31*(6), 905–913. https://doi.org/10.1007/s10096-011-1403-y

Boatin, B. A., Wyatt, G. B., Wurapa, F. K., & Bulsara, M. K. (1986). Use of symptoms and signs for diagnosis of trypanosoma brucei rhodesiense trypanosomiasis by rural health personnel. *Bulletin of the World Health Organization*, *64*(3), 389–395.

Brummett, L. M., Kanost, M. R., & Gorman, M. J. (2017). The immune properties of Manduca sexta transferrin. *Insect Biochemistry and Molecular Biology*, *81*, 1–9. https://doi.org/10.1016/j.ibmb.2016.12.006

Büscher, P., Bart, J. M., Boelaert, M., Bucheton, B., Cecchi, G., Chitnis, N., … Van Reet, N. (2018). Do Cryptic Reservoirs Threaten Gambiense-Sleeping Sickness Elimination? *Trends in Parasitology*, *34*(3), 197–207. https://doi.org/10.1016/j.pt.2017.11.008

Büscher, P., Cecchi, G., Jamonneau, V., & Priotto, G. (2017). Human African trypanosomiasis.

The Lancet, *390*(10110), 2397–2409. https://doi.org/10.1016/S0140-6736(17)31510-6

CABI. (2019). *trypanosomosis.pdf* (p. https://www.cabi.org/isc/datasheet/60779#toPicture). p. https://www.cabi.org/isc/datasheet/60779#toPicture.

Campbell, K., Diao, H., Ji, J., & Soong, L. (2003). DNA Immunization with the Gene Encoding P4 Nuclease of Leishmania amazonensis Protects Mice against Cutaneous Leishmaniasis. *Infection and Immunity*, *71*(11), 6270–6278. https://doi.org/10.1128/IAI.71.11.6270-6278.2003

Capewell, P., Cren-Travaillé, C., Marchesi, F., Johnston, P., Clucas, C., Benson, R. A., … MacLeod, A. (2016). The skin is a significant but overlooked anatomical reservoir for vector-borne African trypanosomes. *ELife*, *5*(September2016), 1–17. https://doi.org/10.7554/eLife.17716

Claes, F., Büscher, P., Touratier, L., & Goddeeris, B. M. (2005). Trypanosoma equiperdum: Master of disguise or historical mistake? *Trends in Parasitology*, *21*(7), 316–321. https://doi.org/10.1016/j.pt.2005.05.010

Clayton, C. (2013). The Regulation of Trypanosome Gene Expression by RNA-Binding Proteins. *PLoS Pathogens*, *9*(11), 9–12. https://doi.org/10.1371/journal.ppat.1003680

Clayton, C. E. (2014). Networks of gene expression regulation in Trypanosoma brucei. *Molecular and Biochemical Parasitology*, *195*(2), 96–106. https://doi.org/10.1016/j.molbiopara.2014.06.005

Cock, P. J. A., Antao, T., Chang, J. T., Chapman, B. A., Cox, C. J., Dalke, A., … De Hoon, M. J. L. (2009). Biopython: Freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics*, *25*(11), 1422–1423. https://doi.org/10.1093/bioinformatics/btp163

Compaoré, C. F. A., Kaboré, J., Ilboudo, H., Thomas, L. F., Falzon, L. C., Bamba, M., … Jamonneau, V. (2022). Monitoring the elimination of gambiense human African trypanosomiasis in the historical focus of Batié, South-West Burkina Faso. *Parasite, 29*. https://doi.org/10.1051/parasite/2022024

Cordon-Obras, C., Cano, J., González-Pacanowska, D., Benito, A., Navarro, M., & Bart, J. M. (2013). Trypanosoma brucei gambiense adaptation to different Mammalian Sera is associated with VSG expression site plasticity. *PLoS ONE, 8*(12), 1–15. https://doi.org/10.1371/journal.pone.0085072

Cordon-Obras, C., García-Estébanez, C., Ndong-Mabale, N., Abaga, S., Ndongo-Asumu, P., Benito, A., & Cano, J. (2010). Screening of Trypanosoma brucei gambiense in domestic livestock and tsetse flies from an insular endemic focus (Luba, Equatorial Guinea). *PLoS Neglected Tropical Diseases*, *4*(6). https://doi.org/10.1371/journal.pntd.0000704

Cornelissen, A. W. C. a., Bakkeren, G. A. M., Barry, J. D., Michels, P. A. M., & Borst, P. (1985). Characteristics of trypanosome variant antigen genes active in the tsetse fly. *Nucleic Acids Research*, *13*(13), 4661–4676. https://doi.org/10.1093/nar/13.13.4661

Corrêa, J. R., Atella, G. C., Batista, M. M., & Soares, M. J. (2008). Transferrin uptake in Trypanosoma cruzi is impaired by interference on cytostome-associated cytoskeleton elements and stability of membrane cholesterol, but not by obstruction of clathrin-dependent endocytosis. *Experimental Parasitology*, *119*(1), 58–66. https://doi.org/10.1016/j.exppara.2007.12.010

Coustou, V., Plazolles, N., Guegan, F., & Baltz, T. (2012). Sialidases play a key role in infection and anaemia in Trypanosoma congolense animal trypanosomiasis. *Cellular Microbiology, 14*(3), 431–445. https://doi.org/10.1111/j.1462-5822.2011.01730.x

Cox, A. P., Tosas, O., Tilley, A., Picozzi, K., Coleman, P., Hide, G., & Welburn, S. C. (2010). Constraints to estimating the prevalence of trypanosome infections in East African zebu cattle. *Parasites and Vectors*, *3*(1), 1–8. https://doi.org/10.1186/1756-3305-3-82

Cuypers, B., Domagalska, M. A., Meysman, P., Muylder, G. De, Vanaerschot, M., Imamura, H., … Dujardin, J. C. (2017). Multiplexed Spliced-Leader Sequencing: A high-throughput, selective method for RNA-seq in Trypanosomatids. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-03987-0

Das, A., Banday, M., & Bellofatto, V. (2008). RNA polymerase transcription machinery in trypanosomes. *Eukaryotic Cell*, *7*(3), 429–434. https://doi.org/10.1128/EC.00297-07

Davaasuren, B., Yamagishi, J., Mizushima, D., Narantsatsral, S., Otgonsuren, D., Myagmarsuren, P., … Suganuma, K. (2019). Draft Genome Sequence of Trypanosoma equiperdum Strain IVM-t1 . *Microbiology Resource Announcements*, *8*(9), 11–13. https://doi.org/10.1128/mra.01119-18

Degneh, E., Shibeshi, W., Terefe, G., Asres, K., & Ashenafi, H. (2017). Bovine trypanosomosis: Changes in parasitemia and packed cell volume in dry and wet seasons at Gidami District, Oromia Regional State, western Ethiopia. *Acta Veterinaria Scandinavica*, *59*(1), 1–8. https://doi.org/10.1186/s13028-017-0327-7

Desquesnes, M, MICHEL, J., DE LA ROCQUE, S., SOLANO, P., MILLOGO, L., BENGALY, Z., & SIDIBE, I. (1999). Enquête parasitologique et sérologique (Elisa-indirect) sur les trypanosomoses des bovins dans la zone de Sidéradougou, Burkina Faso. *Revue d'élevage et de Médecine Vétérinaire Des Pays Tropicaux*, *52*(10), 223–232. https://doi.org/10.19182/remvt.9667

Desquesnes, Marc, Gonzatti, M., Sazmand, A., Thévenon, S., Bossard, G., Boulangé, A., …

Berthier, D. (2022). A review on the diagnosis of animal trypanosomoses. *Parasites and Vectors*, *15*(1), 1–24. https://doi.org/10.1186/s13071-022-05190-1

Desquesnes, Marc, Kamyingkird, K., Vergne, T., Sarataphan, N., Pranee, R., & Jittapalapong, S. (2011). An evaluation of melarsomine hydrochloride efficacy for parasitological cure in experimental infection of dairy cattle with Trypanosoma evansi in Thailand. *Parasitology*, *138*(9), 1134–1142. https://doi.org/10.1017/S0031182011000771

Dieng, M. M., Dera, K. sida M., Moyaba, P., Ouedraogo, G. M. S., Demirbas-Uzel, G., Gstöttenmayer, F., … Abd-Alla, A. M. M. (2022). Prevalence of Trypanosoma and Sodalis in wild populations of tsetse flies and their impact on sterile insect technique programmes for tsetse eradication. *Scientific Reports*, *12*(1), 1–17. https://doi.org/10.1038/s41598-022-06699-2

DP Kateete, C Alezuyo, A Nanteza, C Asiimwe, G. L. (2012). Trypanosoma brucei. *Trends in Parasitology*, *36*(6), 571–572. https://doi.org/10.1016/j.pt.2019.10.007

Ebanga Echi, J., Amadoh Jude, A., Farikou, O., Abba, S., & Oumar, M. (2020). Animal Trypanosomiasis in Cattle of Good Body Condition Score in the Adamawa Region of Cameroon: Hematological and Immunological Implications. *International Journal of Tropical Diseases*, *3*(2), 1–8. https://doi.org/10.23937/2643-461x/1710041

Ekloh, W., Sunter, J. D., & Gwira, T. M. (2023). African trypanosome infection patterns in cattle in a farm setting in Southern Ghana. *Acta Tropica*, *237*(July 2022), 106721. https://doi.org/10.1016/j.actatropica.2022.106721

Ereqat, S., Nasereddin, A., Al-Jawabreh, A., Al-Jawabreh, H., Al-Laham, N., & Abdeen, Z. (2020). Prevalence of Trypanosoma evansi in livestock in Palestine. *Parasites and Vectors*, *13*(1), 1–8. https://doi.org/10.1186/s13071-020-3894-9

Fairlamb, A. H., & Horn, D. (2018). Melarsoprol Resistance in African Trypanosomiasis. *Trends in Parasitology*, *34*(6), 481–492. https://doi.org/10.1016/j.pt.2018.04.002

Fast, B., Kremp, K., Boshart, M., & Steverding, D. (1999). Iron-dependent regulation of transferrin receptor expression in Trypanosoma brucei. *Biochemical Journal*, *342*(3), 691–696. https://doi.org/10.1042/0264-6021:3420691

Fenn, K., & Matthews, K. R. (2007). The cell biology of Trypanosoma brucei differentiation. *Current Opinion in Microbiology*, *10*(6), 539–546. https://doi.org/10.1016/j.mib.2007.09.014

Fermino, B. R., Viola, L. B., Paiva, F., Garcia, H. A., De Paula, C. D., Botero-Arias, R., … Teixeira, M. M. G. (2013). The phylogeography of trypanosomes from South American alligatorids and African crocodilids is consistent with the geological history of South American river basins and the transoceanic dispersal of Crocodylus at the Miocene. *Parasites and Vectors*, *6*(1), 1–14. https://doi.org/10.1186/1756-3305-6-313

Fetene, E., Leta, S., Regassa, F., & Büscher, P. (2021). Global distribution, host range and prevalence of Trypanosoma vivax: a systematic review and meta-analysis. *Parasites and Vectors*, *14*(1), 1–20. https://doi.org/10.1186/s13071-021-04584-x

Fèvre, E. M., Odiit, M., Coleman, P. G., Woolhouse, M. E. J., & Welburn, S. C. (2008). Estimating the burden of rhodesiense sleeping sickness during an outbreak in Serere, eastern Uganda. *BMC Public Health*, *8*, 1–11. https://doi.org/10.1186/1471-2458-8-96

Field, M. C., & Carrington, M. (2009). The trypanosome flagellar pocket. *Nature Reviews Microbiology*, *7*(11), 775–786. https://doi.org/10.1038/nrmicro2221

Franco, Jose R., Cecchi, G., Paone, M., Diarra, A., Grout, L., Ebeja, A. K., … Argaw, D. (2022). The elimination of human African trypanosomiasis: Achievements in relation to WHO road map targets for 2020. *PLoS Neglected Tropical Diseases*, *16*(1), 1–19. https://doi.org/10.1371/JOURNAL.PNTD.0010047

Franco, Jose R., Cecchi, G., Paone, M., Diarra, A., Grout, L., Kadima Ebeja, A., … Argaw, D. (2022). The elimination of human African trypanosomiasis: Achievements in relation to WHO road map targets for 2020. *PLoS Neglected Tropical Diseases*, *16*(1), e0010047. https://doi.org/10.1371/journal.pntd.0010047

Franco, José R., Cecchi, G., Priotto, G., Paone, M., Diarra, A., Grout, L., … Argaw, D. (2020). Monitoring the elimination of human African trypanosomiasis at continental and country level: Update to 2018. *PLoS Neglected Tropical Diseases*, *14*(5), 1–18. https://doi.org/10.1371/journal.pntd.0008261

Franco, Jose R., Simarro, P. P., Diarra, A., & Jannin, J. G. (2014). Epidemiology of human African trypanosomiasis. *Clinical Epidemiology*, *6*(1), 257–275. https://doi.org/10.2147/CLEP.S39728

Fujimoto. (2008). 基因的改变NIH Public Access. *Bone*, *23*(1), 1–7. https://doi.org/10.1111/j.1365-2958.2011.07842.x.Late

Gerrits, H., Mußmann, R., Bitter, W., Kieft, R., & Borst, P. (2002). The physiological significance of transferrin receptor variations in Trypanosoma brucei. *Molecular and Biochemical Parasitology*, *119*(2), 237–247. https://doi.org/10.1016/S0166-6851(01)00417-0

Giordani, F., Morrison, L. J., Rowan, T. G., De Koning, H. P., & Barrett, M. P. (2016). The animal trypanosomiases and their chemotherapy: A review. *Parasitology*, *143*(14), 1862–1889. https://doi.org/10.1017/S0031182016001268

Greca, F. La, & Magez, S. (2011). Vaccination against trypanosomiasis: Can it be done or is the trypanosome truly the ultimate immune destroyer and escape artist? *Human Vaccines*, *7*(11), 1225–1233. https://doi.org/10.4161/hv.7.11.18203

Gruer, M. J., Bradbury, A. J., & Guest, J. R. (1997). Construction and properties of aconitase mutants of Escherichia coli. *Microbiology*, *143*(6), 1837–1846. https://doi.org/10.1099/00221287-143-6-1837

Gudjoncik, A., Guenancia, C., Zeller, M., Cottin, Y., Vergely, C., & Rochette, L. (2014). Iron, oxidative stress, and redox signaling in the cardiovascular system. *Molecular Nutrition and Food Research*, *58*(8), 1721–1738. https://doi.org/10.1002/mnfr.201400036

Günzl, A. (2010). The pre-mRNA splicing machinery of trypanosomes: Complex or simplified? *Eukaryotic Cell*, *9*(8), 1159–1170. https://doi.org/10.1128/EC.00113-10

Günzl, A., Kirkham, J. K., Nguyen, T. N., Badjatia, N., & Park, S. H. (2015). Mono-allelic VSG expression by RNA polymerase I in Trypanosoma brucei: Expression site control from both ends? *Gene*, *556*(1), 68–73. https://doi.org/10.1016/j.gene.2014.09.047

Gutierrez, C., Corbera, J. A., Morales, M., & Büscher, P. (2006). Trypanosomosis in goats: Current status. *Annals of the New York Academy of Sciences*, *1081*, 300–310. https://doi.org/10.1196/annals.1373.040

Habeeb, I. F., Chechet, G. D., & Kwaga, J. K. P. (2021). Molecular identification and prevalence of trypanosomes in cattle distributed within the Jebba axis of the River Niger, Kwara state, Nigeria. *Parasites and Vectors*, *14*(1), 1–12. https://doi.org/10.1186/s13071-021-05054-0

Haldar, K., Henderson, C. L., & Cross, G. A. M. (1986). Identification of the parasite transferrin receptor of Plasmodium falciparum-infected erythrocytes and its acylation via 1,2-diacyl-sn-glycerol. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(22), 8565–8569. https://doi.org/10.1073/pnas.83.22.8565

Hamill, L. C., Kaare, M. T., Welburn, S. C., & Picozzi, K. (2013). Domestic pigs as potential reservoirs of human and animal trypanosomiasis in Northern Tanzania. *Parasites and Vectors*, *6*(1), 1–7. https://doi.org/10.1186/1756-3305-6-322

Headrick, D. R. (2014). Sleeping Sickness Epidemics and Colonial Responses in East and Central Africa, 1900-1940. *PLoS Neglected Tropical Diseases*, *8*(4). https://doi.org/10.1371/journal.pntd.0002772

Hertz-Fowler, C., Figueiredo, L. M., Quail, M. A., Becker, M., Jackson, A., Bason, N., … Berriman, M. (2008). Telomeric expression sites are highly conserved in Trypanosoma brucei. *PLoS ONE*, *3*(10). https://doi.org/10.1371/journal.pone.0003527

Hide, G. (1999). History of sleeping sickness in East Africa. *Clinical Microbiology Reviews*, *12*(1), 112–125. https://doi.org/10.1128/cmr.12.1.112

Hirumi, H., & Hirumi, K. (1989). Continuous Cultivation of Trypanosoma brucei Blood Stream Forms in a Medium Containing a Low Concentration of Serum Protein without Feeder Cell Layers. *The Journal of Parasitology*, *75*(6), 985. https://doi.org/10.2307/3282883

Horn, D. (2014). Antigenic variation in African trypanosomes. *Molecular and Biochemical Parasitology*, *195*(2), 123–129. https://doi.org/10.1016/j.molbiopara.2014.05.001

Huson, L. E. J., Authié, E., Boulangé, A. F., Goldring, J. P. D., & Coetzer, T. H. T. (2009). Modulation of the immunogenicity of the Trypanosoma congolense cysteine protease, congopain, through complexation with α2-macroglobulin. *Veterinary Research*, *40*(6). https://doi.org/10.1051/vetres/2009036

Idehen, C. O., Ishola, O. O., Adeyemi, I. G., Abongaby, G., Olaleye, O. O., Aluma, A. L., … Obaloto, O. B. (2018). Prevalence of African trypanosomosis in cattle and sheep in Bassa local government area of Plateau State, Nigeria. *Sokoto Journal of Veterinary Sciences*, *16*(3), 11. https://doi.org/10.4314/sokjvs.v16i3.2

Imlay, J., & Linn, S. (1988). Damage and Oxygen Radical. *Science*, *240*, 1302–1309.

Jackson, A. P., Allison, H. C., Barry, J. D., Field, M. C., Hertz-Fowler, C., & Berriman, M. (2013). A Cell-surface Phylome for African Trypanosomes. *PLoS Neglected Tropical Diseases*, *7*(3). https://doi.org/10.1371/journal.pntd.0002121

Jackson, A. P., Goyard, S., Xia, D., Foth, B. J., Sanders, M., Wastling, J. M., … Berriman, M. (2015). Global gene expression profiling through the complete life cycle of Trypanosoma vivax. *PLoS Neglected Tropical Diseases*, *9*(8), 1–29. https://doi.org/10.1371/journal.pntd.0003975

Jamonneau, V., Ilboudo, H., Kaboré, J., Kaba, D., Koffi, M., Solano, P., … Bucheton, B. (2012). Untreated human infections by trypanosoma brucei gambiense are not 100% fatal. *PLoS Neglected Tropical Diseases*, *6*(6). https://doi.org/10.1371/journal.pntd.0001691

Jirků, M., Votýpka, J., Petrželková, K. J., Jirků-Pomajbíková, K., Kriegová, E., Vodička, R., … Lukeš, J. (2015). Wild chimpanzees are infected by Trypanosoma brucei. *International Journal for Parasitology: Parasites and Wildlife*, *4*(3), 277–282. https://doi.org/10.1016/j.ijppaw.2015.05.001

Kaplan, J., & Ward, D. M. (2013). The essential nature of iron usage and regulation. *Current Biology*, *23*(15), R642–R646. https://doi.org/10.1016/j.cub.2013.05.033

Kariuki, C. K., Stijlemans, B., & Magez, S. (2019). The trypanosomal transferrin receptor of Trypanosoma brucei—a review. *Tropical Medicine and Infectious Disease*, *4*(4). https://doi.org/10.3390/tropicalmed4040126

Kasozi, K. I., Zirintunda, G., Ssempijja, F., Buyinza, B., Alzahrani, K. J., Matama, K., … Welburn, S. C. (2021). Epidemiology of Trypanosomiasis in Wildlife—Implications for

Humans at the Wildlife Interface in Africa. *Frontiers in Veterinary Science*, *8*(June), 1–15. https://doi.org/10.3389/fvets.2021.621699

Kateregga, J., Lubega, G. W., Lindblad, E. B., Authié, E., Coetzer, T. H. T., & Boulangé, A. F. V. (2012). Effect of adjuvants on the humoral immune response to congopain in mice and cattle. *BMC Veterinary Research*, *8*, 1–10. https://doi.org/10.1186/1746-6148-8-63

Kelly, T. K., Liu, Y., Lay, F. D., Liang, G., Berman, B. P., & Jones, P. A. (2012). Genome-wide mapping of nucleosome positioning and DNA methylation within individual DNA molecules. *Genome Research*, *22*(12), 2497–2506. https://doi.org/10.1101/gr.143008.112

Kelvin, N., Gabriel, S., Emmanuel A, M., Anna B, E., Peter J, H., & Paul S, G. (2020). Influence of Land Cover and Host Species on Trypanosome Infection in Cattle and its Socio-Economic Impacts to Pastoralists of the Maasai Steppe, Tanzania. *Journal of Infectious Diseases and Epidemiology*, *6*(1). https://doi.org/10.23937/2474-3658/1510107

Kennedy, P. G. E. (2013). Clinical features, diagnosis, and treatment of human African trypanosomiasis (sleeping sickness). *The Lancet Neurology*, *12*(2), 186–194. https://doi.org/10.1016/S1474-4422(12)70296-X

Krewulak, K. D., & Vogel, H. J. (2008). Structural biology of bacterial iron uptake. *Biochimica et Biophysica Acta - Biomembranes*, *1778*(9), 1781–1804. https://doi.org/10.1016/j.bbamem.2007.07.026

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D. J., Suzuki, H., … Igarashi, I. (2003). Loop-Mediated Isothermal Amplification for Detection of African Trypanosomes. *Journal of Clinical Microbiology*, *41*(12), 5517–5524. https://doi.org/10.1128/JCM.41.12.5517-5524.2003

Kurup, S. P., & Tewari, A. K. (2012). Induction of protective immune response in mice by a

DNA vaccine encoding Trypanosoma evansi beta tubulin gene. *Veterinary Parasitology*, *187*(1–2), 9–16. https://doi.org/10.1016/j.vetpar.2012.01.009

Lança, A. S. C., de Sousa, K. P., Atouguia, J., Prazeres, D. M. F., Monteiro, G. A., & Silva, M. S. (2011). Trypanosoma brucei: Immunisation with plasmid DNA encoding invariant surface glycoprotein gene is able to induce partial protection in experimental African trypanosomiasis. *Experimental Parasitology*, *127*(1), 18–24. https://doi.org/10.1016/j.exppara.2010.06.017

Landfear, S. M., & Ignatushchenko, M. (2001). The flagellum and flagellar pocket of trypanosomatids. *Molecular and Biochemical Parasitology*, *115*(1), 1–17. https://doi.org/10.1016/S0166-6851(01)00262-6

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Li, S. Q., Yang, W. B., Lun, Z. R., Ma, L. J., Xi, S. M., Chen, Q. L., … Yang, L. Z. (2009). Immunization with recombinant actin from Trypanosoma evansi induces protective immunity against T. evansi, T. equiperdum and T. b. brucei infection. *Parasitology Research*, *104*(2), 429–435. https://doi.org/10.1007/s00436-008-1216-9

Liao, Y., Smyth, G. K., & Shi, W. (2014). FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Lubega, G. W., Byarugaba, D. K., & Prichard, R. K. (2002). Immunization with a tubulin-rich preparation from Trypanosoma brucei confers broad protection against African trypanosomosis. *Experimental Parasitology*, *102*(1), 9–22. https://doi.org/10.1016/S0014-4894(02)00140-6

Macedo, M. F., & de Sousa, M. (2008). Transferrin and the transferrin receptor: Of magic bullets and other concerns. *Inflammation and Allergy - Drug Targets*, *7*(1), 41–52. https://doi.org/10.2174/187152808784165162

MacLean, L. M., Odiit, M., Chisi, J. E., Kennedy, P. G. E., & Sternberg, J. M. (2010). Focus-specific clinical profiles in human African trypanosomiasis caused by trypanosoma brucei rhodesiense. *PLoS Neglected Tropical Diseases*, *4*(12), 1–12. https://doi.org/10.1371/journal.pntd.0000906

Magez, S, Schwegmann, A., Atkinson, R., Claes, F., & Drennan, M. (2008). The Role of B-cells and IgM Antibodies in Parasitemia, Anemia, and VSG Switching in Trypanosoma brucei-Infected Mice. *PLoS Pathog*, *4*(8), 1000122. https://doi.org/10.1371/journal.ppat.1000122

Magez, Stefan, Esteban, J., Torres, P., & Obishakin, E. (2020). *Infections With Extracellular Trypanosomes Require Control by Efficient Innate Immune Mechanisms and Can Result in the Destruction of the Mammalian Humoral Immune System*. *11*(March), 1–19. https://doi.org/10.3389/fimmu.2020.00382

Magez, Stefan, Stijlemans, B., Radwanska, M., Pays, E., Ferguson, M. A. J., & Baetselier, P. De. (1998). This information is current as of March 25, 2013. *The Journal of Immunology*.

Magona, J. W., Mayende, J. S. P., Olaho-Mukani, W., Coleman, P. G., Jonsson, N. N., Welburn, S. C., & Eisler, M. C. (2003). A comparative study on the clinical, parasitological and molecular diagnosis of bovine trypanosomosis in Uganda. *Onderstepoort Journal of Veterinary Research*, *70*(3), 213–218.

Mahoudeau, C. (2021). Trypanosomiasis, human African (sleeping sickness). *World Health Organization*, 21–24.

Maier, A., & Steverding, D. (1996). Low affinity of Trypanosoma brucei transferrin receptor to apotransferrin at pH 5 explains the fate of the ligand during endocytosis. *FEBS Letters*, *396*(1), 87–89. https://doi.org/10.1016/0014-5793(96)01073-3

Manna, P. T., Boehm, C., Leung, K. F., Natesan, S. K., & Field, M. C. (2014). Life and times: Synthesis, trafficking, and evolution of VSG. *Trends in Parasitology*, *30*(5), 251–258. https://doi.org/10.1016/j.pt.2014.03.004

Manta, B., Fleitas, L., & Comini, M. (2000). Iron Metabolism in Pathogenic Bacteria. *Annual Review of Microbiology*, *54*(1), 881–941. https://doi.org/10.1146/annurev.micro.54.1.881

Martínez-Calvillo, S., Vizuet-De-Rueda, J. C., Florencio-Martínez, L. E., Manning-Cela, R. G., & Figueroa-Angulo, E. E. (2010). Gene expression in trypanosomatid parasites. *Journal of Biomedicine and Biotechnology*, *2010*. https://doi.org/10.1155/2010/525241

Masiga, D. K., McNamara, J. J., Laveissière, C., Truc, P., & Gibson, W. C. (1996). A high prevalence of mixed trypanosome infections in tsetse flies in Sinfra, Côte d'Ivoire, detected by DNA amplification. *Parasitology*, *112*(1), 75–80. https://doi.org/10.1017/s0031182000065094

Masumu, J., Geysen, D., & Bossche, P. Van den. (2009). Endemic type of animal trypanosomiasis is not associated with lower genotype variability of Trypanosoma congolense isolates circulating in livestock. *Research in Veterinary Science*, *87*(2), 265–269. https://doi.org/10.1016/j.rvsc.2009.03.003

Matovu, E., Mugasa, C. M., Waiswa, P., Kitibwa, A., Boobo, A., & Ndung'u, J. M. (2020). Haemoparasitic Infections in Cattle from a Trypanosoma brucei Rhodesiense Sleeping

Sickness Endemic District of Eastern Uganda. *Tropical Medicine and Infectious Disease*, *5*(1), 1–10. https://doi.org/10.3390/tropicalmed5010024

Meharenet, B., & Tsegaye, M. (2020). *Bovine Anemia Associated With Trypanosomosis Infection in Local Breed Cattle ' s (Ethiopian Zebu Breeds)*. *7*, 3–7.

Mehlert, A., Wormald, M. R., & Ferguson, M. A. J. (2012). Modeling of the N-glycosylated transferrin receptor suggests how transferrin binding can occur within the surface coat of trypanosoma brucei. *PLoS Pathogens*, *8*(4), 1–11. https://doi.org/10.1371/journal.ppat.1002618

Meyer, A., Holt, H. R., Selby, R., & Guitian, J. (2016). Past and Ongoing Tsetse and Animal Trypanosomiasis Control Operations in Five African Countries: A Systematic Review. *PLoS Neglected Tropical Diseases*, *10*(12), 1–29. https://doi.org/10.1371/journal.pntd.0005247

Mkunza, F., Olaho, W. M., & Powell, C. N. (1995). Partial protection against natural trypanosomiasis after vaccination with a flagellar pocket antigen from Trypanosoma brucei rhodesiense. *Vaccine*, *13*(2), 151–154. https://doi.org/10.1016/0264-410X(95)93128-V

Moreno, C. J. G., Temporão, A., Torres, T., & Silva, M. S. (2019). Trypanosoma brucei interaction with host: Mechanism of VSG release as target for drug discovery for african trypanosomiasis. *International Journal of Molecular Sciences*, *20*(6). https://doi.org/10.3390/ijms20061484

Mugnier, M. R., Stebbins, C. E., & Papavasiliou, F. N. (2016). Masters of Disguise: Antigenic Variation and the VSG Coat in Trypanosoma brucei. *PLoS Pathogens*, *12*(9), 1–6. https://doi.org/10.1371/journal.ppat.1005784

Muhanguzi, D., Mugenyi, A., Bigirwa, G., Kamusiime, M., Kitibwa, A., Akurut, G. G., …

Tweyongyere, R. (2017). African animal trypanosomiasis as a constraint to livestock health and production in Karamoja region: A detailed qualitative and quantitative assessment. *BMC Veterinary Research*, *13*(1), 1–13. https://doi.org/10.1186/s12917-017-1285-z

Mulindwa, J., Fadda, A., Merce, C., Matovu, E., Enyaru, J., & Clayton, C. (2014). Methods to Determine the Transcriptomes of Trypanosomes in Mixtures with Mammalian Cells: The Effects of Parasite Purification and Selective cDNA Amplification. *PLoS Neglected Tropical Diseases*, *8*(4). https://doi.org/10.1371/journal.pntd.0002806

Müllner, E. W., Neupert, B., & Kühn, L. C. (1989). A specific mRNA binding factor regulates the iron-dependent stability of cytoplasmic transferrin receptor mRNA. *Cell*, *58*(2), 373–382. https://doi.org/10.1016/0092-8674(89)90851-9

Musaya, J., Matovu, E., Nyirenda, M., & Chisi, J. (2015). Role of cytokines in Trypanosoma brucei-induced anaemia: A review of the literature. *Malawi Medical Journal*, *27*(2), 45–50. https://doi.org/10.4314/mmj.v27i2.3

Mussmann, R., Engstler, M., Gerrits, H., Kieft, R., Toaldo, C. B., Onderwater, J., … Borst, P. (2004). Factors affecting the level and localization of the transferrin receptor in Trypanosoma brucei. *Journal of Biological Chemistry*, *279*(39), 40690–40698. https://doi.org/10.1074/jbc.M404697200

N'Djetchi, M. K., Ilboudo, H., Koffi, M., Kaboré, J., Kaboré, J. W., Kaba, D., … Jamonneau, V. (2017). The study of trypanosome species circulating in domestic animals in two human African trypanosomiasis foci of Côte d'Ivoire identifies pigs and cattle as potential reservoirs of Trypanosoma brucei gambiense. *PLoS Neglected Tropical Diseases*, *11*(10), 1–16. https://doi.org/10.1371/journal.pntd.0005993

Naessens, J. (2006). Bovine trypanotolerance: A natural ability to prevent severe anaemia and haemophagocytic syndrome? *International Journal for Parasitology*, *36*(5), 521–528. https://doi.org/10.1016/j.ijpara.2006.02.012

Nakayima, J., Nakao, R., Alhassan, A., Mahama, C., Afakye, K., & Sugimoto, C. (2012a). Molecular epidemiological studies on animal trypanosomiases in Ghana. *Parasites and Vectors*, *5*(1), 1–7. https://doi.org/10.1186/1756-3305-5-217

Nakayima, J., Nakao, R., Alhassan, A., Mahama, C., Afakye, K., & Sugimoto, C. (2012b). *Molecular epidemiological studies on animal trypanosomiases in Ghana*. 1–7.

Nantulya, V. M., & Diall, O. (1998). TSETSE AND. *Journal of Protozoology Research*, *8*(3), 29.

Ngomtcho, S. C. H., Weber, J. S., Ngo Bum, E., Gbem, T. T., Kelm, S., & Achukwi, M. D. (2017). Molecular screening of tsetse flies and cattle reveal different Trypanosoma species including T. grayi and T. theileri in northern Cameroon. *Parasites and Vectors*, *10*(1), 1–16. https://doi.org/10.1186/s13071-017-2540-7

Nimpaye, H., Njiokou, F., Njine, T., Njitchouang, G. R., Cuny, G., Herder, S., … Simo, G. (2003). *Prevalence in Domestic Animals of Sleeping Sickness Foci of C Ameroon*.

Njiokou, F., Nimpaye, H., Simo, G., Njitchouang, G. R., Asonganyi, T., Cuny, G., & Herder, S. (2010). *of T Rypanosoma Brucei Gambiense in Sleeping Sickness Foci in C Ameroon*. 61–66.

Ofori, J. A. (2019). *University Of Ghana* (Vol. 7). https://doi.org/10.1080/02724936.1987.11748497

Ofori, J. A., Bakari, S. M., Bah, S., Kolugu, M. K., Aning, G. K., Awandare, G. A., … Gwira, T. M. (2022). A longitudinal two-year survey of the prevalence of trypanosomes in domestic cattle in Ghana by massively parallel sequencing of barcoded amplicons. *PLoS Neglected*

Tropical Diseases, *16*(4), 1–16. https://doi.org/10.1371/journal.pntd.0010300

Omowumi T, K., Charity K, L., & Abolanle AA, K. (2020). Trypanosomiasis: Recent advances in strategies for control. *Global Journal of Infectious Diseases and Clinical Research*, *6*(1), 037–041. https://doi.org/10.17352/2455-5363.000033

Onyilagha, C., & Uzonna, J. E. (2019). Host Immune Responses and Immune Evasion Strategies in African Trypanosomiasis. *Frontiers in Immunology*, *10*(November). https://doi.org/10.3389/fimmu.2019.02738

Pantopoulos, K. (2004). Iron Metabolismo and the IRE/IRP Regulation System. *Annals of the New York Academy of Sciences*, *1012*, 1–13.

Paulnock, D. M., Freeman, B. E., & Mansfield, J. M. (2010). *Modulation of innate immunity by African Trypanosomes*. https://doi.org/10.1017/S0031182010001460

Perdomo, D., Bonhivers, M., & Robinson, D. (2016). The Trypanosome Flagellar Pocket Collar and Its Ring Forming Protein—TbBILBO1. *Cells*, *5*(1), 9. https://doi.org/10.3390/cells5010009

Radwanska, M., Magez, S., Dumont, N., Pays, A., Nolan, D., & Pays, E. (2000). Antibodies raised against the flagellar pocket fraction of Trypanosoma brucei preferentially recognize HSP60 in cDNA expression library. *Parasite Immunology*, *22*(12), 639–650. https://doi.org/10.1046/j.1365-3024.2000.00348.x

Ramey, K., Eko, F. O., Thompson, W. E., Armah, H., Igietseme, J. U., & Stiles, J. K. (2009). Immunolocalization and challenge studies using a recombinant Vibrio cholerae ghost expressing Trypanosoma brucei Ca2+ ATPase (TBCA2) antigen. *American Journal of Tropical Medicine and Hygiene*, *81*(3), 407–415. https://doi.org/10.4269/ajtmh.2009.81.407

Reid, S. A., & Copeman, D. B. (2003). The development and validation of an antibody-ELISA to detect Trypanosoma evansi infection in cattle in Australia and Papua New Guinea. *Preventive Veterinary Medicine*, *61*(3), 195–208. https://doi.org/10.1016/j.prevetmed.2003.07.004

Reyes-López, M., Piña-Vázquez, C., & Serrano-Luna, J. (2015). Transferrin: Endocytosis and cell signaling in parasitic protozoa. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/641392

Reyes-López, M., Serrano-Luna, J. D. J., Negrete-Abascal, E., León-Sicairos, N., Guerrero-Barrera, A. L., & De la Garza, M. (2001). Entamoeba histolytica: Transferrin binding proteins. *Experimental Parasitology*, *99*(3), 132–140. https://doi.org/10.1006/expr.2001.4647

Rock, K. S., Huang, C. I., Crump, R. E., Bessell, P. R., Brown, P. E., Tirados, I., … Peka, M. (2022). Update of transmission modelling and projections of gambiense human African trypanosomiasis in the Mandoul focus, Chad. *Infectious Diseases of Poverty*, *11*(1), 1–13. https://doi.org/10.1186/s40249-022-00934-8

Rodrigues, C. M. F., Garcia, H. A., Rodrigues, A. C., Pereira, D. L., Pereira, C. L., Viola, L. B., … Teixeira, M. M. G. (2020). Expanding our knowledge on African trypanosomes of the subgenus Pycnomonas: A novel Trypanosoma suis-like in tsetse flies, livestock and wild ruminants sympatric with Trypanosoma suis in Mozambique. *Infection, Genetics and Evolution*, *78*(December 2019), 104143. https://doi.org/10.1016/j.meegid.2019.104143

Rodriguez, M. H., & Jungery, M. (1986). A protein on plasmodium falciparum-infected erythrocytes functions as a transferrin receptor. *Nature*, *324*(6095), 388–391. https://doi.org/10.1038/324388a0

Rouault, T., & Klausner, R. (1997). Regulation of Iron Metabolism in Eukaryotes. In *Current*

Topics in Cellular Regulation (Vol. 35). https://doi.org/10.1016/S0070-2137(97)80001-5

Salmon, D., Hanocq-Quertier, J., Paturiaux-Hanocq, F., Pays, A., Tebabi, P., Nolan, D. P., … Pays, E. (1997). Characterization of the ligand-binding site of the transferrin receptor in Trypanosoma brucei demonstrates a structural relationship with the N-terminal domain of the variant surface glycoprotein. *EMBO Journal*, *16*(24), 7272–7278. https://doi.org/10.1093/emboj/16.24.7272

Salmon, Didier, Geuskens, M., Hanocq, F., Hanocq-Quertier, J., Nolan, D., Ruben, L., & Pays, E. (1994). A novel heterodimeric transferrin receptor encoded by a pair of VSG expression site-associated genes in T. brucei. *Cell*, *78*(1), 75–86. https://doi.org/10.1016/0092-8674(94)90574-6

Santos, A. L. S., Branquinha, M. h., D'Avila-Levy, C. M., & Kneipp, L. F. (2014). Proteins and Proteomics of Leishmania and Trypanosoma. In *Subcellular Biochemistry* (Vol. 74). https://doi.org/10.1007/978-94-007-7305-9

Sara, A., Zeelen, J., Gkeka, A., Chandra, M., & Foti, K. (2023). *A Structural Classification of the Variant Surface Glycoproteins of the African Trypanosome*.

Schuster, S., Lisack, J., Subota, I., Zimmermann, H., Reuter, C., Müller, T., … Engstler, M. (2021). Unexpected plasticity in the life cycle of trypanosoma brucei. *ELife*, *10*, 2019–2021. https://doi.org/10.7554/ELIFE.66028

Schwartz, K. J., Peck, R. F., & Bangs, J. D. (2013). Intracellular trafficking and glycobiology of TbPDI2, a stage-specific protein disulfide isomerase in Trypanosoma brucei. *Eukaryotic Cell*, *12*(1), 132–141. https://doi.org/10.1128/EC.00293-12

Silva, M. S., Prazeres, D. M. F., Lança, A., Atouguia, J., & Monteiro, G. A. (2009). Trans-sialidase from Trypanosoma brucei as a potential target for DNA vaccine development against African trypanosomiasis. *Parasitology Research*, *105*(5), 1223–1229. https://doi.org/10.1007/s00436-009-1542-6

Silva, T. M., Olinda, R. G., Rodrigues, C. M., Câmara, A. C., Lopes, F. C., Coelho, W. A., … Batista, J. S. (2013). Pathogenesis of reproductive failure induced by Trypanosoma vivax in experimentally infected pregnant ewes. *Veterinary Research*, *44*(1), 1–9. https://doi.org/10.1186/1297-9716-44-1

Simarro, P. P., Cecchi, G., Franco, J. R., Paone, M., Diarra, A., Ruiz-Postigo, J. A., … Jannin, J. G. (2012). Estimating and Mapping the Population at Risk of Sleeping Sickness. *PLoS Neglected Tropical Diseases*, *6*(10). https://doi.org/10.1371/journal.pntd.0001859

Simarro, P. P., Cecchi, G., Paone, M., Franco, J. R., Diarra, A., Ruiz, J. A., … Jannin, J. G. (2010). The Atlas of human African trypanosomiasis: A contribution to global mapping of neglected tropical diseases. *International Journal of Health Geographics*, *9*, 1–18. https://doi.org/10.1186/1476-072X-9-57

Simarro, P. P., Diarra, A., Postigo, J. A. R., Franco, J. R., & Jannin, J. G. (2011). The human african trypanosomiasis control and surveillance programme of the world health organization 2000-2009: The way forward. *PLoS Neglected Tropical Diseases*, *5*(2). https://doi.org/10.1371/journal.pntd.0001007

Simarro, P. P., Franco, J. R., Cecchi, G., Paone, M., Diarra, A., Ruiz Postigo, J. A., & Jannin, J. G. (2012). Human African trypanosomiasis in non-endemic countries (2000-2010). *Journal of Travel Medicine*, *19*(1), 44–53. https://doi.org/10.1111/j.1708-8305.2011.00576.x

Simarro, P. P., Jannin, J., & Cattand, P. (2008). Eliminating human African trypanosomiasis: Where do we stand and what comes next? *PLoS Medicine*, *5*(2), 0174–0180. https://doi.org/10.1371/journal.pmed.0050055

Simwango, M., Ngonyoka, A., Nnko, H. J., Salekwa, L. P., Ole-Neselle, M., Kimera, S. I., & Gwakisa, P. S. (2017). Molecular prevalence of trypanosome infections in cattle and tsetse flies in the Maasai Steppe, northern Tanzania. *Parasites and Vectors*, *10*(1), 1–11. https://doi.org/10.1186/s13071-017-2411-2

Singh, B., Kalra, I. S., Gupta, M. P., & Nauriyal, D. C. (1993). Trypanosoma evansi infection in dogs: seasonal prevalence and chemotherapy. *Veterinary Parasitology*, *50*(1–2), 137–141. https://doi.org/10.1016/0304-4017(93)90014-E

Smith, D. H., Pepin, J., & Stich, A. H. R. (1998). Human African trypanosomiasis: An emerging public health crisis. *British Medical Bulletin*, *54*(2), 341–355. https://doi.org/10.1093/oxfordjournals.bmb.a011692

Smith, T. K., Bringaud, F., Nolan, D. P., & Figueiredo, L. M. (2017). Metabolic reprogramming during the Trypanosoma brucei life cycle [version 2 ; referees : 4 approved] Referee Status : *F1000Research*, *6*(May), 1–12. https://doi.org/10.12688/f1000research.10342.1

Stadman, E. R. (1993). Oxidation of Free Amino Acids and Metal-Catalyzed Reactions. *Annu. Rev. Biochem.*, *62*, 797–821.

Steverding, D. (1997). Bloodstream forms of Trypanosoma brucei require only small amounts of iron for growth. *Parasitology Research*, *84*(1), 59–62. https://doi.org/10.1007/s004360050357

Steverding, D. (2000). The transferrin receptor of Trypanosoma brucei. *Parasitology International*, *48*(3), 191–198. https://doi.org/10.1016/S1383-5769(99)00018-5

Steverding, D., Sexton, D. W., Chrysochoidi, N., & Cao, F. (2012). Trypanosoma brucei transferrin receptor can bind C-lobe and N-lobe fragments of transferrin. *Molecular and Biochemical Parasitology*, *185*(2), 99–105.

https://doi.org/10.1016/j.molbiopara.2012.06.007

Steverding, D., Sexton, D. W., Wang, X., Gehrke, S. S., Wagner, G. K., & Caffrey, C. R. (2012). Trypanosoma brucei: Chemical evidence that cathepsin L is essential for survival and a relevant drug target. *International Journal for Parasitology*, *42*(5), 481–488. https://doi.org/10.1016/j.ijpara.2012.03.009

Steverding, D., Stierhof, Y. D., Fuchs, H., Tauber, R., & Overath, P. (1995). Transferrin-binding protein complex is the receptor for transferrin uptake in Trypanosoma brucei. *Journal of Cell Biology*, *131*(5), 1173–1182. https://doi.org/10.1083/jcb.131.5.1173

Stijlemans, Benoît, Baral, T. N., Guilliams, M., Brys, L., Korf, J., Drennan, M., … Magez, S. (2007). A Glycosylphosphatidylinositol-Based Treatment Alleviates Trypanosomiasis-Associated Immunopathology. *The Journal of Immunology*, *179*(6), 4003–4014. https://doi.org/10.4049/jimmunol.179.6.4003

Stijlemans, Benoit, Beschin, A., Magez, S., Van Ginderachter, J. A., & De Baetselier, P. (2015). Iron homeostasis and trypanosoma brucei associated immunopathogenicity development: A battle/quest for iron. *BioMed Research International*, *2015*. https://doi.org/10.1155/2015/819389

Stijlemans, Benoit, De Baetselier, P., Magez, S., Van Ginderachter, J. A., & De Trez, C. (2018). African trypanosomiasis-associated anemia: The contribution of the interplay between parasites and the mononuclear phagocyte system. *Frontiers in Immunology*, *9*(FEB). https://doi.org/10.3389/fimmu.2018.00218

Stijlemans, Benoit, Vankrunkelsven, A., Caljon, G., Bockstal, V., Guilliams, M., Bosschaerts, T., … Baetselier, P. (2012). The Central Role of Macrophages in Trypanosomiasis-Associated Anemia:Rationale for Therapeutical Approaches. *Endocrine, Metabolic & Immune*

Disorders - Drug Targets, *10*(1), 71–82. https://doi.org/10.2174/187153010790827966

Sutak, R., Lesuisse, E., Tachezy, J., & Richardson, D. R. (2008). Crusade for iron: iron uptake in unicellular eukaryotes and its significance for virulence. *Trends in Microbiology*, *16*(6), 261–268. https://doi.org/10.1016/j.tim.2008.03.005

Taylor, M. C., & Kelly, J. M. (2010). Iron metabolism in trypanosomatids, and its crucial role in infection. *Parasitology*, *137*(6), 899–917. https://doi.org/10.1017/S0031182009991880

Tewari, A. K., Kurup, S. P., Baidya, S., Barta, J. R., & Sharma, B. (2015). Protective antibody and cytokine responses in mice following immunization with recombinant beta-tubulin and subsequent Trypanosoma evansi challenge. *Parasites and Vectors*, *8*(1), 1–8. https://doi.org/10.1186/s13071-015-1189-3

Tiengwe, C., Bush, P. J., & Bangs, J. D. (2017). Controlling transferrin receptor trafficking with GPI-valence in bloodstream stage African trypanosomes. *PLoS Pathogens*, *13*(5), 1–24. https://doi.org/10.1371/journal.ppat.1006366

Tilg, H., Ulmer, H., Kaser, A., & Weiss, G. (2002). Role of IL-10 for Induction of Anemia During Inflammation. *The Journal of Immunology*, *169*(4), 2204–2209. https://doi.org/10.4049/jimmunol.169.4.2204

Trevor, C. E., Gonzalez-Munoz, A. L., Macleod, O. J. S., Woodcock, P. G., Rust, S., Vaughan, T. J., … Higgins, M. K. (2019). Structure of the trypanosome transferrin receptor reveals mechanisms of ligand recognition and immune evasion. *Nature Microbiology*, *4*(12), 2074–2081. https://doi.org/10.1038/s41564-019-0589-0

Trindade, S., Rijo-Ferreira, F., Carvalho, T., Pinto-Neves, D., Guegan, F., Aresta-Branco, F., … Figueiredo, L. M. (2016). Trypanosoma brucei Parasites Occupy and Functionally Adapt to the Adipose Tissue in Mice. *Cell Host and Microbe*, *19*(6), 837–848.

https://doi.org/10.1016/j.chom.2016.05.002

Tweneboah, A., Rosenau, J., Agyapong, A. K., Addison, T. K., Mahamat, I., Moussa, A., … Badu, K. (2021). *forest zone of Ghana*.

Uilenberg, G. (1998). *A field guide for THE DIAGNOSIS , TREATMENT AND PREVENTION OF AFRICAN*. Retrieved from http://www.fao.org/3/X0413E/X0413E02.htm

Vanhamme, L., Pays, E., McCulloch, R., & Barry, J. D. (2001). An update on antigenic variation in African trypanosomes. *Trends in Parasitology*, *17*(7), 338–343. https://doi.org/10.1016/S1471-4922(01)01922-5

Verloo, D., Holland, W., My, L. N., Thanh, N. G., Tam, P. T., Goddeeris, B., … Büscher, P. (2000). Comparison of serological tests for Trypanosoma evansi natural infections in water buffaloes from north Vietnam. *Veterinary Parasitology*, *92*(2), 87–96. https://doi.org/10.1016/S0304-4017(00)00284-3

Voyiatzaki, C. S., & Soteriadou, K. P. (1992). Identification and isolation of the Leishmania transferrin receptor. *Journal of Biological Chemistry*, *267*(13), 9112–9117.

Waldvogel-Abramowski, S., Waeber, G., Gassner, C., Buser, A., Frey, B. M., Favrat, B., & Tissot, J. D. (2014). Physiology of iron metabolism. *Transfusion Medicine and Hemotherapy*, *41*(3), 213–221. https://doi.org/10.1159/000362888

Walochnik, J., & Duchêne, M. (2016). Molecular parasitology Protozoan Parasites and their Molecules. In *Scientist* (Vol. 10).

Waskom, M. (2021). Seaborn: Statistical Data Visualization. *Journal of Open Source Software*, *6*(60), 3021. https://doi.org/10.21105/joss.03021

Weinberg, E. D. (2010). The hazards of iron loading. *Metallomics*, *2*(11), 732–740. https://doi.org/10.1039/c0mt00023j

WHO. (2012). Accelerating Work to Overcome the Global Impact of Neglected Tropical Diseases: A Roadmap for Implementation (World Health Organizaiton, Geneva, Switzerland, 2012). *Who*, 42. Retrieved from http://www.who.int/neglected_diseases/NTD_RoadMap_2012_Fullversion.pdf

World Health Organization. (2013). Control and surveillance of human African trypanosomiasis. *World Health Organization Technical Report Series*, (984), 1–237.

World Health Organization. (2022). *Sleeping sickness : prioritizing case detection and treatment to achieve elimination as public health problem*. pp. 2021–2022. Retrieved from https://www.who.int/news/item/01-02-2022-sleeping-sickness-prioritizing-case-detection-and-treatment-to-achieve-elimination-as-public-health-problem

Wylie, D., & Lyons, M. (1994). The Colonial Disease: A Social History of Sleeping Sickness in Northern Zaire, 1900-1940. *Canadian Journal of African Studies / Revue Canadienne Des Études Africaines*, *28*(3), 543. https://doi.org/10.2307/485358

Ziegelbauer, K., & Overath, P. (1993). Organization of two invariant surface glycoproteins in the surface coat of Trypanosoma brucei. *Infection and Immunity*, *61*(11), 4540–4545. https://doi.org/10.1128/iai.61.11.4540-4545.1993

APPENDICES

Appendix A: Ethical clearance

Ethical clearance for the study was obtained from the Council for Scientific and Industrial Research (CSIR) Institutional Animal Care and Use Committee (IACUC), Ghana, with approval number RPN012/CSIR-ACUC/2020.

Appendix B: Questions asked to the farmers

1) What is the animal’s age?

2) What is the animal’s breed?

3) When was the last time you administered any anti-trypanosomal drug to the animals?

4) Do all the animals graze the same area

Appendix C: Description of the various livestock samples at different study site

Table C1: Details of cattle sampled at Liprec farms (Ashaley Botwe)

Sample ID	Sex	Breed	Age (Years)
CS1	M	Wash	5
CS2	M	Crossbreed	6
CS3	M	Crossbreed	6
CS4	M	Crossbreed	5
CS5	M	Crossbreed	7
CS6	M	Crossbreed	5
CS7	M	Wash	6
CS8	M	Wash	7
CS9	M	Crossbreed	6
CS10	M	Crossbreed	5
CS11	F	Sanga	5
CS12	F	Sanga	6
CS13	F	Sanga	6
CS14	F	Sanga	5
CS15	F	Sanga	5
CS16	F	Sanga	4
CS17	F	Sanga	5
CS18	F	Sanga	4
CS19	F	Sanga	4
CS20	F	Sanga	6

Notes: CS represents the code used cattle followed by a unique number, F- female and M- male. Wash is West African Shorthorn.

Table C2: Details of sheep sampled at Liprec farms (Ashaley Botwe)

Sample ID	**Sex**	**Breed**	**Age** (years)
SS1	M	Blackhead	2
SS2	M	Blackhead	3
SS3	M	Crossbreed	3
SS4	M	Crossbreed	2
SS5	M	Crossbreed	2
SS6	M	Crossbreed	3
SS7	M	Crossbreed	2
SS8	M	Crossbreed	2
SS9	M	Crossbreed	2
SS10	M	Crossbreed	3
SS11	F	Crossbreed	3
SS12	F	Crossbreed	3
SS13	F	Blackhead	3
SS14	F	Blackhead	3
SS15	F	Blackhead	2
SS16	F	Blackhead	3
SS17	F	Blackhead	2
SS18	F	Blackhead	2
SS19	F	Blackhead	2
SS20	F	Blackhead	2

Notes: *SS represents to code used for sheep; the code is followed by a unique number, F-male and M-male.

Table C3: Details of goats sampled at the CSIR farms (Katamanso)

Sample ID	Sex	Breed	Age (years)
CSG1	F	Crossbreed	2
CSG2	F	Crossbreed	2
CSG3	F	Crossbreed	2
CSG4	F	Crossbreed	2
CSG5	F	Short-Legged	3
CSG6	F	Short-Legged	3
CSG7	F	Short-Legged	3
CSG8	F	Short-Legged	2
CSG9	F	Short-Legged	3
CSG10	F	Short-Legged	3
CSG11	F	Crossbreed	2
CSG12	M	Crossbreed	2
CSG13	F	Crossbreed	2
CSG14	F	Crossbreed	2
CSG15	F	Crossbreed	2
CSG16	F	Short-Legged	3
CSG17	M	Short-Legged	3
CSG18	M	Crossbreed	2
CSG19	F	Crossbreed	2
CSG20	F	Short-Legged	3

Notes: *CSG represents the code used for goats followed by a unique number, F-female and M-male.

Appendix D: Iron parameters in infected and non-infected livestock

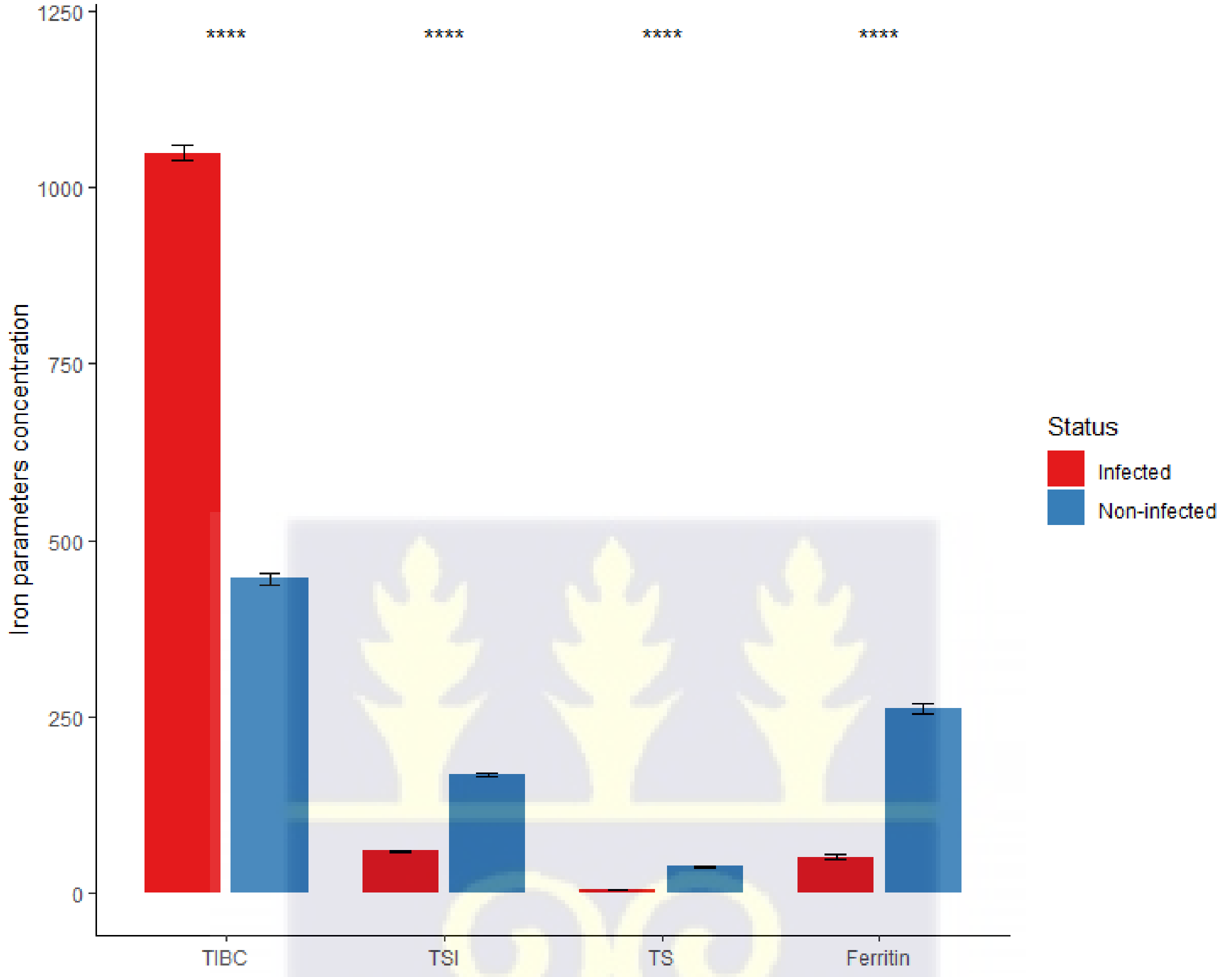


Figure D1: Iron parameters in infected and non-infected sheep. Total iron binding capacity (TIBC), total serum iron (TSI), transferrin saturation (TS) and ferritin concentration. **p-value* < 0.05 and *****p-value* < 0.0001 were considered statistically significant.

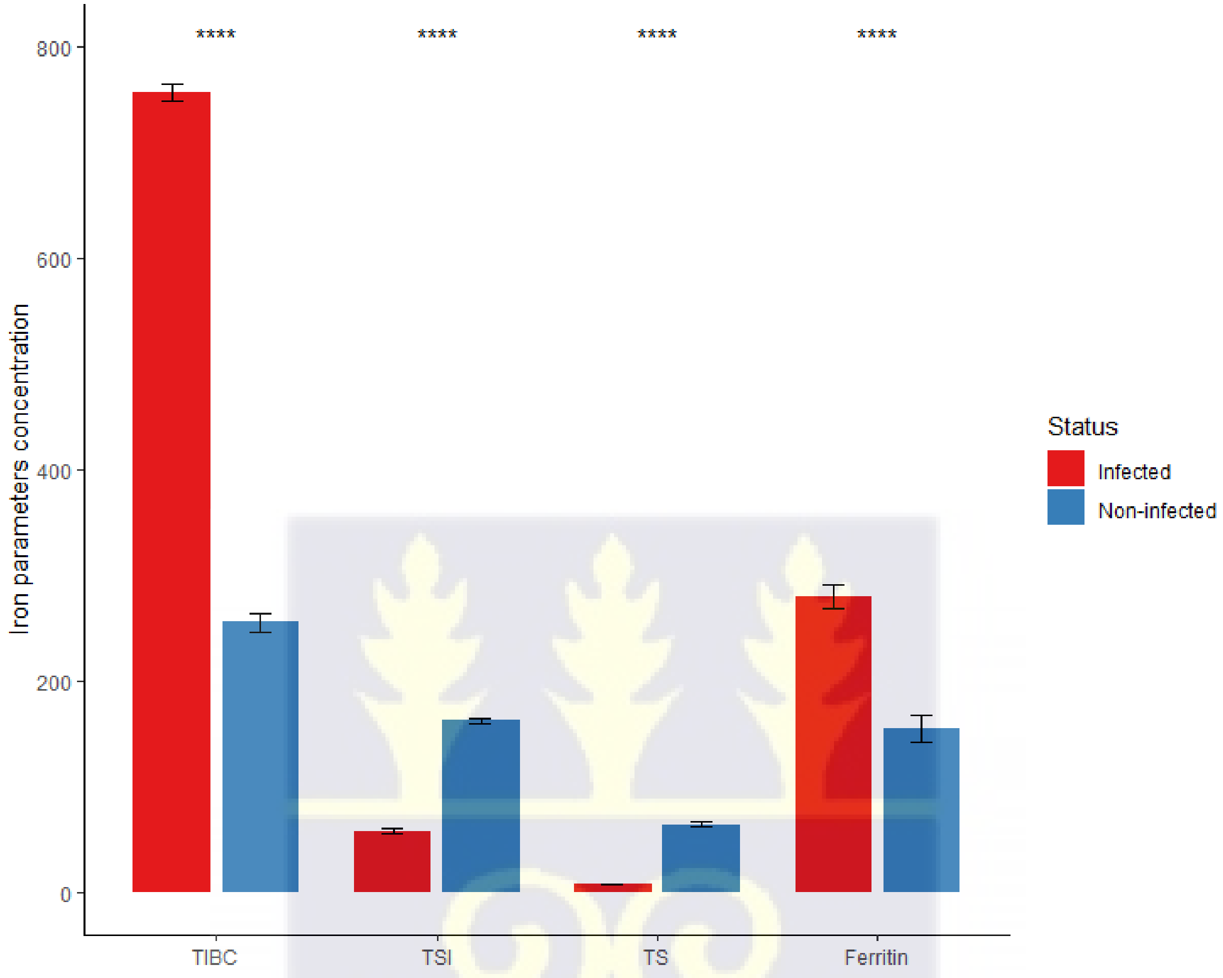


Figure D2: Iron parameters in infected and non-infected cattle. Total iron binding capacity (TIBC), total serum iron (TSI), transferrin saturation (TS) and ferritin concentration. ******p-value* < 0.05 and *****p-value* < 0.0001 were considered statistically significant.

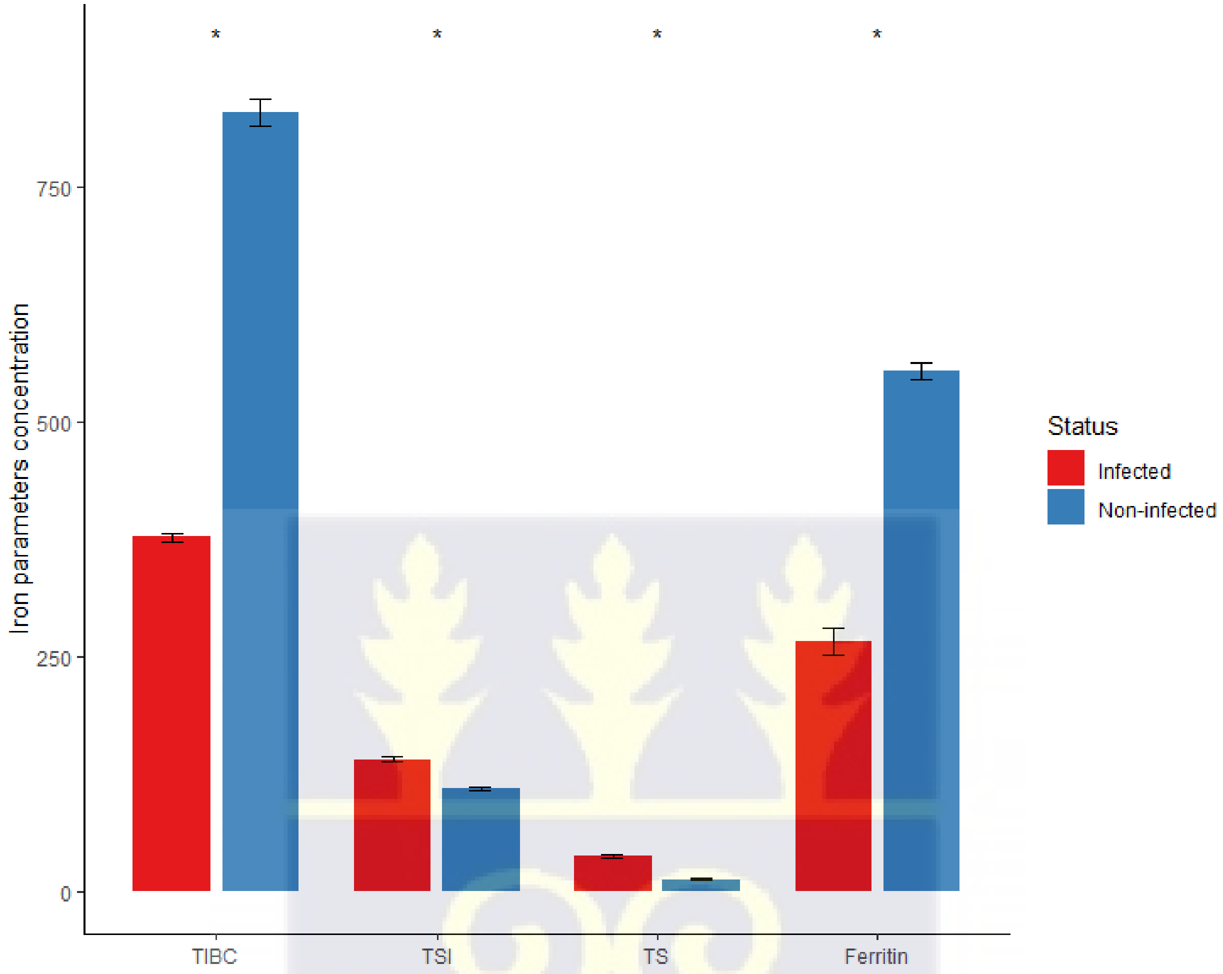


Figure D3: Iron parameters in infected and non-infected goats. Total iron binding capacity (TIBC), total serum iron (TSI), transferrin saturation (TS) and ferritin concentration. ******p-value* < 0.05 was considered statistically significant.

Appendix E: Hematological parameters in infected and non-infected livestock

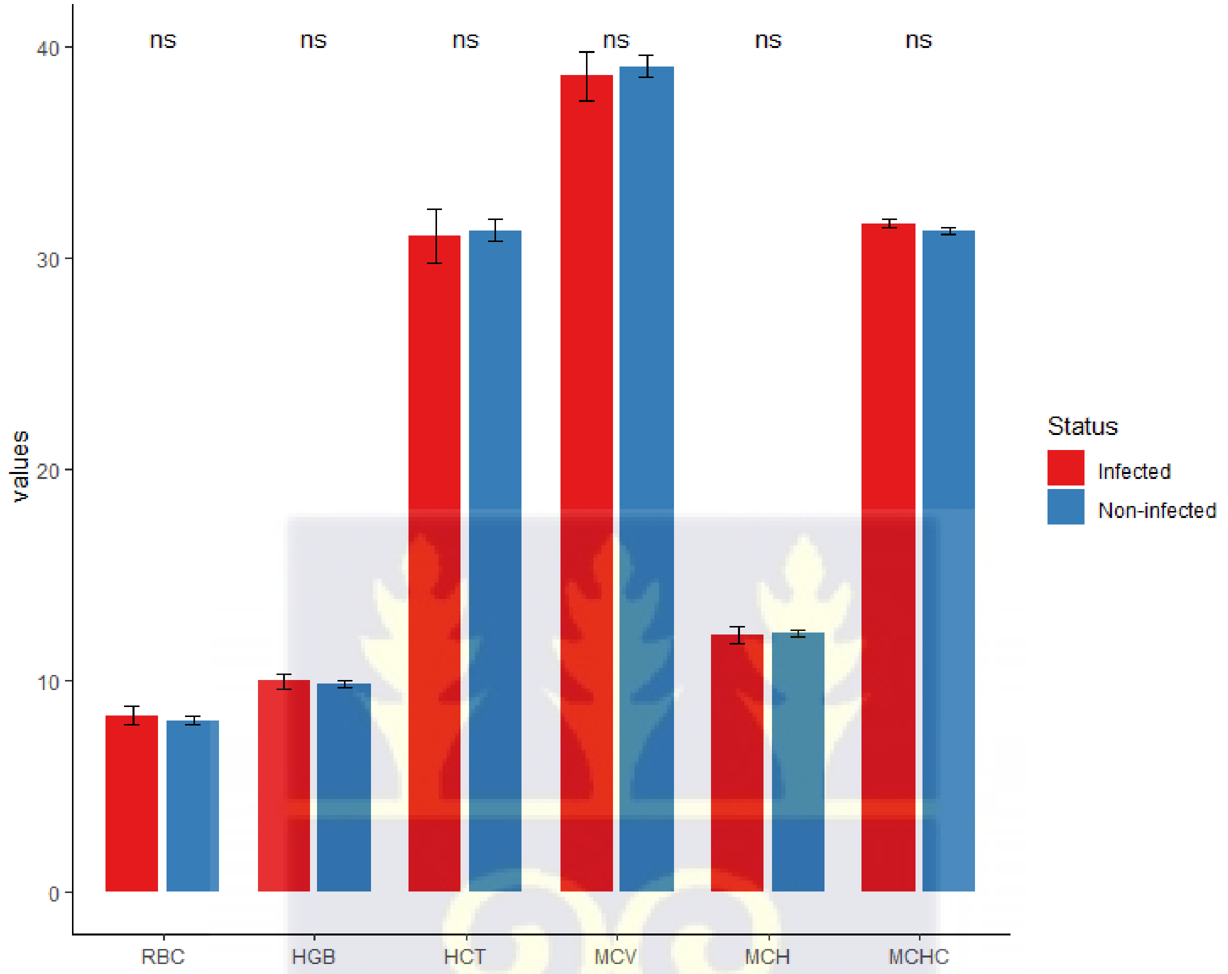


Figure E1: Hematological parameters in infected and non-infected cattle. Red blood cell (RBC), hemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). **p-value* < 0.05 was considered statistically significant, ns (non-significant).

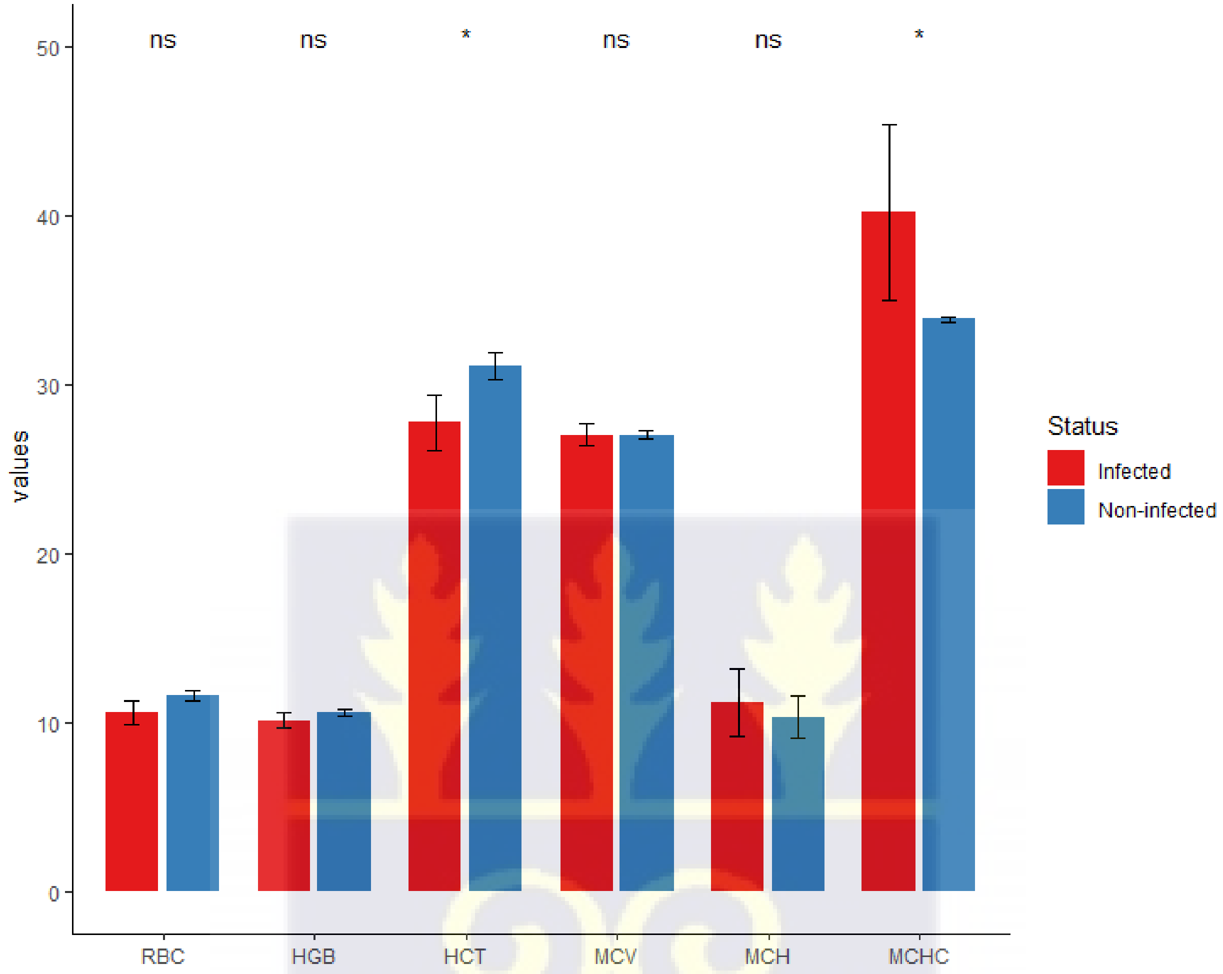


Figure E2: Hematological parameters in infected and non-infected sheep. Red blood cell (RBC), hemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). ***p-value** < 0.05 was considered statistically significant, ns (non-significant).

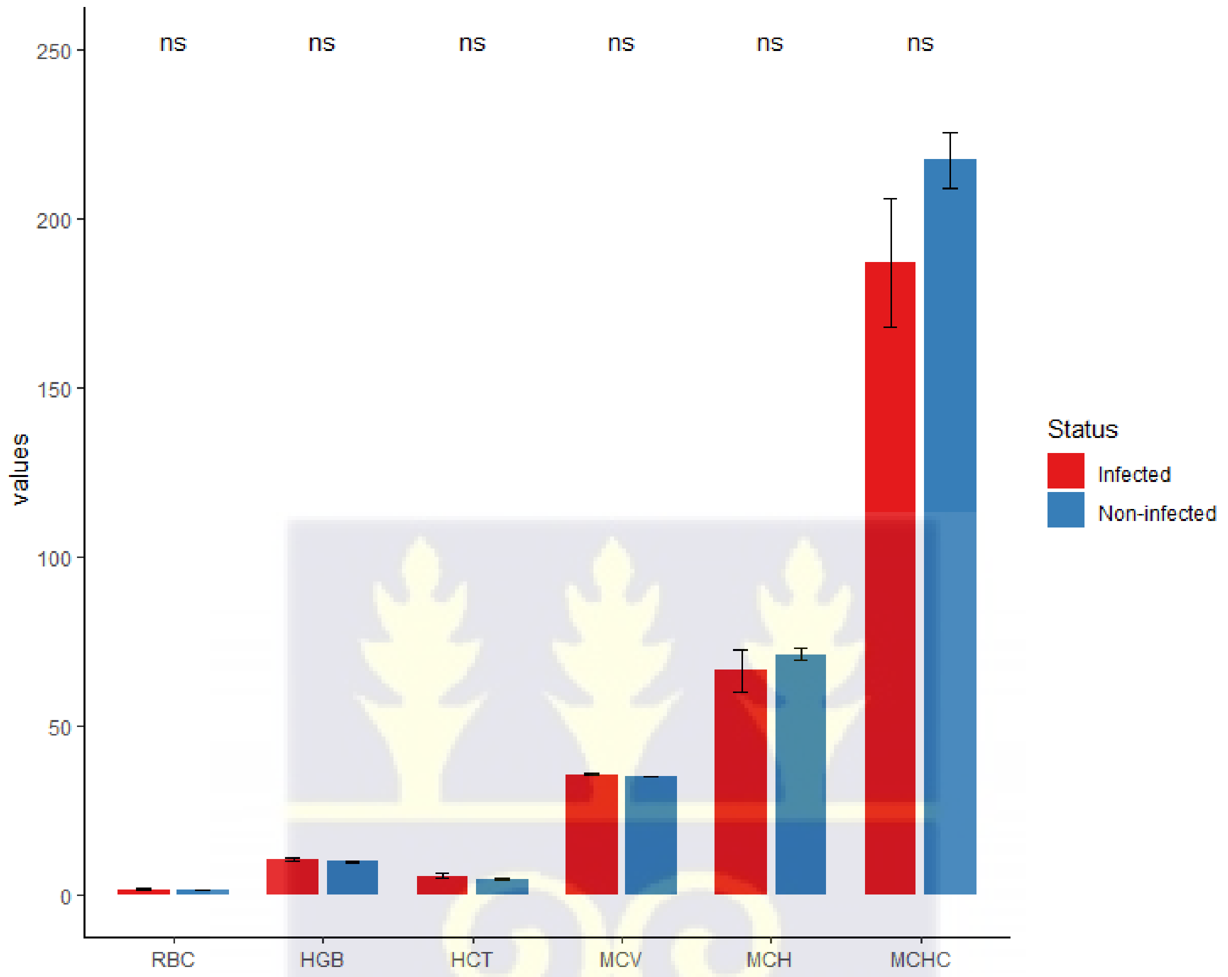


Figure E3: Hematological parameters in infected and non-infected goats. Red blood cell (RBC), hemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). ******p-value* < 0.05 was considered statistically significant, ns (non-significant).

Appendix F: Amplification of TfR in trypanosomes infected livestock and mapping to specific expression sites

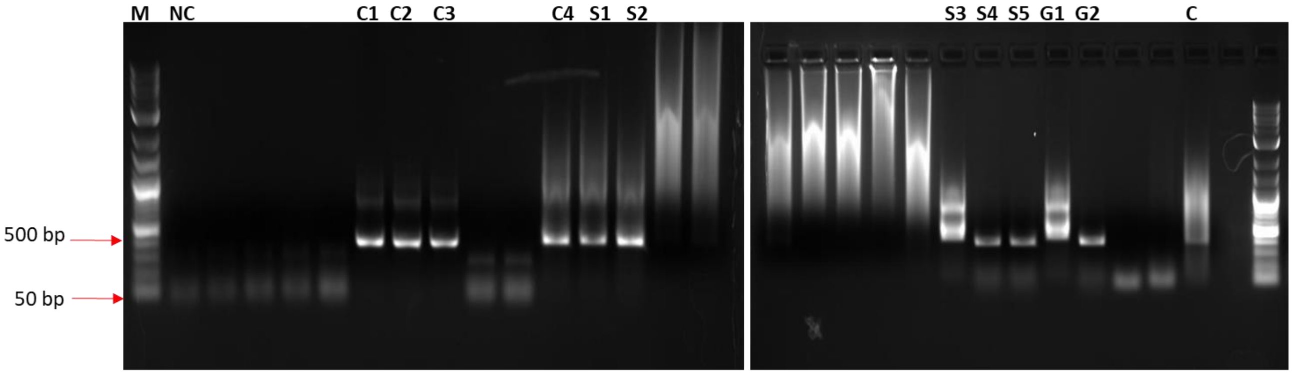
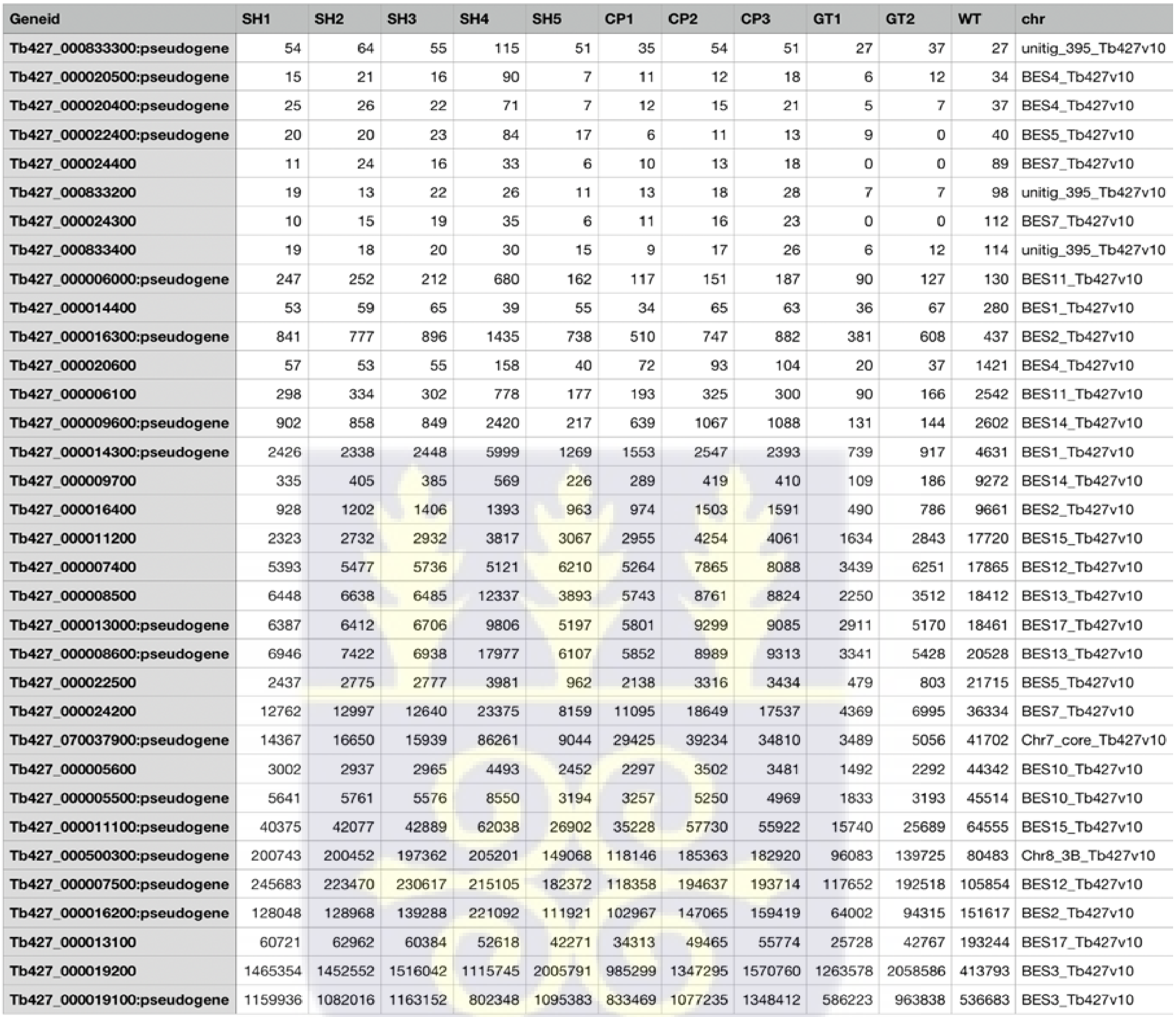


Figure F1: Amplification of TfR from the dsDNA of trypanosomes infected animals. An agarose gel picture shows the bands used to identify TfR in livestock. M: marker, C: wild-type *T. brucei* (GuTat 3.1), NC: PCR H2O and C1-C4: cattle IDs, S1-S5: sheep IDs and G1-G2: goats IDs. Expected band size by PCR was 400 bp.

Table F1: Frequency distribution of mapped reads to the entire genome with Gene IDs



Geneid	SH1	SH2	SH3	SH4	SH5	CP1	CP2	CP3	GT1	GT2	WT	chr
Tb427_000833300:pseudogene	54	64	55	115	51	35	54	51	27	37	27	unitig_395_Tb427v10
Tb427_000020500:pseudogene	15	21	16	90	7	11	12	18	6	12	34	BES4_Tb427v10
Tb427_000020400:pseudogene	25	26	22	71	7	12	15	21	5	7	37	BES4_Tb427v10
Tb427_000022400:pseudogene	20	20	23	84	17	6	11	13	9	0	40	BES5_Tb427v10
Tb427_000024400	11	24	16	33	6	10	13	18	0	0	89	BES7_Tb427v10
Tb427_000833200	19	13	22	26	11	13	18	28	7	7	98	unitig_395_Tb427v10
Tb427_000024300	10	15	19	35	6	11	16	23	0	0	112	BES7_Tb427v10
Tb427_000833400	19	18	20	30	15	9	17	26	6	12	114	unitig_395_Tb427v10
Tb427_000006000:pseudogene	247	252	212	680	162	117	151	187	90	127	130	BES11_Tb427v10
Tb427_000014400	53	59	65	39	55	34	65	63	36	67	280	BES1_Tb427v10
Tb427_000016300:pseudogene	841	777	896	1435	738	510	747	882	381	608	437	BES2_Tb427v10
Tb427_000020600	57	53	55	158	40	72	93	104	20	37	1421	BES4_Tb427v10
Tb427_000006100	298	334	302	778	177	193	325	300	90	166	2542	BES11_Tb427v10
Tb427_000009600:pseudogene	902	858	849	2420	217	639	1067	1088	131	144	2602	BES14_Tb427v10
Tb427_000014300:pseudogene	2426	2338	2448	5999	1269	1553	2547	2393	739	917	4631	BES1_Tb427v10
Tb427_000009700	335	405	385	569	226	289	419	410	109	186	9272	BES14_Tb427v10
Tb427_000016400	928	1202	1406	1393	963	974	1503	1591	490	786	9661	BES2_Tb427v10
Tb427_000011200	2323	2732	2932	3817	3067	2955	4254	4061	1634	2843	17720	BES15_Tb427v10
Tb427_000007400	5393	5477	5736	5121	6210	5264	7865	8088	3439	6251	17865	BES12_Tb427v10
Tb427_000008500	6448	6638	6485	12337	3893	5743	8761	8824	2250	3512	18412	BES13_Tb427v10
Tb427_000013000:pseudogene	6387	6412	6706	9806	5197	5801	9299	9085	2911	5170	18461	BES17_Tb427v10
Tb427_000008600:pseudogene	6946	7422	6938	17977	6107	5852	8989	9313	3341	5428	20528	BES13_Tb427v10
Tb427_000022500	2437	2775	2777	3981	962	2138	3316	3434	479	803	21715	BES5_Tb427v10
Tb427_000024200	12762	12997	12640	23375	8159	11095	18649	17537	4369	6995	36334	BES7_Tb427v10
Tb427_070037900:pseudogene	14367	16650	15939	86261	9044	29425	39234	34810	3489	5056	41702	Chr7_core_Tb427v10
Tb427_000005600	3002	2937	2965	4493	2452	2297	3502	3481	1492	2292	44342	BES10_Tb427v10
Tb427_000005500:pseudogene	5641	5761	5576	8550	3194	3257	5250	4969	1833	3193	45514	BES10_Tb427v10
Tb427_000011100:pseudogene	40375	42077	42889	62038	26902	35228	57730	55922	15740	25689	64555	BES15_Tb427v10
Tb427_000500300:pseudogene	200743	200452	197362	205201	149068	118146	185363	182920	96083	139725	80483	Chr8_3B_Tb427v10
Tb427_000007500:pseudogene	245683	223470	230617	215105	182372	118358	194637	193714	117652	192518	105854	BES12_Tb427v10
Tb427_000016200:pseudogene	128048	128968	139288	221092	111921	102967	147065	159419	64002	94315	151617	BES2_Tb427v10
Tb427_000013100	60721	62962	60384	52618	42271	34313	49465	55774	25728	42767	193244	BES17_Tb427v10
Tb427_000019200	1465354	1452552	1516042	1115745	2005791	985299	1347295	1570760	1263578	2058586	413793	BES3_Tb427v10
Tb427_000019100:pseudogene	1159936	1082016	1163152	802348	1095383	833469	1077235	1348412	586223	963838	536683	BES3_Tb427v10

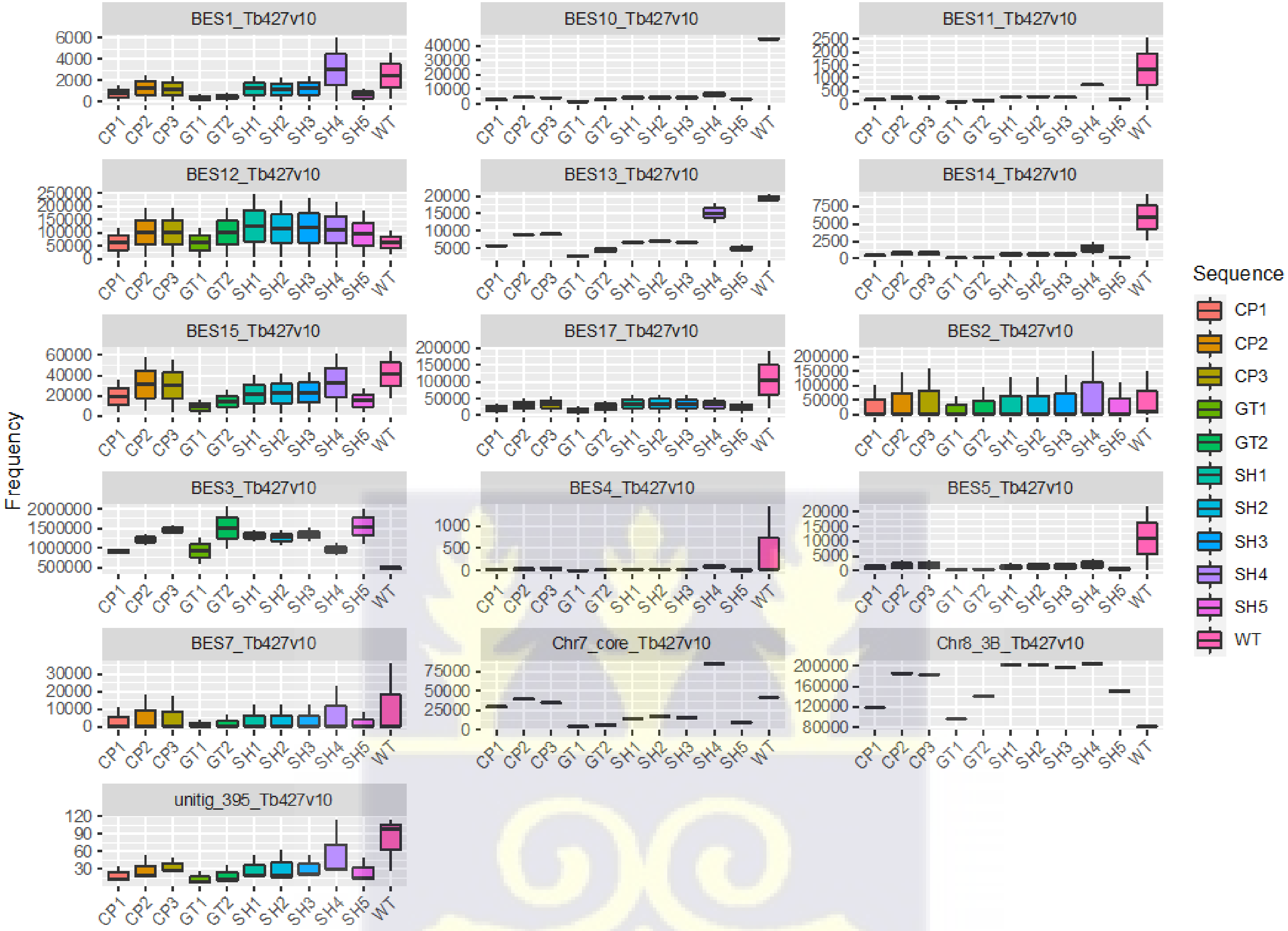


Figure F1: Frequency distribution of specific sequences mapped to all expression sites. Each box shows the distribution of sequences from each animal (x-axis) for each BES. The y-axis shows read counts for different sequences within the ES.

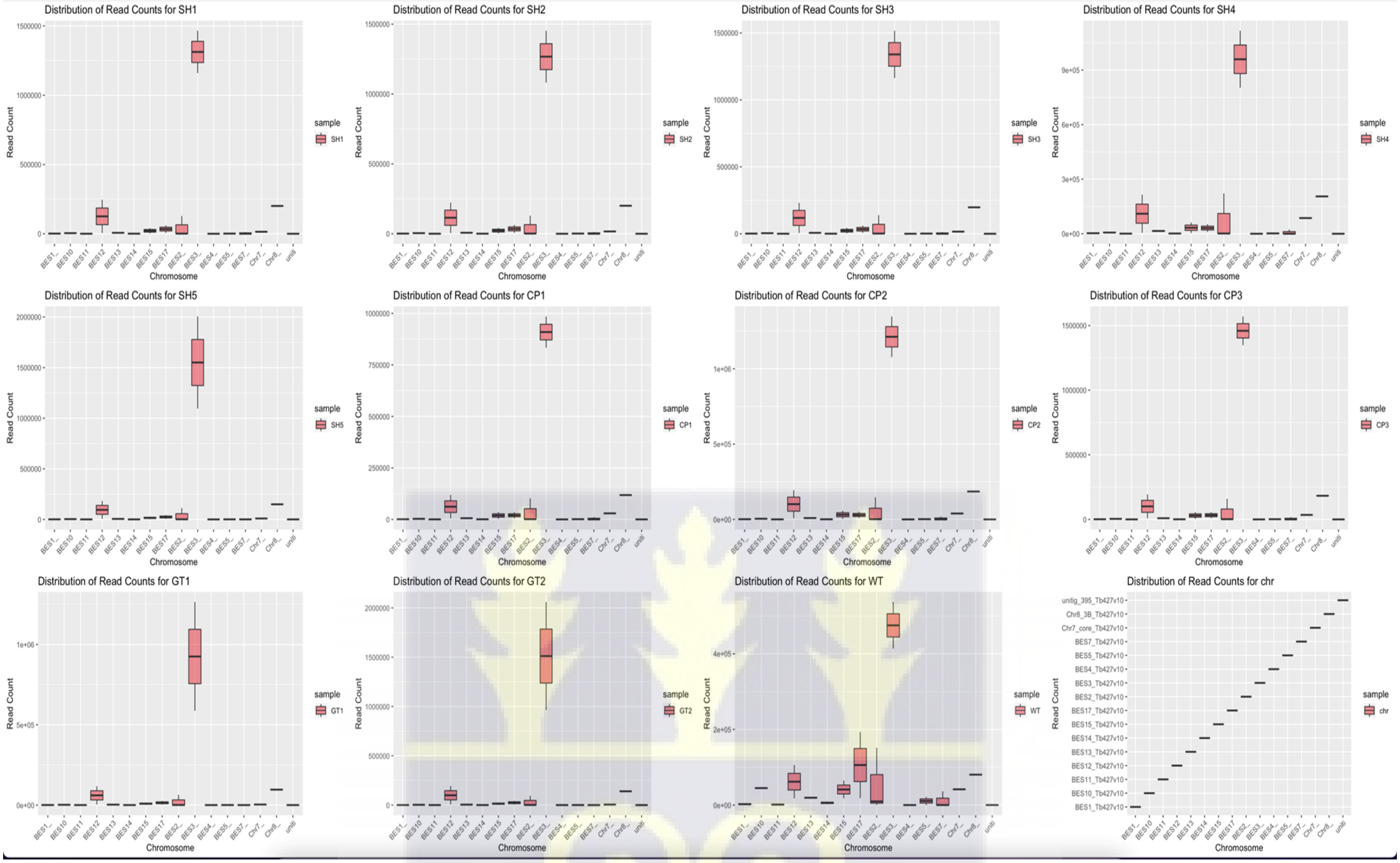


Figure F2: Distribution of read counts for all the samples.

Appendix G: Generation of ESAG7-GPI cell lines and validation of the transfection

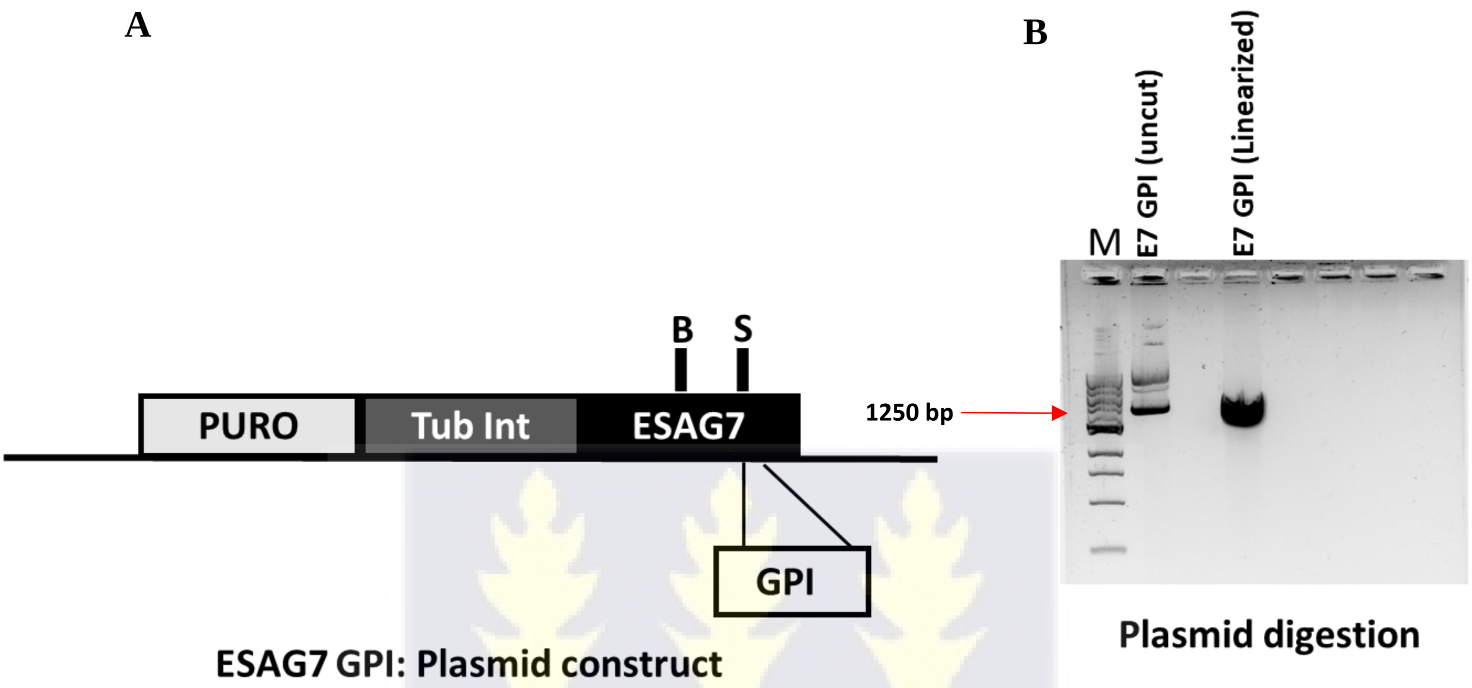


Figure G1: Generation of ESAG7-GPI cell lines. (A) ESAG7-GPI Plasmid construct containing (5’-3’): 5’ upstream targeting sequences; puromycin resistance cassette (PUR); tubulin βα-intergenic region (TubInt); RNAir E7 ORF or a fusion construct of native E7 with the C-terminal GPI signal of E6 from SacI site (S) to stop codon (GPI); 3’ downstream targeting sequences. Underbar indicates recoded RNAir region. **(B)** Representative gel of the plasmid digestion. M: molecular marker 1kb, E7 (ESAG7).

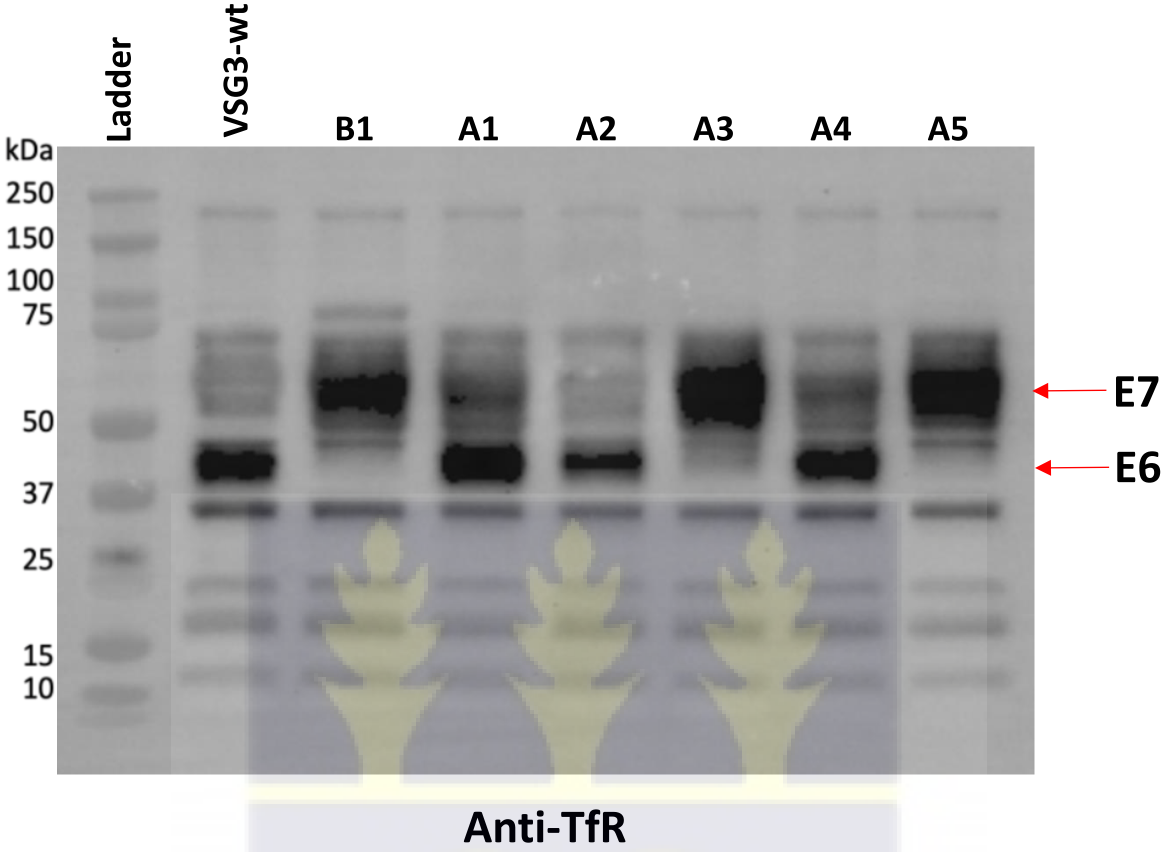


Figure G2: validation of the transfection for the generation of ESAG7-GPI cell lines. Anti-TfR western blot on whole cell protein from VSG3-WT and ESAG7-GPI cells.