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CHARACTERIZATION OF TRYPANOSOMES AND DETERMINATION OF TRYPANOSOME IMMUNOGENIC PROTEINS IN CATTLE IN GHANA

BY

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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 BIOCHEMISTRY

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DECLARATION

I Jennifer Afua Ofori, do hereby declare that the work described in this thesis is my own, carried out under the supervision of Dr. Theresa Manful Gwira, Professors Mark Carrington, Gordon Akanzuwine Awandare and George Kwame Aning and that reference made to the works of others have been duly acknowledged. I certify that no part of this thesis has been previously submitted for a degree or any other qualification. I outline below the various contributions made by partners to this study.

Role	Responsibility
Sample collection, processing and storage	Shared
DNA Extraction and Purification	Sole
Trypanosome tubulin tagged primer design	Shared
Multiplex tagged nested polymerase chain reaction	Sole
Library preparation and illumina sequencing	Commercial
Bioinformatics handling of raw data and analysis	Shared
In vitro culturing of trypanosome cells	Sole
Antibody responses to trypanosome by ELISA	Sole
Statistical analysis of ELISA data	Shared
Identification of immunogenic trypanosome cells by Western blot	Sole
Immunoprecipitation of trypanosome proteins	Sole
Thesis preparation	Sole

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ABSTRACT

Livestock production is one of the farming systems and has a great impact on food and economic security in most developing countries. Animal African trypanosomiasis is a major limitation to livestock production in Africa, particularly cattle production. The disease is caused by *Trypanosoma* spp., a broad range of protozoan parasites of wild and domestic animals through cyclical and mechanical transmission. Cross-sectional studies conducted in Ghana reveal the common species of trypanosomes infecting cattle using molecular techniques as *T. brucei brucei, T. congolense, T. vivax, T. theileri* and *T. simiae.* Despite the seriousness of the disease, no study has been conducted on lifetime trypanosome infections in Ghanaian cattle. Antigenic variation of the variant surface glycoprotein (VSG) of trypanosomes is a hindrance to the successful development of a vaccine against trypanosomiasis. Therefore, alternative approaches such as exploiting the immunogenicity of other trypanosomes proteins and understanding antibody response during lifetime infection with parasites maybe useful in vaccine development. In this study, trypanosome species throughout the natural infection cycle in cattle were characterized over a two year period. In addition, the antibody response in the individual cattle over time and potential trypanosome immunogenic proteins were determined.

Two herds of cattle (20 each) at Accra and Adidome were selected on the basis of geographical location, tsetse fly density, trypanosomiasis prevalence and the breed of cattle at the sites. Blood and serum samples were collected at approximately four to five-week intervals for about two years. The infecting trypanosomes were identified and characterized using an in-house developed tagged multiplex nested polymerase chain reaction (PCR) targeting a portion of the trypanosome tubulin gene cluster and

next generation sequencing. Total GUTat 3.1 *T. brucei* protein were prepared and antibody reactivity against *T. brucei* proteins as well as identified *T. brucei* immunogenic proteins recognized by cattle sera were performed by indirect ELISA, SDS-PAGE, western blot, immunoprecipitation and LC-MS/MS.

Cattle were infected throughout the natural infection cycle at both study sites, Adidome and Accra with cases of mixed infections. *T. vivax, T. brucei, T. theileri* and *T. congolense* were the major infecting species at both study sites with *T. vivax* being predominant [incidence rate of 84% (282 samples) for Adidome and incidence rate of 70.3% (353 samples) for Accra]. *T. vivax* was also the principal trypanosome species existing within both young and old aged cattle, among males and females and the different cattle breeds at both study sites. Cattle sera (215 samples) had high antibody response at Adidome with a seroprevalence of 97% compared to Accra with a seroprevalence of 73% out of 172 sera samples. Mass spectrometric analysis of the *T. brucei* proteins immunoprecipitated by trypanosome immune antibodies identified beta-tubulin and triosephosphate isomerase to be selectively recognized by infected sera in natural infection.

Data from this study has given an insight on the biology of trypanosome infection and informed policy makers of better control measures to be adopted in the infected area. In addition possible biomarkers have been identified for diagnosing cattle trypanosomiasis in Ghana and for potential vaccine development.

DEDICATION

I dedicate this thesis to my Heavenly Father for His blessings, my nuclear and extended families for their support and to all the scientists who promote cutting-edge research in developing countries.

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LIST OF ABBREVIATIONS

AAT: Animal African Trypanosomiasis

ADF: African Development Fund

APOL-1: Apolipoprotein L-1

BARP: Bloodstream Alanine-Rich Protein

BCA: Bicinchoninic Acid

CATT: Card Agglutination Test for Trypanosomiasis

CBPP: Contagious Bovine Pheuro-Pheumonia

CP: Cysteine Protease

CR3: Complement Receptor 3

CSF: Cerebrospinal Fluid

CTD: C-Terminal Domain

DAMPs: Damage-Associated Molecular Patterns

ELISA: Enzyme Linked Immunosorbent Assay

ES: Expression Sites

ESAGs: Expression Sites Associated Genes

EV: Extracellular Vesicles

FAZ: Flagellum Attachment Zone

FP: Flagellar Pocket

GPI: Glycophosphatidylinositol

HAT: Human African Trypanosomiasis

HCT: Hematocrit Centrifuge Test

HDLs: High-Density Lipoproteins

HpHbR: Haptoglobin-Haemoglobin Receptor

Hpr: Haptoglobin-related protein

IFN-ɣ: Interferon Gamma

IgG: Immunoglobulin G

IgM: Immunoglobulin M

ISGs: Invariant Surface Glycoproteins

LAMP: Loop-mediated Isothermal Amplification

LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

LS: Long Slender

NGS: Next Generation Sequencing

NO: Nitric Oxide

ORF: Open Reading Frame

PAMPs: Pathogen-Associated Molecular Patterns

PATTEC: Pan-African Tsetse and Trypanosomiasis Eradication Campaign

PCR: Polymerase Chain Reaction

PCV: Packed Cell Volume

PF: Procyclic Forms

PFR: Paraflagellar Rod

PRRs: Pattern Recognition Receptors

RFLP: Restriction Fragment Length Polymorphism

RNAPI: RNA Polymerase I

RNAPII: RNA Polymerase II

rRNA: Ribosomal RNA

SELDI-TOF: Surface Enhanced Laser Desorption Ionization- Time-of-Flight

SL: Splice Leader

SS: Short Stumpy

TgsGP: *Trypanosoma brucei gambiense* specific Glycoprotein

Th1: T-helper 1

TLFs: Trypanosome Lytic Factors

TNF-α: Tumor Necrosis Factor-alpha

VSGs: Variant Surface Glycoproteins

CHAPTER ONE

INTRODUCTION

African trypanosomiasis (sleeping sickness) affects both human (Human Trypanosomiasis) and livestock mainly cattle (Animal Trypanosomiasis) in most sub-Saharan rural poor populations in Africa (Nkegbe and Beyuo, 2011). The disease is caused by *Trypanosoma* species, which are extracellular flagellate protozoans (Yaro *et al.,* 2016). Animal Trypanosomiasis is a well-known constraint to livestock production and a major threat to food security in Africa (Vreysen, 2006). The economies of 36 trypanosomiasis endemic countries in Africa are dependent on agricultural and livestock production (Nkegbe and Beyuo, 2011). Over US$ 4.5 billion dollars annual losses have been reported to be associated with Animal African Trypanosomiasis through indirect and direct means (Yaro *et al.,* 2016; Dagnachew and Bezie, 2015).

African trypanosomes are regarded as flagellated protozoan parasites which are resident in the extracellular unit of the mammalian host, and are transmitted between mammalian hosts by bites from infected tsetse fly (Nakayima *et al.,* 2012). They are found in many parts of the continent about 15^{o}N and 20^{o}S of the equator (Nakayima *et al.,* 2012; Donelson, 2003). Trypanosomiasis is caused by the following species: *T. godfreyi, T. vivax, T. theileri, T. congolense, T. simiae,* and *T. brucei s.l* and its derivatives, *T. evansi, T. equiperdum,* (Ahmed *et al.,* 2013; Cox *et al.,* 2005; Van Den Bossche, 2001). *T. evansi* is transmitted largely through mechanical means by other biting flies and not tsetse flies. Mechanical transmission by other biting flies has intensify continuous infection in domestic animals with especially the virulent species types. The three subspecies of *T. brucei s.l.* are *T. brucei rhodesiense, T. brucei gambiense* and *T. brucei brucei.* The human infective types, *T. brucei rhodesiense* and

T. brucei gambiense are known to cause the East African acute form and the West African chronic form of the disease respectively (Welburn *et al.,* 2001) while *T. brucei brucei* is mildly pathogenic in domestic animals such as cattle, sheep, goats and pigs, but mostly cattle (Magona *et al.,* 2004). Another species frequently found in cattle worldwide is *T. theileri* which is typically non-pathogenic (Cox *et al.,* 2010). In Africa, *T. congolense, T. brucei brucei and T. vivax* are the three most significant trypanosome species responsible for livestock morbidity and great production losses (Geerts *et al.,* 2001). The most common trypanosome species detected in cattle in Ghana are *T. brucei, T. congolense, T. vivax, T. theileri* and *T. simiae* (Bakari *et al.,* 2017; Cox *et al.,* 2010).

In animal trypanosomiasis epidemiological studies, accurate diagnosis and identification of trypanosome species in livestock is important for the development of good treatment and control strategies against the disease for high productivity and quality meat and diary products (Thumbi, *et al.,* 2008). Therefore, rapid, sensitive and specific molecular diagnostic techniques are used for trypanosome detection. Diagnostic polymerase chain reaction (PCR) is one of such techniques which is highly sensitive and specific (Aradaib and Majid, 2006).

Due to the extracellular nature of African trypanosomes, the parasite encounters an immunologically hostile environment of the host bloodstream: both the innate and adaptive immune responses contribute to the elimination of trypanosomes (Namangala, 2012; Stijlemans *et al.,* 2016). However, trypanosomes persist in the host bloodstream by evading immune responses and establishing an infection. The evasion of host antibody responses by the parasite population is achieved mostly through antigenic variation of the variant surface glycoprotein (VSG) leading to parasite survival and the

establishment of chronic infections (Matthews *et al.*, 2015). Although the VSG is highly immunogenic, it prospects of being used in diagnosis and vaccine development is minimal due to its complexity and high frequency of switching (Vincendeau and Bouteille, 2006; Higgins and Carrington, 2014; Schwede *et al.*, 2015).

Rationale of the study

A number of investigations have shown the prevalence of trypanosome species that affect cattle in Africa. A study in Western Oromia State, Ethiopia, showed a trypanosome prevalence of 8.57% out of which 7.24%, 1.06% and 0.27% correspond to *T. congolense*, *T. vivax* and mixed infection of *T. congolense* and *T. vivax* respectively. This indicates that two trypanosome species were existing in the study area with *T. congolense* being the most prevailing (Tasew and Duguma, 2012). Also, Takeet and colleagues in 2013, sampled 411 Nigerian cattle to survey pathogenic trypanosome infection circulating in these naturally infected animals using microscopy and polymerase chain reaction (PCR). It was observed that out of 15.1% positive infection detected by microscopy, there was either *T. brucei, T. vivax or T. congolense.* PCR detected 4.4%, 48.7%, 26.0% and 0.5% of *T. brucei, T. congolense, T. vivax* and *T. evansi* infections respectively. Except for two *T. congolense* forest-type infections detected, all of the others were savannah-type. Of the mixed infections, a prevalence rate of 13.9%, for *T. congolense* and *T. vivax* and 1.5% of *T. evansi, T. vivax* and *T. congolense* were observed. In the same study, significant differences were found between the prevalence of *T. vivax* and mixed infections between younger (less than 1 year old) and old animals (above one year) with a higher prevalent of infection in younger animals (Takeet *et al.*, 2013).

In a study carried out in Ghana using PCR assays, 248 pigs, 219 tsetse flies, and 146 cattle blood samples were collected and analyzed from Koforidua and Adidome in 2010 for the presence of trypanosomes. The study revealed an overall trypanosome prevalence of 28.6% (71/248) for pigs, 17.4% for tsetse flies (38/219) and 57.5% (84/146) for cattle (Nakayima *et al.,* 2012). Also, *T. congolense* forest (66.0%) and *T. congolense* savannah (52.6%) were the endemic *T. congolense* subgroup determined in Ghana using subgroup-specific PCR with 18.6% being mixed infections. In a single tsetse fly, *T. evansi* was detected. There was no human infective species of trypanosomes detected in the tested samples (Nakayima *et al.,* 2012). All of the above studies are cross-sectional providing scanty information on the trypanosomes infection pattern over the lifetime of the host. Therefore, it is important to investigate the lifetime trypanosome infection in cattle in Ghana to aid better understanding of the disease. In addition, competition among trypanosomes within a single animal over time is not well understood and whether this competition leads to variations in antibody production elicited by the trypanosome species.

Drugs used to treat animal trypanosomiasis are effective only if properly applied but not sustainable because of the high cost associated with the purchase of trypanocidal drugs by peasant farmers and drug resistance development (Delespaux and De Koning, 2007; Murray *et al.,* 1990). Antigenic variation of the variant surface glycoprotein (VSG) of trypanosomes has obstructed the successful vaccine development against trypanosomiasis. However, researchers are targeting invariant surface antigens and tubulin which are accessible to the immune system of the host (Stijlemans *et al.,* 2007; Lubega *et al.,* 2002). Alternative approaches such as exploiting the immunogenicity of other trypanosomes proteins and understanding antibody response during lifetime

natural infection with parasites is useful in the design of a diagnostic marker and vaccine development.

1.1 Overall Hypothesis

The competition among different trypanosome species in cattle over time may lead to either single or mixed population of species with the resultant production of antibodies against the prevailing species.

Aim

To characterize trypanosome species in cattle in Ghana over a two year period and determine potential immunogenic trypanosome proteins for diagnostic or vaccine development.

Hypothesis for objectives 1, 2 and 3: Competition among different trypanosome species and strains over a lifetime period in different sex and breed of cattle may lead to the emergence and clearance of the trypanosome species or strains consequently affecting the trypanosome species existing at a particular age of the cattle.

Objective 1: To determine the molecular genotype of trypanosome species from selected cattle.

Objective 2: To determine the dynamics of infection over a two year period.

Objective 3: To determine the predominant infecting trypanosome species based on age, sex and breeds of cattle.

Hypothesis for objectives 4 and 5: Cattle infected with *T. brucei* may produce antibody against *T. brucei* parasite proteins (antigen).

Objective 4: To evaluate the correlation and specificity of the *T. brucei* detected with antibody production.

Objective 5: To identify immunogenic *T. brucei* proteins.

1.2 Significance of the study

Data obtained from the study provides information on incidence of animal African trypanosomiasis and the different trypanosome strains in cattle in Ghana and suggest possible strategies for treatment to reduce emergence of drug resistance. In addition, antibody response to trypanosome species and identification of the immunogenic trypanosome proteins provides information on a possible biomarker for diagnosing cattle trypanosomiasis in Ghana and may serve as possible target for vaccine development.

CHAPTER TWO

LITERATURE REVIEW

2.1 African Trypanosomiasis

African trypanosomiasis is a disease caused by blood and tissue dwelling protozoan parasites of the genus *Trypanosoma* and transmitted by the tsetse fly (Fasanmi *et al.*, 2014). It is a disease of public health importance for both animals and humans in sub-Saharan Africa (Koffi *et al.*, 2014). Human African trypanosomiasis (HAT), also referred to as sleeping sickness is an endemic parasitic sickness caused by *Trypanosoma brucei s.l* (Koffi *et al.*, 2014). On the other hand, Animal African Trypanosomiasis (AAT) is caused by *T. congolense, T. godfreyi, T. vivax, T. theileri, T. simiae* and *T. brucei s.l* along with *T. evansi, T. equiperdum* (Ahmed *et al.*, 2013; Cox *et al.*, 2005; Van Den Bossche, 2001) specific to cattle and other ruminants.

2.2.1 Distribution and epidemiology of African Trypanosomiasis

African trypanosomiasis occurrence overlaps with the spread of tsetse fly vectors in the tsetse fly "belt." This "belt" include the regions between latitudes 14^0 North and 29^0 South covering about 10 million km^2 and 37 countries (Hursey and Slingenbergh, 1995). All the 33 species and subspecies of tsetse fly (*Glossina* spp) existing have the tendency to transmit pathogenic trypanosomes; however, only a few are vectors of trypanosomiasis. These are *Glossina morsitans morsitans*, *G. m. centralis*, *G. pallidipes*, *G. palpalis palpalis*, *G. fuscipes fuscipes* and *G. tachinoides* (Gooding and Krafsur, 2005). World Health Organization estimated cases of Human African Trypanosomiasis (HAT) is about 20,000 and poses a risk to a population of about 70 million people (WHO, 2013). Also, Animal African Trypanosomiasis (AAT), one of the principal infection disease constraints to livestock rearing in sub-Saharan Africa is

responsible for economic losses of US$ 4.5 million annually, with 3 million cattle dying (Giordani *et al.,* 2016; Yaro *et al.,* 2016; Gilbert *et al.,* 2002; Swallow, 1999). These economical losses are mostly due to a combined result of different factors such as political, sociocultural, environmental, entomological and livestock management (Baker, *et al.,* 2013; Bouyer *et al.,* 2013; Delespaux and De Koning, 2007).

2.1.2 Human African Trypanosomiasis (HAT)

2.1.2.1 Epidemiology

Sleeping sickness or HAT is one of the major neglected tropical diseases (NTDs) globally (Kennedy, 2013). The disease is caused by two morphologically indistinguishable subspecies of *T. brucei s.l*; *Trypanosoma brucei gambiense*, the causative organism of chronic HAT and *T. b. rhodesiense,* the causative agent of the acute form of the HAT disease (Welburn and Maudlin, 2012). The HAT disease causes extensive morbidity and mortality with an estimate of 1.6 million disability adjusted life years (DALY) lost annually across the tsetse fly belt of sub-Saharan Africa, (Fèvre *et al.,* 2008). The HAT disease occurs in distinct geographical locations in Africa. The *gambiense* HAT is endemic in West and Central Africa, responsible for 98% of all human infection whereas the *rhodesiense* HAT is endemic in East and Southern Africa (Franco *et al.,* 2014; WHO, 2013; WHO, 1998). In both cases of the HAT disease, there are human as well as animal reservoirs, which hamper the control and eradication of the disease (WHO, 2013; Simarro *et al.,* 2011; Njiokou *et al.,* 2010).

2.1.2.2 Pathogenesis and diagnosis of HAT

Human African Trypanosomiasis clinically develops in two stages. However, the first symptoms start at the site of the tsetse fly's bite within the first five days leading to a

localized skin reaction known as a trypanosomal chancre (Franco *et al.*, 2014; Stich *et al.,* 2002). In the first (early) stage of the disease, parasite dwells in the lymphatic system and bloodstream (hemo-lymphatic stage) (WHO, 2013; Brun *et al.,* 2010). After, years of the early stage in *gambiense* HAT and months in the *rhodesiense* HAT, a second (late stage) commences when the blood-brain barrier is traversed by the trypanosome and the central nervous system is invaded; this is referred to as the meningo-encephalitic stage associated with neurological damage and mental changes (Franco *et al.*, 2014; WHO, 2013; Brun *et al.,* 2010; Stich *et al.*, 2002). Often, the HAT diseases if left untreated is usually fatal and can advance to a coma leading to severe organ deterioration and death (Franco *et al.,* 2014; Jamonneau *et al.*, 2012).

For effective control and surveillance of sleeping sickness, early diagnosing and new improved tests of HAT is important (WHO, 2017; Njiru *et al.,* 2008). Diagnosis should be cheap, rapid, reliable, sensitive, simple to implement at any health structure level and requiring less expertise and equipment (WHO, 2017). Obtaining a positive diagnosis of HAT is essential due to the toxic nature of treatment administered hence the parasite presence must be ascertained (Wastling and Welburn, 2011).

As part of the parasitological method, clinical confirmation of HAT infection is dependent on the accurate visualization of parasites in the blood, lymph or cerebrospinal fluid (CSF) (Wastling and Welburn, 2011), Giemsa-stained thin or thick blood films (Brun *et al.,* 2010). It is often difficult to detect *T. b. gambiense* in blood but not *T. b rhodesiense* (CDC, 2012). Also, the card agglutination test for trypanosomiasis (CATT) is employed as the primary serological method to diagnose HAT. Unfortunately, CATT is only applicable to *T. b. gambiense* infections (WHO,

2017). However, there are limitations for this method, which includes the high frequency of equivocal results and limited sensitivity (Brun *et al.,* 2010; Truc *et al.,* 2002). Another alternative serological method to CATT is the LATEX/*T. b. gambiense* test (Büscher *et al.,* 1999), which uses a mix of three surface antigens (LiTat 1.3, 1.5 and 1.6), which shows similarity in sensitivity but more specific than CATT (Chappuis *et al.,* 2005). Furthermore, other anti-trypanosome antibody detection methods such as immunofluorescence assays, ELISA and immune trypanolysis can also be useful where there are good laboratory facilities (Chappuis *et al.,* 2005).

With the evolving techniques in the field of science especially in biotechnology, many molecular diagnostics have been developed to detect parasites (Deborggraeve and Büscher, 2010). Conventional, low-throughput and high- throughput polymerase chain reaction (PCR) are designed for accurate detection and speciation of trypanosomes (Deborggraeve and Büscher, 2010). The loop-mediated isothermal amplification (LAMP) that is a promising, highly specific and sensitive method for staging HAT disease can be used on the field (Bonnet *et al.,* 2015). This is because the target DNA is amplified at constant temperature and requires minimal equipment (Bonnet *et al.,* 2015). Furthermore, blood samples (either fresh or dried smears or spots can be used. Moreover, positive results are visually identified by fluorescence action (white precipitate or color change) without gel electrophoresis (Bonnet *et al.,* 2015). Multiplex PCR-based methods in which more than one pair of primers are used together in one reaction leading to simultaneous amplification of different sizes of DNA fragments are more efficient (Tao *et al.,* 2012). The PCR detection of mixed pathogens infection, with respective single primers separately is time consuming and costly (Biswas *et al.,* 2013).

These molecular diagnostic techniques are not yet applicable at the primary-care level and in the field setting (Bonnet *et al.,* 2015; Deborggraeve and Büscher, 2010).

The use of PCR and related DNA-amplification-based diagnostics have been promising and enable strain diversity, however, they are expensive and require specific training for their implementation in the field (Kuboki *et al.,* 2003). Also, antibody cross reactivity to non-pathogenic trypanosomes remains a challenge in serological methods which subsequently affects their specificity and the reliability of antibody titres distinguishing between the previous and recent infection (Kuboki *et al.,* 2003). Papadopoulos and colleagues (2004) reported the tendency of a close association between circulating proteome and disease states. Also, another study reported human plasma to have over 1000 distinct gene products human (Anderson *et al.,* 2004) even though limited number of diagnostic assays are currently being used.

The use of surface-enhanced laser desorption–ionization time-of-flight mass spectrometry (SELDI-TOF MS) has revolutionized the high-throughput proteomic profiling of serum (Issaq *et al.,* 2002). Serum proteomic fingerprinting in infectious diseases became attractive and promising when it was realized that the proteomic contributions from both the host response and pathogen might be integrated to generate more sensitive and specific signatures (Agranoff *et al.,* 2005). In the case of HAT, parasite-derived proteins suffices for diagnosis (Agranoff *et al.,* 2005). Also, Papadopoulos and co-workers (2004), showed that West African form of HAT have a highly discriminatory serum proteomic fingerprint. In their research, they employed the use of a decision-tree classifier, neural network and a genetic algorithm for data-mining in a bid to differentiate between sera mass-spectra obtained from HAT patients and a

series of controls using SELDI-TOF MS. In addition, they extracted a proteomic pattern that is indicative of HAT with 100% sensitivity and 98.6% specificity in independent set of controls and cases (Papadopoulos *et al.,* 2004).

Patients infected with other protozoa such as toxoplasmosis, *Plasmodium falciparum* and bacterial infections, like tuberculosis served as controls in the study. Apart from its remarkable accuracy, this technology does not require major prior assumptions about the protein chemistry and structure which contribute to the signature even though its diagnostic performance is independent on the knowledge of their identities (Agranoff *et al.,* 2005; Papadopoulos *et al.,* 2004). This work stands as a prerequisite for the development of better diagnostics and disease staging and provides a better understanding of the disease pathophysiology (Agranoff *et al.,* 2005; Papadopoulos *et al.,* 2004). Presently, the cost and complexity associated with the technique prevents its widespread use in HAT diagnosis in an African setting. However, the work by Papadopoulos and colleagues (2004) suggests that proteome profile could constitute a potential great discovery tool in the design and improvement of diagnosis and staging tests for HAT.

2.1.3 Animal African Trypanosomiasis (AAT)

Animal African Trypanosomiasis (AAT) is an arthropod-borne diseases of importance in relation to domestic animals and wide range of wildlife species (Laohasinnarong *et al.,* 2015; Anderson *et al.,* 2011). These animals, mostly serve as infection reservoirs for both the human and animal trypanosomes (Anderson *et al.,* 2011). The disease has a great impact on livestock productivity in sub-Saharan Africa. Mostly, cattle and small ruminants are affected, with 50 million and 70 million being at risk respectively and

costing cumulatively about US$ 4.5 billion dollars annually (Yaro *et al.,* 2016; Kristjanson *et al.,* 1999).

The trypanosomes, which mostly causes AAT in domestic ruminants are *T. brucei species, T. vivax* and *T. congolense* (Van den Bossche *et al.,* 2011; Simukoko *et al.,* 2007; Snow *et al.,* 1996). However, *T. brucei* subspecies are reported to have low pathogenicity in domestic ruminants with *T. brucei brucei* being the animal infective species (Laohasinnarong *et al.,* 2015). Another trypanosome species affecting cattle is the *T. theileri* which is non-pathogenic and generally causes subclinical infection (Fisher *et al.,* 2013). However, the disease caused by this parasite is seldom reported because the disease or death caused by this species is often related to the stressed nature of the cattle or with intercurrent infections such as bovine leucosis (Matsumoto *et al.,* 2011; Vairamuthu *et al.,* 2011; Levine, 1985).

2.1.3.1 Pathogenesis and clinical manifestation of AAT

During tsetse bite, parasites multiply locally in the skin and elicit an inflammatory response that shows as indurated skin lesion called chancre (Bezie *et al.,* 2014). Later, the parasites gain entry into the blood circulation through lymph vessels, transform into trypomastigotes and may finally migrate to the brain causing a broad spectrum of pathologies such as cachexia, weight loss, severe anaemia and abortion (Stijlemans *et al.,* 2015; Bezie *et al.,* 2014; Šíma *et al.,* 2011; Matovu *et al.,* 2001). Although *T. brucei* remains extracellular, it has the capacity to penetrate the walls of capillaries and invade interstitial tissues (Bezie *et al.,* 2014; Banks, 1980). On the other hand, *T. congolense* is an extracellular intravascular blood parasite that is unable to leave the circulation (Bezie *et al.,* 2014; Banks, 1980). Therefore, it has the tendency to bind to walls of

capillaries and small vessels (Bezie *et al.,* 2014; Banks, 1980). Millions of untreated trypanosomiasis related cases lead to the death of livestock each year (Smetko *et al.,* 2015). Sleeping disorders, emaciation, splenomegaly, pica, infertility, paralysis, neuroendocrine dysfunctions and coma are other clinical signs associated with AAT (Courtin *et al.,* 2008; Steverding, 2008).

2.1.3.2 Diagnosis, treatment, prevention and control of AAT

2.1.3.2.1 Diagnosis of AAT

For effective diagnosis and treatment of AAT, there is a need for improved diagnostic tools, which are highly sensitive and specific. The adverse effect of misdiagnosis as a result of false positive and false negative results obtained from less sensitive diagnostic tools include wrong drug administration, the death of animals and other economic loses to farmers (Moti *et al.,* 2014; Kennedy, 2013; WHO, 2013). Endemicity of the sub-region is usually the initial presumptive diagnosis approach used by veterinarians and farmers. The higher the tsetse fly density in the region the greater the incidence of AAT. Sub-Saharan African is reported to have the high incidence of AAT (Kennedy, 2013; Aksoy, 2003). Similar diagnosis methods are employed for the diagnosis of AAT. Clinical symptoms such as fever, loss of appetite, loss of weight and sleeping disorders give indications to the farmers as to the health status of the farm animal. However, this is not usually enough evidence to enable commencement of treatment because laboratory confirmations are required (Moti *et al.,* 2014; WHO, 2013) Routinely, for research and medicinal purposes, parasitological, serological and molecular based techniques are used together usually to complement the limitations of each of the methods (Moti *et al.,* 2014).

2.1.3.2.1.1 Parasitological method

Generally, this method is considered to have a high specificity but relatively low sensitivity in relation to the detection of parasites in infected animals. Preparation of Giemsa-stained thin and thick smears and observed microscopically is the gold standard of diagnosing of animal trypanosomes by parasitological method (Moti *et al.,* 2014; Kennedy, 2013) but this technique requires the judgment of an expert to accurately identify the parasites. Another limitation is that it takes a long time to analyze one sample. In another example of this method, the animal's blood is centrifuged in a capillary tube and followed by a microscope observation of parasites at the white blood cell layer level (buffy coat) (Woo, 1969). The capillary tube is further broken and the buffy coat mounted on a microscope slide for examination, where differential diagnosis is needed (Murray *et al.,* 1977).

Hematocrit centrifuge test (HCT) is also another parasitological method of trypanosome diagnosis. A study by Moti and colleagues (2014), revealed that using HCT compared to polymerase chain reaction (PCR) as a confirmation for trypanosome infection were not significantly different. Therefore, HCT used alongside with PCR is a better detection tool since samples that could possibly be negative with HCT would be PCR positive. Mostly, highly virulent parasites greatly affect the packed cell volume (PCV) value of the hosts in endemic areas, hence, HCT is a more robust and efficient tool in determining clinical cases on the field (Moti *et al.,* 2014). Also, studies have shown that HCT positive test correlates with the incidence of anemia in animals and this gives farmers and veterinarians an indication of the animals that need treatment (Van Den Bossche and Rowlands, 2001; Trail *et al.,* 1990) Often, these applied

parasitological methods fail to reveal low levels of trypanosomes in the bloodstream (Masake and Nantulya, 1991).

2.1.3.2.1.2 Serological method

This method usually exploits the detection of immune proteins (antigen, antibody and other biomarkers) in serum or plasma of the infected animal. Card agglutination test for trypanosome is a serodiagnostic method, which is a fast and simple assay for African trypanosome detection (Kennedy, 2013; WHO, 2013) but could be used to detect AAT in some livestock. This test kit is designed as similar to Rapid Diagnostic Test. It has been used for diagnosis of *T. evansi* (Bajyana Songa *et al.*, 1987). CATT has been shown to have a sensitivity of 91% and specificity of 97%, hence can be used for large-scale screen of HAT and AAT (Rodrigues *et al.*, 2014; Kennedy, 2013). Enzyme immunosorbent assay (ELISA) is another serodiagnostic tool for AAT diagnosis which could be antibody or antigen-based (Büscher, 2002). These serological methods are limited by the fact that they cannot differentiate between passive and active infection. Moreover, cross-reactivity of monoclonal antibodies mostly in cases of co-infection with other infections result in false positive results (WHO, 2013). In cases where parasitemia is low, there are usually false negative results when employed (WHO, 2013). These challenges limit the practical uses of these methods.

2.1.3.2.1.3 Molecular based method

Polymerase chain reaction (PCR) based technique offers an alternative for detection and identification of trypanosome and other parasites. Molecular techniques remain the best alternative and one of the high throughput method for effective diagnosis of most diseases (Eiras *et al.*, 2008). In 1992, Masiga and co-workers developed PCR for

detection of *T. vivax* by amplifying trypanosome DNA using species-specific primers, which has been very useful for research purposes.

PCR has the ability to detect trypanosome infection in unpurified DNA from cattle whole blood (Desquesnes, 1997). However, the presence of PCR inhibitors in blood makes it expedient for DNA purification to enhance efficiency. The RFLP-PCR method is also greatly used for species-specific identification of trypanosome (Karimuribo *et al.*, 2011). Several PCR assays such as the conventional and nested PCR targeting different regions such as the internal transcribed spacer on the rRNA locus have been used to detect several subtypes of trypanosomes (Cox *et al.*, 2010) and reported to be highly specific and sensitive in the laboratory (Gibson, 2009; Cox *et al.*, 2005). PCR is relatively expensive tool compared to the parasitological methods. Apart from the usual PCR, another molecular tool being considered is the loop-mediated isothermal amplification (LAMP), which is time-saving and for which less expertise is needed. Recently, the LAMP was used to screen dogs in Mambwe district, eastern Zambia for canine African trypanosomiasis (Lisulo *et al.*, 2014). In addition to the study, it was revealed that some dogs served as potential reservoirs for the human infective *T. b. rhodesiense* (Lisulo *et al.*, 2014). Multiplex PCR is another time-saving and more cost-effective PCR method, designed to detect different targets simultaneously than the standard PCR (Lau *et al.*, 2008; Carvalho *et al.*, 2007).

2.1.3.2.2 Treatment, prevention and control of AAT

Effective treatment and control of AAT has been a major challenge due to the high incidence of drug resistance in regions where the disease is endemic (Finelle, 1983). However, success in the treatment and control of AAT has been realized over the last

few decades. Some drugs and prophylactic drugs used to combat the occurrences of AAT were discovered around the 1940s and these were significantly useful in the control of AAT (Uilenberg, 1998). Currently, the available veterinary trypanocides as shown in (Table 1) are not adequate and outdated but six are licensed. These drugs have narrow spectrum activity restricting their use, especially when even low-level resistance arises. Up to date, diminazene aceturate and isometamidium chloride are still the most widely used anti-trypanocidal drugs against animal trypanosomiases in Africa, and suramin relatively used to treat *T. b. evansi* infections (Giordani *et al.*, 2016).

Unfortunately, despite efforts put in place to combat AAT in the world, it still remains a burden for developing countries. This results from the fact that production and marketing of trypanocidal drugs is not economically lucrative for pharmaceutical companies due to the high cost of drug discovery, production, registration, and marketing. Researchers are however, trying to develop novel drugs and vector control approaches (Giordani *et al.*, 2016; Meyer *et al.*, 2016; Connor, 1992)

Table 1: Available veterinary trypanocides against animal trypanosomiasis

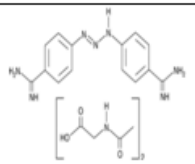
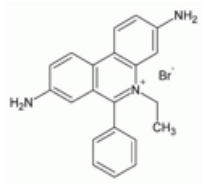
Name	Trade name[a]	Structure	Administration routes	Action	Dosage (mg Kg^{-1})[b]	Indication /animal	Adverse effects/ other information	Treatment of relapses
Diminazene aceturate	Berenil, Veriben, Pirocide, Ganaseg, Azidin, Trypan		IM, SC	T	3.5 (up to 8 for resistant trypanosomes, 5-10 for *T. b. evansi*)	*T. congolense, T. vivax* (less active on *T. b. brucei*, *T. b. evansi*)/ Cattle, sheep, goats, dogs	Toxic to horses, donkeys, dogs and camels. Also babesicidal	Isometamidium chloride
Homidium bromide Homidium bromide	Ethidium Novidium		IM (deep cattle), IV (sheep, goats, pigs)	T, (P)	1	*T. vivax, T. congolense* (less active on *T. b. brucei*)/ Cattle, sheep, goats, pigs	IM toxic to horses. Potentially carcinogenic	Diminazene aceturate, Isometamidium chloride

Table 1: (continued)

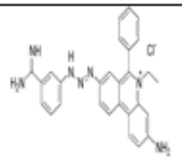
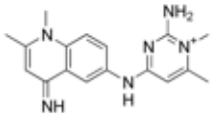
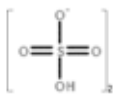
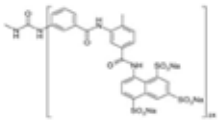
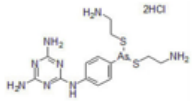
Name	Trade name[a]	Structure	Administration routes	Action	Dosage (mg Kg^{-1})[b]	Indication /animal	Adverse effects/ other information	Treatment of relapses
Isometamidium chloride	Trypamidium, Samorin, Veridium, Securidium		IM (deep)	P, T	0.25-1 (T), 0.5-1 (P)	*T. congolense, T. vivax* (less active on *T. b. brucei, T. b. evansi*)/Cattle, sheep, goats, horses, camels	Toxic above 2 mg Kg^{-1}. Avoid subcutaneous administration. Highly irritant. Possible local reactions in cattle	Diminazene aceturate
Quinapyramine sulphate	Antrycide, Trypacide, Noroquin, Quintrycide, Tribexin, Triquin-S, M7555,		SC	T	3.5 (T) (20-40 for T. simiae) (Camels, horses, pigs, dogs: dose divided and given at 6 h intervals), 7.4 (P)	*T. b. evansi, T. vivax, T. congolense, T. brucei, T. b. equiperdum, T. simiae*/Camels, horses, pigs, dogs, cattle (discouraged)	Toxic at high doses. Fast resistance acquisition	Isometamidium chloride, Suramin sodium
Quinapyramine sulphate:chloride (3:2 w/w)	Trypacide prosalt			P				
Suramin sodium	Naganol, Bayer 205, Germanin		IV	T(P)	10 (horses: 3 doses/1 week)	*T. b. evansi, T. b. brucei, T. b. equiperdum*/ Camels, horses	IM can cause severe necrosis at injection site. May be toxic to horses	Quinapyramine sulphate

Table 1: (continued)

Name	Trade name[a]	Structure	Administration routes	Action	Dosage (mg Kg^{-1})[b]	Indication /animal	Adverse effects/ other information	Treatment of relapses
Melarsomine dihydrochloride	Cymelarsan, Mel, Cy		SC, IM	T	0.25-0.5 (0.5 for cattle)	*T. b. evansi, T. b. brucei, T. b. equiperdum*/ Camels, buffalo, goats, pigs, horses, cattle		

IM, intramuscular; IV, intravenous; SC, subcutaneous; T, therapeutic action; P, prophylactic action.
Note: Products used in animals producing meat or milk for human consumption should only be used in full compliance with product labels including withdrawal periods.
[a] The list of the trade names is not complete
[b] Dosages are for single administration except were stated otherwise

Source: Giordani *et al.*, 2016

WHO and other Integrated Pest Control Programmes such as Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) have tried controlling AAT by reducing or eliminating tsetse fly populations with traps and insecticides (Torr and Vale, 2015; Hill *et al.*, 2005; Holmes, 1997). In addition, the selection of trypanotolerant (animals resistance to AAT with accompanying low mortality rate in the absence of trypanocidal drugs) breeds of cattle can lessen the impact of trypanosomiasis (Figure 1) (Yaro *et al.*, 2016; Namangala, 2012).

The use of trypanotolerance animals could have a major positive effect on long term food security for the sub-Saharan African region (Yaro *et al.*, 2016; Osaer *et al.*, 1994). Earlier reports have suggested that the host's adaptive immunity in the form of VSG-specific B- and T- lymphocytes were mainly responsible for trypanotolerance in trypanotolerant breeds (Taylor, 1998). However, more recent evidence points to the host's innate immunity in the form of activated macrophages as key to trypanotolerance (Liu *et al.*, 2015; Mansfield *et al.*, 2014). Generally, trypanotolerant livestock thrive better with low to medium trypanosome infection challenge than with high intensity parasite challenge (Holmes, 1997). In parts of west and central Africa, where the AAT challenge is very high, diminazene chemotherapy has been used to help trypanotolerant breeds maintain desirable levels of production (Peregrine and Mamman, 1993). The high intensity of the disease challenge in these parts of Africa has completely excluded the farming of trypanosusceptible breeds, given the enormous cost of the trypanocides that would be required (Peregrine and Mamman, 1993). The trypanotolerant trait is beneficial in terms of reducing the disease burden in domestic animals. However, when relocating trypanotolerant breeds to trypanosomiasis-free regions of the continent, there would be some risk of introducing the disease to these regions since they serve as

parasite reservoirs (Yaro *et al.*, 2016). However, under this scenario the risk will still be minor because AAT is mainly transmitted by the tsetse fly vector and other biting insects, the distribution for which is known to be confined to only the trypanosomiasis endemic region (Yaro *et al.,* 2016). In addition, if standard screening and quarantine precautions are applied to ensure that the animals are not harbouring any trypanosomes, the risk will be minimized (Yaro *et al.,* 2016).

Attempts to achieve genetic introgression of trypanotolerance through indiscriminate crossbreeding with trypanosusceptible breeds is common in livestock production systems in many parts of sub-Saharan Africa, but leads to the dilution of the trait (Alvarez *et al.,* 2012; Geerts *et al.,* 2009; Kosgey and Okeyo, 2007; Bradley *et al.,* 1994). Mwai and others in 2015, reported that, most of indigenous African trypanotolerant livestock breeds are now endangered due to indiscriminate crossbreeding and breed replacement leading to a high risk of these adaptive traits being diluted and lost forever since the farmers cross-breed without the specific knowledge of the trypanotolerant quantitative trait loci (QTL) or genes (Berthier *et al.,* 2016). Also, it has been reported that the occurrence of mixed infection presents an important hindrance to exploring trypanotolerance in most livestock production systems in sub-Saharan Africa (Alvarez *et al.,* 2012).

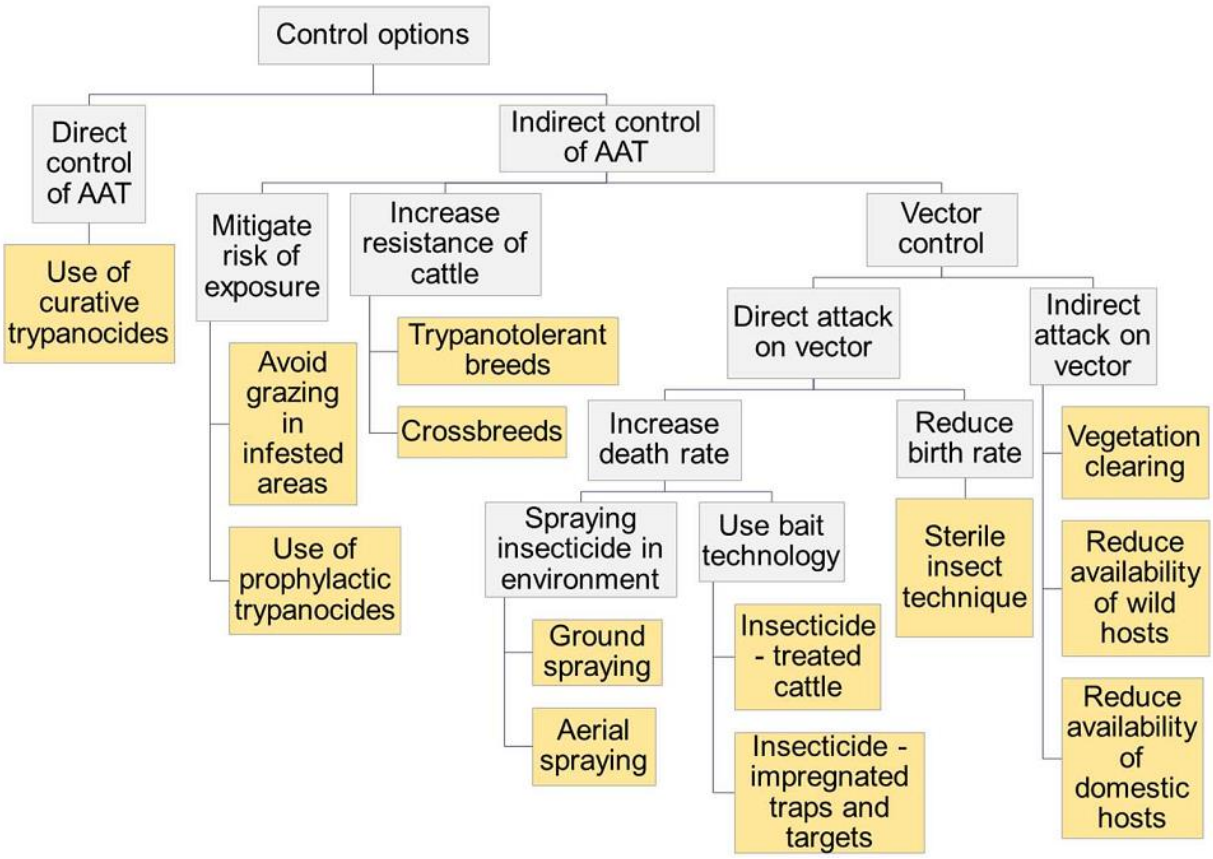


Figure 1: Summary of the techniques available to control tsetse flies and AAT.
Source: Meyer *et al.*, 2016

2.2 The Parasite: *Trypanosoma*

Trypanosomes are considered as unicellular flagellated protozoa parasites belonging to the Class Zoomastigophorea, Order Kinetoplastida and Family Trypanosomatidae (Baral, 2010). Members of Order Kinetoplastida are mostly slender, elongated, have a single nucleus and a kinetoplast close to the origin of a single flagellum (Stuart *et al.*, 2008). The genus *Trypanosoma* includes African trypanosomes, which are pathogenic. Within the genus *Trypanosoma*, exist two major groupings. The first group is the Stercoraria (American trypanosomes), which include the causative agent of Chagas disease; *T. cruzi*, along with leech-transmitted parasites of aquatic vertebrates (Hughes and Piontkivska, 2003). The other group is the Salivaria trypanosomes, also known as the African trypanosomes. This group includes the agent that causes African sleeping

sickness (*T. brucei)*, along with other African parasites of mammals (Hughes and Piontkivska, 2003).

2.2.1. Life cycle of Trypanosome

African trypanosomes' life cycle is a digenetic form, alternating between the mammalian host and the tsetse fly vector, where they exist as trypomastigote and procyclic forms, respectively (Stijlemans *et al.,* 2017; Rodrigues *et al.,* 2014). The life cycle begins when a biting trypanosome-infected tsetse fly takes a blood meal from a mammalian host. The infective parasites (metacyclic parasites) with tsetse fly saliva components are then injected into the host's dermis and this plays a vital role in the host early immune response modulation (Figure 2) (Stijlemans *et al.,* 2017; Caljon *et al.,* 2016; Van Den Abbeele *et al.,* 2010; Caljon *et al.,* 2006). The parasites then move into the blood circulation through the lymphatics from the dermal infection site (Caljon *et al.,* 2016). Afterwards, these infective parasites divide into proliferating long slender bloodstream forms expressing a heterogenous metacyclic VSG coat. These slender forms also express exceptional VSG coat and can persist in highly oxygenated and blood glucose-rich environment of the host (Figure 2) (Stijlemans *et al.,* 2017). Furthermore, these slender forms proliferate quickly, giving rise to a first parasitemia peak, subsequently, and through a quorum sensing mechanism differentiating into a non-proliferating and pre-adapted short stumpy forms for parasite survival in the vector (Stijlemans *et al.,* 2017; Mony *et al.,* 2014) (Figure 2).

The short stumpy form differentiates in the midgut region of the insect into the procyclic form. This leads to an expression procyclin coat and the procyclic forms' survival is dependent on an oxygen-deficient and a proline-rich (carbon source)

environment (Stijlemans *et al.*, 2017). Alimentary tract, mouthparts and salivary glands are important parts of the tsetse fly, where the procyclic forms undergo multiple stages of differentiation (Stijlemans *et al.*, 2017; Rotureau and Van Den Abbeele, 2013; Sharma *et al.*, 2009).

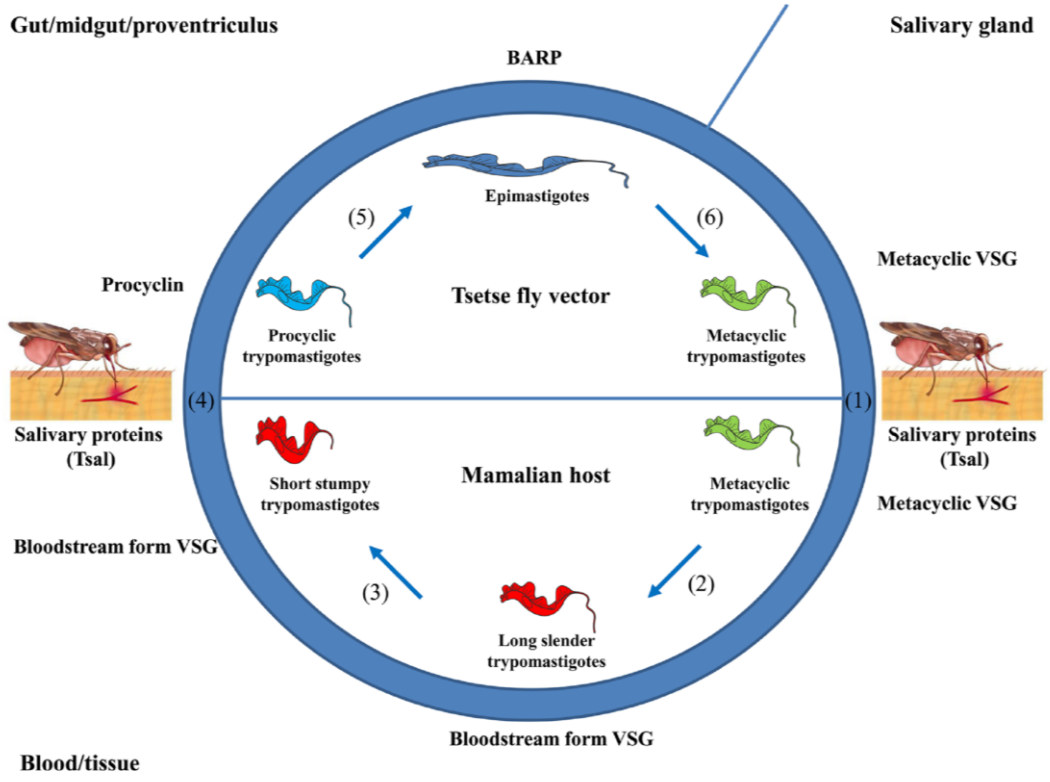


Figure 2: Life cycle of African trypanosomes. "(1) The life cycle is initiated in the mammalian host upon the bite of a trypanosome-infected tsetse fly, metacyclic trypomastigotes and saliva proteins such as Tsal are injected. (2) The metacyclic trypomastigotes expressing a heterogeneous metacyclic variable surface glycoprotein (VSG) differentiate into dividing long slender (LS) forms. (3) These LS parasite forms at the peak of parasitemia, differentiate into non-dividing short stumpy (SS) forms that are pre-adapted to be taken up by the vector. (4) The SS forms are then ingested upon the tsetse fly taking blood meal, and in the midgut, these parasites differentiate into procyclic forms (PF) with procyclin coat. (5) When migrating to the proventriculus, the PF differentiate into epimastigote forms expressing a bloodstream alanine-rich protein coat (BARP). (6) The epimastigote parasites then divide into metacyclic forms that are ready to complete their life cycle after migration to the salivary glands". **Source**: **Stijlemans *et al.*, 2017**

2.2.2 Cell morphology of trypanosome

Trypanosomes have an extended microtubule cytoskeleton, which is highly polarized (Figure 3) (Matthews, 2005). The microtubules are a spiralled corset interlinked forms that underline the plasma membrane that defines the shape of the trypanosome (Sharma *et al.,* 2009; Robinson *et al.,* 1995). The microtubule corset is highly resilient to structural damage, hence providing a protective adaptation for survival of the parasite irrespective of variation chemical or physical features of the environment (Ooi and Bastin, 2013). The microtubules exhibit a uniform polarity with minus ends regarded as anterior whiles plus ends are posterior (Matthews, 2005; Robinson *et al.,* 1995). *T. brucei* are made up of a number of single-copy organelles: flagellum, flagellar pocket, mitochondrion, nucleus and kinetoplast situated within the cytoskeletal corset. (Ooi and Bastin, 2013; Matthews, 2005).

The flagellum drives locomotion toward the direction from posterior to anterior where the flagellum extends beyond the cell body (Ooi and Bastin, 2013). The flagellum arises from the cell and is connected to the cell body along most of the parasite's length through the orifice of the flagellar pocket (FP) located at the posterior end (Sunter and Gull, 2016; Matthews, 2005; Overath and Engstler, 2004). The flagellar pocket is the only site for endocytosis and exocytosis which is of a major importance in bloodstream form (Engstler *et al.,* 2004; Allen *et al.,* 2003). This is simply because the change of the variant surface glycoproteins (VSG) coat is a function of endocytosis (Ooi and Bastin, 2013). Endocytosis in cultured procyclic trypomastigote stage is however, considered insignificant (García-Salcedo *et al.,* 2004).

Flagellum attachment is facilitated by the flagellum attachment zone (FAZ) which consist of a filament and four specialized microtubules. The filament is an electron-dense structure subtending the flagellum (Sherwin and Gull, 1989). The kinetoplast is linked to the flagellum basal body through filament structures which traverses the mitochondrial membranes and the cytoplasm (Gluenz *et al.*, 2011; Ogbadoyi *et al.*, 2003; Robinson and Gull, 1991). Trypanosome cells termed as epimastigotes have their kinetoplast anterior to the nucleus while trypomastigotes have kinetoplast which is posterior to the nucleus (Ooi and Bastin, 2013). Paraflagellar rod (PFR) is a typical feature found in the trypanosome flagellum, which is a crystalline structure which extends across the parasites' flagellum (Ooi and Bastin, 2013).

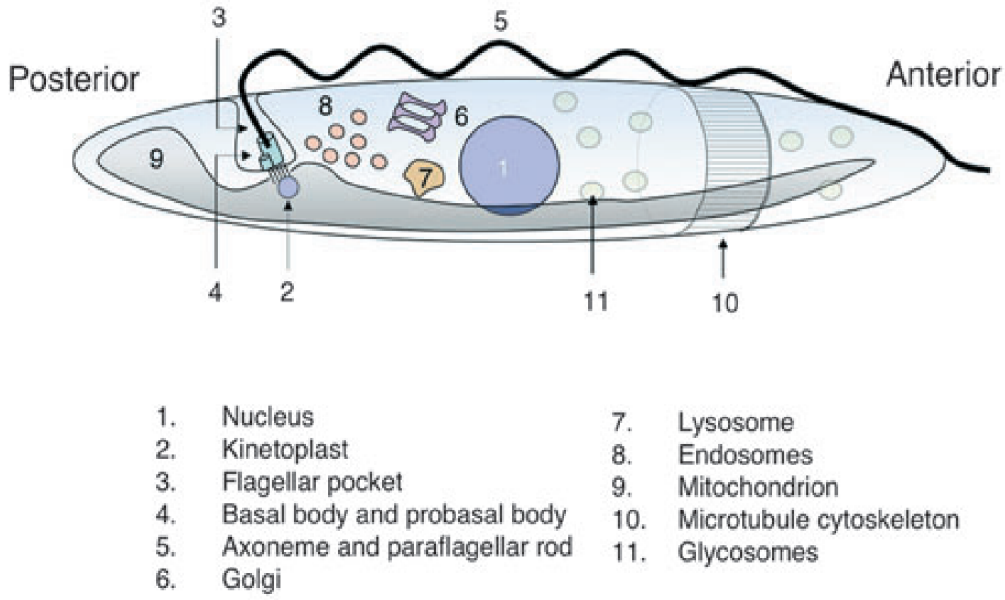


Figure 3: African trypanosome cell structure. A simplified representation of the location of the major structural features of the trypanosome cell. **Source: Matthews, 2005**

2.2.3 Genetic organization of trypanosome

Due to success in the completion of a number of genome sequencing projects over the last decades, there have been improvement in the elucidation of most eukaryote genomes. These include the trypanosomes: *T. cruzi* and *T. brucei* (Berriman *et al.,* 2005; El-Sayed *et al.,* 2005).

Trypanosomes are diploid organisms. *T. brucei* has a haploid genome size of ~35 Mbp and is embedded within a nucleus at a diameter of ~2.5 µm (Ogbadoyi *et al.,* 2000; Ersfeld *et al.,* 1999). Trypanosome gene arrangement involves densely packed protein-coding genes related to bacterial operons, which is within directional clusters in one strand divided by strand-switch (Teixeira *et al.,* 2012) (Figure 4). The 'housekeeping' part of *T. brucei* genome is made up of 11 pairs of megabase-size chromosomes per genome (Figure 5). Intermediate chromosomes and minichromosomes are the remaining parts of the trypanosome nuclear genome (Alsford *et al.,* 2001).

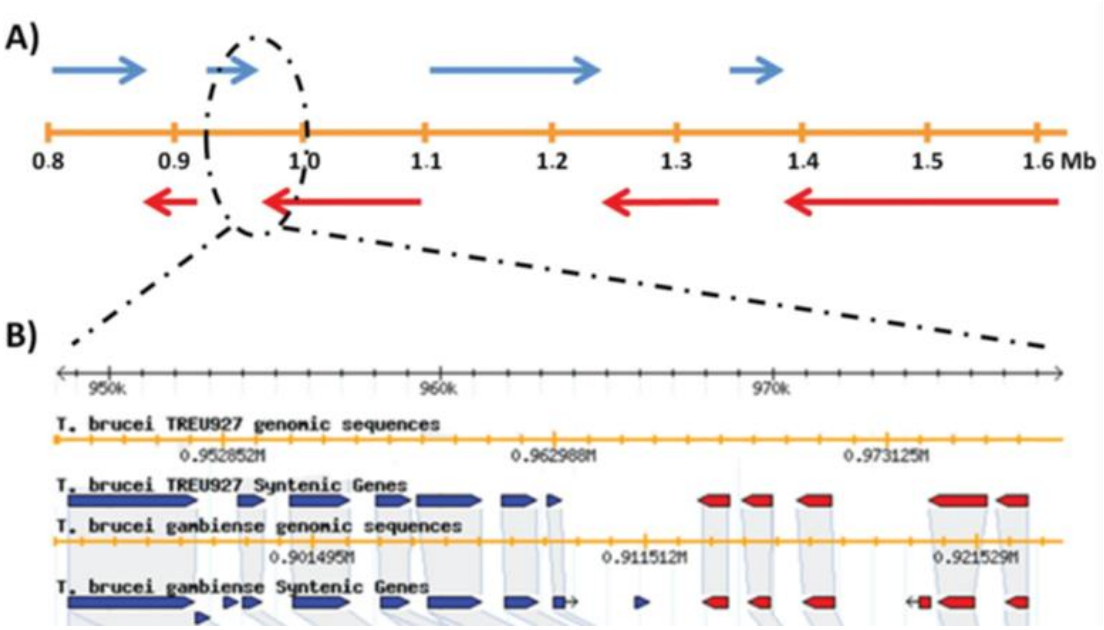


Figure 4: Gene organization in the trypanosomes genome. "The gene distribution in a 0.8 Mb region of *T. brucei* chromosome V with eight large polycistronic transcription units (blue arrows: plus (+) strand encoded open reading frames or ORFs; red arrows: minus (-) strand encoded ORFs). B) A 30 kbp region at around 960 kb is magnified to show the gene synteny in the genome of *T. brucei* (blue and red boxes correspond to + and – strand-encoded ORFs, respectively). The orange line in both panels corresponds to the chromosome position. Sequence information used to draw panel A and the graphic representation in panel B were obtained from the Tri-Tryp database". **Source: Aslett *et al.*, 2009**

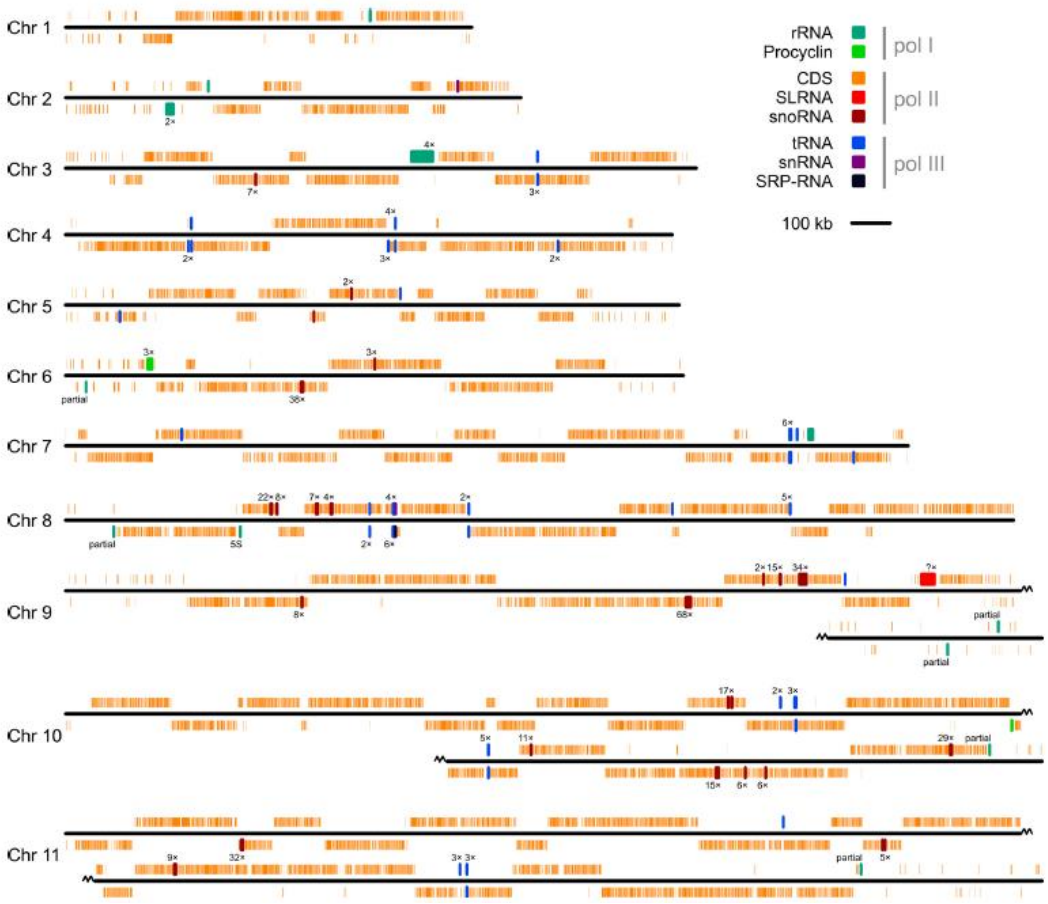


Figure 5: Arrangements of genes according to their class and transcribing polymerase in T. brucei genome. **"**The map is based on the v4 annotation available at *www.genedb.org.* The chromosomes 9 to 11 have been split across two lines. Coloured bars indicate the positions and lengths of different genetic elements relative to the chromosome backbone (black line). Bars above the lines indicate transcription toward the right; bars below the lines indicate transcription toward the left. When a number of similar elements are present in close proximity in the genome, this is indicated by *n*_ next to bar (where *n* is the number of elements). Noncoding RNA genes have been given a minimum bar length to facilitate visualization. Only the largest assembled contig is represented for each chromosome". **Source: Daniels *et al.*, 2010**

Trypanosomal genome arrangements and nuclear gene expression regulation have several aspects which are distinct when compared to eukaryotic model organisms (Martínez-Calvillo *et al.*, 2010; Palenchar and Bellofatto, 2006; Campbell *et al.*, 2003). These unique aspects include: 1) an extensive RNA editing which produce functional mRNAs transcripts from mitochondrial genes (Hajduk *et al.*, 1993), 2) RNA trans-splicing to generate mature and capped mRNAs (Lebowitz *et al.*, 1993), 3) RNA

polymerase I-mediated transcription of some protein-coding genes (Günzl *et al.,* 2003) and 4) polycistronic transcription of majority of the genomes (Martínez-Calvillo *et al.,* 2010).

The enzyme responsible for the transcription of protein-coding genes in eukaryote is DNA-dependent RNA polymerase II whereas eukaryotic DNA-dependent RNA polymerase I transcribes ribosomal RNA (Teixeira, 1998). In trypanosomes, synthesis of mRNA by RNA polymerase II (RNAP II) occurs through polycistronic transcription (Van der Ploeg, 1986). The co-transcriptional processing to individual monocistronic mRNAs is mediated by trans-splicing of a 39-nucleotide capped exon from the splice leader (SL) RNA to the 5´ end of all protein-coding genes. The trans-splicing reactions have been found to operate more often in trypanosomatids, euglena, nematode worms and other protists (Bitar *et al.,* 2013).

The only known RNAPI found in African trypanosome species transcribes both protein-coding mRNA and rRNA genes (Günzl *et al.*, 2003). These essential protein-coding genes include those that code for the procyclin, the major surface protein of the procyclic and the variable surface glycoprotein (VSG) which covers the surface of the bloodstream-form cell (Günzl *et al.*, 2003). In other eukaryotes, transcription of the 45S rRNA precursor is by trypanosomal pol I. The addition of the poly (A) tail at the 3' end and a methylated G nucleotide at the 5' end of each mRNA is essential for an efficient translation by the ribosomes. In trypanosomatids, no consensus signal sequence for polyadenylation has been identified and the cap has a unique, highly methylated structure (Lee and Van der Ploeg, 1997). Studies have provided several lines of proof indicating that polyadenylation and cap addition are dependent events, which occurs

immediately after transcription, but are part of a cut-and-paste system (Teixeira, 1998) (Figure 6).

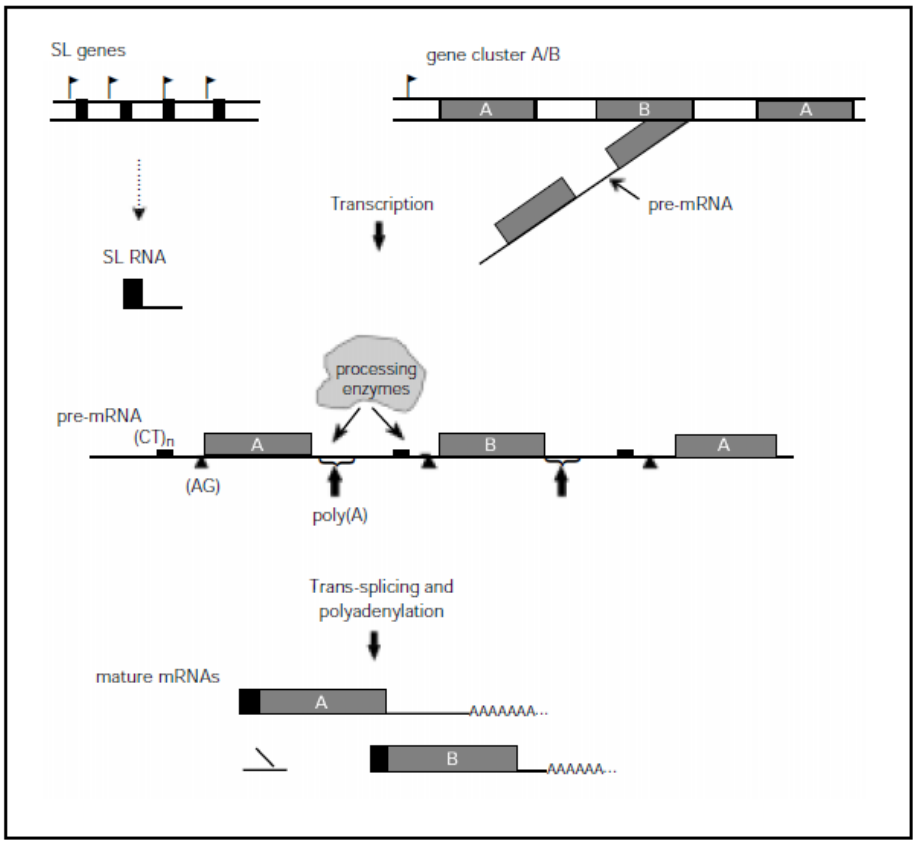


Figure 6: Gene expression in trypanosomatids. "The shaded boxes (representing two copies of an A gene and one copy of a B gene) represent the gene organization in tandem repeats of most trypanosome and *Leishmania* species. At another chromosomal location, there are several hundred tandem direct repeats encoding SL RNAs (dark boxes). In some cases, a single promoter is present upstream of the first gene in the cluster whereas in the SL gene cluster each repeat is presumed to bear a transcriptional promoter (small flags). After transcription, the polycistronic pre-mRNA is processed by *trans*-splicing and polyadenylation, which require signals present within the intergenic regions: a polypyrimidine-rich sequence or (CT)n (small boxes), the spliced leader addition site or AG (arrow heads), and a polyadenylation addition site or poly(A) (arrows). The bottom diagram of the figure represents mature mRNAs which consist of the coding region and 5'UTR (shaded boxes), the SL sequence (dark boxes), 3'UTR and poly (A) tail (thin lines with small "A" letters)." **Source: Teixeira, 1998**

2.3 Host-parasite interactions of African trypanosome infections

Trypanosomes are extracellular parasites found in the blood and oppose the innate immune responses. However, the persistence in resistance to the humoral immune responses of their mammalian hosts is also important (Matthews *et al.*, 2015). The evasion of host antibody responses by the parasite population is achieved mostly through antigenic variation. This leads to parasite survival hence establishing chronic infections, which enhance transmission and dissemination of the disease (Matthews *et al.*, 2015). Over the last three decades, several reviews have been reported on trypanosome antigenic variation (Matthews *et al.*, 2015; Horn, 2014; Glover *et al.*, 2013; Rudenko, 2011; Morrison, *et al.*, 2009). In recent years, the understanding of the complexity of the parasites in relation to infection chronicity and increased transmission capacity is well understood revealing the complex interaction between the evasion strategies of the parasite within the immune system of the host, their developmental transmission control and the contributions of host immunity under continuous exposure to trypanosome in a field setting (Matthews *et al.*, 2015; Gjini *et al.*, 2013; MacGregor *et al.*, 2012; MacGregor *et al.*, 2011; Gjini *et al.*, 2010; Lythgoe *et al.*, 2007). The parasite-host interactions provide information about infection epidemiology and evolution over time and in different geographical environments (Matthews *et al.*, 2015).

2.3.1 Immune evasion of African trypanosomes

In mammalian host, the chronicity of trypanosomiasis established by the parasite, is normally associated severe forms of pathogenicity such as liver injury, weight loss and anaemia. For complete parasites elimination, the innate immune response is insufficient, and, hence, will require supplementation by the adaptive immune response

to fight infections. Nonetheless, adaptive immune response is most likely to be influenced by the innate immune response modulation (Stijlemans *et al.,* 2016).

2.3.1.1 Evasion of innate immunity

In the vertebrate host, the skin is a vital anatomical barrier that the parasite has to succumb in order to launch infection after the metacyclic form, is co-injected with saliva intradermally by the tsetse fly (Stijlemans *et al.,* 2016). Within this microenvironment, both pharmacological and immunological processes may occur to prevent the pathogen from developing. Afterwards, immune cells such as myeloid phagocytes, lymphocytes and keratinocytes detect presence of damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) through unique different pattern recognition receptors (PRRs). This immune recognition then secretes pro-inflammatory cytokines, type-I interferon-gamma, chemokines, reactive oxygen and nitrogen species, and antimicrobial molecules (Harder *et al.,* 2013; Kennedy-Crispin *et al.,* 2012; Akira *et al.,* 2006). With time, the skin is now a significant border for arthropod-borne diseases, in which the pathogen and some components of the saliva of vectors converts the skin barrier into an immuno-tolerant organ thus supporting the parasite to develop (Bernard *et al.,* 2015; Bernard *et al.,* 2014; Frischknecht, 2007). For trypanosome infection, the presence of tsetse fly saliva permits a faster onset of the disease, which is accompanied by a reduction in inflammatory molecules at the location of infection and affects the action of tsetse fly saliva on host hemostatic reactions (Telleria *et al.,* 2014; Caljon *et al.,*2006).

After infection has been established by the parasite by overcoming the skin barrier, the host's innate immunity to the parasite in the bloodstream is due to an expression of uniquely high-density lipoproteins (HDLs) which are referred to as trypanolytic factors

(TLFs) (Namangala, 2011). The human TLFs are HDL particles that are made up of lytic component known as apolipoprotein L-1 (APOL-1) (Vanhollebeke and Pays, 2006; Vanhamme *et al.,* 2003). Apolipoprotein L-1 circulates in the form of a HDL-bound TLF termed TLF1 and a large lipid-poor IgM or apolipoprotein A-1 complex called TLF2 (Vanhollebeke and Pays, 2010). The two toxic complexes enter the trypanosomes using different uptake mechanisms but kill the parasites in the same way (Namangala, 2011).

Trypanolytic factor- 1 is mostly taken up by trypanosome haptoglobin–haemoglobin receptor (HpHbR) through a haptoglobin-related protein (Hpr) that binds free haemoglobin in blood (Namangala, 2011; Vanhollebeke and Pays, 2010; Wheeler, 2010; Molina-Portela *et al.,* 2008; Vanhollebeke *et al.,* 2008; Vanhollebeke and Pays, 2006; Vanhamme *et al.,* 2003). For TLF2 mediated killing, two different models are proposed; (i) TLF2 kills *T. brucei* in a manner similar to TLF1, which depends partly on the uptake of the TbHpHbR receptor since the particle contains both ApoL1 and Hpr (Stijlemans *et al.,* 2016; Bullard *et al.,* 2012; Stephens *et al.,* 2012). However, using the competitive inhibitor of TLF1 (Hpr), TLF2 was unperturbed indicating varying roles and possible internalization mechanism (Stijlemans *et al.,* 2016; Molina-Portela *et al.,* 2008; Raper *et al.,* 1999); (ii) Following the lytic activity of ApoL1 on *T. brucei*, TLF2 association with ApoL1 could facilitate it interaction with the VSG coat. The TLF2 has been shown to be associated with IgM which also could impact its function (Vanhollebeke and Pays, 2010).

Till date little or no data has proven the mechanistic role of TLF2 action on *T. brucei* (Stijlemans *et al.,* 2016). What has been clearly demonstrated, is individuals harbouring

ApoL1 deficiency have no trypanolytic activity in their sera; hence are more susceptible to animal-specific trypanosome species (Namangala, 2011; Vanhollebeke *et al.,* 2006). Most of the animal African trypanosomes do not establish infections in humans because they are lysed. However, the human-infective trypanosomes (*T. b. rhodesiense* and *T. b. gambiense*) has been reported to undergo selective pressure which could favour its survival by escaping attack by the innate immune system (Namangala, 2011).

The parasites are able to evade the lethal action of TLF particles as a result of *T. b. rhodesiense* expressing a serum resistance antigen (SRA) and *T. b. gambiense* expressing a specific glycoprotein (TgsGP) counteracting ApoL1 activity (Stijlemans *et al.,* 2016; Capewell *et al.,* 2013; Uzureau *et al.,* 2013; Van Xong *et al.,* 1998). Also, TLF 1 uptake in *T. b. gambiense* is low because this parasite exhibits low-level HpHbR expression and harbours an amino acid substitution (L210S) in HpHbR (Capewell *et al.,* 2013; DeJesus *et al.,* 2013; Uzureau *et al.,* 2013; Capewell *et al,* 2011; Kieft *et al.,* 2010; Vanhamme *et al.,* 2003; Van Xong *et al.,* 1998). The transfer of SRA from *T. b. rhodesiense* to *T. b. brucei* was recently demonstrated to be achieved through membranous nanotubes from the flagellar membrane disassociating into free extracellular vesicles (EV) (Szempruch *et al.,* 2016). This exchange of virulence factors could confer resistance to innate immune response.

Classical and alternative pathways of complement activation during trypanosomiasis improves the efficiency of parasite and immune-complex removal during innate immunity (Namangala, 2011; Pan *et al.,* 2006; Russo *et al.,* 1994; Takayanagi *et al.,* 1987). Activation of the alternative pathway, which occurs in the absence of specific humoral antibodies, may potentially play a crucial role in parasite clearance during the

early stage of infection (Namangala, 2011). However, according to Ferrante and Allison (1983), bloodstream *T. congolense* and *T. b. brucei,* through their VSGs, have been shown to specifically inhibit the activation of the alternative pathway. Devine and co-workers (1986) demonstrated that *T. b. gambiense* infection induced trypanolysis is by partial activation of the alternative pathway without generating terminal immune complexes (C5-C9).

Serum levels of total haemolytic complement of the alternative complement pathway and C3 of the classical pathway are reduced in *T. congolense* and *T. vivax*-infected cattle (Nieslen, 1985). In the absence of antibodies, a study has shown that trypanosomes induce complement depletion by directly cleaving complement components (Sheppard *et al.,* 1978), possibly for their own survival (Namangala, 2011).

2.3.1.2 Evasion of adaptive immunity

Although trypanosomes use antigenic variation of their VSG coat as an efficient way to escape host adaptive immunity as mentioned earlier, B cells and T cells are also directly/indirectly affected by trypanosomes as an alternative mechanism to escape elimination.

Macrophages are highly phagocytic and play a significant role in the characteristic immunosuppression found in AAT (Flynn and Sileghem, 1991) and HAT (Greenwood *et al.,* 1973). Cellular (T cell) and humoral (B cell) responses are suppressed during African trypanosome infection mediated by activated macrophages through their discharge of several pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α), reactive oxygen, prostaglandin E2 and nitrogen intermediates (Magez *et al.,*

1999; Sternberg, 1998; Schleifer and Mansfield, 1993; Sternberg and McGuigan, 1992). Immunosuppression is yet another way for African trypanosomes to prolong survival in the host but a balance has to be struck as it also increases host susceptibility to trypanosomes and secondary infections. Interferon gamma (IFN-γ) produced by T-helper 1 (Th1) cells, activated by parasite antigens is believed to serve as a strong activation signal for macrophages (Barkhuizen *et al.,* 2007; Sternberg and Mabbott, 1996).

Complement receptor 3 (CR3)-dependent phagocytosis of IgM or IgG opsonized parasites by activated macrophages and Kupffer cells (liver macrophages) results in TNF-α production and downregulation of nitric oxide (NO) synthesis (Stijlemans *et al.,* 2007; Pan *et al.,* 2006; Hertz *et al.,* 1998). It is possible that the TNF-α produced may also kill parasites (Magez *et al.,* 2001), although this observation remains controversial (Kitani *et al.,* 2002). Recently, Trindade and colleagues in 2016 discovered that *T. brucei* also accumulates in the adipose tissue of the mammalian host, which may provide an advantage for the parasite to evade adaptive immunity because of the inefficient elimination of the parasite by adipose tissue-specific immune response.

2.3.1.2.1 Antigenic variation

African trypanosome cells have their surface covered by a uniform protein coat composed of a single VSG type in the mammalian bloodstream (Matthews *et al.,* 2015; Cross, 1975). The VSG, a glycophosphatidylinositol (GPI)-anchored glycosylated protein protects the parasite from the alternative pathway of complement activation (Vincendeau and Bouteille, 2006). Therefore, playing a critical role in the parasites' evasion of host immune system due to high immunogenic nature of the VSG. Common

and invariant antigens resident on parasite surface are also shielded from the immune system by the VSG (Matthews *et al.,* 2015; Horn, 2014). Epitopes resident on the exposed N-terminal domain of the VSG elicit antibody response leading to parasite lysis through the classical pathway of complement activation (Higgins and Carrington, 2014). However, the infection is often not cleared due to part of the parasite population switching to express antigenically distinct VSG, which is not known by the antibodies raised to earlier antigen types (Matthews *et al.,* 2015).

Antigenic variation is best described in two strains of *T. brucei* whose VSG repertoire have been characterized (Cross *et al.,* 2014; Marcello *et al.,* 2007; Berriman *et al.,* 2005), alongside *T. vivax* and *T. congolense* in animals (Jackson *et al.,* 2012). The *T. brucei* genome has more than 2000 VSG genes (more than 20% of the coding genome) even though the VSG cataloguing is still incomplete. Majority of these VSG genes exist in transcriptionally silent subtelomeric arrays, although a significant portion are found in aneuploidy minichromosomes (Matthews *et al.,* 2015).

High dynamics of VSG repertoire, with changes in VSG numbers and identities during strain propagation (Cross *et al.,* 2014), and larger scale rearrangements leads to chromosome size variation within and between strains (Callejas *et al.,* 2006). In comparison of the VSGs among the different trypanosome species, *T. brucei* has A-type VSG and B-type VSG which are derived from a single lineages with a common C-terminal domain (CTD); *T. congolense* combines multiple ancestral B-type VSG lineages, each with a distinct C-terminal domain (15–20 different CTD) and *T. vivax* has the most structurally diverse repertoire of VSGs, comprising A- type VSG, B-type VSG, and two additional unique to *T. vivax* but with low (∼20%) protein sequence

similarity to known VSG (Jackson *et al.,* 2012; Gardiner *et al.,* 1996; Rausch *et al.,* 1994).

The expression of unique antigenic forms of trypanosome VSGs is limitless due to the huge repository of VSG genes and highly flexible 'switching' mechanisms, which supports new VSGs activation in the event of antigenic variation (Figure 7) (Matthews *et al.,* 2015; Stockdale *et al.,* 2008). The specific location of a VSG gene within an active telomeric VSG expression site informs its expression in the trypanosome genome, which range about 15 – 25, each with a different VSG (Hertz-Fowler *et al.,* 2008; Young *et al.,* 2008). A single expression site remains active per time (Kassem *et al.,* 2014) in unique association with a sub-nuclear transcription factory, which is the expression site body (López-Farfán *et al.,* 2014; Navarro and Gull, 2001). The diversity of VSG expression sites could occur by transcriptional switch leading a new VSG gene, thus silencing the previously active site (Matthews *et al.,* 2015).

However, the most prominent way of VSG protein switching includes recombination with about 90% of switching processes (Robinson *et al.,* 1999), principally through gene conversion events; whereby a silent VSG gene is copied and used to replace the expressed one within the expression site. This VSG switching event is strictly responsible for cases of prolonged infections, thereby generating a VSG diversity, which exceeds the initial number of VSG genes in the genome archive (Matthews *et al.,* 2015). Transposition of VSG from subtelomeric loci to the telomeric expression site is by recombination mechanism (Morrison *et al.,* 2009; Pays, 2005). The scale of recombination varies between the species due to their compositional differences, this

occurs frequently in *T. congolense* and *T. brucei* VSG than in *T. vivax*, and more predominant among *T. brucei* VSG than in *T. congolense* (Jackson *et al.*, 2012).

For segmental VSG conversion, involving recombination directed by the coding region of the VSGs, VSG pseudogenes are utilized hence, several VSG open reading frames (intact, pseudogene or gene fragments) can be recombined (Figure 7), generating new antigens (mosaic VSGs). This therefore significantly augments the potential of the expressed VSG diversity well beyond the limitations of the existing genome's VSG repertoire (Hall *et al.,* 2013; Marcello and Barry, 2007). *T. brucei VSGs* sequence mosaics which are generated gives an inclusive source of diversity among expressed VSG (Marcello and Barry, 2007) but limited information is available on the occurrence of dynamic assortment of VSG in other species (Jackson *et al.*, 2012). The parasite also undergo transcriptional switching of their VSG, possibly as a channel to create active transcription of the appropriate expression site for the host (Bitter *et al.*, 1998).

Similarly, telomeric location have a tendency of promoting recombinogenic interactions (Barry *et al.*, 2003). The VSG mosaics assembly initiates as a result of VSG switching and independent advancement in each infection, different hosts become exposed to totally new antigen repertoires which will obstruct the tendency of generating significant immunity to the invading parasites or new co-infecting trypanosome parasites (Matthews *et al.,* 2015). Also, mosaic VSGs could play an important role in establishing chronic infection, which are sustained over longer periods in the wild, but insufficient information is available on the subject of the mechanism of their formation (Mugnier *et al.*, 2016).

The VSGs are known to “shield” invariant surface proteins from attachment by host antibody due to their size and through steric hindrance (Schwede *et al.*, 2015; Grünfelder *et al.*, 2002). *T. brucei* is known to have the invariant surface antigens with >100-fold abundance less than the VSG (El Hassan, 2014; Overath *et al.*, 1994) and organized in between the VSG proteins on the surface of the trypanosome parasite (El Hassan, 2014). Structural integrity and nutrient uptake signaling are functions of the invariant surface proteins, thus secured from the host’s adaptive immune system by the VSG in an unspecified mechanism (Schwede and Carrington, 2010).

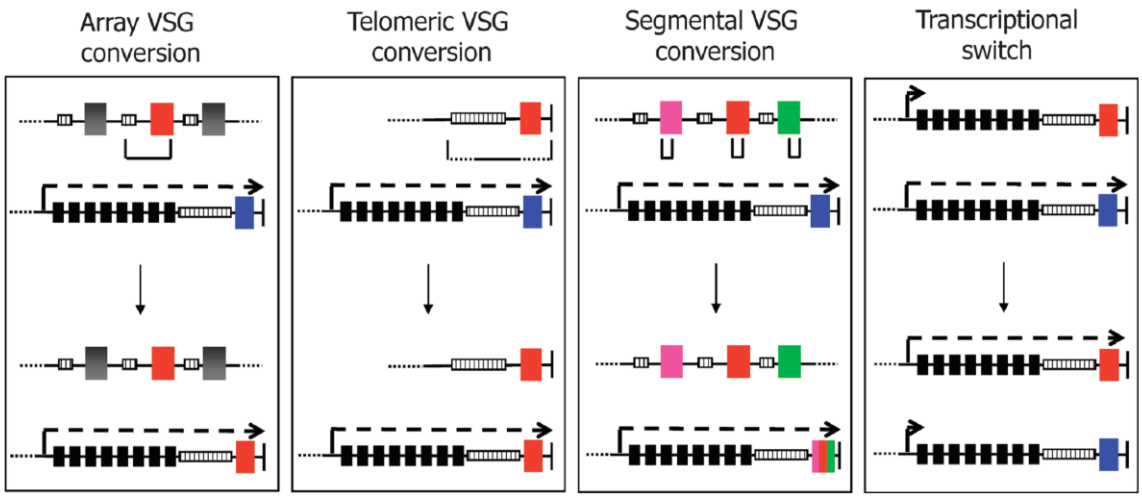


Figure 7: Mechanisms of VSG switching during antigenic variation in T. brucei. "The VSG gene expressed prior to a switch (indicated by a blue box) is transcribed from an expression site (ES) that is found at the telomere (vertical black line) of a chromosome (horizontal black line); active transcription of the ES is indicated by a dotted arrow, expression site associated genes (ESAGs) are depicted by black boxes, and 70-bp repeat sequence is shown as a hatched box. Mechanism of array VSG conversion occurs when a silent VSG (red box) is copied from a subtelomeric VSG array into the ES, where it replaces the active VSG. Telomeric VSG conversion involves a telomeric VSG (including 70 bp repeat sequence upstream and telomere downstream) replacing the active VSG in the ES. Segmental VSG conversion involves the copying of sequence from multiple inactive VSG genes (pink, red, or green boxes) and combined into a novel mosaic VSG in the ES. In transcriptional VSG switching, a non-recombination based mechanism that activates a new (previously silent) VSG ES (indicated by a small arrow), while inactivating the previously active ES." **Source: Stockdale *et al.*, 2008**

2.4 Vaccine development for AAT

Most mammals in sub-Saharan African are capable of dealing with parasites including trypanosomes by their immune system. This implies that in the event of exposure to trypanosomes; anti-parasite response can be launched by the immune system under optimal conditions. This, together with the fact that complete annihilation of the trypanosome reservoir is unlikely, suggesting anti-trypanosome vaccination as the main focus to battle AAT (La Greca and Magez, 2011). So far, finding an effective potential vaccine candidate for use in a field setting has not been attained. This is mainly due to antigenic variation of the surface proteins, allowing the parasite to evade the immune

response until the host succumbs (Horn, 2014; La Greca and Magez, 2011; Horn and McCulloch, 2010).

Although, antigenic variation of the VSGs is a problem for vaccine development against African trypanosomes, other common structural features of the trypanosomes have been considered as potential application in vaccine development in the past. An invagination of the membrane at the base of the flagellum (flagellar pocket) is a common architectural feature of trypanosomes with proteins which were considered as potential vaccine candidates. This flagellar pocket is readily engaged in cell division, protein trafficking, exocytic and endocytic processes, and most importantly, virulence and immune evasion (La Greca & Magez, 2011; Field and Carrington, 2009).

A study involving invariant antigen localized at the FP in the immunization of cattle revealed a partial protection against trypanosome infection, in relation to varying disease incidence (Mkunza *et al.,* 1995). Another similar study involved the immunization of Balb/c susceptible mice with FP preparation (Radwanska *et al.,* 2000). The study revealed a survival rate of about 60% in parasite-challenged mice with a delayed parasitemia onset and doubled survival time presented by the mice that succumbed to trypanosome infection (Radwanska *et al.,* 2000). In spite of these 'positive' findings, induced protection obtained at low dose of trypanosome infection are substantive, when mice were subsequently challenged with higher parasite load (inoculum of 10^3 parasites or more). The authors therefore concluded that FP vaccination is a less effective approach (La Greca and Magez, 2011; Radwanska *et al.,* 2000). Similar approach targeting a number of molecular targets of invariant trypanosome proteins, including several invariant surface glycoproteins (ISGs) and the

transferrin receptors ESAG6/7 have been proposed (Steverding *et al.,* 1994; Jackson *et al.,* 1993; Ziegelbauer and Overath, 1993; Ziegelbauer and Overath, 1992). A study by Lança and co-workers (2011), reported a partial protection of mice (40%) and increased IgG2a antibody levels during vaccination with a DNA plasmid encoding a bloodstream-stage specific ISG.

Subcellular proteins of the cytoskeleton (actin and tubulin) are other structural molecules suggested for use as vaccination candidates. Actin plays a crucial role in cell division, movement and morphology of bloodstream forms of *T. brucei* but most importantly, it is essential for coated vesicles formation from the FP membrane (La Greca and Magez, 2011; García-Salcedo *et al.*, 2004). Also, tubulin was targeted because of its involvement in various intracellular functions within the cytoskeleton, which include cell motility, transport and maintenance of cellular architecture (Lubega *et al.,* 2002; Schneider *et al.,* 1987). An immunization study published by Li and colleagues (2009) involving the use of recombinant *T. evansi* actin displayed homology alongside *T. b. brucei, T. equiperdum* and *T. cruzi*. It was reported that actin immunized mice were protected to different degrees according to the trypanosome species of challenge, and no experience autoimmune reactions was observed. Another study by Li and colleagues (2007), supported the earlier results, in which recombinant β-tubulin of *T. evansi* used in the immunization of mice protected them from fatal challenge with *T. evansi, T. b. brucei* and *T. equiperdum*. This suggests that antibody-mediated protection was displayed, and would reach its target by internalization but the mechanism is unknown (Li *et al.*, 2007).

However, with low parasite dose of 1,000 parasites, the parasite challenge was done only 6 days after the third vaccine boost. (Li *et al.,* 2007). Another anti-tubulin vaccination study by Lubega *et al.,* (2002), reported partial protection against African trypanosomiasis similar to the results of the vaccination approach by Li and others (2007). At the end of the study, 60–80% sterile protection was observed in tubulin immunized mice in addition to heterologous challenges of *T. brucei, T. congolense* and *T. b. rhodesiense* (Lubega *et al.,* 2002). However, as with the studies conducted by Li and others, the short period between parasite challenge and vaccination boosts remains inconclusive on the definite functional involvement of immunological memory (La Greca and Magez, 2011). Also, no clear assumption was made about the protective intracellular mode of action of the presumed antibodies (La Greca and Magez, 2011). Finally, neither the tubulin nor actin vaccine studies were followed up in the field setting (La Greca and Magez, 2011).

Trypanosome cation pumps and trans-sialidases are other membrane-related candidates identified for vaccine development. The movement of sialic acid from sialylated glycoconjugates from the host's cell surface to acceptor molecules on the surface of the parasite is carried out by the membrane-associated enzyme, sialidases (La Greca and Magez, 2011; Schenkman *et al.,* 1991). Limited information of the enzyme study in *T. brucei* is available, although extensive studies have been done in *T. cruzi*. However, a study was done using *T. brucei* by Silva *et al.,* (2009), in which mice immunized with a plasmid encoding the C- and N-terminal domain of the enzyme and challenged with low dose of 500 *T. b. brucei* parasites, a 60% protection was observed.

Alternative approach has been proposed towards solving the challenges associated with the limited success in earlier described anti-trypanosome vaccinations. This new approach gives hope for a possible development of a vaccine, which targets infection-related pathology instead of the parasite itself (La Greca and Magez, 2011). A key target of this approach is that the glycosylphosphatidylinositol (GPI) anchor attaches the VSG molecules to the parasite's membrane. Induction of TNF-α activity is closely associated with the anchor and the relation between initiation of disease-associated immunopathology and this cytokine has been well established (La Greca and Magez, 2011; Magez *et al.,* 1999; Sileghem *et al.,* 1994). Another approach involves a cysteine protease (CP) such as congopain, which elicits high IgG response in trypanotolerant animals, though not in cattle which are susceptible. The role of this molecule was suggested in the area of pathogenicity back in 1993 (Authié *et al.,* 1993). Authié *and* co-workers (2001) reported the first vaccination trial in cattle. A summary shown in Table 2 display information on earlier discussed potential vaccine candidates (La Greca and Magez, 2011).

Table 2: Summary of vaccine candidates reported in literature

Type of vaccine	Antigen	Antigen preparation	Boosts	Time lapse last boost-challenge	Parasite load	Immunological outcome	Reference
Intramuscular	FP	Parasite isolated	3	14 d or more	Natural exposure in a field	Partial protection	Mkunza *et al.*, 1995
I.p.	FP	Parasite isolated	3	NI	500 x 10^3	Partial/no protection	Radwanska *et al.*, 2000
I.p.	ISG65, ISG75	Recombinant protein	3	11 d	10^4	No protection	Ziegelbauer *et al.*, 1992
I.p.	ISG	Plasmid DNA	1	175 d	500	Partial protection	Lança *et al.*, 2011
Subcutaneous	Actin	Recombinant protein	3	6 d	10^3	Partial protection	Li *et al.*, 2009
Subcutaneous	Tubulin	Recombinant protein	3	6 d	10^3	Partial protection	Li *et al.*, 2007
Subcutaneous	Tubulin	Parasite isolated	3	NI	10^3	Partial protection	Lubega *et al.*, 2002
intramuscular	Sialidase	Plasmid DNA	1	175 d	500	Partial protection	Silva *et al.*, 2009
I.p.	Cation ATPases	Recombinant protein	3	6 weeks	10^6	No protection	Ramey *et al.*, 2009
I.p.	GPI	Liposomes	2	3 weeks	5 x 10^3	Partial protection	Stijlemans *et al.*, 2007
Subcutaneous	CP	Recombinant protein	4	1 month	Experimental tsetse fly challenge	Partial protection	Authié *et al.*, 2001

"I.p.: intraperitoneal; FP: flagellar pocket; ISG: invariant surface glycoprotein; GPI: glycosylphosphatidylinositol; CP: cysteine protease; NI: not indicated." **Source: La Greca and Magez, 2011**

2.5 Immunogenic trypanosome proteins associated with AAT as diagnostic markers

In order to identify new diagnostic techniques, comprehensive understanding of host/parasite interaction is crucial to provide signs of infection, disease progression, effective chemotherapy and potential target antigens for vaccine production against trypanosomes (El Hassan, 2014).

Since, the VSG of trypanosome undergoes antigenic variation, most researchers are targeting other trypanosome proteins as diagnostic markers. One of such proteins is the invariant surface glycoproteins (ISG) and other membrane proteins. Many of the ISGs have been identified and their functions identified. Some ISGs function as glucose transporters (Bringaud and Baltz, 1992; Parsons and Nielsen, 1990). The ISG 100 identified is embedded in the endosomal system and recognized as a polytopic protein (Pal *et al.*, 2002; Nolan *et al.*, 1997). Other identified ISGs with unknown functions include a 77 kDa protein isolated from the coated endocytotic vesicles of *T. brucei* (Webster and Shapiro, 1990).

Two other invariant surface glycoproteins (65 and 75 kDa) found by Ziegelbauers and Overath, (1992) in the bloodstream stage of *T. brucei* have large extracellular domains relatively close to the size of the VSGs and are well distributed on the trypanosome's surface (Ziegelbauer and Overath, 1993). These ISGs were originally considered as the most abundant surface proteins with the exception of VSG (Overath *et al.,* 1994). Also, these ISGs were not considered to be accessible to antibodies and do not protect against reinfection as well as anti-ISG 75 antibodies showing a weak reaction in live trypanosomes, (Ziegelbauer *et al.*, 1992) Nevertheless, Lança and co-workers (2011)

reported partial protection in Balb/C mice immunized by the intramuscular approach with a single dose of a DNA plasmid encoding ISG 65 against a lethal dose of *T. brucei* (Lança *et al.*, 2011). An intracellular pool of the ISG 65 protein was also detected despite its occurrence on the trypanosome cell surface (Chung *et al.*, 2004). Later, in 2014, El Hassan also reported the presence of an ISG of 52kDa in *T. evansi* and its been characterized for probable use as diagnostic marker but this study was preliminary (El Hassan, 2014).

2.6 Animal African Trypanosomiasis (AAT) in Ghana

Agriculture plays a central role in the social and economic development in Ghana. Livestock production is a key aspect of Ghana's agricultural sector because of its contribution towards food needs and revenue generation, forming an estimated 7% of agricultural GDP (ADF, 2001). In northern Ghana, cattle production plays an important role in farming systems, where about 50% of farmers use bullocks for ploughing in the Upper West and Upper East Regions (ADF, 2001). The prevalence of tsetse flies which transmit trypanosomiasis in the transitional and humid forest zones is responsible for the sparse population of cattle in these zones (ADF, 2001). Animal health has been the major constraints to livestock production in Ghana. For cattle, rinderpest and anthrax diseases have been eradicated (Mahama *et al.*, 2003; ADF, 2001) but other diseases such as Contagious Bovine Pheuro-Pneumonia (CBPP), the main zoonoses (tuberculosis, brucellosis, and rabies), Foot and Mouth Disease, trypanosomiasis and tick-borne diseases (dermatophilosis, babesiosis and anaplasmosis) (ADF, 2001) are dangerous and still persisting.

In Ghana, a cross-sectional study by Nakayima and others (2012), using molecular techniques to survey animal trypanosomiasis in Adidome and Koforidua reported that overall occurrence of trypanosomes was 17.4%, 57.5% and 28.6% in tsetse flies, cattle and pigs, respectively. Also, the study identified *T. congolense* savannah (52.6%) and *T. congolense* forest (66.0%) as the endemic subgroups in Ghana with 18.6% being mixed infections. An earlier epidemiological study of bovine trypanosomiasis was investigated in two districts of Northern Ghana (Savelugu and West Mamprusi) by (Mahama *et al.,* (2004). Parasitological and serological techniques were employed for the study. Using both techniques the bovine trypanosomiasis prevalence was significantly higher in West Mamprusi than Savelugu district (Mahama *et al.,* 2004).

Another, parasitological and serological cross-sectional surveys of bovine trypanosomiasis in the Upper West Region of Ghana was conducted (Adam *et al.,* 2012). In the study, 1800 cattle of the Sanga, Zebu and West African Shorthorn breeds from 36 randomly selected grid cells covering the study area were sampled. The average parasitological prevalence was estimated at 2.5% with *T. vivax* causing majority of the infections (Adam *et al.,* 2012). Enzyme linked immunosorbent assay (ELISA) gave a seroprevalence of 19% (Adam *et al.,* 2012). Again, the study reported that many infected cattle were found close to the major river systems and cattle with anti-trypanosomal antibodies were found throughout the study area using the ELISA (Adam *et al.,* 2012). Furthermore, there is no publishable data yet on trypanosomiasis prevalence in Accra. From all these studies it is concluded that bovine trypanosomiasis still is prevalent in Ghana and is affecting livestock production. Food security is threatened and the economy of Ghana is affected. Consequently, there is the need to investigate and generate more scientific information about bovine trypanosomiasis and

other domestic animal trypanosome infection status in Ghana using high throughput molecular techniques over a lifetime period.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

The longitudinal study was carried out at Adidome and Accra, selected on the basis of geographical location, tsetse fly density, trypanosomiasis prevalence and the breed of cattle at the sites with approval from the Council for Scientific and Industrial Research (CSIR) Institutional Animal Care and Use Committee (IACUC), Ghana (Certificate number in appendix). Twenty cattle were tagged and used at each study area. Animals at Adidome were periodically treated (1 to 3 months intervals) with anti-trypanocidal drugs (Isometamidium chloride and Diminazene aceturate) but no treatment was administered to animals at Accra.

Blood samples were collected at approximately four to five-week intervals for two years. DNA was extracted to characterize trypanosome species in cattle in Ghana using molecular techniques. In addition, sera were prepared to determine antibody response to trypanosome proteins as well as potential immunogenic trypanosome proteins for diagnostic or vaccine development using ELISA, western blot, immunoprecipitation and mass spectrometry.

3.2 Study sites

This study was carried out at two sites of different cattle trypanosomiasis endemicity: University of Ghana Livestock and Poultry Research Farm on the Accra Plains in the Greater Accra Region and a privately owned cattle farm at Adidome in the Central Tongu District, Volta Region (Figure 8). The University of Ghana Livestock and Poultry Research

Farm is situated at latitude 5° 40' 29.20" North and longitude 0° 06' 14.95" West with an average altitude of 68 m. The vegetation is savanna type with a low tsetse fly challenge and an average annual rainfall of about 730 mm. The major rainy season begins in April and ends in mid-July and the minor season occurs in October. The breeds of cattle in this area include Sanga, Sanga Cross-breds and West African Shorthorn (WASH) (Figure 9). Adidome is located about 200 km east of Accra and lies within latitude 6° 04' 25.97" North and longitude 0° 29' 59.88" East with an average altitude of 53 m. The vegetation type is that of mixed savanna and forest. It has a high tsetse fly challenge and an average annual rainfall between 900 mm and 1100 mm. The major rainy season starts from mid-April to early July and the minor season is from September to November. Sanga is the major cattle breed found at this site (Figure 9).

3.3 Experimental animals and sample collection

The animal populations randomly selected for the study were 40 cattle made up of 20 ear-tagged cattle from each study site with ages between 2 to 7 months (Figure 9). Table 3 is summary information on the sex, breed and age of the cattle selected at both study sites. Some animals were replaced and no samples were collected at some time points at both sites due to death of previous animals and challenges with taking samples from animals.

Peripheral blood samples were taken from cattle at approximately four to five weeks interval from both study sites over 2 years (from 2014 to 2016; funds allocated for the study was from 2013-2017). Selection of 2 years was chosen because animals are normally reared, sold and slaughtered within 3 years. The blood samples from each animal (5 ml

each) were collected by jugular venipuncture into two separate S-monovette blood collection tubes (Sarstedt AG & Co, Nümbrecht, Germany), with and without EDTA and transported on ice to the Molecular Biology Laboratory for analysis. A sum of 635 blood samples were collected over the study period.

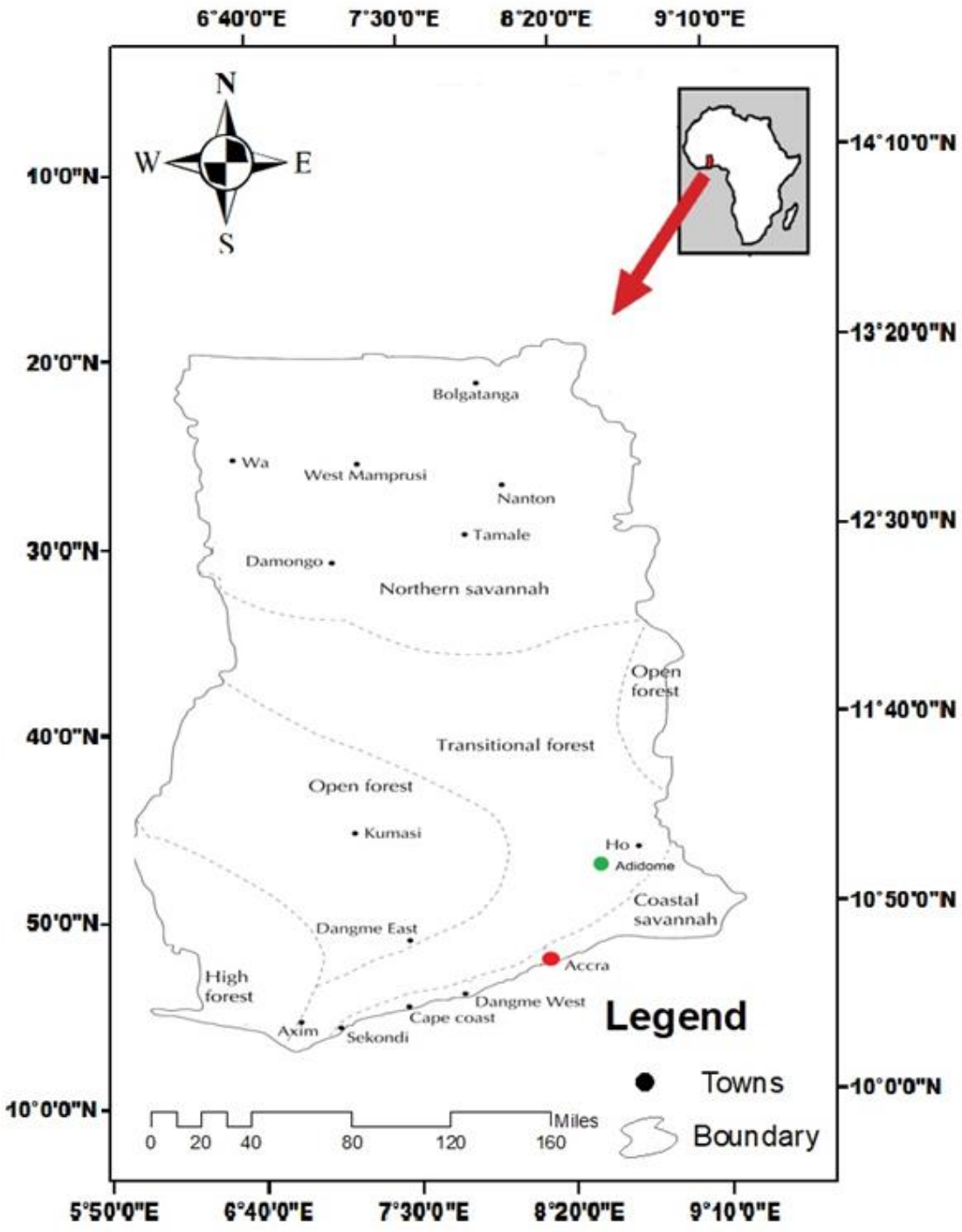


Figure 8: Map of Ghana showing sampling sites. The University of Ghana Livestock and Poultry Research Farm in Accra, Greater Accra region (🔴). A privately owned cattle ranch in Adidome, Volta region (🟢). Adapted from **Mahama *et al.*, 2003** with modifications

Table 3: Characteristics of selected cattle at Accra and Adidome study sites

Selected cattle ID	Sex	Breed	Age (months)
		Accra	
UG-160	Bull	Sanga	7
UG-184	Heifer	WASH	6
UG-153	Bull	WASH	7
UG-159	Bull	Sanga	7
UG-182	Heifer	Sanga Cross	6
UG-161	Heifer	Sanga	7
UG-169	Heifer	Sanga Cross	7
UG-179	Heifer	WASH	6
UG-166	Heifer	Sanga Cross	6
UG-164	Heifer	Sanga Cross	6
UG-178	Bull	WASH	6
UG- 158	Bull	Sanga	7
UG-165	Heifer	Sanga Cross	7
UG-185	Heifer	WASH	6
UG-156	Bull	WASH	6
UG-181	Heifer	Sanga Cross	6
UG-177	Bull	Sanga Cross	6
UG-168	Bull	Sanga Cross	6
UG-176	Heifer	Sanga Cross	6
UG-155	Bull	WASH	7
UG-1555	Bull	Sanga	3
UG-1545	Bull	Sanga Cross	8
		Adidome	
AD-7741	Bull	Sanga	4
AD-7742	Heifer	Sanga	3
AD-7743	Heifer	Sanga	4
AD-7744	Bull	Sanga	4
AD-7745	Bull	Sanga	4
AD-7746	Bull	Sanga	3
AD-7747	Heifer	Sanga	4
AD-7748	Heifer	Sanga	4
AD-7749	Bull	Sanga	3
AD-7751	Bull	Sanga	3
AD-7752	Bull	Sanga	4
AD-7753	Bull	Sanga	2
AD-7754	Heifer	Sanga	3
AD-7755	Heifer	Sanga	4
AD-7756	Heifer	Sanga	2
AD-7757	Heifer	Sanga	4
AD-7758	Heifer	Sanga	3
AD-7759	Heifer	Sanga	3
AD-7782	Heifer	Sanga	4
AD-7783	Bull	Sanga	4
AD-3780	Heifer	Sanga	6
AD-3737	Bull	Sanga	7
AD-7781	Heifer	Sanga	8

*UG and AD prefix followed by a number represents unique tags placed on individual cattle selected.

Figure 9: Selected experimental cattle from Accra and Adidome study sites. (June 10, 2015) **Photocredit: Mr. Samuel Adadey**

3.4 Isolation of DNA from cattle whole blood

The DNA was extracted from the cattle blood using Qiagen QIAmp DNA blood maxi kit (spin protocol) (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. In brief, 5 ml of cattle whole blood was transferred into 50 ml centrifuge tubes and 500 µl of QIAGEN protease added and the contents of the tubes mixed. The lysis buffer (AL (6 ml)) was added and the contents mixed by inversion for about 15 times followed by vigorous shaking for about 1 minute. The contents were then incubated at 70ºC for 10 minutes after which, 5 ml of ethanol was added and then mixed by inverting the tubes about 10 times, followed by vigorous shaking for about 1 minute. The mixtures were then transferred onto a QIAamp Maxi column in 50 ml centrifuge tubes and centrifuged at 4000 rpm for 3 minutes with Eppendorf 5804 centrifuge (Hamburg, Germany). The QIAamp Maxi column filtrates were discarded and the columns washed by adding 5 ml of buffer AW1 and spun at 5000 rpm for 1 minute and the filtrate from the wash discarded. A second washing was done using 5 ml of buffer AW2 and centrifuged at 5000 rpm for 15 minutes. After washing, the QIAamp Maxi columns were transferred into clean 50 ml centrifuge tubes. The DNA was eluted from the washed column with 1 ml of elution buffer (AE) by

applying it directly onto the membrane of the QIAamp Maxi columns, equilibrated at room temperature for 5 minutes and centrifuged at 5000 rpm for 2 minutes. For highly concentrated DNA, the flow through was reloaded onto the QIAamp Maxi columns, incubated at room temperature for 5 minutes and then centrifuged at 5000 rpm for 5 minutes. Aliquots (200 µl) of eluted DNA from each tube were stored in 1.5 ml Eppendorff tubes and kept at -20ºC until ready for use.

3.5 Serum preparation

Blood samples in plain S-monovette tubes (without EDTA) were allowed to clot for 3 hours and serum separated by centrifugation at 3000 rpm for 10 minutes. The sera were aliquoted into four 1.5 ml Eppendorf tubes for each sample and stored at -80°C for later use.

3.6 PCR primer design

In-house-tagged nested multiplex primers were designed from available databases to enable the identification of already existing and new trypanosomes species using nested PCR (Tables 4, 5, 6, and 7). All of the designed primers bind to part of the tubulin gene cluster (Figure 10). These primers are multiplexed, more sensitive, more specific, less expensive as compared to other published primers (Cox *et al.,* 2010; Thumbi *et al.,* 2008; Cox *et al.,* 2005) for trypanosome identification.

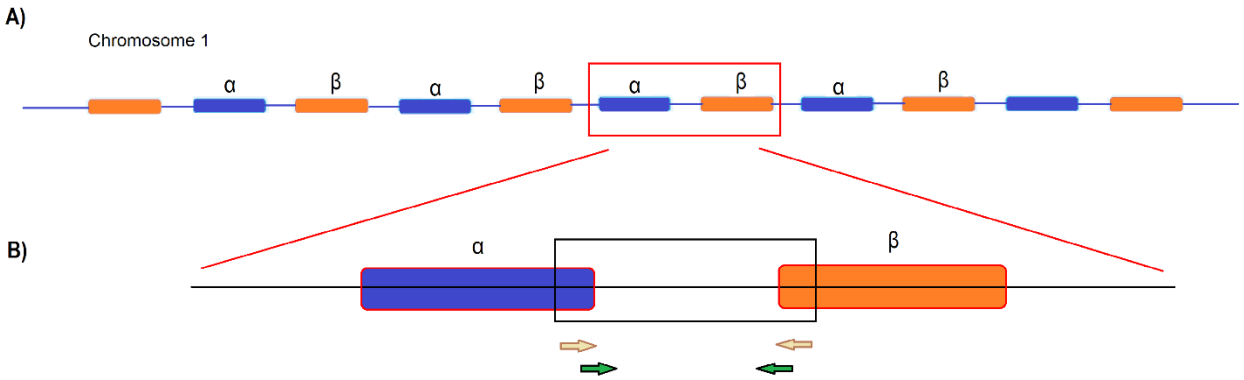


Figure 10: A representation of the tubulin gene cluster in T. brucei. A) *Trypanosoma brucei* tubulin genes are arranged in a tandem repeat of alternating alpha and beta genes and there are ~20 repeats in the genome. An alpha beta repeat is 3800 base pairs. B) Region on the tubulin gene where primers amplify for nested PCR. The primer sequences amplify the alpha to beta intergenic region from any of the trypanosome species likely to be present in cattle whole blood DNA.

Table 4: Sequences of outer primers designed for first round PCR

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

Table 5: Sequences of inner forward primers designed for nested PCR for Adidome cattle

Primer	Primer sequence (5' to 3')
	Inner forward
AD 37	TCGA CTGATCACYTCCCAGAACTT
AD 41	TCAG CTGATCACYTCCCAGAACTT
AD 42	TCGG CTGATCACYTCCCAGAACTT
AD 43	TCAA CTGATCACYTCCCAGAACTT
AD 44	CTGA CTGATCACYTCCCAGAACTT
AD 45	CTAG CTGATCACYTCCCAGAACTT
AD 46	CTGG CTGATCACYTCCCAGAACTT
AD 47	CTAA CTGATCACYTCCCAGAACTT
AD 48	TTGA CTGATCACYTCCCAGAACTT
AD 49	TTAG CTGATCACYTCCCAGAACTT
AD 51	TTGG CTGATCACYTCCCAGAACTT
AD 52	TTAA CTGATCACYTCCCAGAACTT
AD 53	CCGA CTGATCACYTCCCAGAACTT
AD 54	CCAG CTGATCACYTCCCAGAACTT
AD 55	CCGG CTGATCACYTCCCAGAACTT
AD 56	CCAA CTGATCACYTCCCAGAACTT
AD 57	GATC CTGATCACYTCCCAGAACTT
AD 58	GACT CTGATCACYTCCCAGAACTT
AD 59	GATT CTGATCACYTCCCAGAACTT
AD 80	GACC CTGATCACYTCCCAGAACTT
AD 81	AGTC CTGATCACYTCCCAGAACTT
AD 82	AGCT CTGATCACYTCCCAGAACTT
AD 83	AGTT CTGATCACYTCCCAGAACTT

*AD prefix and numbers represent cattle identification number for Adidome study site.

Table 6: Sequences of inner forward primers designed for nested PCR for Accra cattle

Primer	Primer sequence (5' to 3')
Inner forward	
UG 160/UG 1555	AGCC CTGATCACYTCCCAGAACTT
UG 184	AATC CTGATCACYTCCCAGAACTT
UG 153	AACT CTGATCACYTCCCAGAACTT
UG 159	AATT CTGATCACYTCCCAGAACTT
UG 182	AACC CTGATCACYTCCCAGAACTT
UG 161	GGTC CTGATCACYTCCCAGAACTT
UG 169	GGCT CTGATCACYTCCCAGAACTT
UG 179	GGTT CTGATCACYTCCCAGAACTT
UG 166	GGCC CTGATCACYTCCCAGAACTT
UG 164	GCAT CTGATCACYTCCCAGAACTT
UG 178	GCTA CTGATCACYTCCCAGAACTT
UG 158/UG 1545	CGAT CTGATCACYTCCCAGAACTT
UG 165	CGTA CTGATCACYTCCCAGAACTT
UG 185	TAGC CTGATCACYTCCCAGAACTT
UG 156	TACG CTGATCACYTCCCAGAACTT
UG 181	CATG CTGATCACYTCCCAGAACTT
UG 177	CAGT CTGATCACYTCCCAGAACTT
UG 168	GTAC CTGATCACYTCCCAGAACTT
UG 176	GTCA CTGATCACYTCCCAGAACTT
UG 155	TGAC CTGATCACYTCCCAGAACTT

*UG prefix and numbers represent cattle identification number for Accra study site.

Table 7: Sequences of inner reverse primers designed for nested PCR for Adidome and Accra cattle

Primer	Primer sequence (5' to 3')
	Inner Reverse
time point 1	TCGA GAGGAGGAYGTKGGAGGAGTACTA
time point 2	TCAG GAGGAGGAYGTKGGAGGAGTACTA
time point 3	TCGG GAGGAGGAYGTKGGAGGAGTACTA
time point 4	TCAA GAGGAGGAYGTKGGAGGAGTACTA
time point 5	CTGA GAGGAGGAYGTKGGAGGAGTACTA
time point 6	CTAG GAGGAGGAYGTKGGAGGAGTACTA
time point 7	CTGG GAGGAGGAYGTKGGAGGAGTACTA
time point 8	CTAA GAGGAGGAYGTKGGAGGAGTACTA
time point 9	TTGA GAGGAGGAYGTKGGAGGAGTACTA
time point 10	TTAG GAGGAGGAYGTKGGAGGAGTACTA
time point 11	TTGG GAGGAGGAYGTKGGAGGAGTACTA
time point 12	TTAA GAGGAGGAYGTKGGAGGAGTACTA
time point 13	CCGA GAGGAGGAYGTKGGAGGAGTACTA
time point 14	CCAG GAGGAGGAYGTKGGAGGAGTACTA
time point 15	CCGG GAGGAGGAYGTKGGAGGAGTACTA
time point 16	CCAA GAGGAGGAYGTKGGAGGAGTACTA
time point 17	GATC GAGGAGGAYGTKGGAGGAGTACTA
time point 18	GACT GAGGAGGAYGTKGGAGGAGTACTA

*time points 1 to 18 represents time points (4-5 weeks intervals) for sample collection.

3.7 Identification of trypanosome species using nested PCR

A species diagnostic multiplex nested PCR was performed by amplifying part of the tubulin gene. A PCR reaction mixture of 30 µl total volume was used. The initial PCR screening was performed by adding 1 µl of unpurified cattle whole blood DNA to a master reaction mixture of 29 µl. The master reaction mixture was made up of 1X Mango *Taq* buffer with 2.5 mM $MgCl_2$, 0.3 µM outer primers (forward and reverse, Table 4), 0.3 mM dNTPs, and 2.5 U Mango *Taq* polymerase (Bioline Reagents Ltd, London, UK). The thermal cycling condition was done using an initial denaturation step of 94°C for 5 minutes, followed by 30 cycles plus 3 seconds per cycle of 94°C for 45 seconds, 61°C for 45 seconds, 72°C for 45 seconds, and a final extension of 72°C for 5 minutes and cooling at 4°C. The second round of PCR (nested step) was done using a reaction mixture of 50 µl made up of 1 µl of the first PCR product and 49 µl of the master reaction mix. The components of second reaction mixture was the same as the first except that the outer primers for the tubulin alpha-beta intergenic region were replaced with inner primers (Tables 5, 6 and 7). The thermal cycling condition used was the same as that for the initial PCR. The PCR product (10 µl) obtained after amplification was then run on 1.5% agarose gel at 100 V for 1 hour. The gels were stained with ethidium bromide and visualised by ultravoilet (UV) light using GE Healthcare Amersham Image600 gel dock (Little Chalfont, UK) and trypanosome identification was based on the length of the PCR product. Further identification and characterization was done by nucleotide sequence of the amplicons using high throughput illumina sequencing.

3.8 NGS library preparation and targeted amplicon sequencing

Amplicons generated from a multiplex tagged *Trypanosoma* tubulin nested PCR were pooled together for next generation sequencing. The inner primers (nested primers) for the PCR amplification contained MiSeq adapter sequences at the 5′ end for the sequencing library preparation. Each sample was uniquely indexed with Illumina Nextera XT indices (Illumina, San Diego, USA), and the final sequencing library was pooled and prepared using the Nextera XT DNA Sample Preparation Kit according to the instructions from the manufacturer. The library was sequenced on an Illumina MiSeq using a 500-cycle kit V2 kit (Illumina, San Diego, USA) according to the manufacturer's instructions to produce millions of individual sequences (300 bp paired-end reads) in FASTQ files format. All sequencing was carried out at the sequencing facility at the Department of Biochemistry, University of Cambridge.

3.9 Analyses of NGS data

During generation of PCR amplicon for sequencing, each cow was tagged with a four unique nucleotide sequences and amplicons for each time point (18-time points) consisting of all the cows were pooled for sequencing using Illumina paired end sequencing. In-house python scripts were used to de-multiplex each time into individual cows based on their unique four nucleotide bases. Quality control (QC) was done using the FastQC which provided a QC report for each sample for quality assurance. Paired-end reads were then mapped to different Trypanosoma reference genomes using Bowtie2 using default settings (Li and Durbin, 2009). The following genomes; *T. brucei*, *T. congolense* and *T. vivax* were obtained from the TriTrypDB (www.tritrypdb.org) (Figure 11). *T. theileri* genome is not

in the TriTrypDB, therefore for *T. theileri*, the sequence reads were mapped to the transcriptome (Kelly *et al.,* 2017). The read mapping was done in order to find locations of the newly sequenced reads with respect to the reference genomes and identify the differences in the DNA sequences and quantify the mapped reads for different species. The quality of the sequence alignment mapping (SAM) files was assessed using SAMtools flagstat to examine the mapping statistics. The mapped read counts were then obtained from the SAM files using SAMtools (Li *et al.,* 2009) (Figure 11). The percentage mapped read were then estimated in Microsoft excel package. The percentage mapped reads were organized and edited by eliminating artefacts reads generated such as PCR products of some individual cattle which were not included for the illumina sequencing and time points without replacement of dead animals. Reorganized percentages mapped reads were then used to generate stacked bar charts using GraphPad® Prism version 6 (GraphPad® Sofware Inc., California, USA) (Figure 11). Trypanosome species with the highest mapped read in proportion to the total mapped read within individual cow represented the most predominant species (Appendices 1 and 2).

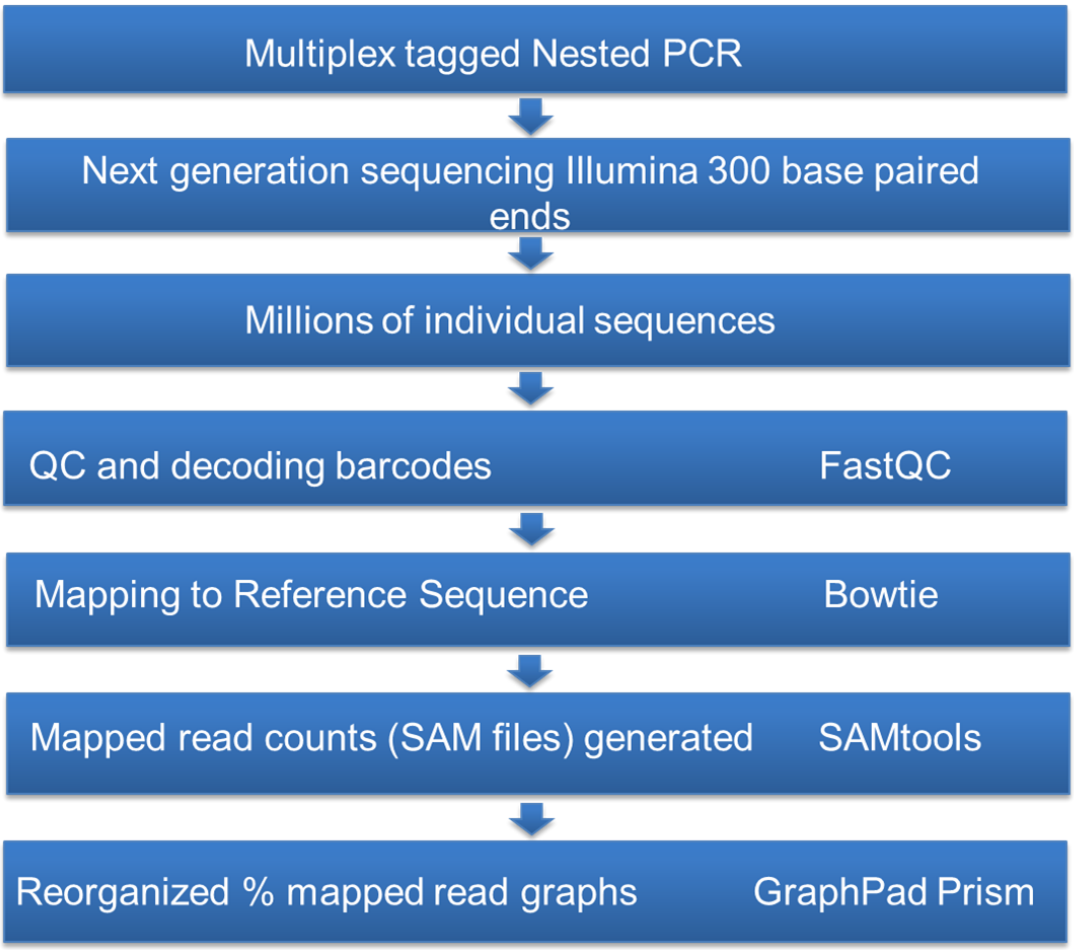


Figure 11: Workflow for illumina sequencing and analysis. Multiplex tagged nested PCR and a high throughput sequencing (illumina sequencing) was performed to characterize trypanosomes circulating in cattle in Ghana. The fastq files generated after sequencing were trimmed and quality control done using FastQC. The reads were mapped to trypanosome reference genomes using Bowtie and mapped read counts generated using SAMtools. Percentage mapped reads were estimated and reorganized in Microsoft excel. Stacked bar graphs were generated with the reorganized percentage mapped counts using GraphPad® Prism.

3.10 Preparation of trypanosome lysate

All proteomic studies were done using *in vitro* cultivated *T. b. brucei*, strain GUTat 3.1. Bloodstream-form trypanosomes (1 x 10^8 cells) were centrifuged 13 000 rpm for 10 minutes at 4°C and the supernatant (media) discarded. Parasite were lysed in

immunoprecipitation (IP) lysis/wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4) in the presence of a protease inhibitor cocktail (complete Mini, EDTA-free; Roche Diagnostics, GmbH, Mannheim, Germany), mixed thoroughly to homogeneity and stored at -80°C overnight for subsequent processing. The lysis buffer aided in preparation of the total proteome of the parasite including the membrane proteins. The sample was then thawed and centrifuged at 13000 rpm for 10 minutes at 4°C and the supernatant (400 ul) transferred in 200 µl aliquots each into two 1.5 ml Eppendorf tubes for determination of protein concentration and other analysis.

3.11 Bicinchoninic acid (BCA) protein assay

Protein concentration was determined using the Thermo scientific 23225 Pierce BCA protein assay kit (Rockford, USA) according to manufacturer's protocol. Serially diluted bovine serum albumin (BSA) standards were prepared over a range of 25-2000 µg/ml. The total volume of working reagent (WR) required for the BCA assay was determined using the formula below:

(# standards + # unknowns) x (# replicates) x (volume of WR per sample)

The WR was prepared by mixing BCA reagent A with BCA reagent B in a 50:1 ratio. The microplate procedure was employed, where 200 µl of WR was required for each sample so the sample to WR ratio was 1:8. The unknown samples (*T. brucei* protein lysate) were diluted in ratios of 1:10, 1:50 and 1:100 and 25 µl of each pipetted into microplate wells in triplicates. The standards were also pipetted (25 µl) into the microplate wells in duplicates and 200 µl of WR added to each well and mixed thoroughly on an Eppendorf thermomixer

C (Hamburg, Germany) for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. After the incubation period, the mixture in the microplate was allowed to cool and the absorbance measured at 562 nm using a Thermo Scientific Varioskan Lux plate reader (Rockford, USA). A standard curve was plotted for each blank-corrected absorbance for each BSA standard and its concentration (µg/ml) and used to determine the protein concentration of each unknown sample.

3.12 Determination of antibody responses of trypanosome-infected cattle sera to whole *T. brucei* lysate

Antibody levels in trypanosome-infected cattle sera to whole *T. brucei* lysates (crude antigen) were determined using indirect Enzyme Linked Immunosorbent Assay (ELISA).

Nunc polystyrene 96-well plates were coated with 100 µl per well of crude *T. brucei* lysate at a concentration of 5 µg/ml in coating buffer (1X PBS, pH 7.2) and incubated overnight at 4°C. The plates were washed thrice with 100 µl per well of Phosphate buffered saline, 0.01% Tween-20 (PBS-T) using a Biotek ELISA plate washer (Winooski, Vermont, US) and plates blotted on tissue paper after washing. Blocking buffer (5% BSA in PBS) was added to the wells (100 µl/ well) and the plates incubated for 1 hour at room temperature on a Stuart mini gyro-rocker (London, UK). Three-fold serial dilutions of bovine pooled sera standard (1:20 dilution) was added in the first two columns and the last wells in the columns left as reagent blank wells. Serum samples from both study sites, diluted 1:100 in 1% BSA in PBS, was added to the wells in duplicate (100 µl/well) and incubated for 2 hours at room temperature. The plates were then washed as described earlier. The detection

antibody, rabbit anti-bovine IgG (whole molecule) peroxidase conjugate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was diluted in 1% BSA in PBS (1:5000), and added to the wells (100 µl/well) and incubated at room temperature for 1 hour. After incubation, the plates were washed three times with PBS-T as earlier. After washing, 3, 3', 5, 5'-Tetramethylbenzidine (TMB) plus substrate (Kem-En-Tec Diagnostics, Taastrup, Denmark) was added (100 µl/well) and plates incubated at room temperature for 10 minutes in the dark. The reaction was stopped by addition of 0.2 M H_2SO_4 and optical density was measured at 450 nm using a Biotek plate reader (Winooski, Vermont, US). Bovine serum (uninfected serum) from a 2-week old calf was used as negative control and antibody against alpha-paraflagellar rod was used as positive control. All samples were run in duplicate and two independent ELISA runs were performed. Antibody titres were extrapolated from calibration curves constructed for bovine sera pool standards using the measured optical densities (ODs) in an Auditable Data Analysis and Management System for ELISA (ADAMSEL, Ed Remarque®). The cut off for seropositivity was calculated as the mean titre of the negative control serum plus 3 times the standard deviation [mean (control) + 3xSD].

3.13 IgG purification from Adidome cattle sera pool

The IgG purification was performed using the HiTrap protein G HP column following the manufacturer's (GE Healthcare, Little Chalfont, UK) protocol with modifications. Only cattle that showed antibody response was further analyzed.

Cattle sera from Adidome which showed positive antibody responses by ELISA were pooled (50 ml) and diluted 10-fold using 10 mM Tris pH 7.2. The diluted sera were applied

to a 1 ml HiTrap protein G HP column (GE Healthcare, Little Chalfont, UK) equilibrated in 10 mM Tris pH 7.2. The GE Healthcare AKTA pure protein purification system (Little Chalfont, UK) was used for the purification. The bound IgG antibodies were washed with 20 column volumes of 10 mM Tris pH 7.2 and eluted with 100 mM glycine-HCl buffer, pH 2.5. Buffer (1M Tris), pH 8.0 was introduced into eluate collection tubes to neutralize the elution buffer The eluates were collected in 3 ml fractions, aliquoted (500 µl) into 1.5 ml Eppendorf tubes and kept at -20°C for further analysis. Immunoglobulin G was also purified from commercial fetal calf serum and used as a control since it was not possible to obtain naïve cattle or cattle sera that are uninfected with trypanosomes from the sample sites. The eluates were run on 12% SDS-PAGE gel under non-reducing and reducing conditions. The antibody concentration was determined by BCA method as described earlier.

3.14 SDS-PAGE and western blotting

Crude trypanosome lysate (3.9 x 10^6 cells) was loaded and separated by a 12% one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 2½ hours at 100 volts. The gels were then transferred onto Millipore PVDF membrane, 0.45 µm (Bellerica, USA) for 1½ hours at 180 mA. Further processes for Western blotting were followed as described by Mendoza-Palomares *et al.*, (2008) with modifications.

Blots were blocked by soaking in 1.5% BSA in PBS overnight at 4°C and washed once with 0.05% PBS-T for 30 minutes after incubation. The blots were then washed 3 times with 1X PBS pH 7.4 at 20 minutes intervals with gentle shaking. Dilutions of primary antibodies [crude cattle sera from Adidome positive sample pool (1:1000); total IgG

purified from Adidome positive sample pool (1:2500); commercial fetal calf serum, non-heat inactivated (1:1000); negative control, naive calf sera (1:1000) and positive control, anti-PFR (anti-paraflagellar rod antibodies, 1:1000)] in 1.5% BSA in PBS were then added to each blot and incubated for 2 hours with gentle shaking. The blot was washed once with 0.05% PBS-T for 20 minutes and three times with 1X PBS pH 7.4 at 10 minutes intervals. The secondary antibody, rabbit anti-bovine IgG (whole molecule) peroxidase conjugate (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) diluted (1:10000) in 1.5% BSA in PBS was then added to each blots and incubated for 1 hour with gentle shaking. After an hour incubation, antigen-antibody interactions were revealed with the Millipore Immobilon Western chemiluminescence horseradish peroxidase substrate (Millipore Corporation, Billerica, USA) and imaged using the GE Healthcare Amersham Image600 gel dock (Little Chalfont, UK).

3.15 Immunoprecipitation of *T. brucei* protein

Parasites were lysed in IP lysis/wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4) and incubated on ice for 1 hour, vortexing every 10 minutes. The samples were centrifuged at a speed of 13000 rpm for 30 minutes at 4°C and the supernatant was transferred into a new tube. *T. brucei* native proteins from the crude lysate were enriched by anion exchange chromatography. The eluates were collected in fractions of 8 ml. The immunoprecipitation procedures were done following protocol by Hastings, (2012) with slight modifications. To reduce background recognition and non-specific binding in the eluates, they were pre-cleared with 150 µl of Pierce Protein A/G Agarose resin (Thermo scientific, Rockford, USA) for 1 hour, rotating at 4°C and

centrifuged at 13000 rpm at 4°C. The supernatant was collected and split into 2 tubes; to the first 50 µl of purified IgG antibody from the Adidome sera pool was added and to the second 50 µl of non-heat inactivated FCS. The incubation lasted overnight, rotating at 4°C. To pull-down the antibody/protein complexes, 50 µl of Pierce Protein A/G Agarose resin (Thermo scientific, Rockford, USA) was added and rotated at 4°C for 1 hour. The samples were centrifuged at 13000 rpm for 2 minutes at 4°C. Supernatant was discarded and the pellet washed 6 times with IP lysis/wash buffer and eluted with IP elution buffer pH 2.8 but 1M Tris buffer, pH 8.0 was introduced to neutralize the elution buffer. The eluting complex was subjected to SDS-PAGE separation for mass spectrometry (MS) analysis.

3.16 SDS-PAGE analysis of immunoprecipitation eluates

Eluates were resolved by electrphoresis on a 12% sodium dodecyl sulfate polyacylamide gel after ethanol precipitated. The immunoprecipitation eluates for running on the SDS-PAGE were prepared by adding 4X sample buffer (1M Tris-HCl pH 6.8, SDS, 0.1% bromophenol blue, 100% glycerol, 14.3 M β-mercaptoethanol, double distilled H_2O) to make a final solution of 1X and heated at 100ºC for 7 minutes. The samples were allowed to cool at room temperature before applying to the gel. The SDS-PAGE was run at 100 V for 2 hours and stained with Coomassie brilliant blue R-250 and detained with destaining solution (40% methanol, 10% glacial acetic acid and 50% distilled water).

3.17 Protein (antigen) identification by mass spectrometry

The eluates from the immunoprecitation for the IgG purified from the Adidome cattle pool and the commercial fetal calf serum (control) against the *T. brucei* antigens were prepared

for sequencing by the liquid chromatography coupled with tandem-mass spectrometry (LC MS/MS) to identify immunoprecipitated proteins. Liquid chromatography tandem mass spectrometry (LCMS/MS) was performed by the Proteomic Facility at the University of Dundee. The eluates obtained from immunoprecipitation were subjected to LC-MS/MS on an UltiMate 3000 RSLCnano System (Thermo Scientific) coupled to an LTQ OrbiTrap velos Pro (Thermo Scientific) (De Pablos *et al.*, 2017). The mass spectra were analyzed using Maxquant-Dev v. 1.6.1.0 (Cox and Mann, 2008) searching the *T. brucei* TREU 927 annotated protein database and Lister 427 together with the *B. taurus* proteome from TriTrypDB (http://tritrypdb.org/tritrypdb/). Minimum peptide length was set at six amino acids and false discovery rate (FDR) was set at 0.01% for peptides and proteins. The "match between runs" (MBR) option was allowed with a match time window of 0.7 minutes and an alignment time window of 20 minutes. Label-free quantification (LFQ) was done using both unique and razor peptides for each protein. At least 2 such peptides were required for LFQ. Bioinformatic analysis of the MaxQuant workflow output and the analysis of the abundance of the identified proteins were performed.

3.18 Data and statistical analyses

Statistical analyses were done for ELISA data using GraphPad® Prism Software version 6.0 (GraphPad® Sofware Inc., California, USA) and R software version 3.4.3. The data obtained for the various groups compared in the study were largely non-parametric. The antibody response dynamics in individual cattle across the time points were statistically compared by the Kruskal Wallis test and subsequently by Bonferroni post-hoc tests where appropriate. The effects of geographical location and sex on antibody response were

assessed using Mann Whitney test. Proportion of male to female sample size was tested by Chi-square tests. The effects of cattle breed and age on antibody responses were assessed using Kruskal Wallis rank sum test with Bonferroni post-hoc test were necessary. Differences between groups were considered statistically significant at $p < 0.05$. Multivariate regression analysis was done using SPSS® software version 20 to determine the contribution of different variables to antibody response levels at Accra. The seroprevalence was determined by calculating the period prevalence; the percentage number of cattle that gave antibody response in a given period divided by the number of cattle in the population during that period.

CHAPTER FOUR

RESULTS

4.1 Molecular genotypes of trypanosomes in cattle over 2 years

To determine the molecular genotype of trypanosome species circulating in cattle herds at the two different study sites longitudinally, DNA extracted from blood samples collected from all the cattle were analyzed by nested PCR targeting the tubulin gene of trypanosomes. Trypanosomes were then identified by next generation sequencing (Illumina). In some of the cattle from both study sites, infection were not detected by nested PCR but were subsequently detected by illumina sequencing (Appendices 1 and 2).

At Adidome, the animals were predominantly infected with *T. brucei* and *T. vivax* with *T. vivax* being the highest with infection frequency of 237 out of 282 (84%) samples collected (Tables 8 and 9). Also, *T. vivax* was higher at Accra with infection frequency of 248 out of 353 (70.3%) samples collected (Tables 8 and 9) although the circulating species were *T. vivax* and *T. theileri. T. congolense* infection was relatively higher at Adidome compared to Accra [infection frequency of 7/282 (2.5%; Adidome) versus 2/353 (0.6%; Accra)] (Tables 8 and 9).

Table 8: Incidence of predominant trypanosome species identified by next generation sequencing of PCR products

	Adidome					Accra				
Time point	**Number of cattle infected**					**Number of cattle infected**				
	T. brucei	*T. congolense*	*T. vivax*	*T. theileri*	Number of sample tested	*T. brucei*	*T. congolense*	*T. vivax*	*T. theileri*	Number of sample tested
1	2	0	14	0	16	2	0	13	5	20
2	6	1	9	0	16	3	0	15	2	20
3	1	0	11	4	16	2	0	14	4	20
4	3	0	13	0	16	4	0	14	3	20
5	1	0	15	0	16	1	0	14	4	19
6	2	0	14	0	16	1	0	15	3	19
7	0	1	12	3	16	3	0	11	5	19
8	0	0	15	0	15	2	0	13	5	20
9	1	1	12	0	14	0	0	18	2	20
10	1	2	12	0	15	3	1	12	4	20
11	2	0	13	1	16	1	1	14	4	20
12	0	1	15	0	16	3	0	15	2	20
13	1	0	14	0	15	0	0	14	6	20
14	1	0	14	1	16	2	0	11	7	20
15	1	0	13	1	16	0	0	12	8	20
16	2	0	13	1	16	0	0	16	3	19
17	1	1	14	0	16	1	0	16	2	19
18	1	0	14	0	15	0	0	13	5	18

Time points were ~4 to 5 weeks intervals for about two years

Table 9: Incidence of infection of predominant trypanosome species identified over the study period

	Adidome		Accra	
Trypanosome	**Sample tested**	**Incidence of infection**	**Sample tested**	**Incidence of infection**
T. brucei	282	26 (9.2%)	353	29 (8.2%)
T. congolense	282	7 (2.5%)	353	2 (0.6%)
T. vivax	282	237 (84%)	353	248 (70.3%)
T. theileri	282	12 (4.3%)	353	74 (21%)

4.2 Dynamics of infection with trypanosomes over 2 years

To determine the dynamics of infection of trypanosomes over the 2 years in cattle herds at the two different study sites, the same individual animals were followed over the time of the study; however some animals died and were replaced at some time points. Results obtained from the study shows that individual cattle at both study sites were infected throughout the 18 time points (Tables 10, Appendices 1 and 2). There were also instances of mixed infections (double or triple bands) observed in cattle at both study sites at particular time points over the 2 years (Table 10, Appendices 1and 2). The incidence of lifetime infections with trypanosomes in individual cattle was higher at Adidome although the animals were treated with isometamidium hydrochloride at time points 3, 6 and 15 and diminazene aceturate at time points 6, 10, 13, 17 and 18 (Table 10 and Appendix 1). At Accra, incidence of lifetime infections with trypanosomes in individual cattle was also high but animals were not treated against trypanosomes (Table 10 and Appendix 2).

Table 10: Incidence of lifetime infections with trypanosomes in individual cattle

Cattle ID	**Adidome**					**Cattle ID**	**Accra**				
	T. brucei	*T. congolense*	*T. vivax*	*T. theileri*	Time point period		*T. brucei*	*T. congolense*	*T. vivax*	*T. theileri*	Time point period
AD-3737	1	0	2	2	5	UG-160	0	0	1	4	5
AD-7741	0	0	18 (1)*	0	18	UG-184	2	0	15	1	18
AD-7742	2	0	16	0	18	UG-153	3	0	8	5	16
AD-7743	2	1	15 (2)*	0	18	UG-159	0	0	14	3	17
AD-7744	0	0	8	1	9	UG-182	0	0	10	8 (1)*	18
AD-7745	3	4 (1)*	5	0	12	UG-161	2	0	10	5	17
AD-7746	0	0	17 (1)*	1	18	UG-169	0	0	15	3	18
AD-7747	2	0	14	2	18	UG-179	0	0	18	0	18
AD-7748	2	1	13	1	17	UG-166	0	0	6	12	18
AD-7749	1	1	12	2	16	UG-164	1	0	3	14 (2)*	18
AD-7751	1	0	16	1	18	UG-178	0	0	18 (1)*	0	18
AD-7752	1	0	7	0	8	UG-158	0	0	10 (1)*	0	10
AD-7753	1	0	16	0	17	UG-165	4	0	10	4	18
AD-7754	0	0	18	0	18	UG-185	3	0	14	1	18
AD-7755	0	0	18	0	18	UG-156	1	0	17	0	18
AD-7756	3	0	15	0	18	UG-181	0	0	18	0	18
AD-3780	3	0	5	0	8	UG-177	3	1	11	3 (1)*	18
AD-7781	1	0	9	0	10	UG-168	2	0	11	5	18
AD-7783	3	0	13	2	18	UG-176	3 (1)*	0	12	3 (1)*	18
						UG-155	4 (1)*	0	14	0	18
						UG-1555	1	1	9	0	11
						UG-1545	0	0	4	3	7

* Values in parentheses represent the number of mixed infections

4.3 Predominant infecting trypanosomes species based on age, sex and breed of cattle

In order to determine the effect of age, sex and breed of cattle on the predominantly infecting trypanosome species, the trypanosomes identified were compared between Adidome and Accra. Adidome recorded *T. vivax* as the most prevailing species within both young (aged <1 year) and adult (aged ≥1 year) cattle (Table 11). Similar observation was seen at Accra but with *T. theileri* existing as well (Table 11). Mixed infections were also observed at both study sites. *T. vivax* and *T. brucei* mixed infection was observed at Adidome and *T. vivax* and *T. theileri* mixed infection observed at Accra (Table 11). For the prevailing trypanosomes existing among males and females at both study sites, *T. vivax* was the highest for both sexes (Table 11). At Adidome, there was only one cattle breed type (Sanga) and all the animals were infected predominantly with *T. vivax* (Table 11). On the other hand, there were three different cattle breed types at Accra (Sanga, WASH and Sanga Cross). In all these breed types, the most predominant trypanosome species was *T. vivax* but with *T. theileri* and mixed infections existing (Table 11).

Table 11: Prevailing trypanosome species in relation to age, sex and breed of cattle

Factors		Adidome		Accra	
		Number of cattle examined	**Predominant trypanosome species identified**	**Number of cattle examined**	**Predominant trypanosome species identified**
Age	**< 1 year**	19	14 *T. vivax* 4 *T. vivax* + *T. brucei** 1 *T. vivax* + *T. theileri**	22	17 *T. vivax* 4 *T. theileri* 1 *T. vivax* + *T. theileri**
	≥ 1 year	18	16 *T. vivax* 1 *T. congolense* 1 *T. vivax* + *T. brucei**	22	17 *T. vivax* 3 *T. theileri* 2 *T. vivax* + *T. theileri**
Sex	**Male**	10	10 *T. vivax*	11	8 *T. vivax* 1 *T. theileri* 2 *T. vivax* + *T. theileri**
	Female	9	8 *T. vivax* 1 *T. vivax* + *T. brucei**	11	8 *T. vivax* 2 *T. theileri* 1 *T. vivax* + *T. theileri**
Breed	**Sanga**	19	19 *T. vivax*	5	4 *T. vivax* 1 *T. theileri*
	WASH	None		7	6 *T. vivax* 1 *T. vivax* + *T. theileri**
	Sanga Cross	None		10	6 *T. vivax* 2 *T. theileri* 2 *T. vivax* + *T. theileri**

The asterisk (*) represents mixed infections

4.4 Antibody response to *T. brucei* among individual cattle in two different regions of Ghana

In order to identify immunogenic trypanosome proteins, sera were collected from individual cattle at Adidome in the Volta region and Accra in the Greater Accra regions at intervals over one year (from time points 7 to 18). The sera were screened against *T. brucei* (GUTat 3.1 strain) total protein lysate by ELISA to determine their respective antibody response. *T. brucei* was the only parasite used due to inaccessibility to laboratory adapted *T. vivax, T. congolense* and *T. theileri*. During this study, there were several attempts to adapt the field isolates to *in vitro* culturing but were unsuccessful. There was high antibody response to *T. brucei*, GUTat 3.1 strain at Adidome (97% seroprevalence) throughout the time points of collection compared to Accra (seroprevalence of 73%) (Figures 12A-F and 13A-F). Most of the cows had antibody responses above the cut-off value (naïve sera) at Adidome (Figure 12A-F) whiles in Accra, majority were below the cut-off values (Figure 13A-F). Although the individual animals were periodically treated with trypanocidal drugs (isometamidium chloride and diminazene aceturate) at Adidome, this did not seem to impair the antibody response raised against *T. brucei* proteins (Figure 12A-F).

Due to differences in levels of previous exposure at Accra and Adidome (Figure 14), antibody levels were compared at each time point in reference to the initial sampling period. There was no significant difference in the antibody response observed for the cows at some time point in reference to the initial sampling period at Adidome (Figure 14B). However, there were significant differences observed in the antibody responses to *T. brucei* antigens for others (Figure 14B). Although, the antibody responses were relatively stable

from time points 3 to 12, time points 1 compared to 4, showed significant difference in the antibody response (p= 0.0088, Bonferroni post-hoc test) as well as the time point 5 compared to time point 1 (p= 0.0025, Bonferroni post-hoc test) (Figure 14B). Also, the anti-trypanosome antibody response significantly increased from the initial sampling period compared to time point 6 (p= 0.0198, Bonferroni post-hoc test) and time point 8 (p= 0.0131, Bonferroni post-hoc test) (Figure 14B). Again, there was a significant difference observed in the antibody response from time point 1 compared to time points 9 (p= 0.0085, Bonferroni post-hoc test) and 10 (p= 0.0078, Bonferroni post-hoc test) (Figure 14B). The antibody response to *T. brucei* proteins also significantly increased from the initial sampling period compared to time point 11 (p= 0.0000, Bonferroni post-hoc test) and time point 12 (p= 0.0166, Bonferroni post-hoc test (Figure 14B). Although the individual animals were periodically treated with trypanocidal drugs (isometamidium chloride and diminazene aceturate) at Adidome, this did not seem to impair the antibody response raised against *T. brucei* proteins (Figures 14B).

At the Accra study site, individual cattle's antibody responses against the *T. brucei* proteins across the time points were statistically not significant in reference to the initial sampling period (Figure 14A).

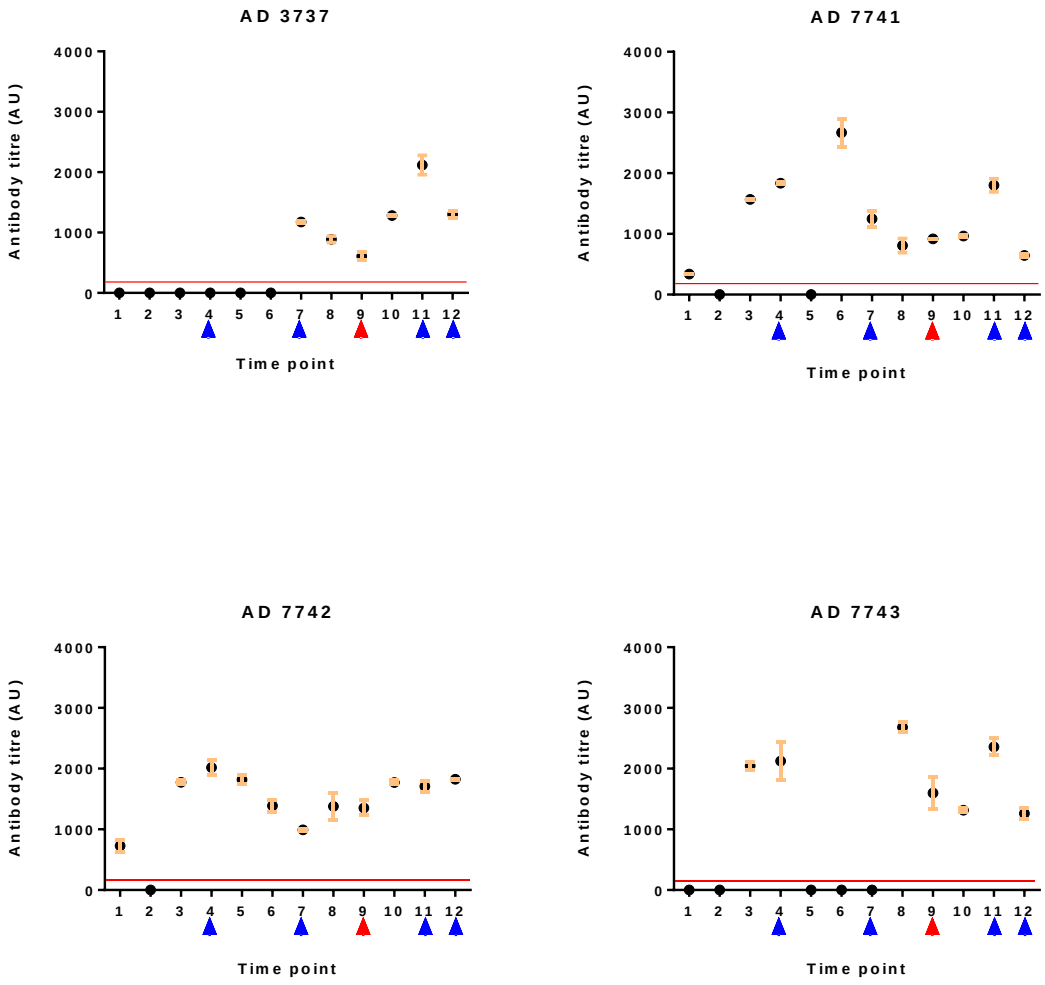


Figure 12A: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 3737, 7741, 7742 and 7743 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

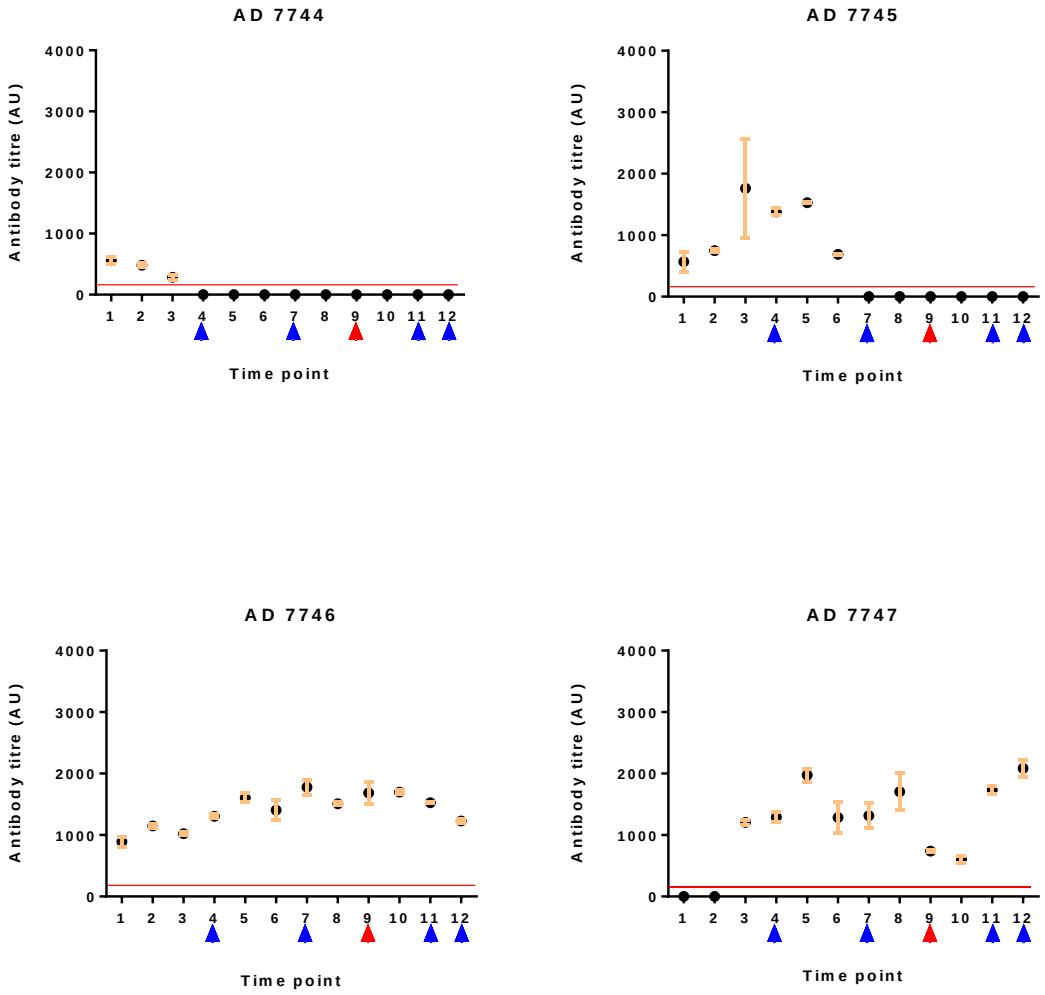


Figure 12B: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 7744, 7745, 7746 and 7747 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

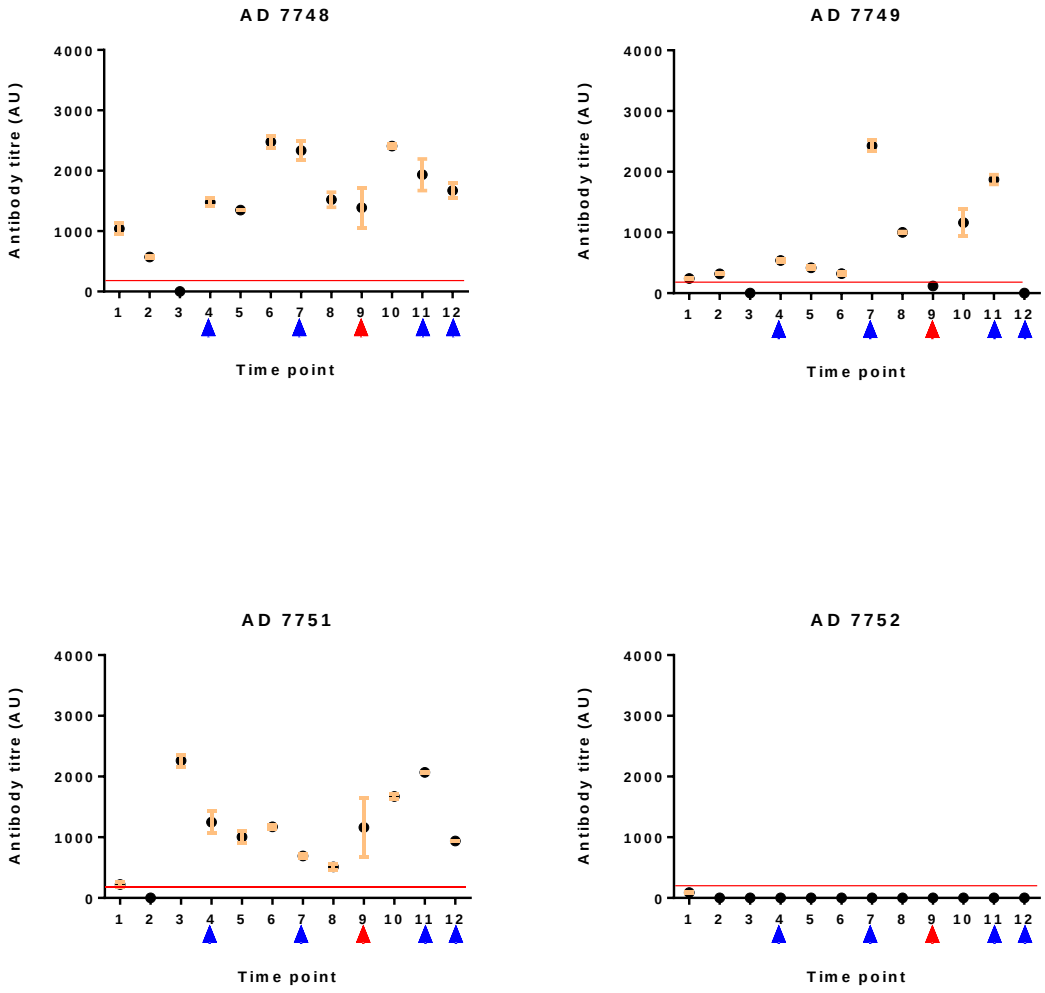


Figure 12C: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 7748, 7749, 7751 and 7752 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

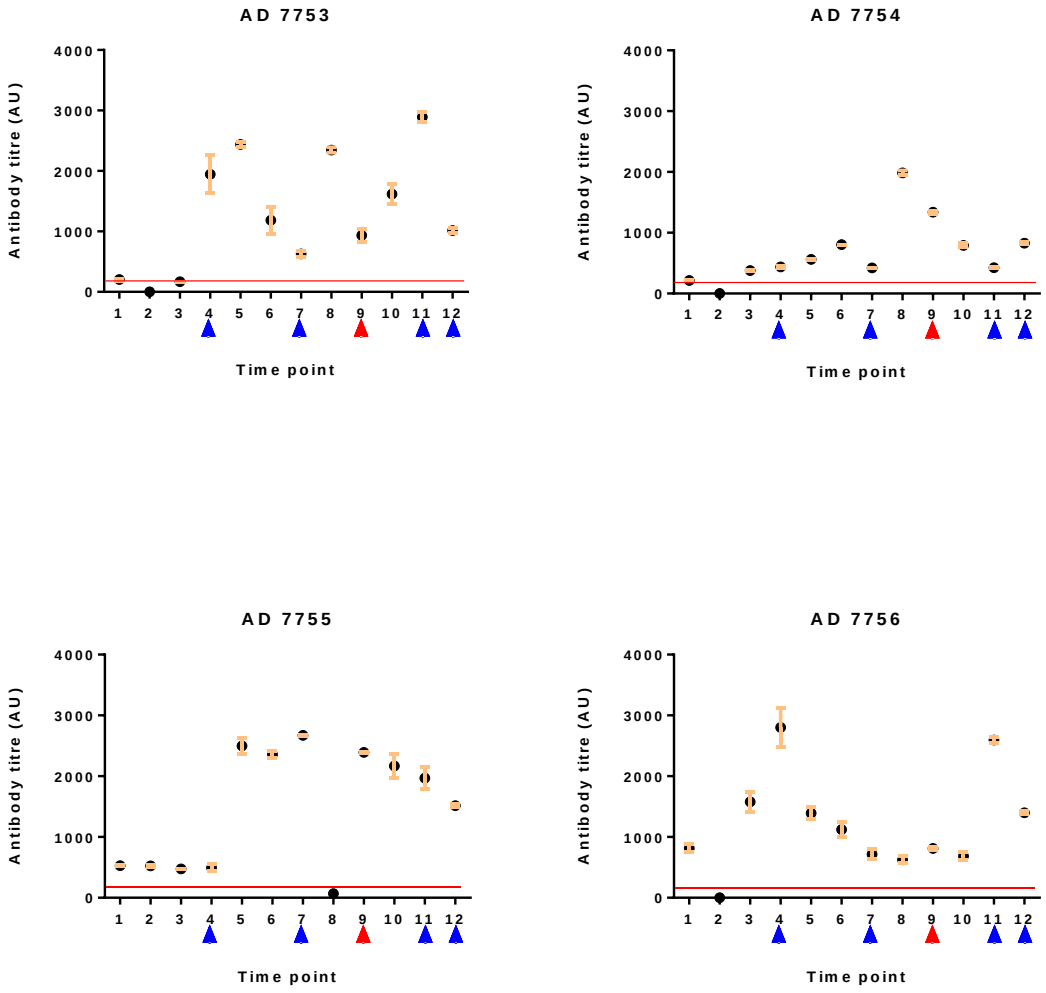


Figure 12D: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 7753, 7754, 7755 and 7756 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

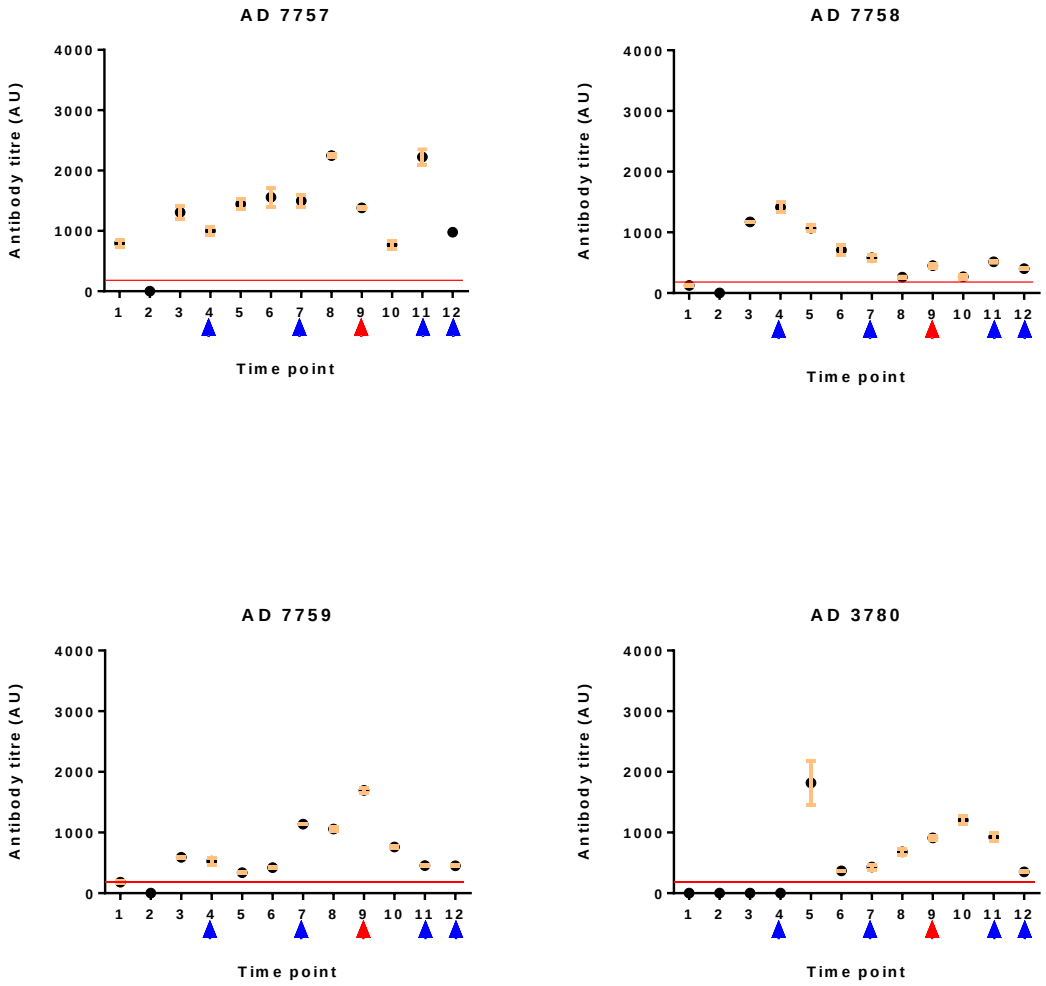


Figure 12E: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 7757, 7758, 7759 and 3780 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

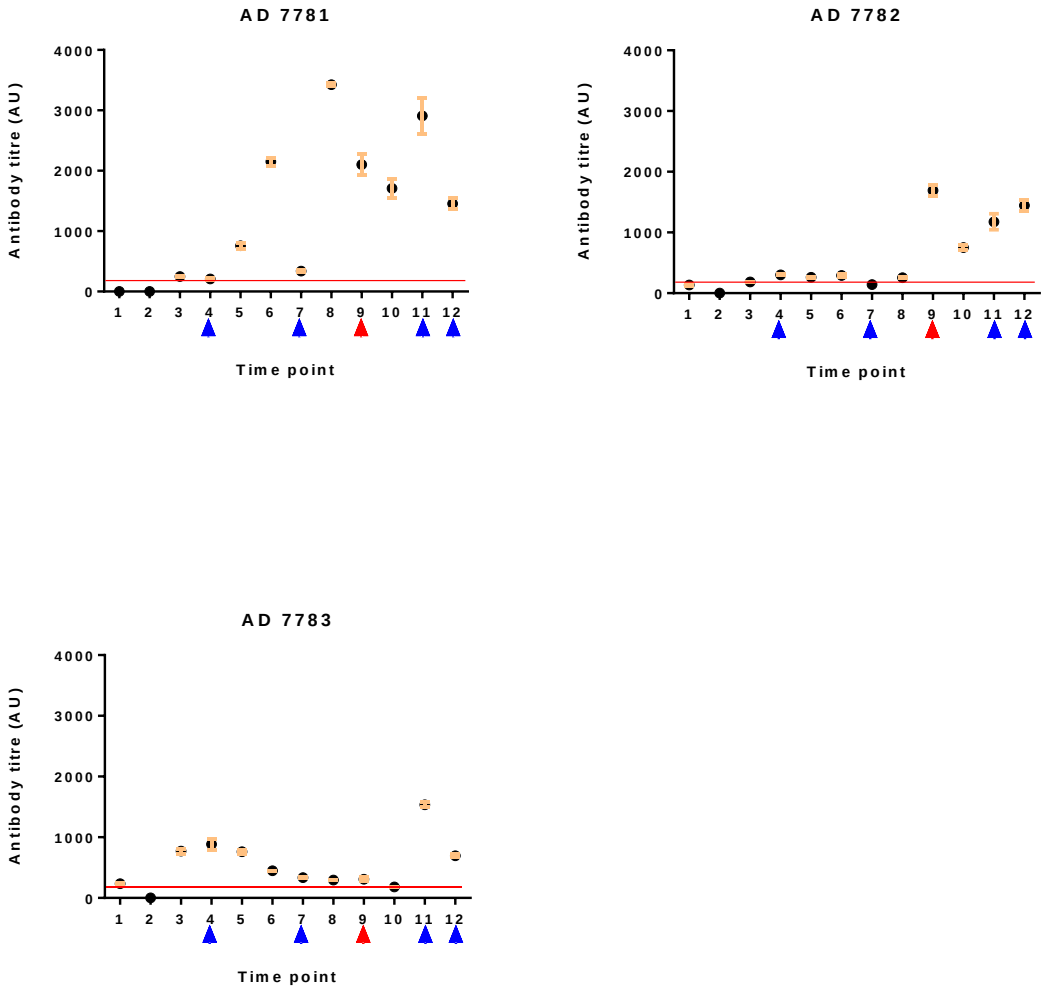


Figure 12F: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Adidome. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are presented as mean ± SD on the scatter plots. The red horizontal bar represents the cut-off using naive sera (negative control). AD 7781, 7782 and 7783 represent cattle identification numbers at the Adidome study site. Numbers with arrows represent treatment period with trypanocides for each time. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively.

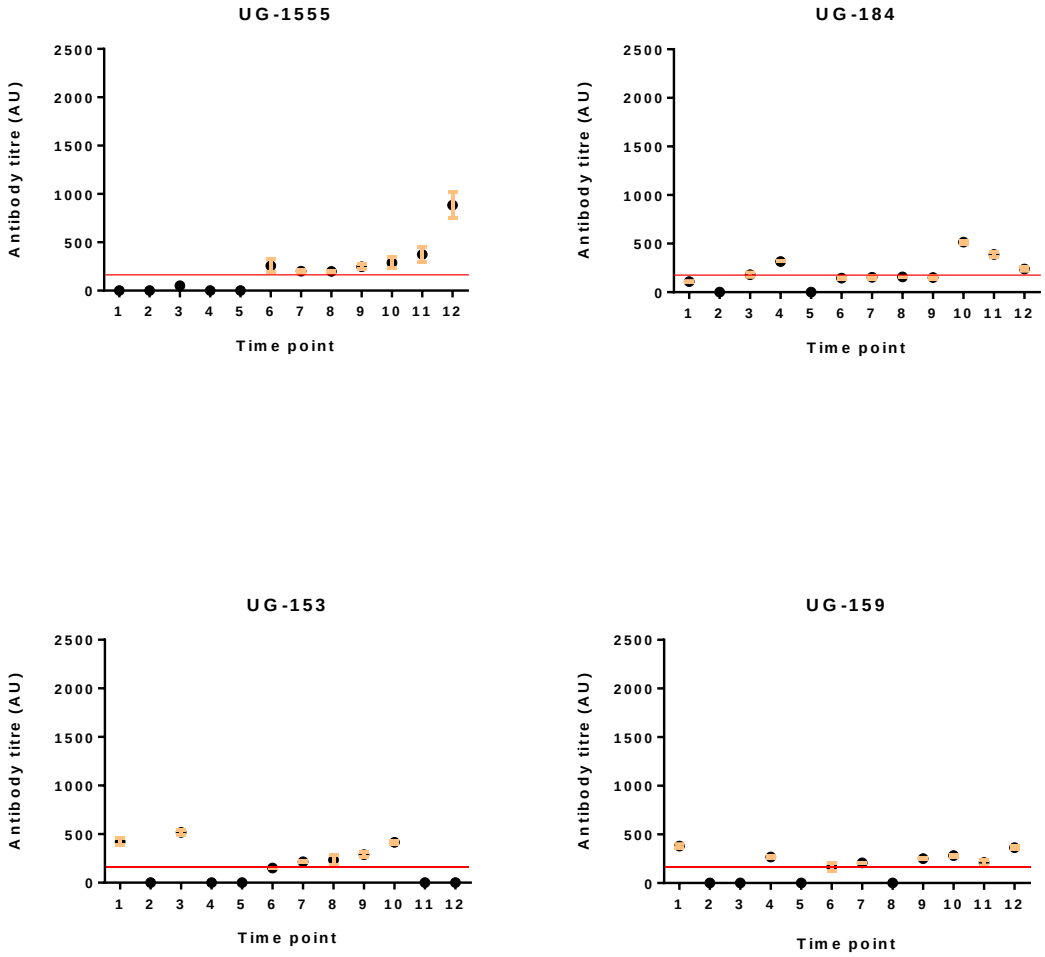


Figure 13A: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 1555, 184, 153 and 159 represent Accra study site cattle identification number.

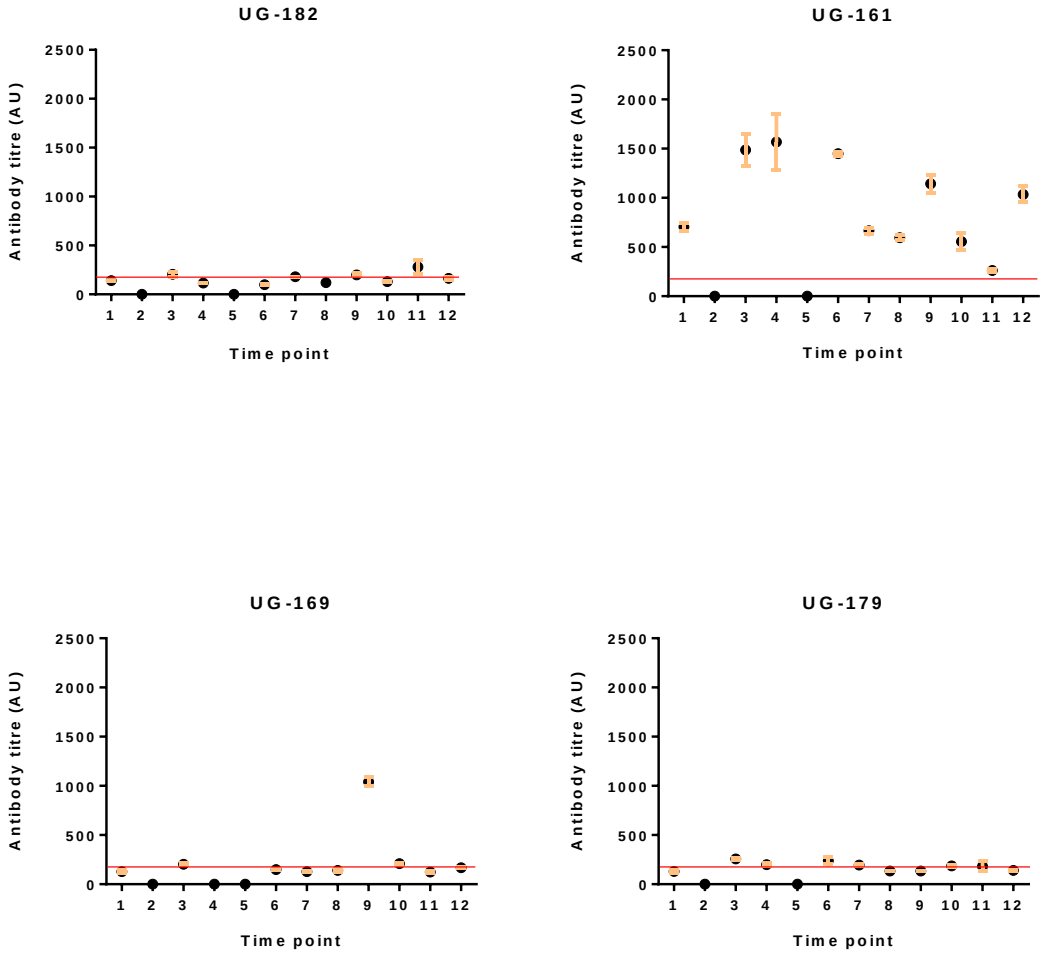


Figure 13B: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 182, 161, 169 and 179 represent Accra study site cattle identification number.

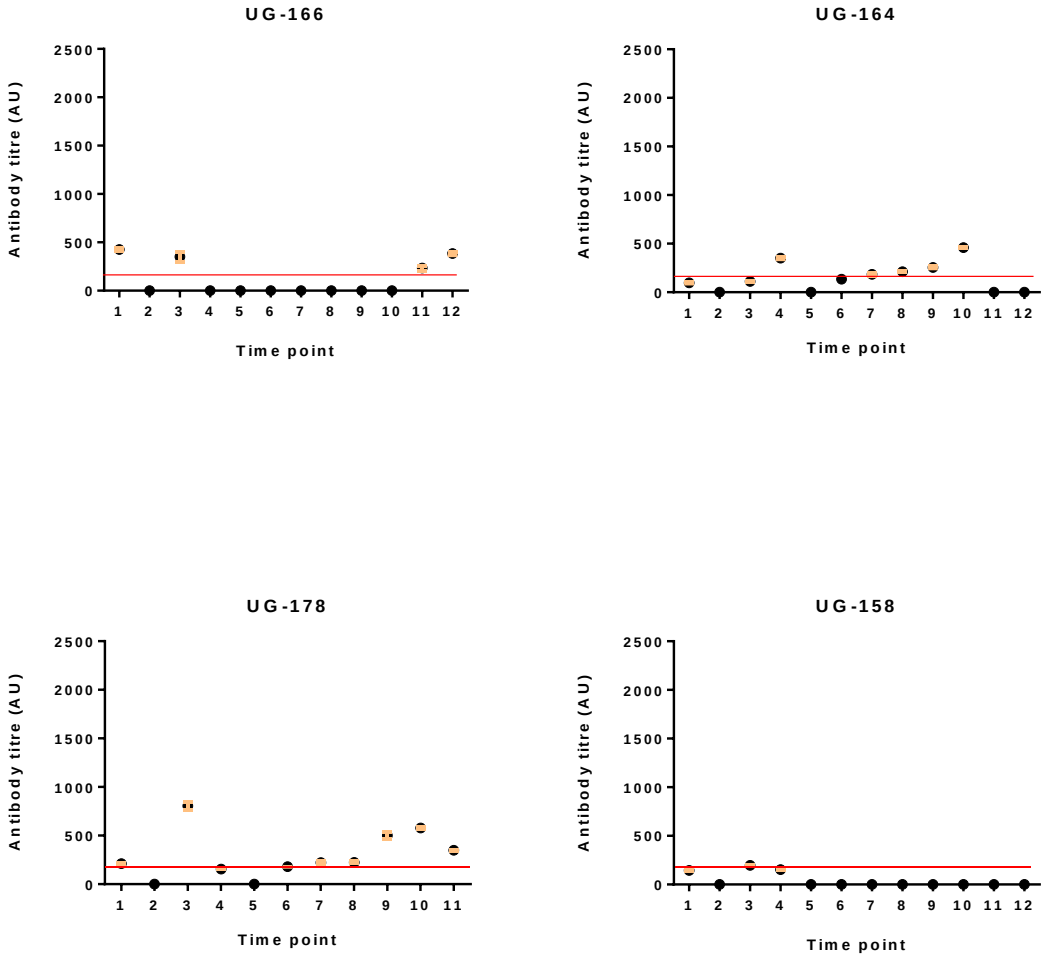


Figure 13C: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 166, 164, 178 and 158 represent Accra study site cattle identification number.

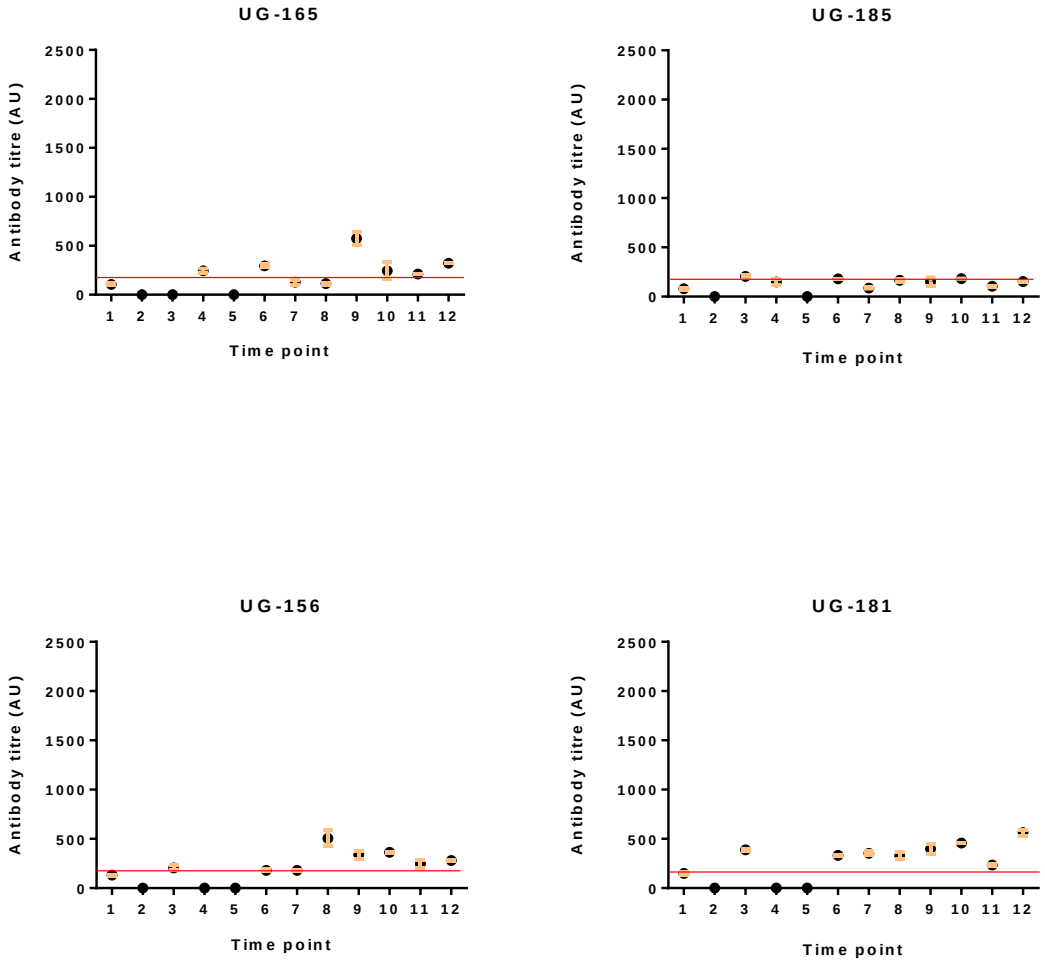


Figure 13D: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 165, 185, 156 and 181 represent Accra study site cattle identification number.

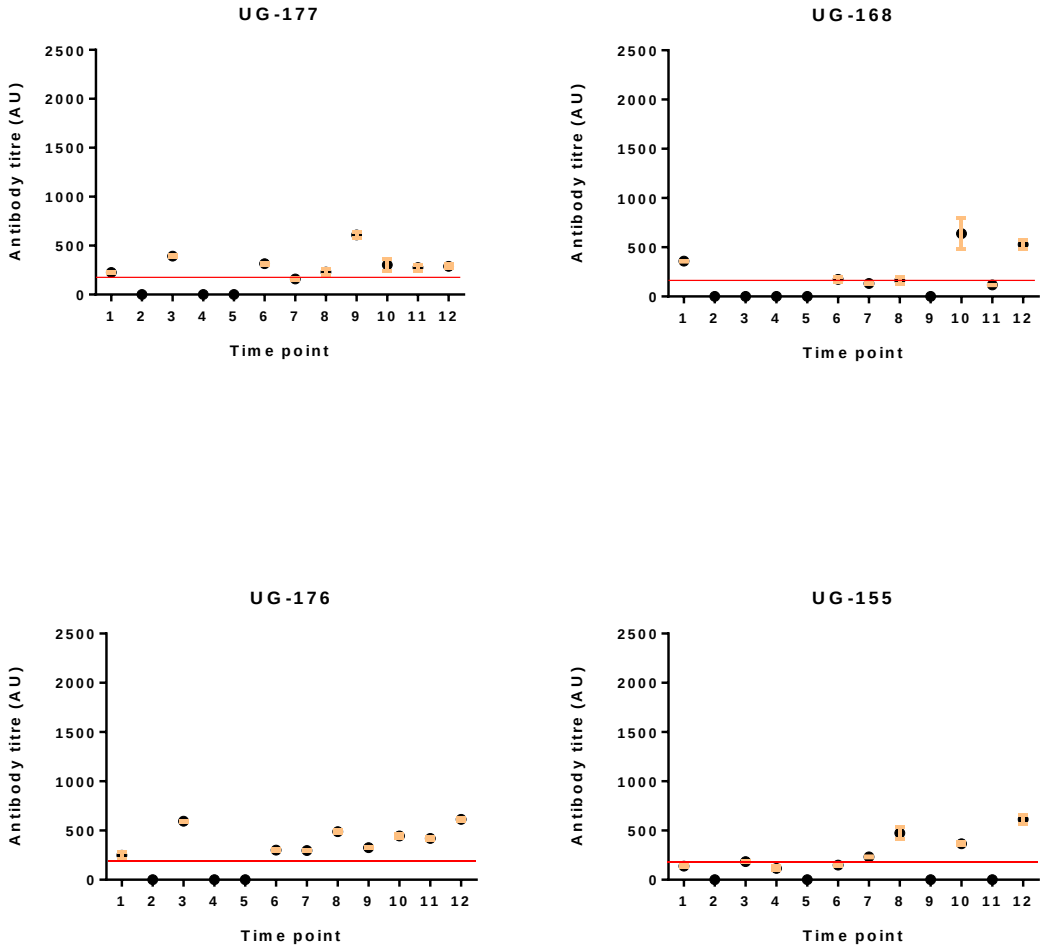


Figure 13E: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 177, 168, 176 and 155 represent Accra study site cattle identification number.

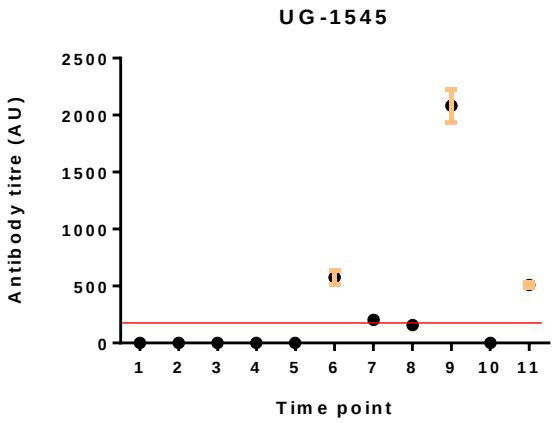


Figure 13F: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at Accra. Samples were collected at different time points (4-5 weeks intervals) for individual cows. Data are plotted as presented as mean ± SD on scatter plot. The red horizontal bar represents the cut-off using naive sera (negative control). UG 1545 represents Accra study site cattle identification number.

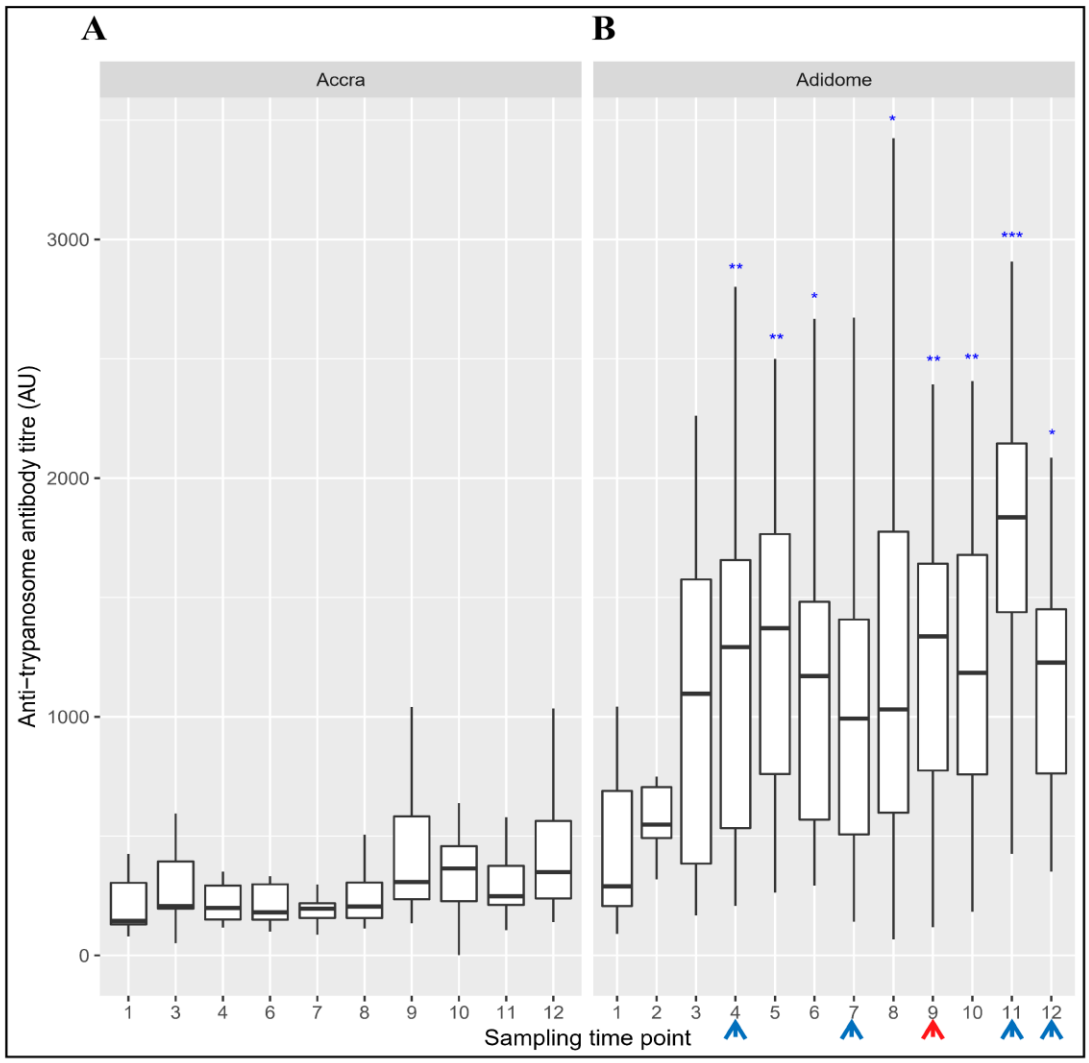


Figure 14: Antibody response of trypanosome-infected cattle sera to T. brucei proteins at A) Accra and B) Adidome. Samples were collected at different time points (4-5 weeks intervals). Data are presented as box and whisker plots, which indicate the median, lower and upper quartiles. No samples were collected at time points 2 and 5 for the Accra study site due to challenges with taking samples from animals. Numbers with arrows represent treatment period with trypanocides for each time points at Adidome. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Statistical significance was tested using Bonferroni post-hoc test for antibody response at each time point in reference to the initial sampling period, *p< 0.05, **p< 0.01, ***p< 0.001.

4.4.1 Effect of geographical location, breed and sex on antibody response of trypanosome-infected cattle to *T. brucei* proteins

As a results of the differences in the trypanosome transmission intensity at the two study site, the differences in the antibody responses against *T. brucei* at the two location were assessed. The median titres of the antibody responses for cattle at Adidome and Accra over the one-year period were compared and was significant ($p < 0.0001$, Mann-Whitney test) (Figure 15). Again, the potential effect of the ages of the animals on the measured antibody responses was analyzed. There was no significant effect of age on the levels of antibody reactivity at both study sites (Adidome: $p = 0.20$ and Accra: $p = 0.53$, Kruskal-Wallis rank sum test).

The potential effect of cattle breed on antibody response was also assessed by comparing the respective antibody titres between breeds at Accra alone since all cattle at the Adidome site were the Sanga breed. The Sanga breed showed significant antibody response to *T. brucei* proteins as compared to the WASH cattle breed ($p = 0.0038$, Bonferroni post-hoc test, Figure 16). There was no significant difference in anti-trypanosome antibody titres between Sanga and Sanga Cross cattle breed ($p = 0.07$, Bonferroni post-hoc test) as well as between Sanga Cross and WASH ($p = 0.47$, Bonferroni post-hoc test) (Figure 16). To further understand the potential effect of cattle breed on the antibody reactivity at Accra, the antibody responses among the cattle breed for each time point were compared. However, there was no significant difference in antibody titres among the cattle breeds for each time point (Figure 17).

Further, the effect of sex of animals on antibody response by comparing the proportions between male (bull) and female (heifer) cattle at the two study sites was assessed. The proportion of females was significantly higher compared to males at Adidome ($p < 0.001$, Chi-squared test). However, there was no significant difference in antibody response to *T. brucei* proteins between females and males at both sites (Adidome: $p = 0.37$, Accra $p = 0.21$; Mann-Whitney test, Figure 18). Also multivariate regression analysis revealed the cattle sex and breed type have impact on the level of antibody responses at Accra, with the breed type being significant ($p < 0.0001$).

Since we only observed high levels of antibody titres with sera at Adidome compared to cattle from Accra, the rest of the work was done using cattle sera from Adidome.

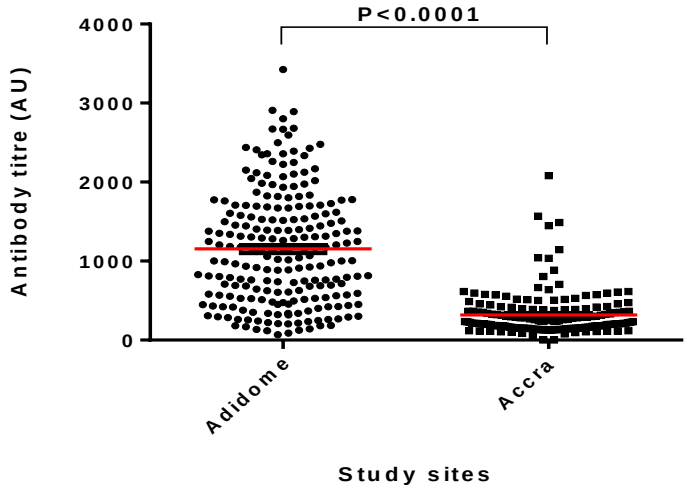


Figure 15: Comparison of antibody response of trypanosome-infected cattle sera to T. brucei at Adidome and Accra. Dots represent antibody titres for individual cow on the scatter plot. The red horizontal line represents the median of antibody titres. Statistical significance was tested using Mann-Whitney test, $p < 0.05$.

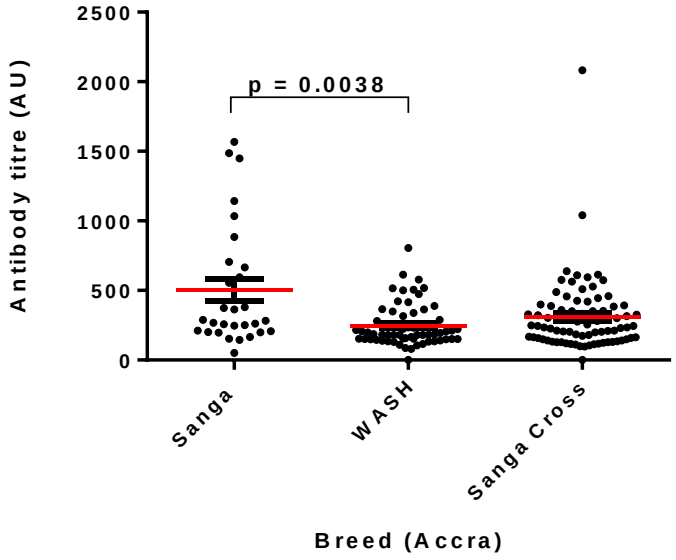


Figure 16: Comparison of T. brucei lysate-specific antibody levels among different cattle breeds at Accra. Dots represent antibody titres for individual cow breed at Accra on the scatter plot. The red horizontal line represents the median of the antibody titres.
WASH represents West African Shorthorn breed of cattle. Statistical significance was tested using Kruskal-Wallis rank sum test, $P < 0.05$.

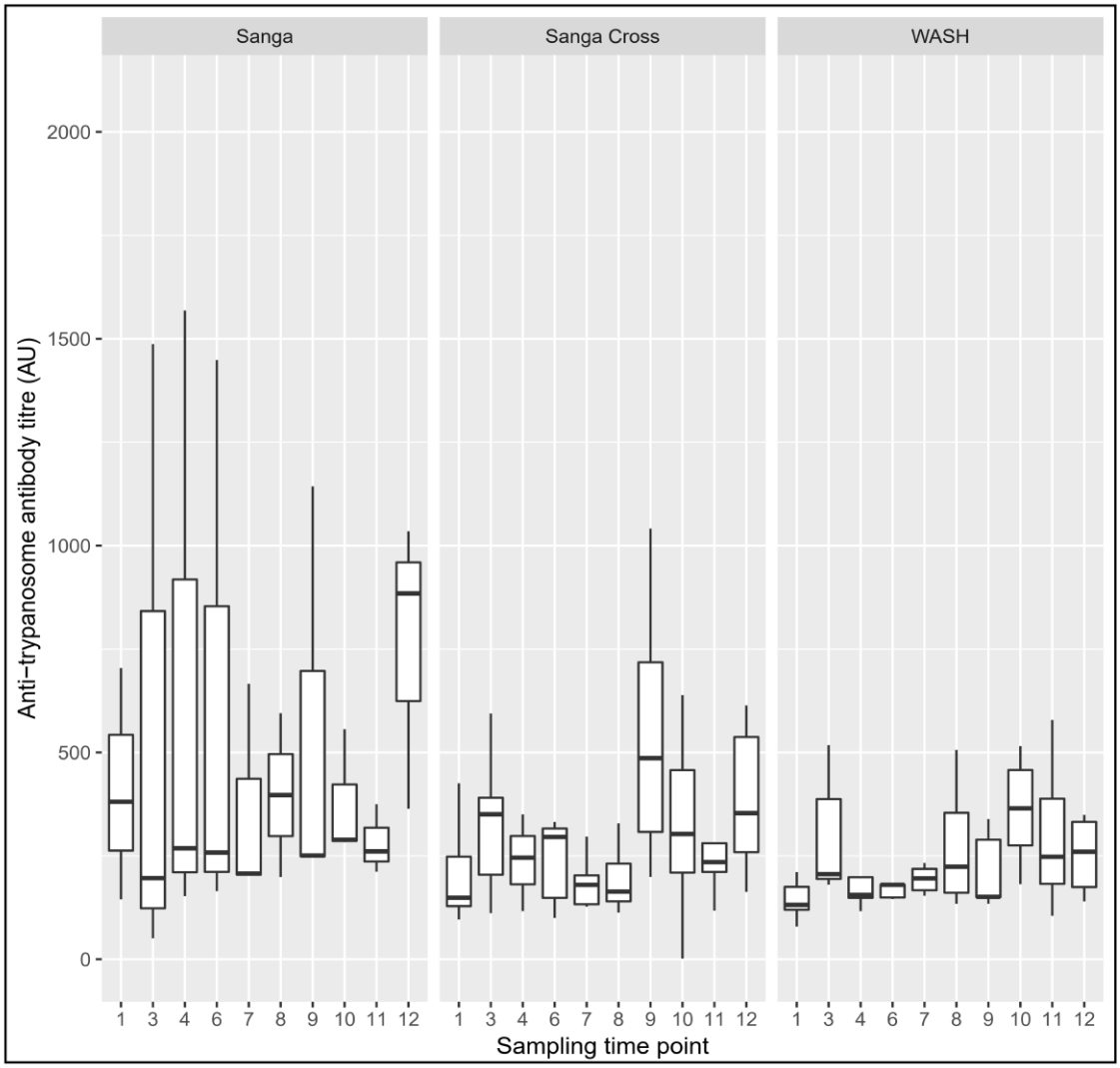


Figure 17: Impact of cattle breed on antibody response to T. brucei proteins across time points at Accra. . Data are presented as box and whisker plots. The black thick horizontal line in the box plot represents the median of antibody titres. No samples were collected at time points 2 and 5 for Accra study site due to challenges with taking samples from animals. WASH - West African Shorthorn. Statistical significance was tested using Kruskal-Wallis rank sum test, $p < 0.05$.

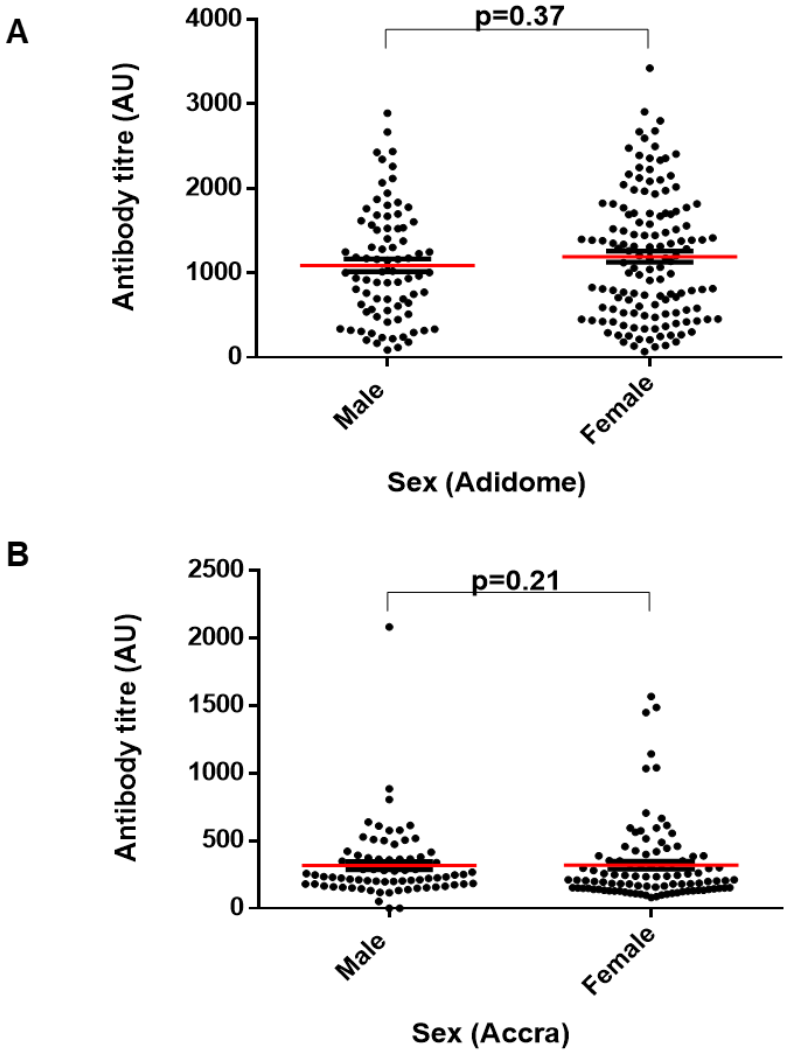


Figure 18: Comparison of antibody response of trypanosome-infected cattle to T. brucei among males and females at A) Adidome and B) Accra. Dots represent antibody titres for individual cow breed at Accra on the scatter plot. The red horizontal line represents the median of the antibody titres. Statistical significance was tested using Mann-Whitney test, $p < 0.05$.

4.5 Identification of immunogenic *T. brucei* proteins

4.5.1 Detection of immunogenic *T. brucei* proteins by Western blotting

To detect immunogenic *T. brucei* proteins other than the variant surface proteins, whole *T. brucei* lysate was enriched by anion exchange chromatography, and the eluates ethanol were precipitated (Peak 3, Figure 19A) and resolved on 12% SDS-PAGE (Figure 19B). The resolved *T. brucei* lysate displayed a profile of both low (about 15 KDa) and high (≥ 200 kDa) molecular weight proteins (Figure 19B).

Total IgG was purified from the seropositive pooled cattle sera from the Adidome study site (Figure 20A) for the immunoprecipitation assays. Firstly, the purified IgG was resolved under reducing and non-reducing conditions to establish its stability. Thus, 150 kDa band observed under non-reducing condition and 50 kDa band (2 heavy chains) and 25 kDa band (2 light chain) observed under reducing condition. (Figure 20B). The purified IgG was then probed against the whole *T. brucei* lysate enriched by anion exchange chromatography along with controls including the crude pooled infected cattle sera from Adidome, naïve calf sera, positive control (paraflagellar rod protein antibody) and commercial fetal bovine serum (non-heat inactivated), for detection of the immunogenic *T. brucei* proteins (Figure 21). *T. brucei* serum-reactive proteins were detected in both crude pooled sera, purified IgG from infected cattle pooled sera from Adidome, the naïve sera and the positive control (Figure 21). However, *T. brucei* proteins bands between 80 and 58 kDa were recognized by the purified IgG from Adidome study but not by the crude trypanosome-infected cattle sera. Also, low molecular weight protein (about 50 kDa and 25 kDa) appeared prominent for the purified IgG from Adidome study site as compared to the crude Adidome

seropositive pooled cattle sera (Figure 21). Notably, there was no immunogenic protein signal observed for the commercial fetal calf serum (FCS) but protein signals were observed for the naïve sera with molecular weight between 245 kDa and 22 kDa. Also, the positive control, anti-paraflagellar rod antibody recognized *T. brucei* protein with molecular weight of about 50 kDa and 25 kDa.

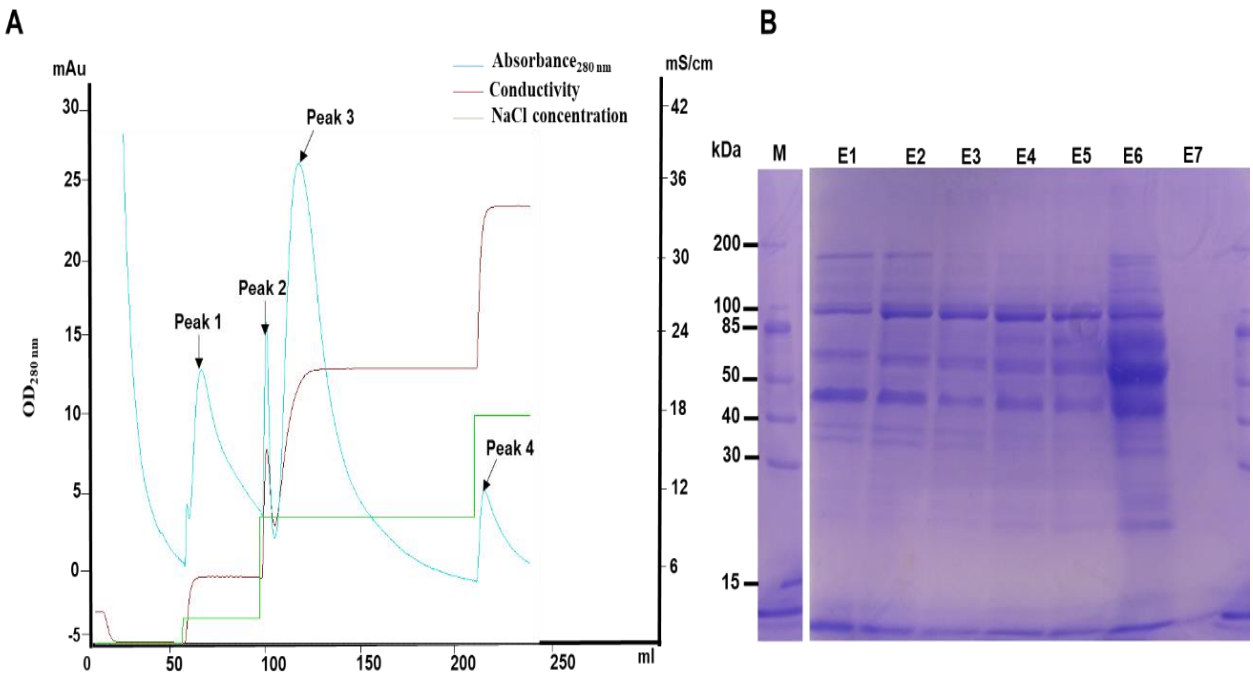


Figure 19: T. brucei lysate separation by anion-exchange chromatography. (A) Anion-exchange chromatogram of *T. brucei* lysate eluates were collected in fractions of 8 ml. Peaks 1, 2 and 4 represent washes done. Peak 3 is the *T. brucei* lysate eluates collected from the volumes 110 to 170 ml. (B) Coomassie-stained gel of ethanol precipitated *T. brucei* lysate eluates. E1-E7 represents the different eluates collected. M is Protein marker (Biolabs broad range marker).

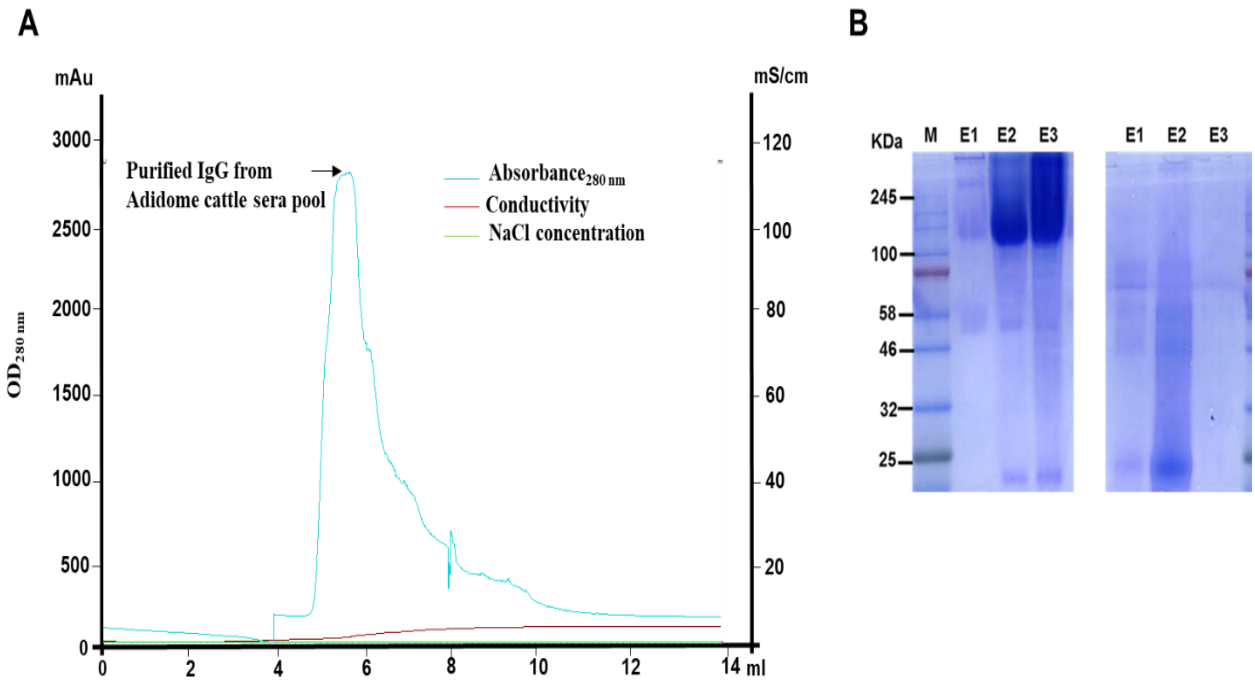


Figure 20: Purification of total IgG from pooled seropositive cattle sera collected from Adidome. (A) Chromatogram of purified IgG from Adidome cattle sera pool collected in fractions of 3 ml from the volumes 4 to 12 ml. (B) Coomassie-stained gel eluates after total IgG purification under reducing (right) and non-reducing (left) conditions. E1, E2, E3 are eluates collected after total IgG purification; M is Protein marker (Biolabs broad range marker).

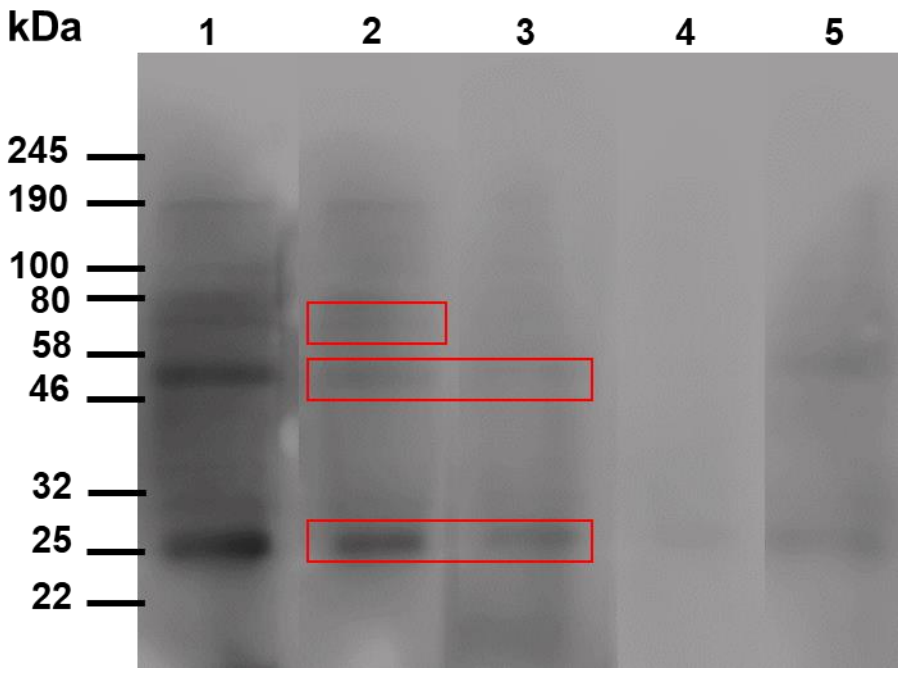


Figure 21: Interaction of cattle sera with T. brucei proteins. T. brucei (GUTat 3.1) lysate eluate after anion exchange was resolved on a 12% SDS-PAGE gel and western blotting performed using 1) naïve calf serum (~2 weeks old calf) diluted at 1:1000; 2) purified total IgG from Adidome seropositive cattle sera pool diluted at 1:2500; 3) crude Adidome seropositive cattle sera pool diluted at 1:1000; 4) commercial fetal calf serum (non-heat inactivated) diluted at 1:1000 and 5) paraflagellar rod protein antibody diluted at 1: 1000.

4.5.2 Identification of immunogenic *T. brucei* proteins by mass spectrometry

To identify the immunogenic *T. brucei* proteins, immunoprecipitation was performed with the immune trypanosome antibodies from the cattle sera (purified IgG) and the pre-immune FCS (non-heat inactivated) after the detection of immunocomplexes by western blotting (Figure 22). The protein immunoprecipitated eluates were further identified by LC-MS/MS.

Three similar protein bands were differentially observed in both immunoprecipitated trypanosome-infected cattle sera and control (FCS) with molecular weight of about 45, 28 and 10 kDa. However, two protein bands with the molecular weight of about 45 and 28 KDa were enriched in the trypanosome-infected cattle sera as compared to the control (FCS) and *T. brucei* protein lysate profile (Figure 22). There was also less intense protein bands observed between about 60 kDa and 200 kDa for trypanosome-infected cattle sera as compared to the control FCS and *T. brucei* protein lysate profile (Figure 22). Mass spectrometric analysis of the proteins immunoprecipitated by the trypanosome immune antibodies in comparison to those immunoprecipitated by the pre-immune FCS control antibodies identified several proteins with no significant score, however, beta- tubulin and triosephosphate isomerase were identified with significant ratios.

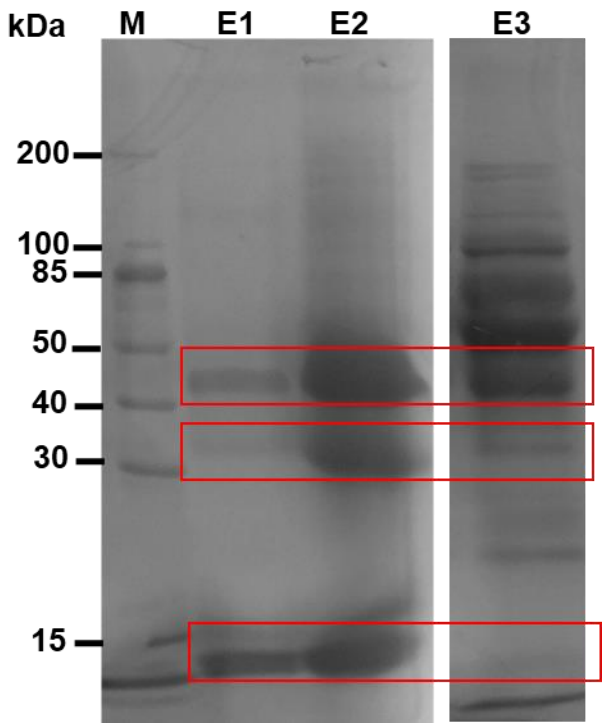


Figure 22: Identification of potential immunogenic T brucei protein by immunoprecipitation. Immunoprecipitation was performed with *T. brucei* lysate after anion exchange chromatography. Immunoglobulin G (IgG) purified from pooled trypanosome-infected cattle sera and fetal calf serum (FCS) were cross-linked to agarose beads separately and used to identify IgG-specific *T. brucei* proteins. The eluates obtained were subjected to SDS-PAGE and Coomassie-stained. E1 represents commercial fetal calf serum (not heat inactivated, control) against *T. brucei* (GUTat 3.1 strain) whole protein lysate; E2 represents Purified IgG from Adidome seropositive pooled sera against *T. brucei* (GUTat 3.1 strain) whole protein lysate and E3 represents ethanol precipitated eluate of *T. brucei* lysate after anion exchange chromatography for immunoprecipitation.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Animal African trypanosomiasis is a major constraint to livestock production in Africa, particularly impacting cattle (Nkegbe and Beyuo, 2011). This study was conducted to provide information on Animal African Trypanosomiasis and the different trypanosome strains in cattle in Ghana and suggest possible strategies for potential treatment. In the study, DNA samples were extracted from cattle whole blood over 18 months periods (4 to 5 weeks interval) and analyzed by nested PCR and next generation sequencing (NGS) to identify the circulating trypanosomes present at two different study sites in Ghana. For the nested PCR, in-house primers were designed targeting the tubulin gene of the trypanosome.

The PCR reaction was very sensitive for trypanosome species identification based on their band size. However, the detection level of the ethidium bromide stained gel was limited, thus a high throughput method (NGS) was used to identify and characterize the trypanosome. In both study sites, *T. vivax* was identified as the predominant infecting trypanosome species by both nested PCR and NGS. This high incidence of *T. vivax* infection could be associated with the possible mechanical transmission by other biting flies such as the genera *Tabanus* and *Stomoxys*, enabling a worldwide distribution even outside the tsetse belt of Africa (Nakayima *et al.*, 2012). This may be a contributing factor as reported to occur as well in *T. evansi* and *T. vivax* and to a certain extent *T. congolense* (Nimpaye *et al.*, 2011).

High *T. vivax* infection recorded at Adidome corroborates other studies in Ghana (Bakari *et al.,* 2017; Nakayima *et al.,* 2012). Although, *T. vivax* infection was predominate at Adidome, *T. brucei* was also identified. *T. brucei* co-existing with *T. vivax* could be as a result of its lower pathogenicity feature compared to *T. vivax* and *T. congolense* (Mbewe *et al.,* 2015). Also, *T. brucei* is reported to produce a higher parasitemia but restricted by quorum sensing and the host, presumably due to innate and adaptive immune factors (Kelly *et al.,* 2017). This indicates that the *T. brucei* density is lower in the host. Thus, *T. brucei* is believed to persist for long periods during an infection leading to chronic infection. Therefore, *T. vivax* being pathogenic will thrive in terms of competition with interaction with the host's immune system. In Accra, *T. theileri* was also identified as one of the circulating trypanosome species apart from *T. vivax*. *T. theileri* as a non-pathogenic parasite is well tolerated by the cattle (Mott *et al.*, 2011) leading to chronic infection, and therefore may not cause any significant pathology during infection, which then allows *T. vivax* to thrive.

Mixed infections were also observed at either study sites, which could be either two or three different or same trypanosome species co-existing. Similar observations were reported in an earlier study by Bakari and others (2017) in Ghana and elsewhere in Africa (Tchamdja *et al.,* 2017; Angwech *et al.,* 2015; Takeet *et al.,* 2013).These mixed infections are probably as a result of continual re-infection. In addition, the animals were being treated but still had infection, thus, the drug being ineffective and probably leading to the emergence of drug resistance. Mostly, mixed infections consist of both pathogenic species, such as *T. vivax* (more pathogenic) and *T. brucei* (less pathogenic), and non-pathogenic

species, such as *T. theileri* occurring at different time points. Therefore, there is possibly no competition among these species hence the ability of the species to co-exist (Peacock *et al.,* 2007).

The high incidence of lifetime infections with trypanosomes in individual cattle at Adidome confirms its high tsetse fly challenge and cattle trypanosomiasis as reported in most studies in Ghana (Bakari *et al.,* 2017; Nakayima *et al.,* 2012; Mahama *et al.,* 2003). On the other hand, Accra has a low tsetse fly density and relatively high cattle trypanosomiasis (Bakari *et al.,* 2017; Mahama *et al.,* 2003). Thus, a high incidence of lifetime infections with trypanosomes observed in individual cattle suggests a high rate of mechanical transmission. Although trypanocidal drugs (diminazene aceturate and isometamidium chloride) were administered to the cattle at Adidome periodically (1 to 3 months intervals) while those in Accra were not, there was no observed reduction in the infection at Adidome over the period of the study. This observation indicates a possible existence of drug resistance and/or continual re-infection of animals with parasites and this corroborates earlier reports in other African countries of the existence of trypanocidal drug resistance (diminazene aceturate and isometamidium chloride) among cattle (Tchamdja *et al.,* 2017; McDermott *et al.,* 2003).

On the other hand, the low infection frequency of *T. congolense* at both study sites could be as a result of being out-competed by *T. vivax.* In addition, *T. congolense* is mainly transmitted cyclically by the tsetse fly but to a lesser extent by mechanical means (Nimpaye *et al.,* 2011) thus, the lower frequency of the parasite existing in Accra with a low tsetsefly

challenge than at Adidome. Furthermore, this could be related to lower parasitemias of *T. congolense* (pathogenic) infections existing at both study sites. However, the low parasitemia of *T. congolense* observed at Adidome could be as a result of the parasite responding better to the trypanocidal drugs, diminazene aceturate and isometamidium chloride given to the animals at 1 to 3 months intervals.

The high incidence of *T. vivax* infection observed in cattle at young age (<1 year) and adult age (≥1 year) at both study sites could be as a result of the continual mechanical transmission of this parasite at these study sites. Also, the cattle at both study sites have similar grazing practice (free range), therefore, it is possible calves and older animals get exposed to the trypanosome vectors along their grazing route. Also, calves may be exposed to the trypanosome as a result of parasite transfer from mothers to calves by tsetse fly or other biting flies during milk suckling. There was the presence of mixed infections in females at Adidome and both sexes at Accra which indicates that some other host-environmentally-related factors may play a role in influencing the existence of a particular trypanosome.

Infection of cattle in Adidome (all Sanga) with *T. vivax* could possibly be due to the similarity in their genetic make-up, although there could be differences in body conditions among the animals. Accra had different cattle breeds and in all of these breed types, *T. vivax* was the most prevailing species although *T. theileri* was found. The animals sampled at Accra included both trypanotolerant (WASH) and cross-breds with trypanotolerant breeds (Sanga and Sanga cross). Trypanotolerance is likely playing a role in the animals

through controlling the levels of parasitemia (Orenge *et al.,* 2012) and probably co-existence of parasites.

The study also longitudinally analyzed naturally acquired antibodies to *T. brucei* proteins in cattle from two geographically distinct regions in Ghana– Adidome and Accra. In Adidome, endemicity of cattle trypanosomiasis is higher than in Accra (Bakari *et al.,* 2017).The combined effects of higher *T. brucei* prevalence and higher parasite densities at Adidome explains the relatively higher antibody reactivity towards *T. brucei* proteins measured in most cattle at Adidome compared to Accra.. The reduced antibody response towards *T. brucei* proteins in Accra cattle could be due to the lower prevalence and density of the *T brucei* parasites in the Accra cattle.

The characterization study at Adidome showed that most of the cattle were re-infected even after treatment with trypanocidal drugs (diminazene aceturate and isometamidium chloride). Thus, the antibody responses raised against the *T. brucei* antigens were not surprising. However, some of the animals had no bands for PCR but tested positive for the *T. brucei* whole protein lysate indirect ELISA. This could be due to the persistence of antibodies after treatment. Studies by Van Den Bossche and others (2000) and Authiée *et al.* (1993) have shown that antibodies against whole trypanosome lysate can persist for 10-13 months post-treatment.

Most studies that have reported cattle antibody responses against trypanosomes have been cross-sectional (Manful *et al.,* 2010; Eyford *et al.,* 2013; Sullivan *et al.,* 2013; Sengupta *et al.,* 2014; Luciani *et al.,* 2018), and the dynamics of antibody production that occur

during natural infection have rarely been studied. In this study we have looked at whether cattle immune response to natural trypanosomes infection changes over a one year period in reference to the baseline *T. brucei*-specific antibody response. The significant differences observed in some time points in relation to the initial sampling time at Adidome may suggest persistence of antibody production to *T. brucei* antigens. This observation affirms the fact that animals at this site were more frequently exposed to tsetse flies infected with *T. brucei* parasites and their immune systems were consistently challenged by the high parasite burden to maintain high levels of antibodies over time. *T. brucei* is reported to produce a higher parasitemia but restricted by quorum sensing in the host, presumably due to innate and adaptive immune factors (Kelly *et al.,* 2017). Thus, *T. brucei* infections more easily establish as chronic infections and persist for long periods. The chronicity of infection at Adidome over time, despite animals being regularly treated, may indicates a possible existence of drug resistance and/or continual re-infection of animals with parasites (McDermott *et al.,* 2003; Bakari *et al.,* 2017; Tchamdja *et al.,* 2017).

Certain factors such as geographical location age, sex, and breed may be associated with the extent of immune challenge during an infection. There was a significant negative correlation observed between the antibody responses of the trypanosome-infected cattle to *T. brucei* proteins at Adidome and Accra locations ($p < 0.0001$). This may be due to the vegetation type in the study sites correlating with the tsetse fly density leading to the existence of a particular trypanosome parasite. Adidome with a mixed savannah and forest type has a high tsetse fly density corresponding to the high prevalence of *T. brucei*. This probably explains the higher antibody response generated against *T. brucei* in Adidome

compared to Accra, which has only savannah vegetation type and a low tsetse fly challenge. This may also explain the higher incidence of *T. theileri* at Accra since *T. theileri* is mechanically transmitted and does not rely on tsetse flies. However, firm conclusions cannot be made, due to the unavailability of other *in vitro* cultured trypanosome species to determine their antibody responses.

Cattle breed types such as Sanga, WASH and Sanga cross were present at the Accra study site whereas Sanga was the only breed type in Adidome study site. In Accra, the cattle breed types differed significantly in their antibody response to *T. brucei* proteins. Some breeds of cattle are trypanotolerant probably due to robust natural selection over several centuries (Hill *et al.*, 2005; Murray *et al.*, 1990). Trypanotolerant animals are able to limit parasitemia, resulting in low levels of anaemia and preservation of body weight (Paling *et al.*, 1991a; Paling *et al.*, 1991b). Generally, the main mechanism mediating the control of parasitemia by the trypanotolerant host, is the uninterrupted interaction between the parasite surface proteins and antibodies which may lead to chronic infection and persistent antibody production against the parasite in a host (Magez *et al.*, 2008; Vanhamme *et al.*, 2001), as seen at Accra. However, there was persistent antibody production against *T. brucei* antigens observed in Sanga breed as reflected in the high antibody titres compared to WASH breed, which is more trypanotolerant. This observation may probably be as a result of antibody response produced by mixed infections of different strains or species of trypanosome other than *T. brucei.*

Females have been shown to be more trypanotolerant than males among different genetic cattle breeds, and under field conditions, the females have the ability to withstand trypanosomiasis unlike their male counterparts (Orenge *et al.*, 2012). This implies that the females would have the trypanosomes-specific serum antibody persisting for longer periods than males. Surprisingly, there were no significant differences in the levels of *T. brucei* specific serum antibodies observed between male and female cattle at both Adidome and Accra. This is in line with multivariate regression analysis which showed that cattle breed was a significant contributing factor to antibody response levels at Accra. The observation that sex did not seem to affect antibody response levels could be explained by the fact that trypanotolerant and trypanosusceptible cattle may fall within the same sex category, thus affecting any direct effect of sex on the immune response.

Many trypanosome parasites have developed strategies to evade immunity such as presenting variety of different surface proteins to the immune system leading to antigenic variation while hiding highly conserved sites or proteins that are essential for protein function (Matthews *et al.*, 2015; Schwede and Carrington, 2010). The characteristic antigenic variation of the parasite poses a tough challenge towards the development of a vaccine to target the parasite. Thus, it is important to target other immunogenic trypanosome proteins apart from the VSGs to serve as vaccine candidate as well as a diagnostic biomarker.

T. brucei lysates enriched by anion exchange chromatography were probed with seropositive crude and purified total IgG from pooled cattle sera at Adidome,

immunoprecipitated and analyzed by mass spectrometry to deduce the identity of the immunoprecipitated proteins. The differential reactivity of crude and purified IgG from Adidome pooled cattle sera to the *T. brucei* proteins indicate the immune responses mounted against the trypanosomes in the cattle. The antigen recognition of these sera was directed toward protein bands between 22 and 245 kDa. However, a prominent band was observed for the blot probed with the naïve calf serum. This may be due to passive transfer of antibody from maternal to calf since the serum was collected from a 2 week old calf at the Accra study site.

Protein bands of molecular weight about 50 kDa and 25 kDa recognized by both crude and purified IgG from seropositive Adidome cattle pooled sera were suspected to be alpha/beta tubulin and triosephosphate isomerase (with approximate weights of 50 kDa and 27 kDa), respectively (Bourguignon *et al.,* 1998; Ludueña, 1998). These proteins are reported to be abundant in trypanosomes (Nanavaty *et al.*, 2016; Baral, 2010). Additionally, protein bands between 80 and 58 kDa recognized by the purified IgG from seropositive Adidome cattle pooled sera but not the crude, could be as a result of the pure and specific antibody being used without other impurities present to reduce the recognition signals. Subsequently, commercial fetal calf serum was used as control, which resulted in no immunogenic *T. brucei* protein targeted by this sera. In Adidome, there were predominantly *T. vivax* and *T. brucei* identified with *T. vivax* being the highest, thus probably the reason for the observation of the weak bands for the seropositive pooled crude Adidome cattle sera. This could be as a result of *T. brucei* being out-competed by *T. vivax*, hence, antibody raised against *T. vivax* higher than antibody produced against *T. brucei.* The positive control (anti-

paraflagellar rod antibody) also reacted with the *T. brucei* antigens with band observed at about 50 kDa and 25 kDa. The expected band sizes for the positive control were 73 and 69 kDa, which are two closely related proteins (PFR 1 and PFR 2), resulting in two distinct bands in SDS-gel electrophoresis (Schlaeppi *et al.*, 1989). Therefore, the different protein band sizes obtained in this study compared to the expected sizes could be as a result of cross-reactivity of the antibody.

Proteomic analysis by immunoprecipitation and mass spectrometry has been widely used to identify specific parasites antigens during serodiagnostic test and vaccine development (Reddy *et al.,* 2015; Dietz *et al.,* 2014). Nevertheless, there have been few proteomic studies focused on identifying potential diagnostic markers in trypanosomes in Africa (Eyford *et al.,* 2013). In the present study, serum samples from individual trypanosome-infected cattle from Adidome were used to identify novel biomarkers of *T. brucei* infection using one-dimensional SDS-PAGE coupled with western blotting, immunoprecipitation and mass spectrometry analysis. The protein bands enriched by the immunoprecipitation using the trypanosome-infected cattle sera compared to the control FCS and the *T. brucei* lysate profile suggest antibodies produced against these specific antigens and could be unique diagnostic markers for the animals in Ghana.

Based on the mass spectrometric findings of the immunoprecipitated protein eluates, beta-tubulin and triosephosphate isomerase were the only proteins identified with significant ratios, which confirms the protein bands (about 50 kDa and 25 kDa) recognized by the crude Adidome seropositive pooled sera and the purified IgG. Glycosomes, a key feature

in protozoans, harbour the enzyme triosephosphate isomerase which is involved in the glycolytic pathway (Baral, 2010). It plays a key role in isomerizing dihydroxyacetone phosphate into glyceraldehyde 3-phosphate in the glycosomes ensuring the complete catabolism of hexose units (carbohydrate metabolism) for the parasite's survival in its host (Helfert *et al.,* 2001). For tubulin (alpha and beta), it is also an abundant conserved cytoskeleton protein and plays an essential role during trypanosome cell division (Nanavaty *et al.,* 2016; Bhargava & Chatterji, 2014). The fast population doubling rate of trypanosomes makes them highly dependent on tubulin polymerization or depolymerization (Werbovetz, 2002). More significantly, tubulin is very critical for trypanosome locomotion, which is a vital function for trypanosomes to survive (Nanavaty *et al.,* 2016).

These two proteins are known to be targets for vaccine development. (Nanavaty *et al.,* 2016; Bhargava & Chatterji, 2014; Galland *et al.,* 2010; Verlinde *et al.,* 2001). This suggests that these two proteins as probable biomarkers could be targets for designing a diagnostic tool for diagnosing trypanosome-infected cattle in Ghana, thus understanding the biology and pathogenesis of the disease.

5.1.1 Limitations of study

A limitation of this study is the inability to adapt other trypanosomes to *in vitro* culturing in order to measure antibody levels against lysates of these parasites and determine immunogenic proteins. The low *T. brucei* protein coverage obtained from the mass spectrometric analysis may suggest that the antibodies produced from the trypanosome-

infected cattle was not predominantly against *T. brucei*. The antibodies may probably be against other species such as *T. vivax*, the most predominant species identified at both study sites.

5.2 Conclusions

The current study sought to characterize trypanosome species in cattle over a two-year period using nested PCR and next generation sequencing data from two areas in Ghana with differing vegetation type, tsetse fly challenge and trypanosomiasis endemicity. The sequencing data analyzed revealed that there was high trypanosome prevalence during lifetime infection at Adidome as compared to Accra. The trypanosome species identified were *T. vivax, T. brucei, T. theileri* and *T. congolense* with *T. vivax* as the most prevailing parasite at both study sites. *T. vivax* was also the predominant parasite existing based on age, sex and breed of cattle.

In addition, the study investigated the antibody response to *T. brucei* and identification of immunogenic *T. brucei* proteins. Cattle sera (215 samples) had high antibody response at Adidome with a seroprevalence of 97% compared to Accra with a seroprevalence of 73% out of 172 sera samples. This confirms the correlation and specificity between the *T. brucei* characterized at both study sites with its antibody production. Mass spectrometric analysis of the *T. brucei* proteins immunoprecipitated by trypanosome immune antibodies identified beta-tubulin and triosephosphate isomerase to be selectively recognized by infected sera in natural infection.

5.3 Recommendations

5.3.1 Recommendations from the study

The study used target sequencing to identify and characterize the circulating trypanosomes in Ghana. This could have missed out information on the single nucleotide polymorphisms (SNPs), which may be observed in the entire genome of the different trypanosome species or different strains of the same trypanosome species identified. Whole genome sequencing could be employed to identify these SNPs to inform the uniqueness of the African trypanosome existing in Ghana, which may contribute to a better understanding of the biology of the parasite. In the study, there were mixed infections observed at both Adidome and Accra which could probably be as a result of drug resistance or re-infection. Therefore, a drug resistance study could be done to ascertain the extent of the resistance and the genomes of the identified trypanosomes could also be sequenced to identify mutated genes conferring resistance. In addition, policy makers in the veterinarian community should be informed and educated on the usage and drug administration which probably lead to the emergence of drug resistance. Therefore challenging the usefulness of the trypanocidal drugs.

To identify immunogenic trypanosome proteins, the immune response associated with pathogenesis of the trypanosome infection needs to be clearly understood. Thus, seroprevalence of the study areas and other tsetse fly challenge zones could be done using other trypanosome species proteins for the indirect ELISA. Also, in identifying immunogenic trypanosome biomarkers, trypanosome-infected cattle sera could be tested against *T. vivax* proteins as this species was predominantly present.

5.3.1 Recommendations for further studies

This study focused mainly on samples from two ecologically contrasting communities, Adidome and Accra with differing vegetation type, tsetse fly challenge and cattle trypanosomiasis endemicity. Future studies in the country could utilize samples from all the known tsetse fly zones of Ghana to provide a meta-analysis of the lifetime trypanosome infection in cattle in Ghana. Also, with the ever increasing advancement in sequencing technologies, genomic data could be generated from each of these tsetse flies-challenged areas to serve as a database for the animal trypanosome endemic areas in Ghana.

This study also characterized only trypanosome species in the cattle but there could be a confounder that is co-infection with other pathogens such as *Babesia* species and *Theileria* species. Therefore, to understand the disease burden of the animal trypanosomiasis in cattle, co-infection of trypanosome with other pathogens could be studied. This could provide information to develop a model for disease burden.

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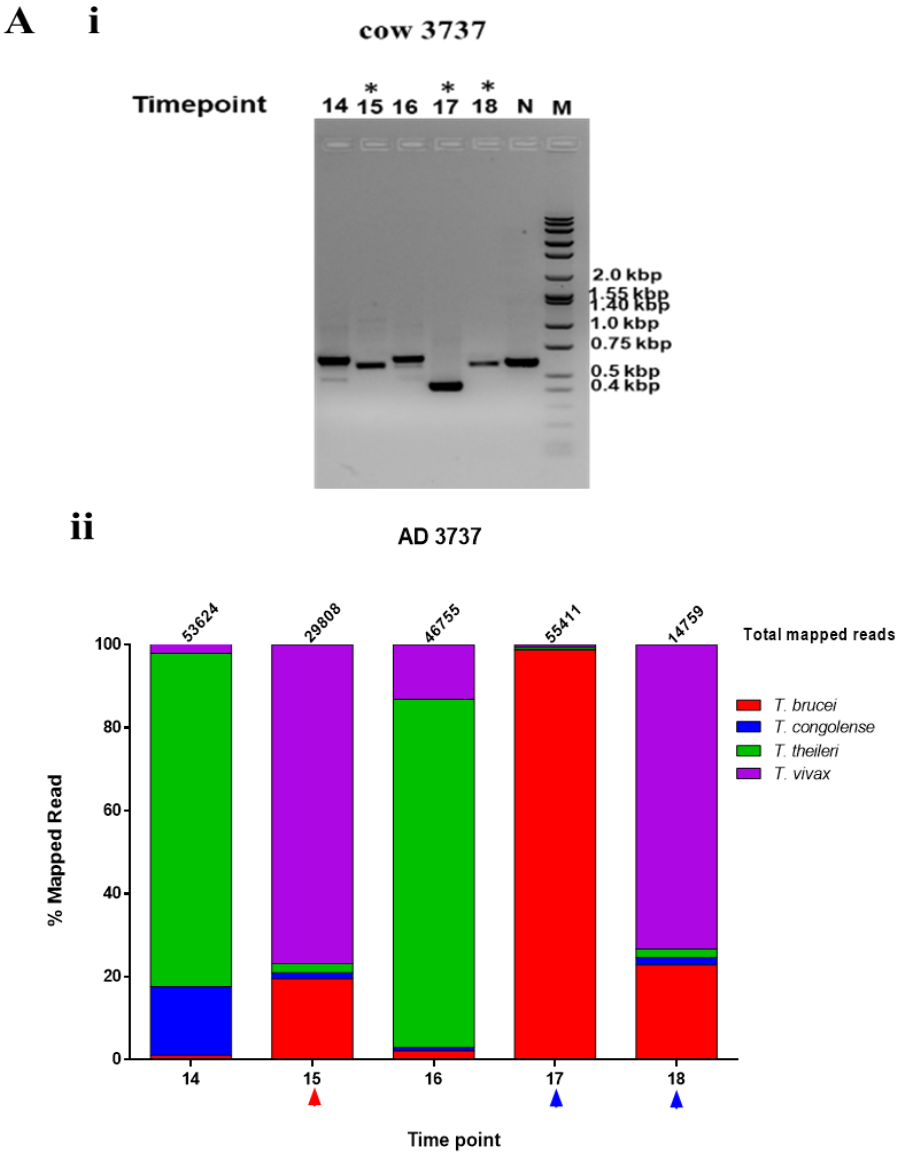
APPENDIX

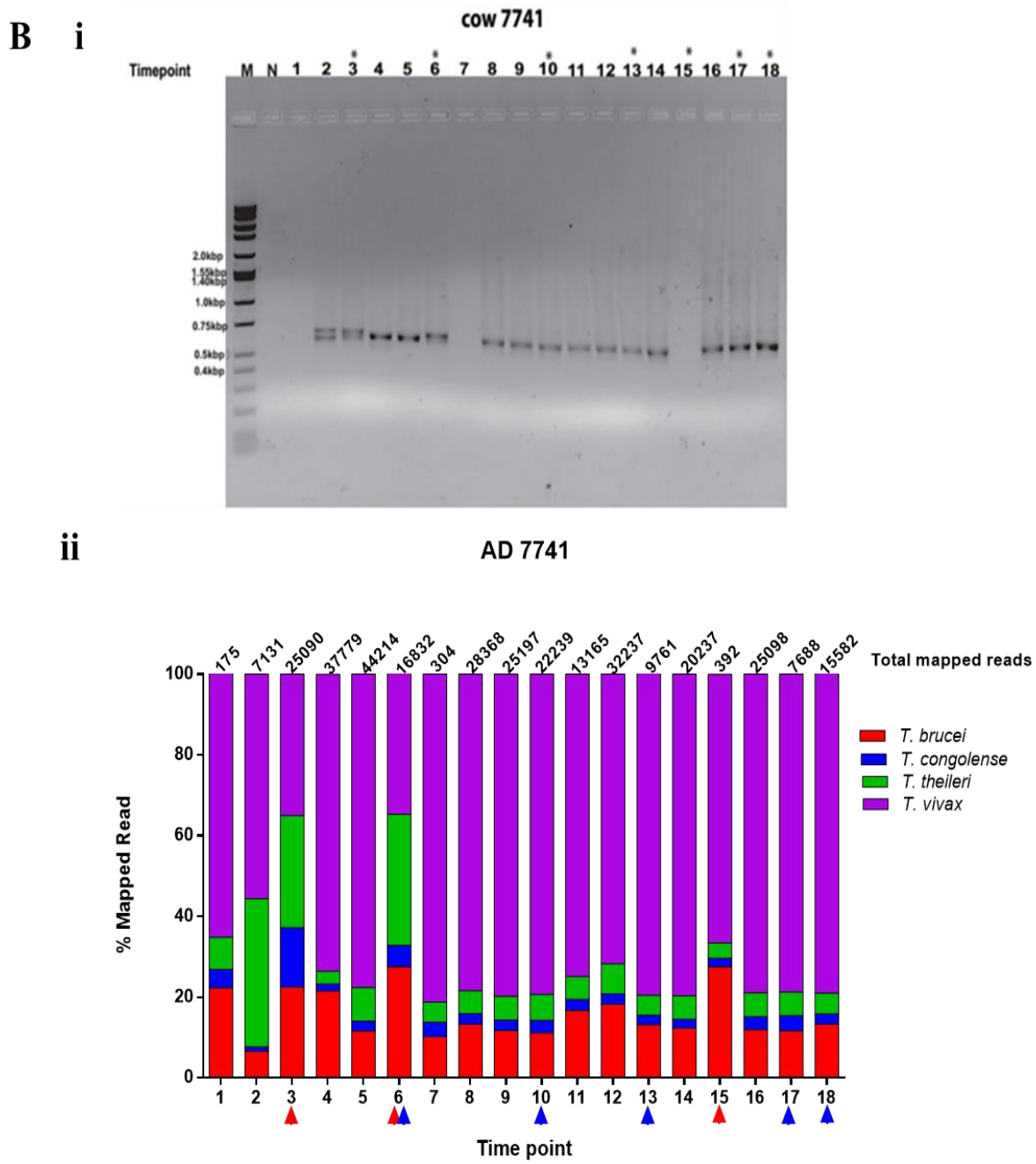
Ethical approval

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 RPN 001/CSIR-IACUC/2013.

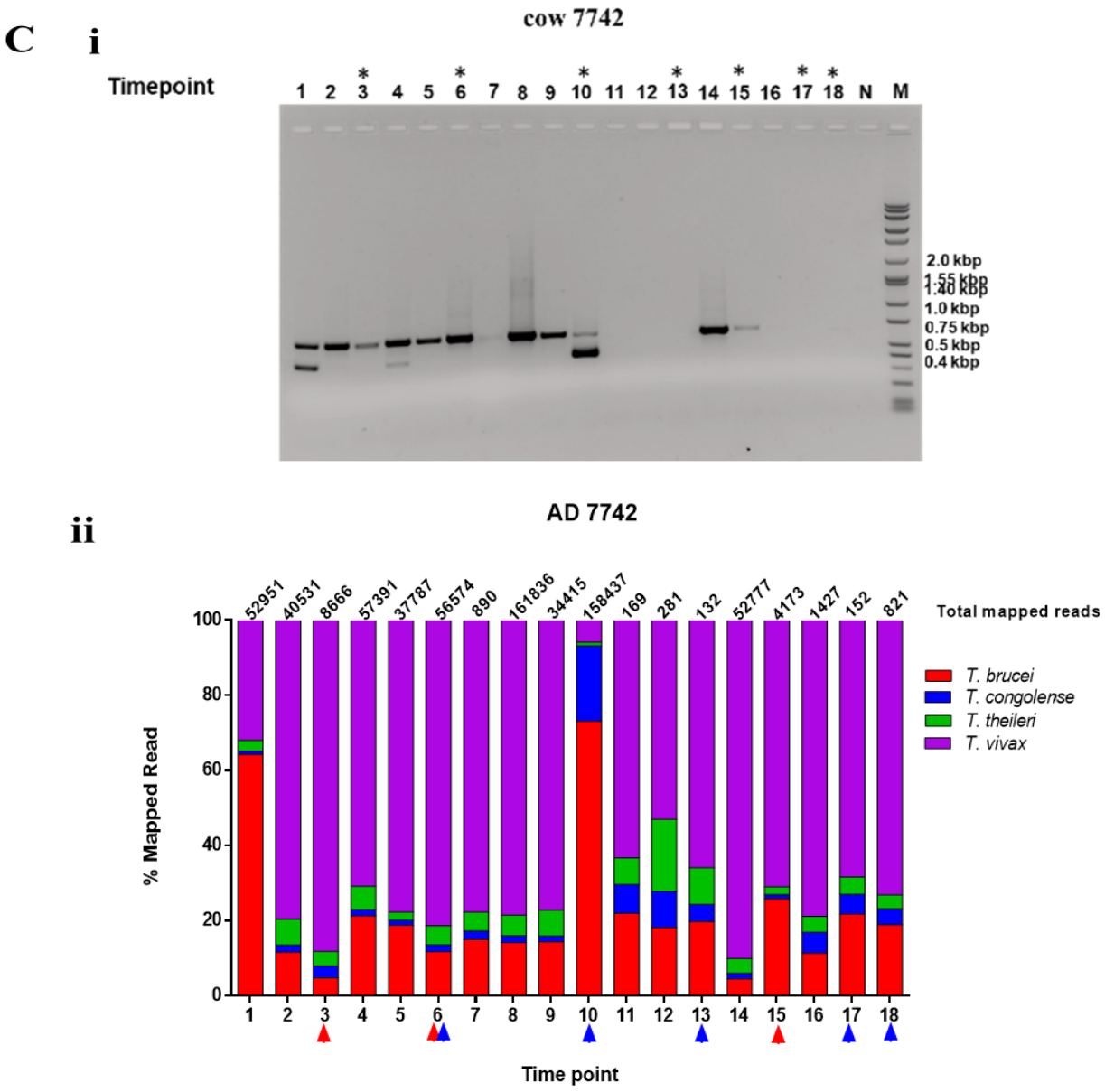
Characterization of lifetime trypanosome infection in individual cattle in Ghana



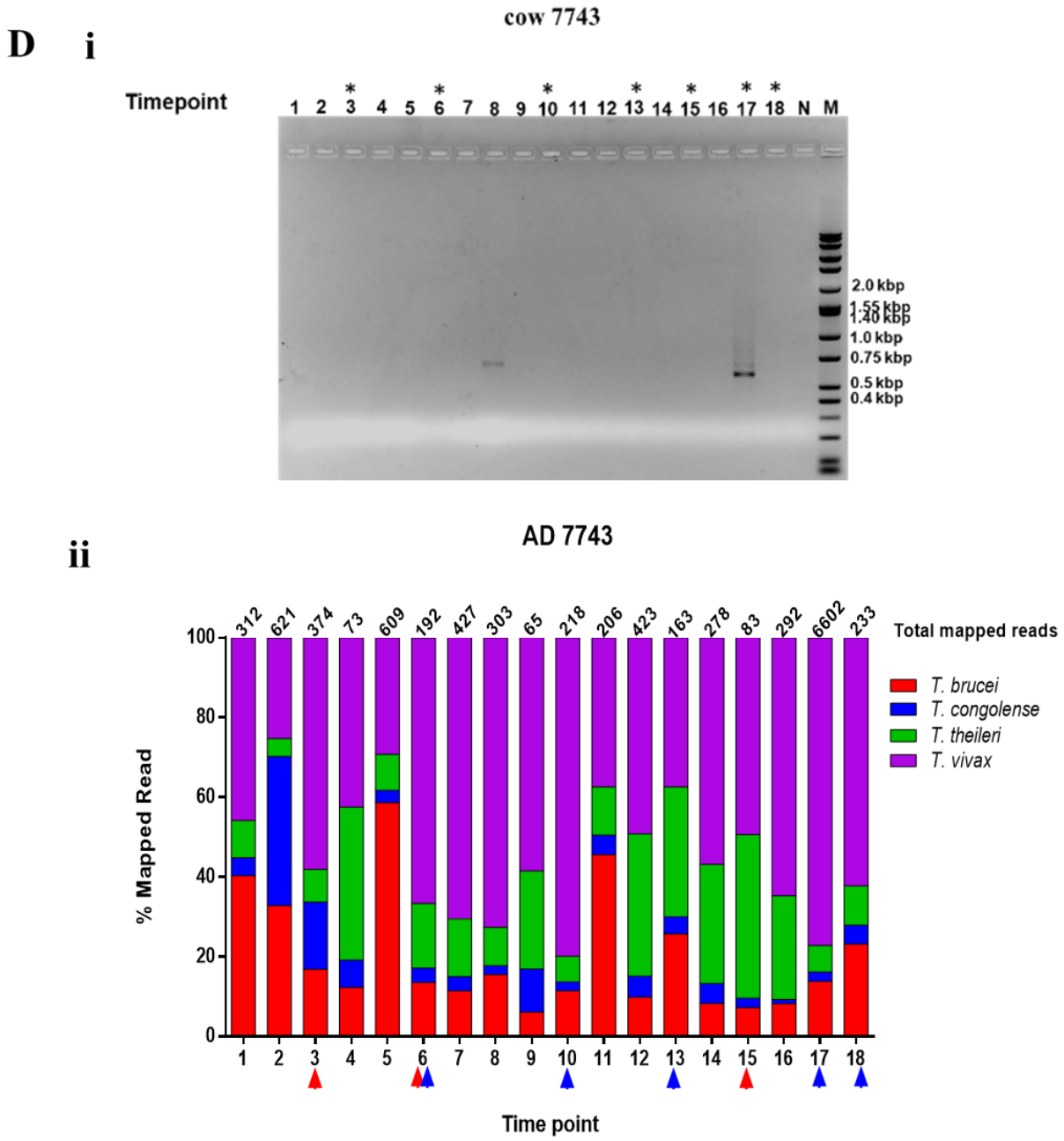
Appendix 1A: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



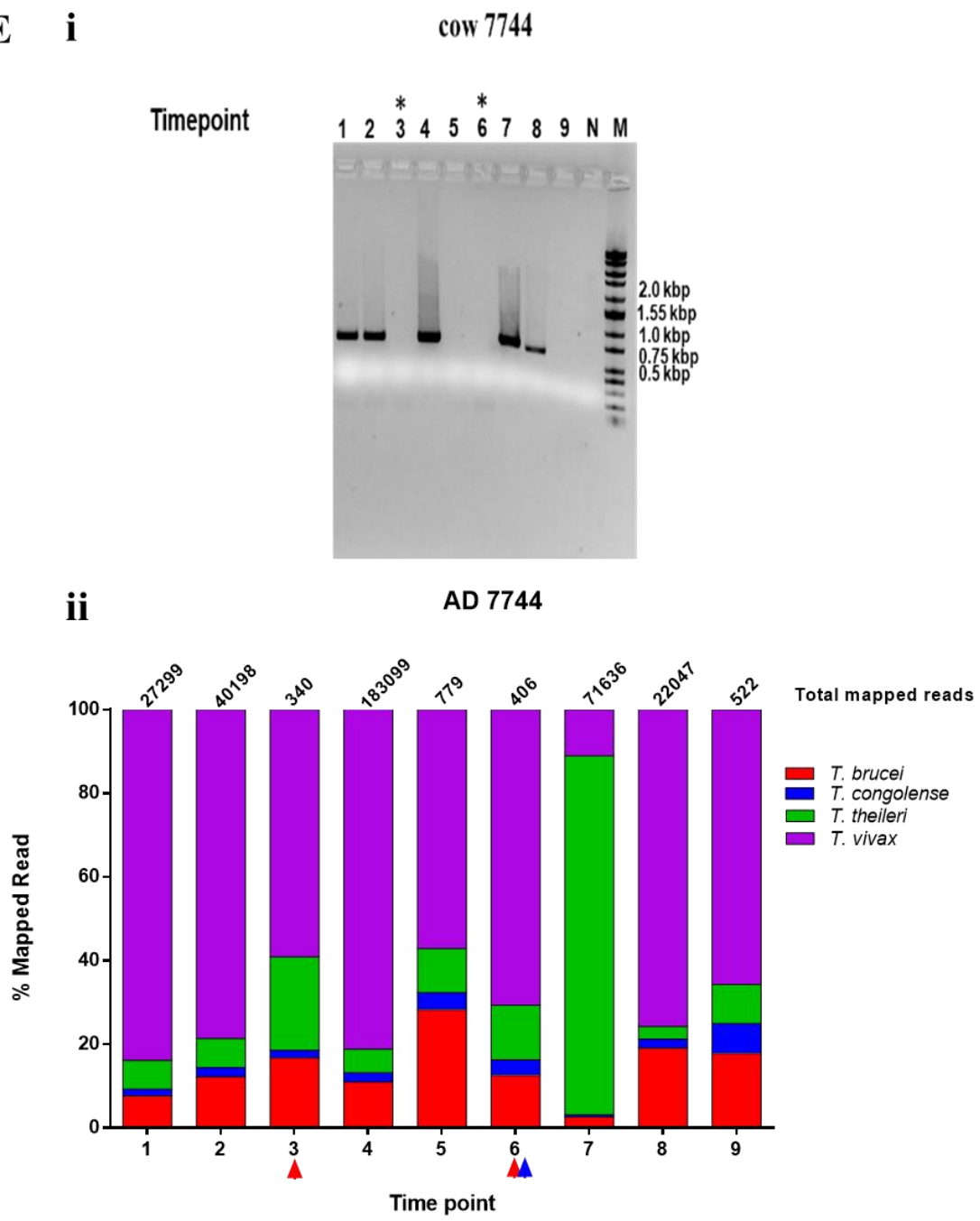
Appendix 1B: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



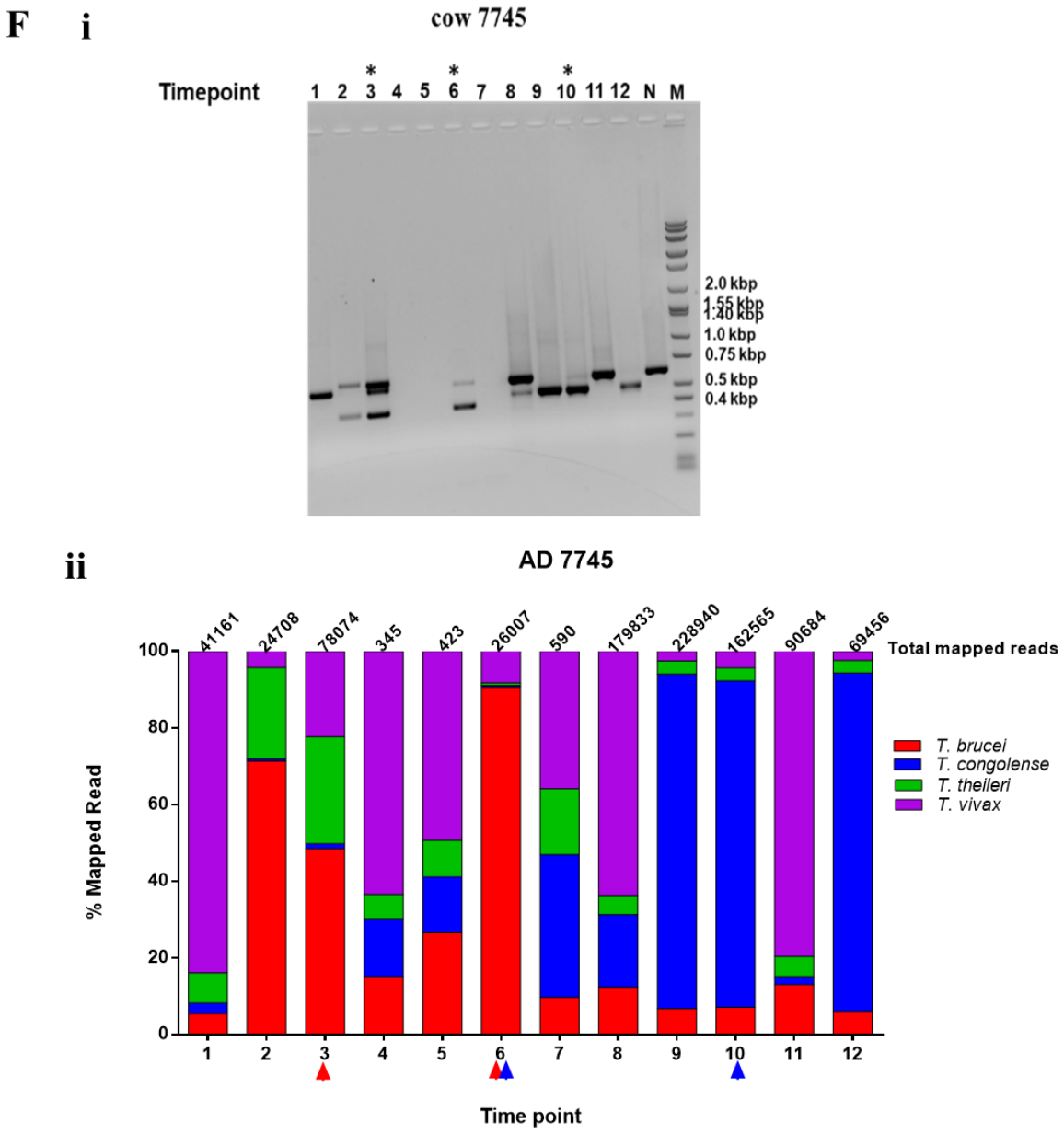
Appendix 1C: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



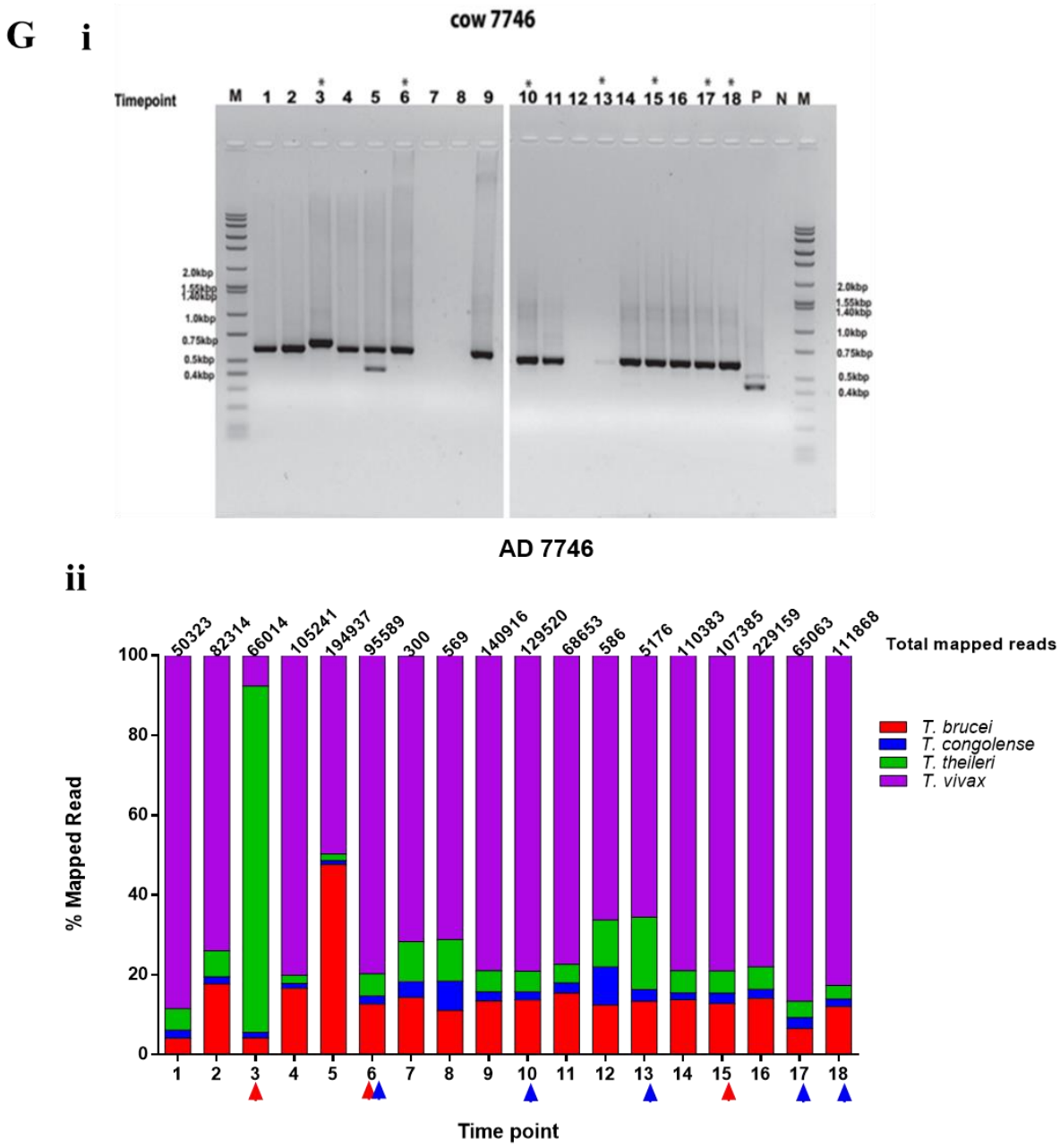
Appendix 1D: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



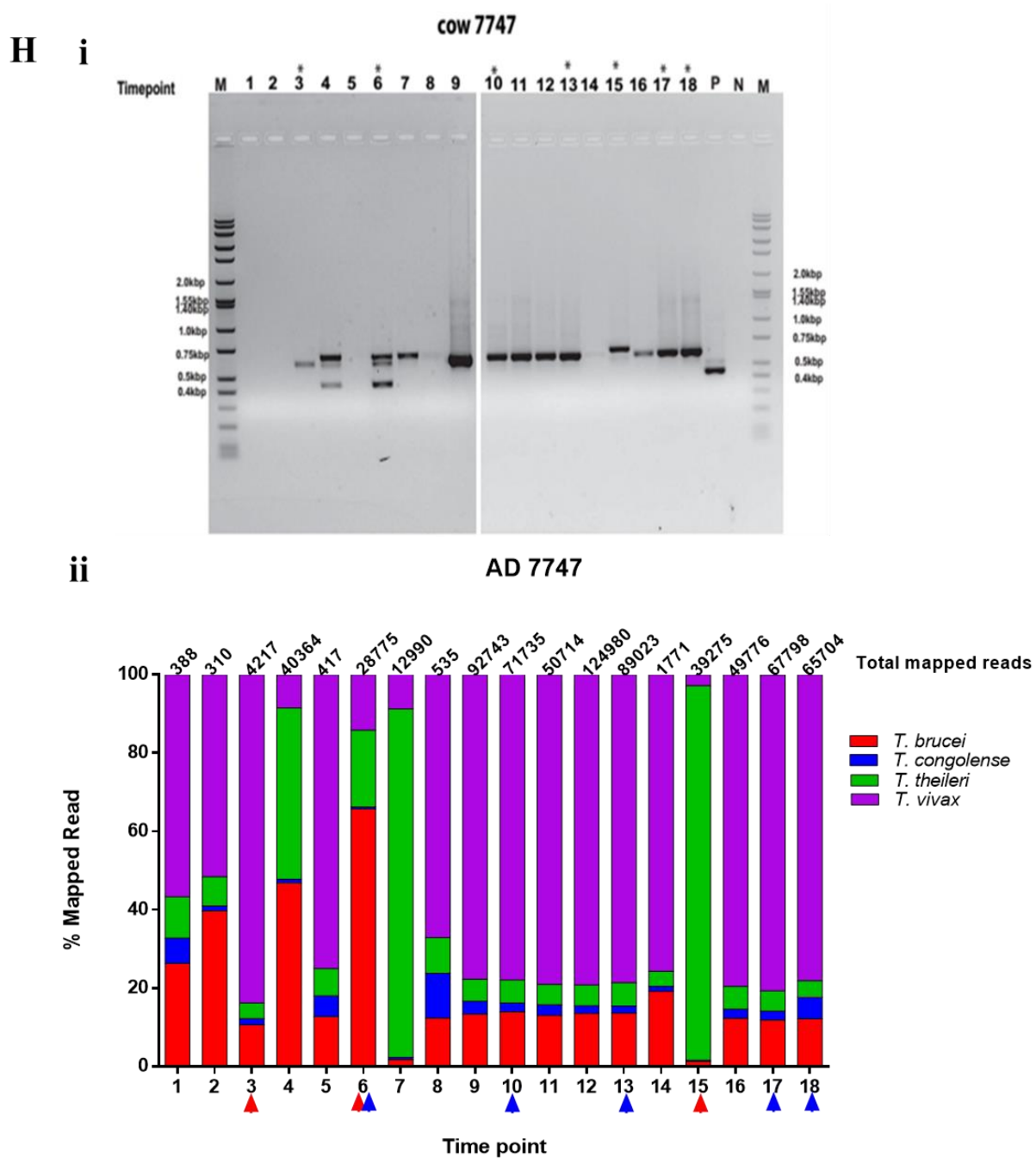
Appendix 1E: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



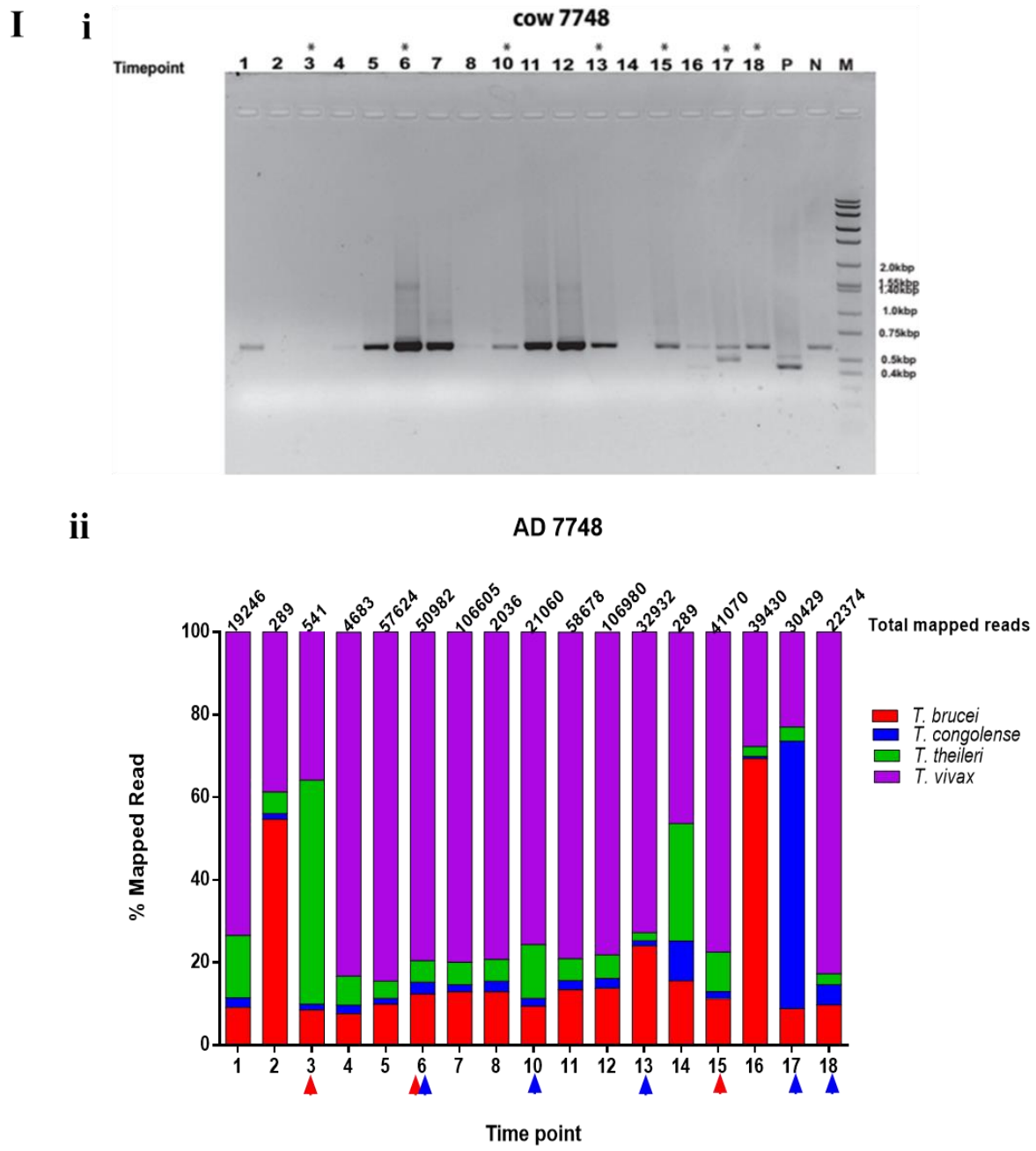
Appendix 1F: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



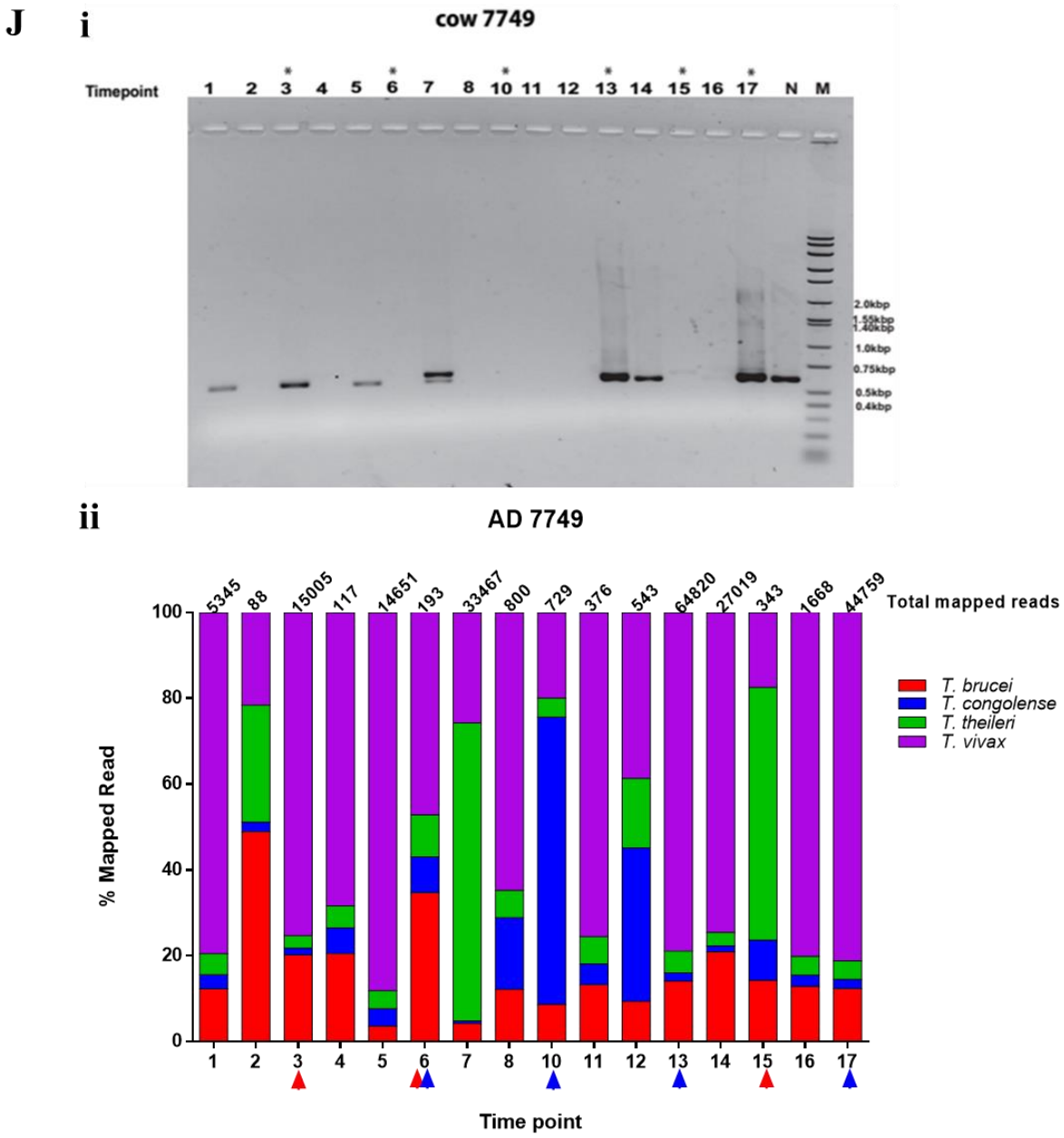
Appendix 1G: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



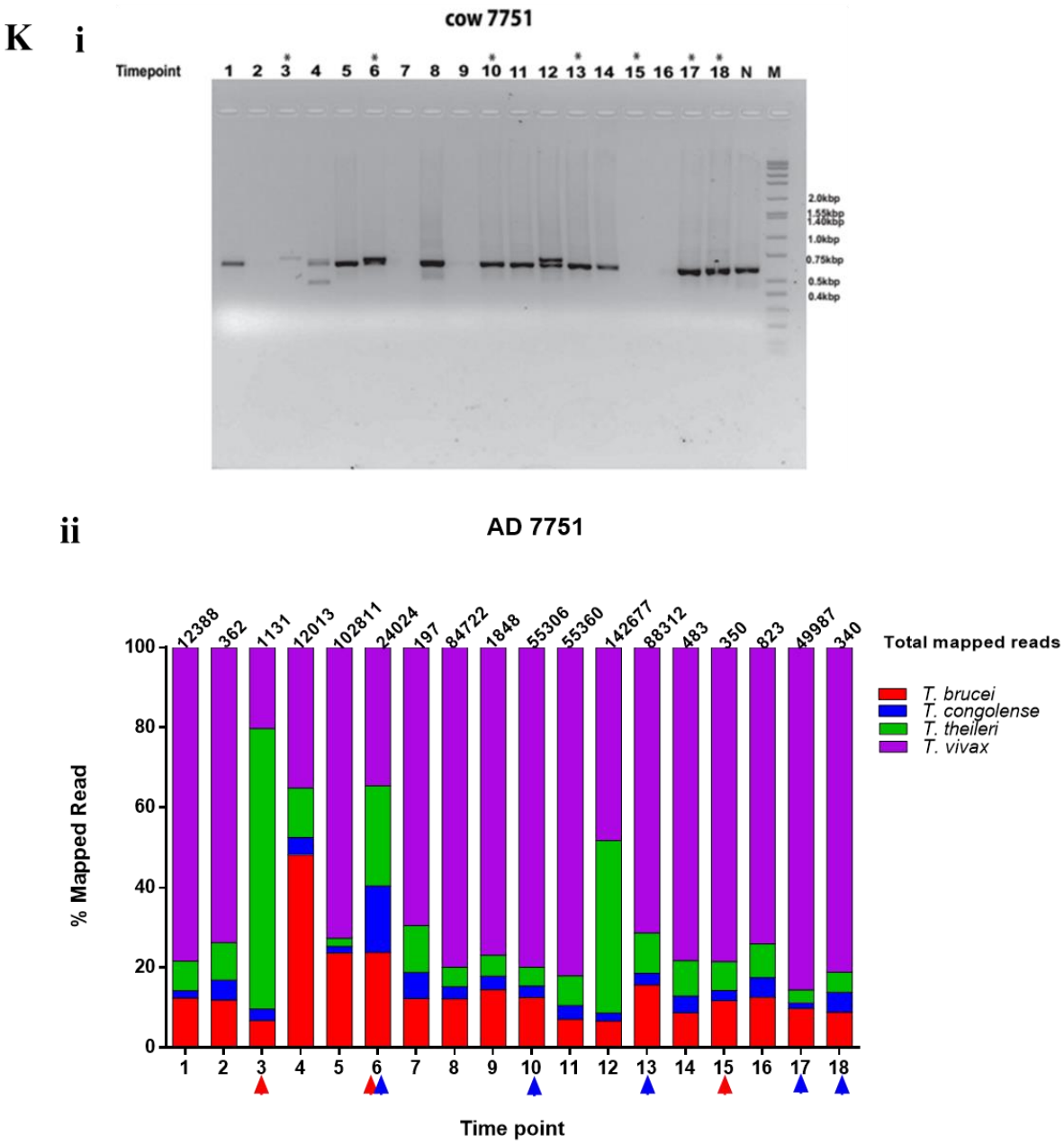
Appendix 1H: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



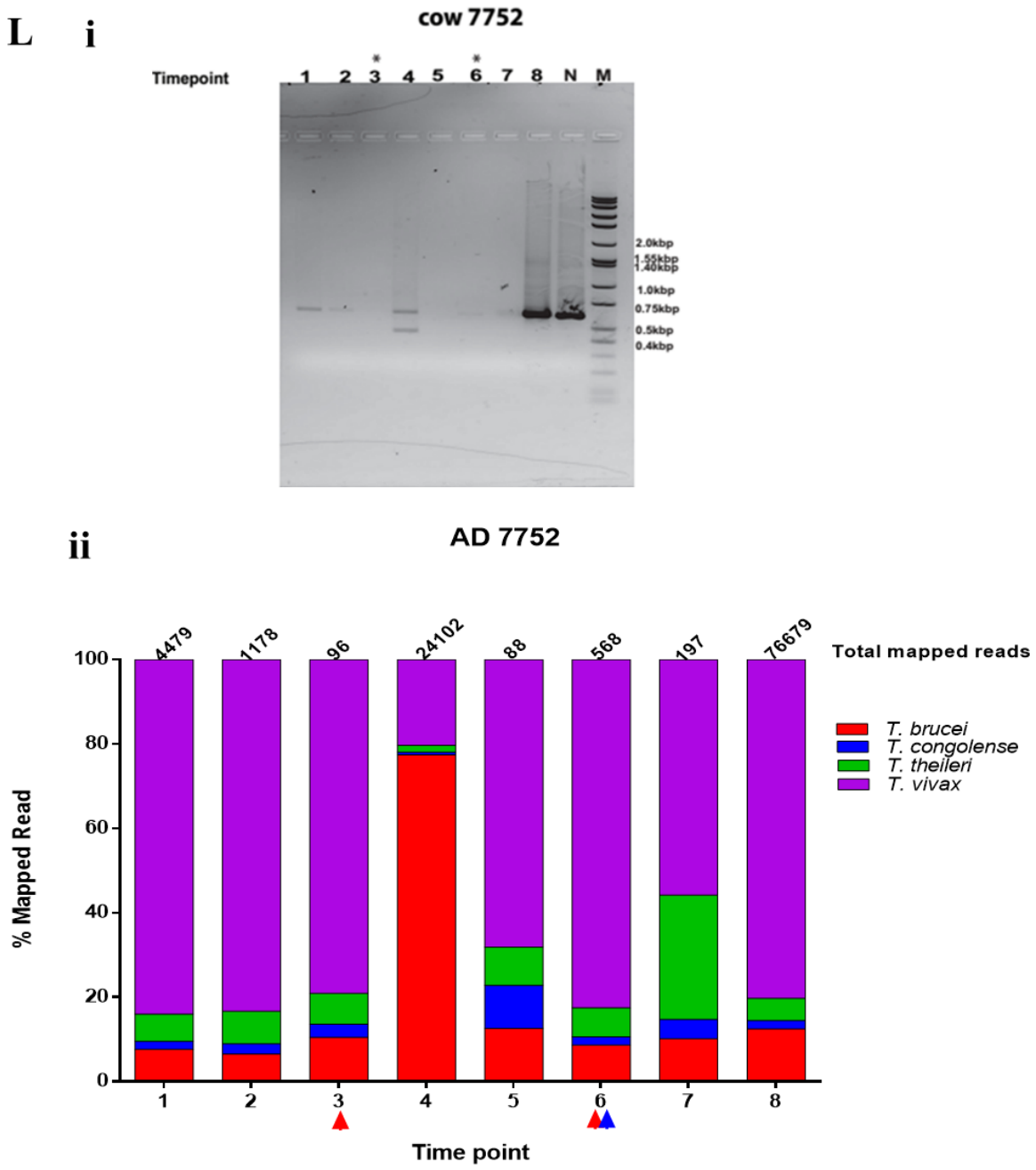
Appendix 1I: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



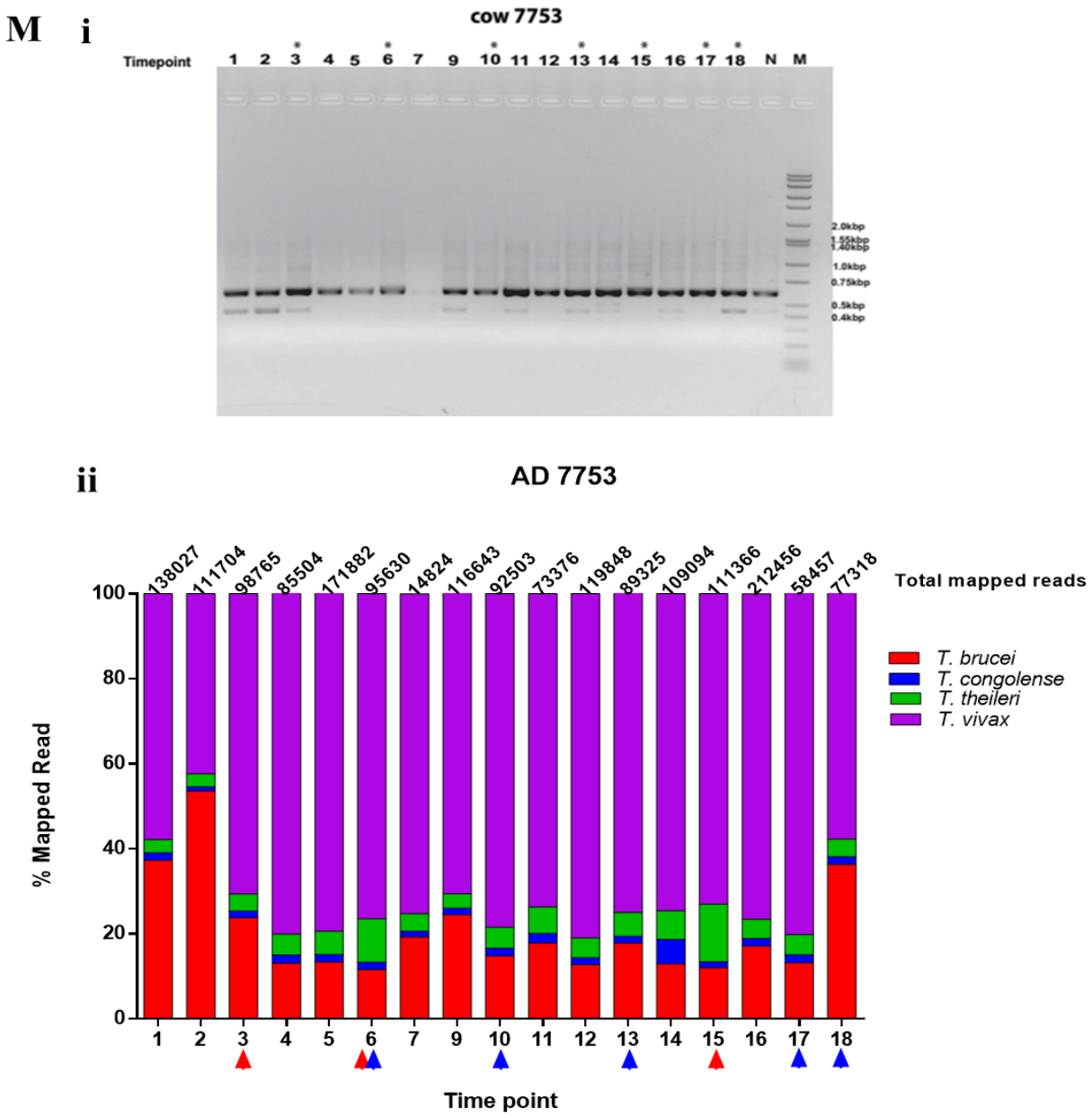
Appendix 1J: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



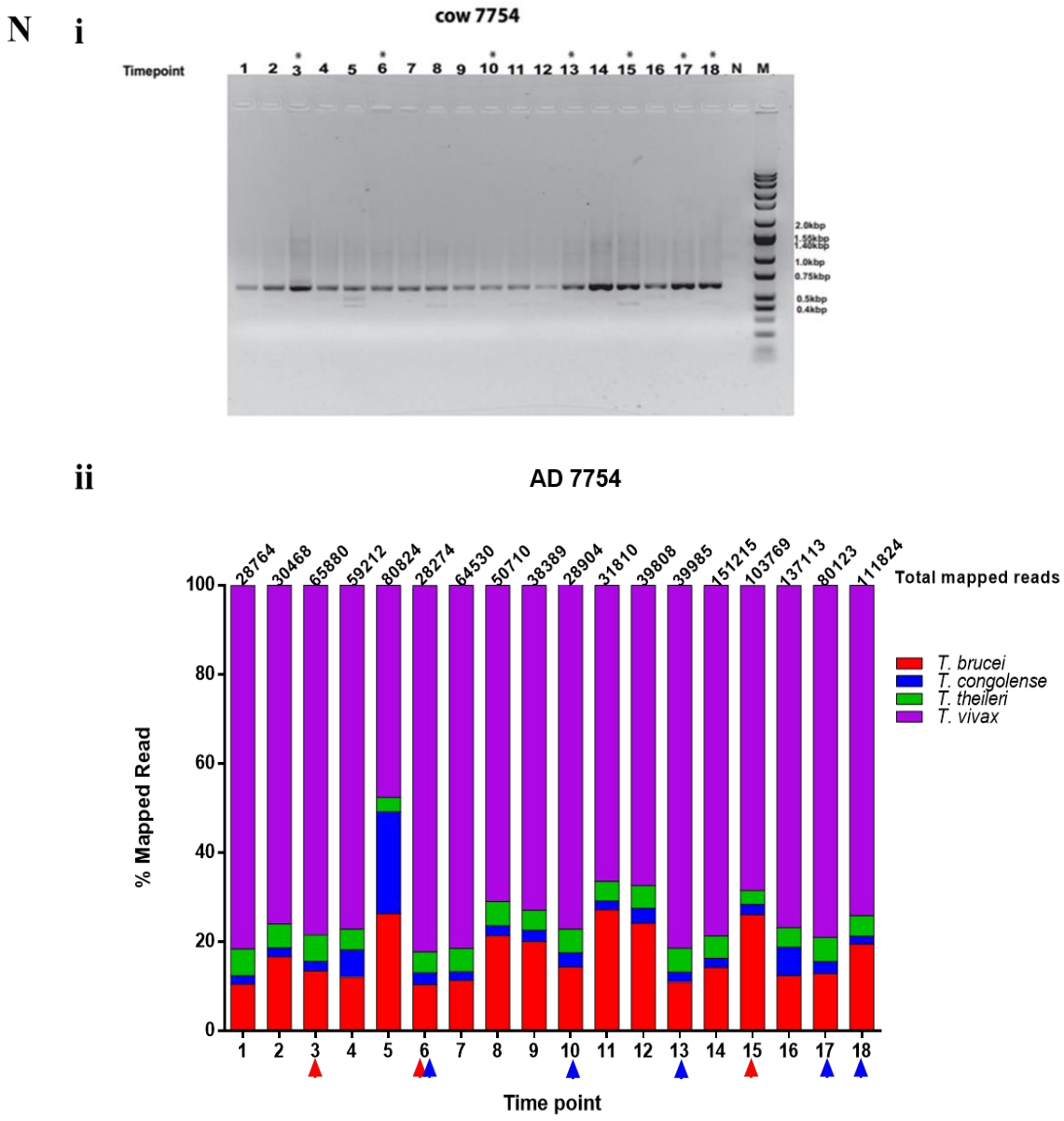
Appendix 1K: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



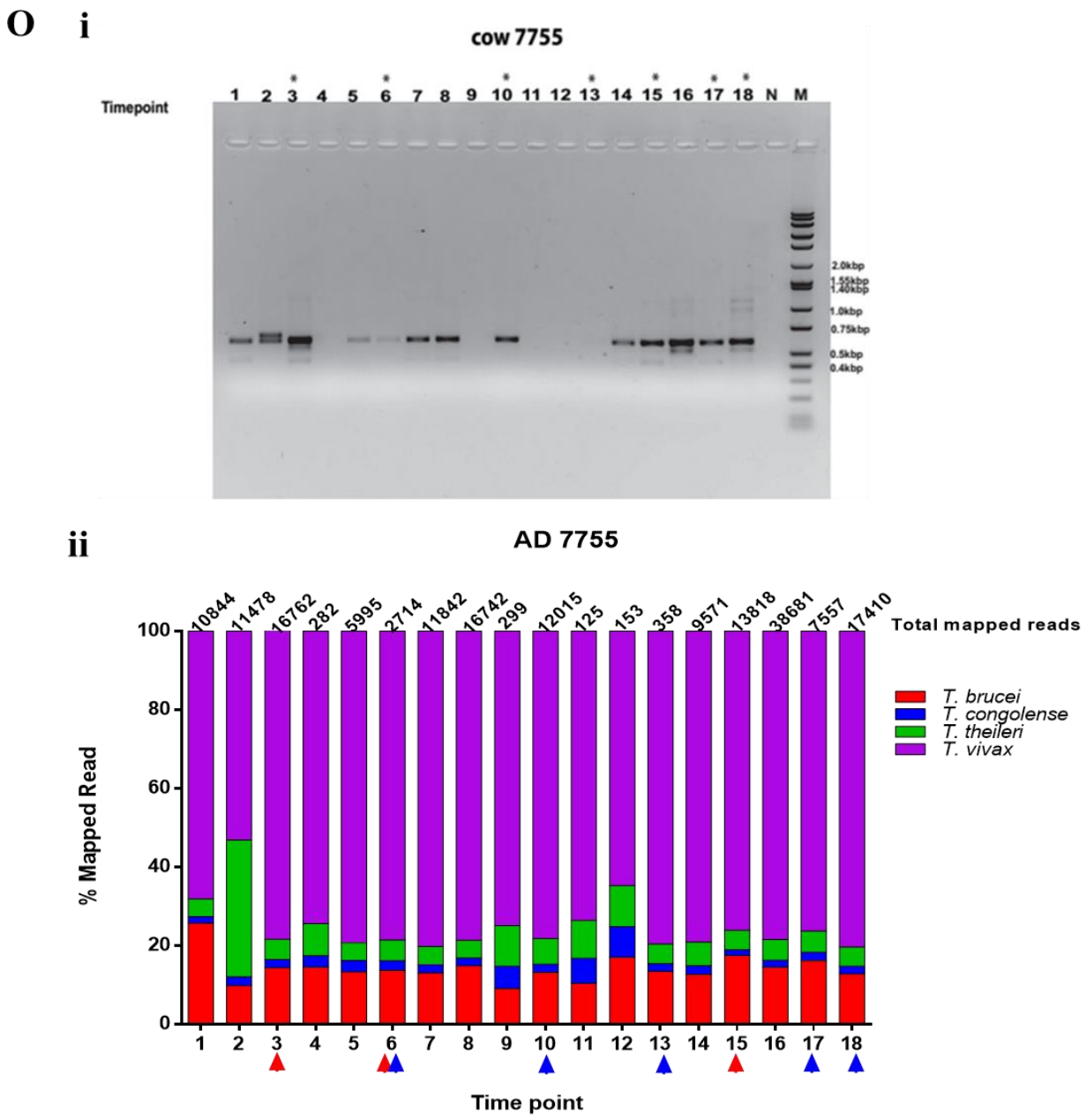
Appendix 1L: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



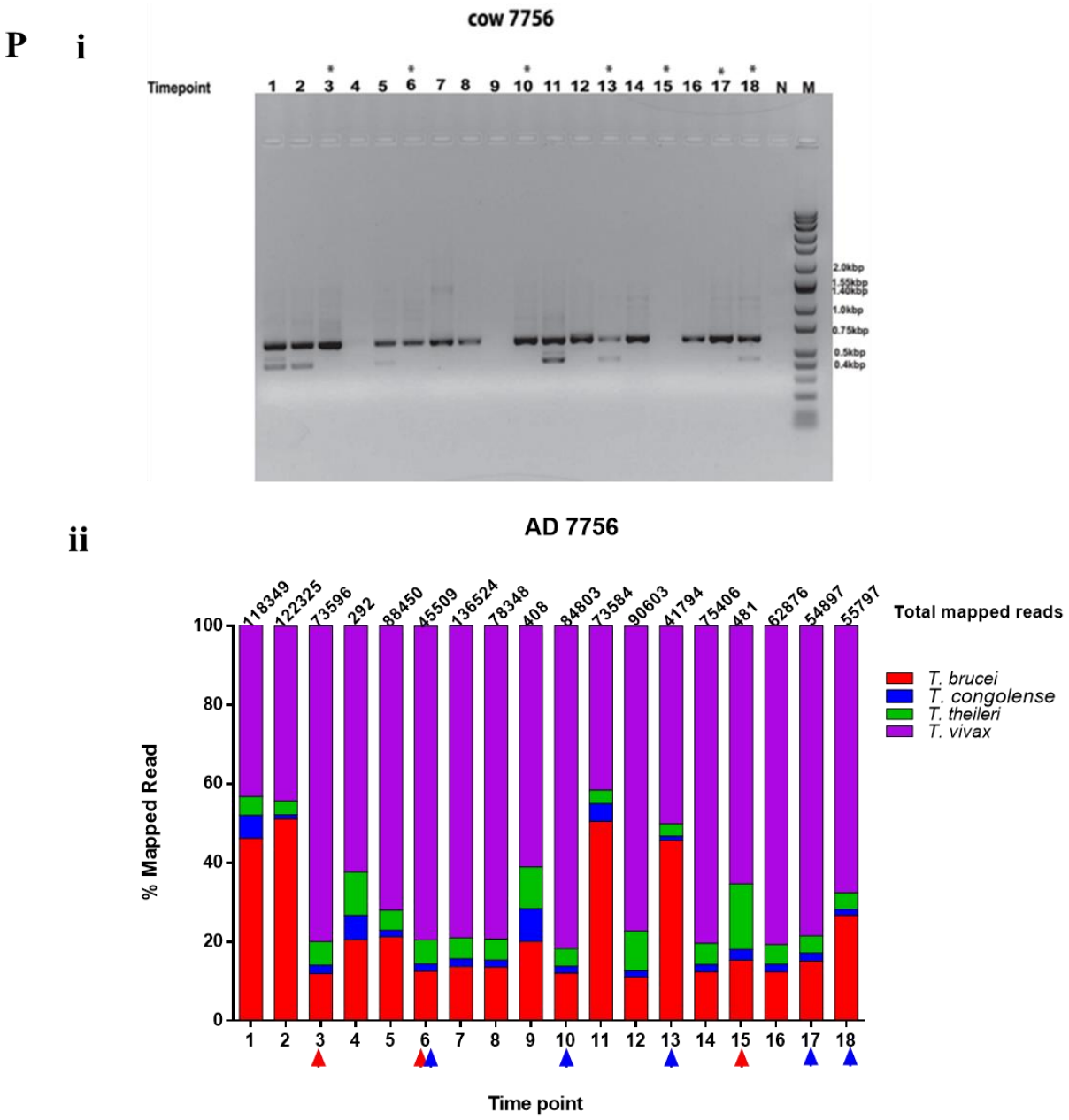
Appendix 1M: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



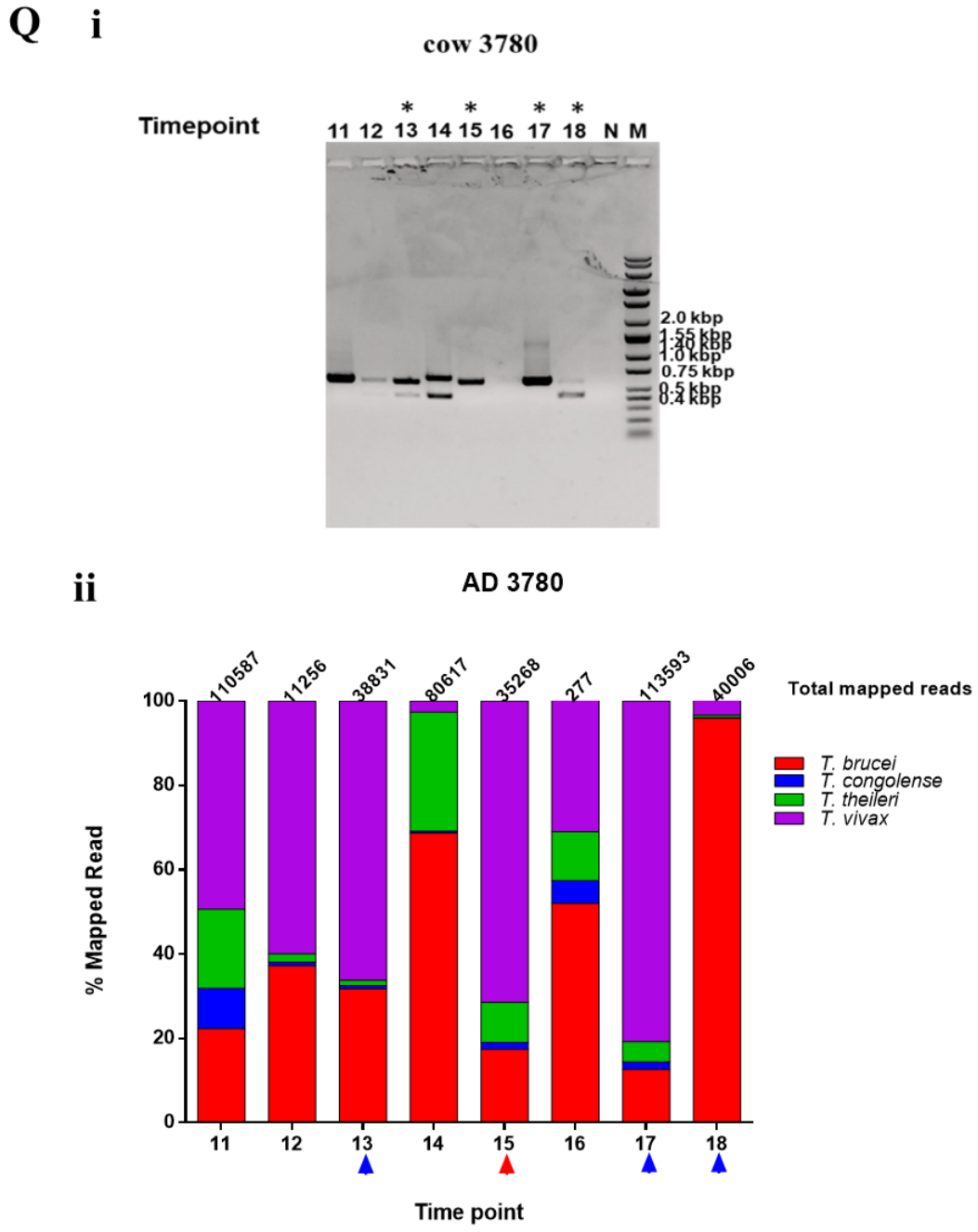
Appendix 1N: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



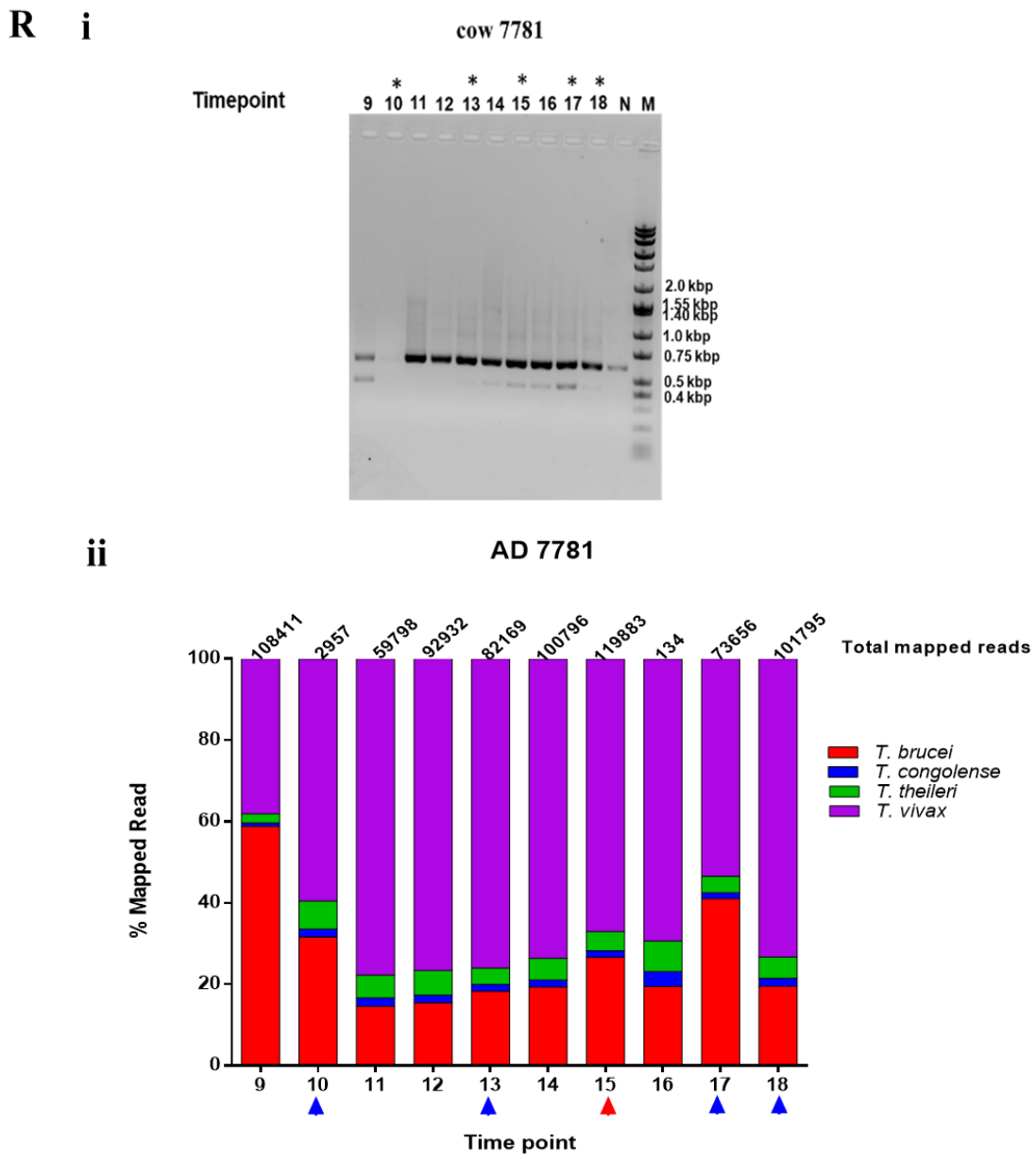
Appendix 1O: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



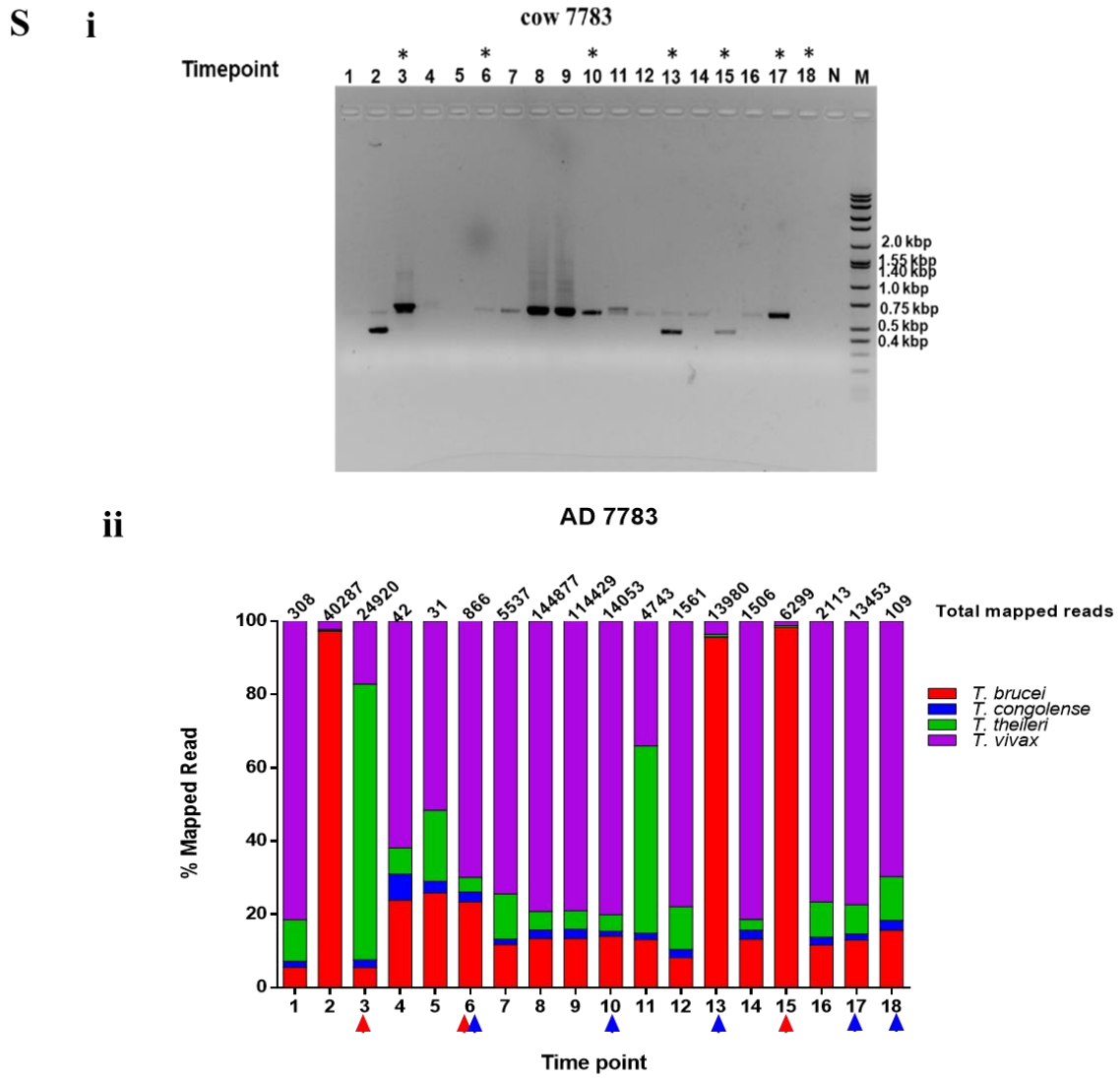
Appendix 1P: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



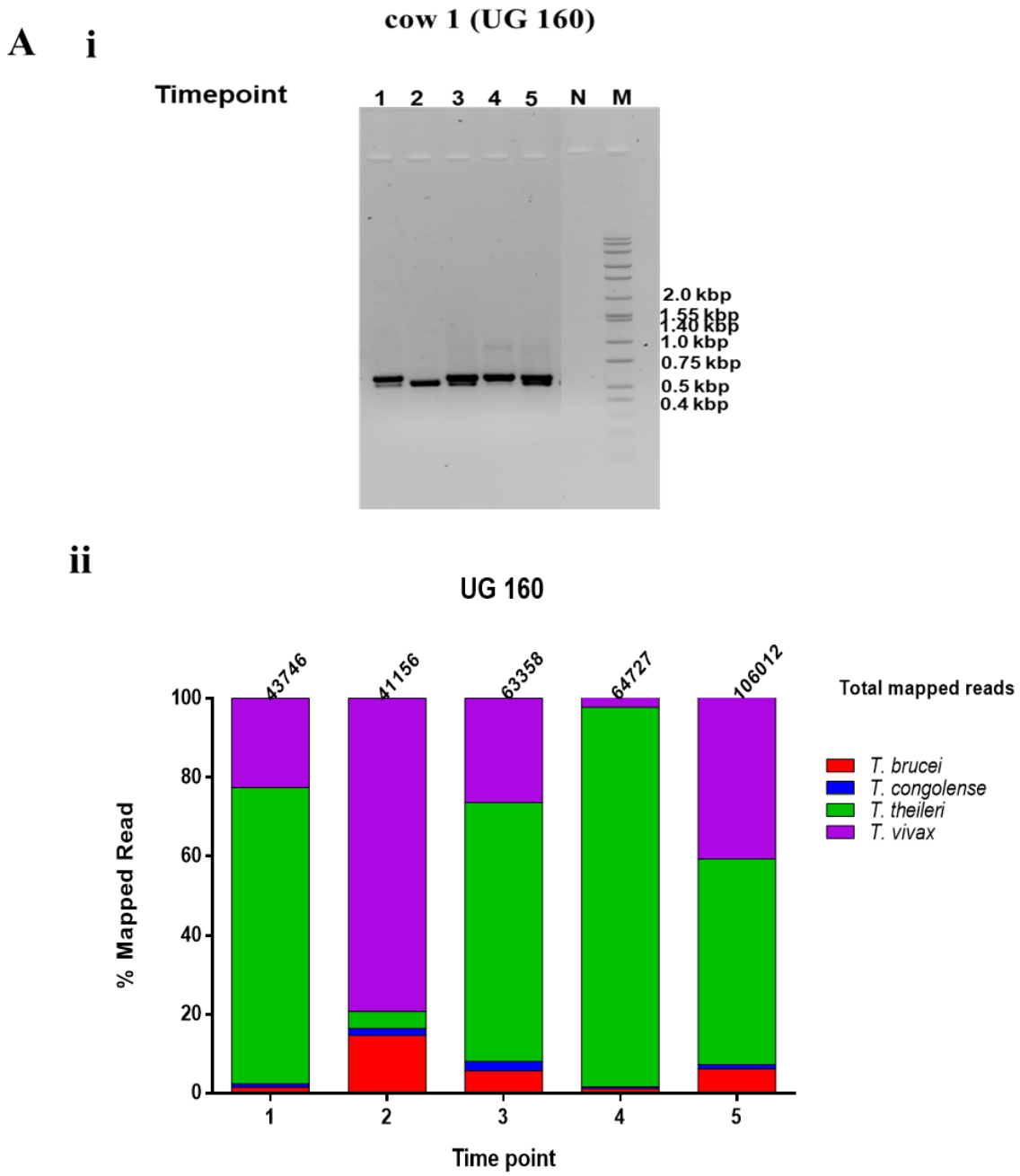
Appendix 1Q: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



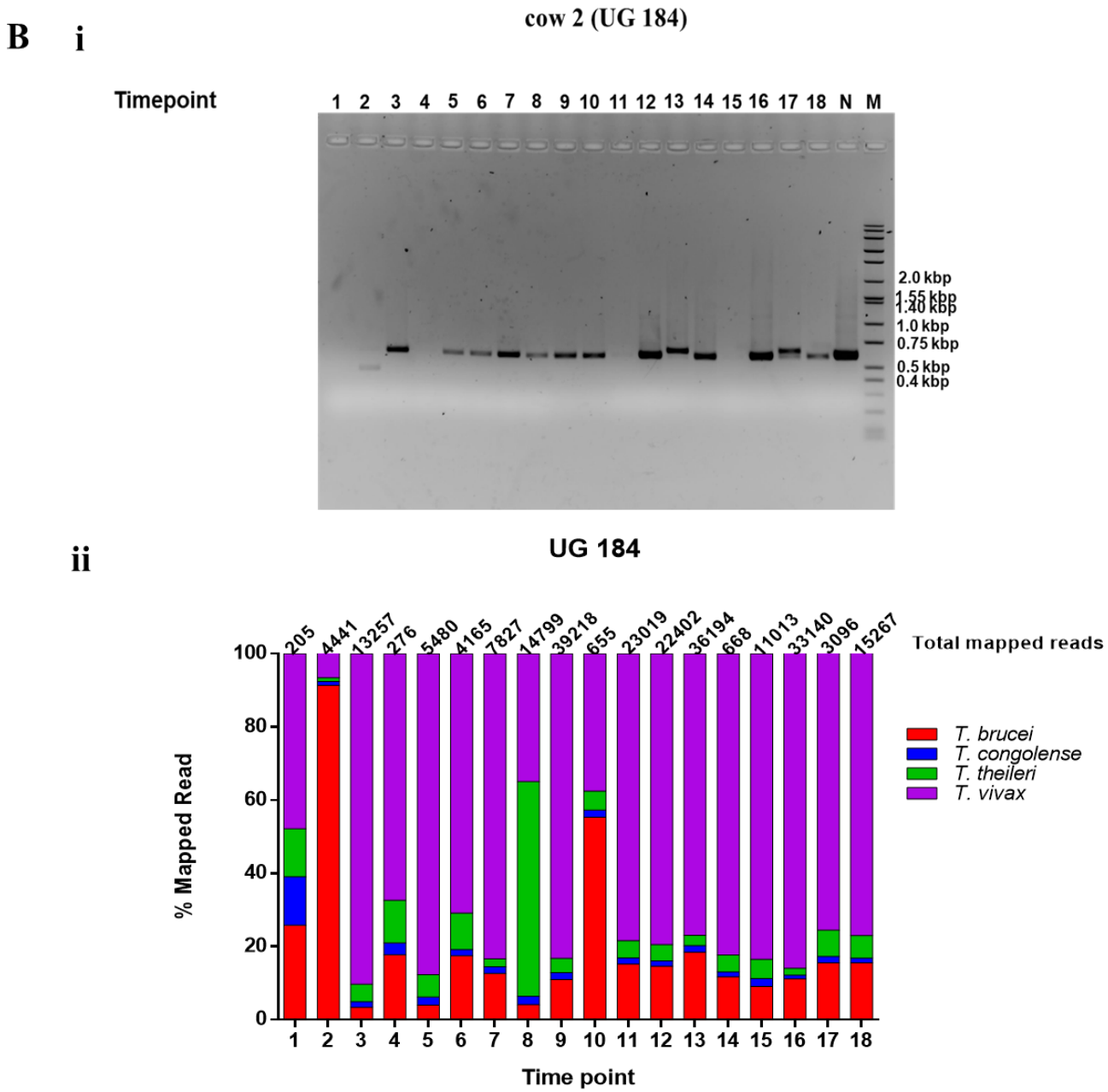
Appendix 1R: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



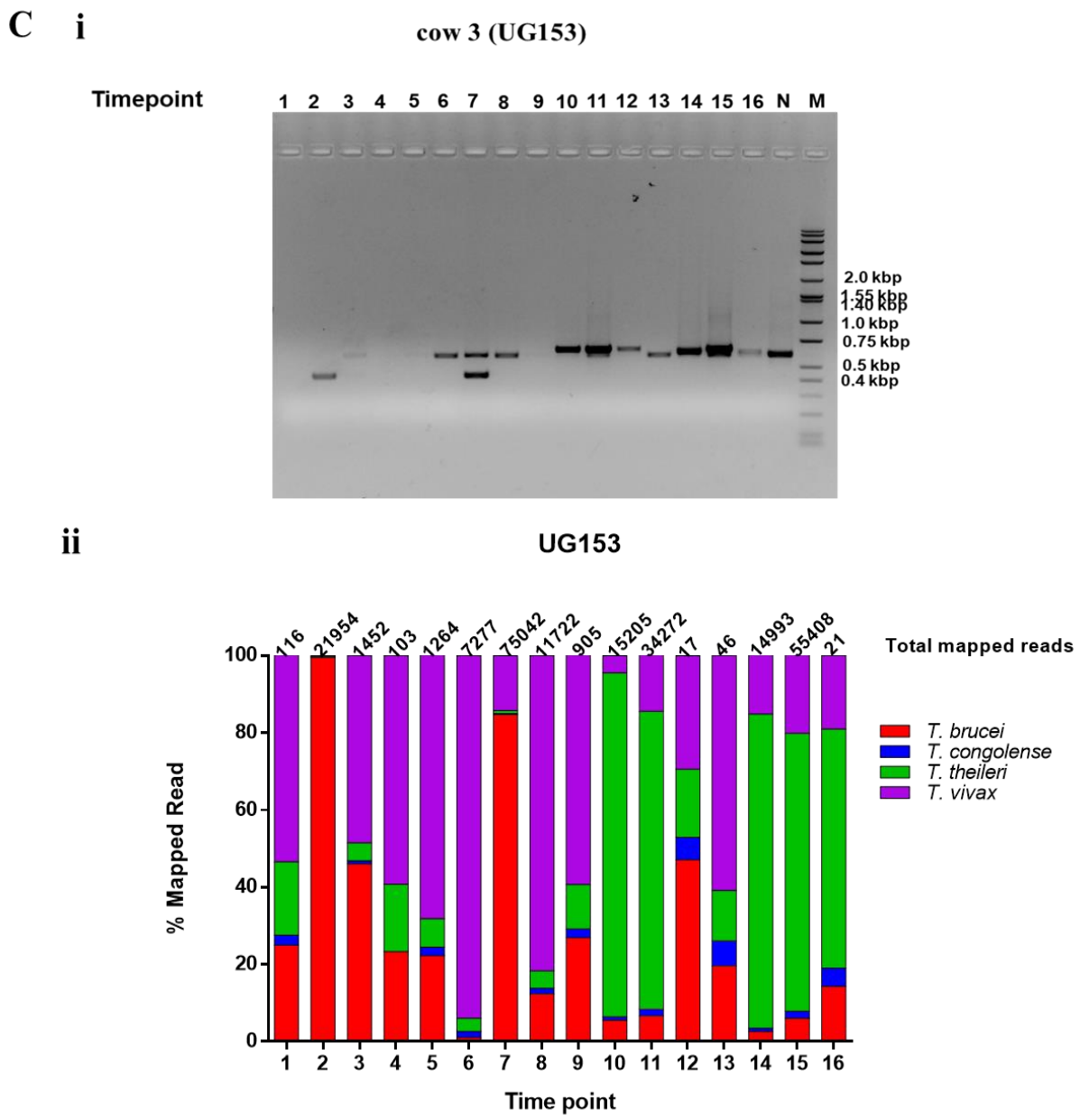
Appendix 1S: Characterization of trypanosome lifetime infections in individual cattle at Adidome study site using (i) nested PCR and (ii) next generation sequencing. Numbers with arrows and asterisks (*) represent treatment period with trypanocides for each time point. The blue and red arrows represent treatment with diminazene aceturate and isometamidium chloride, respectively. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



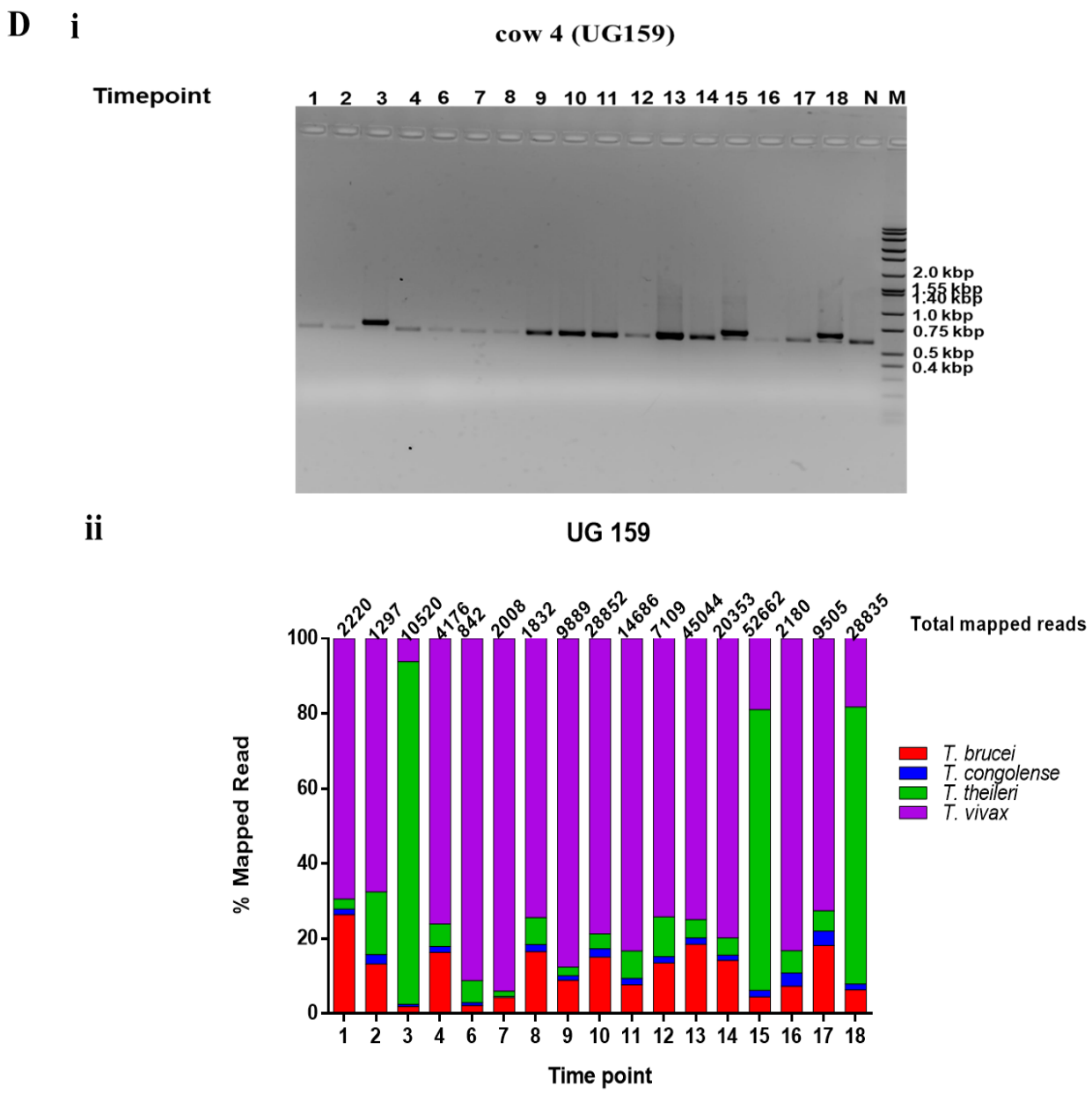
Appendix 2A: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



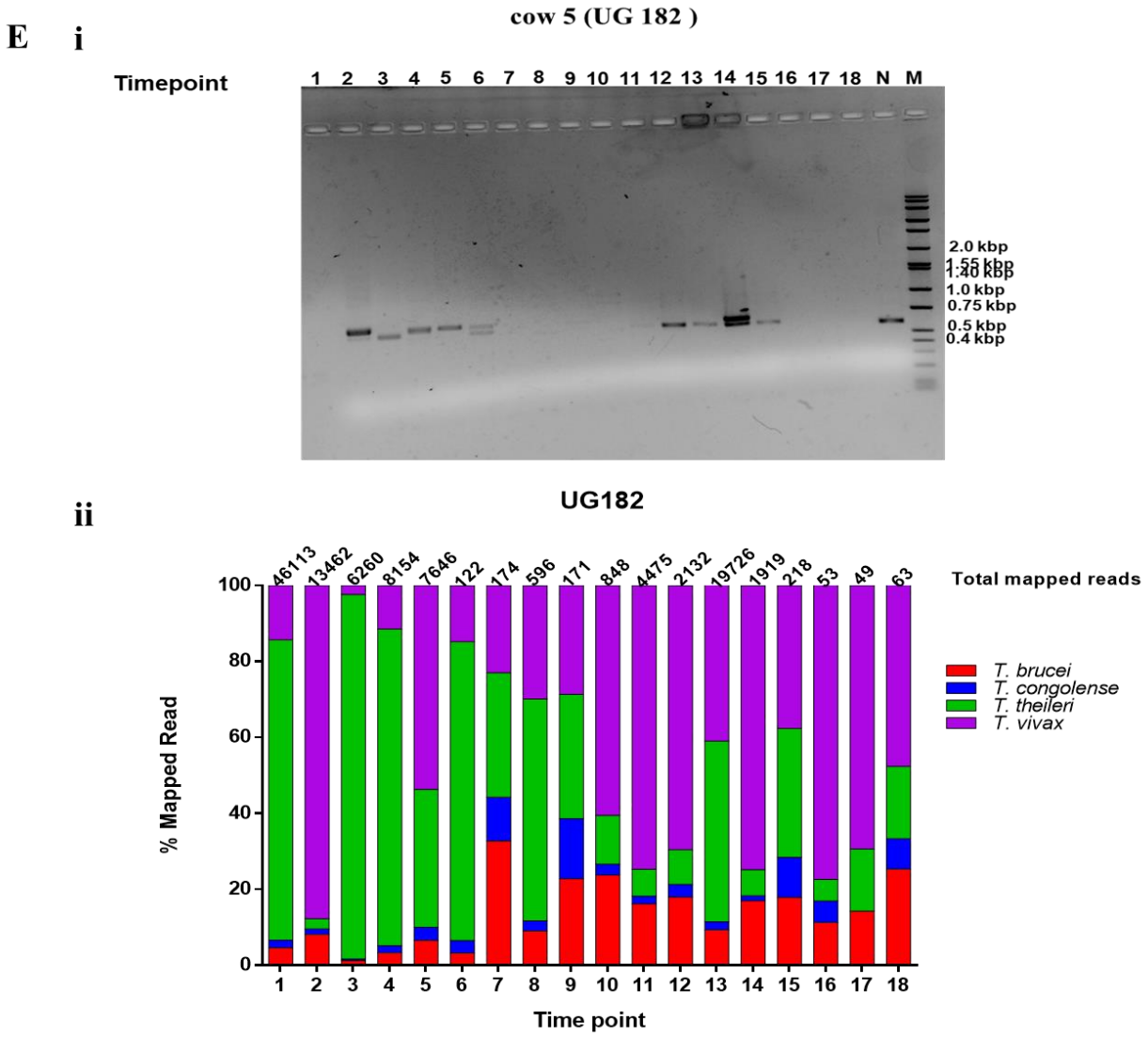
Appendix 2B: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



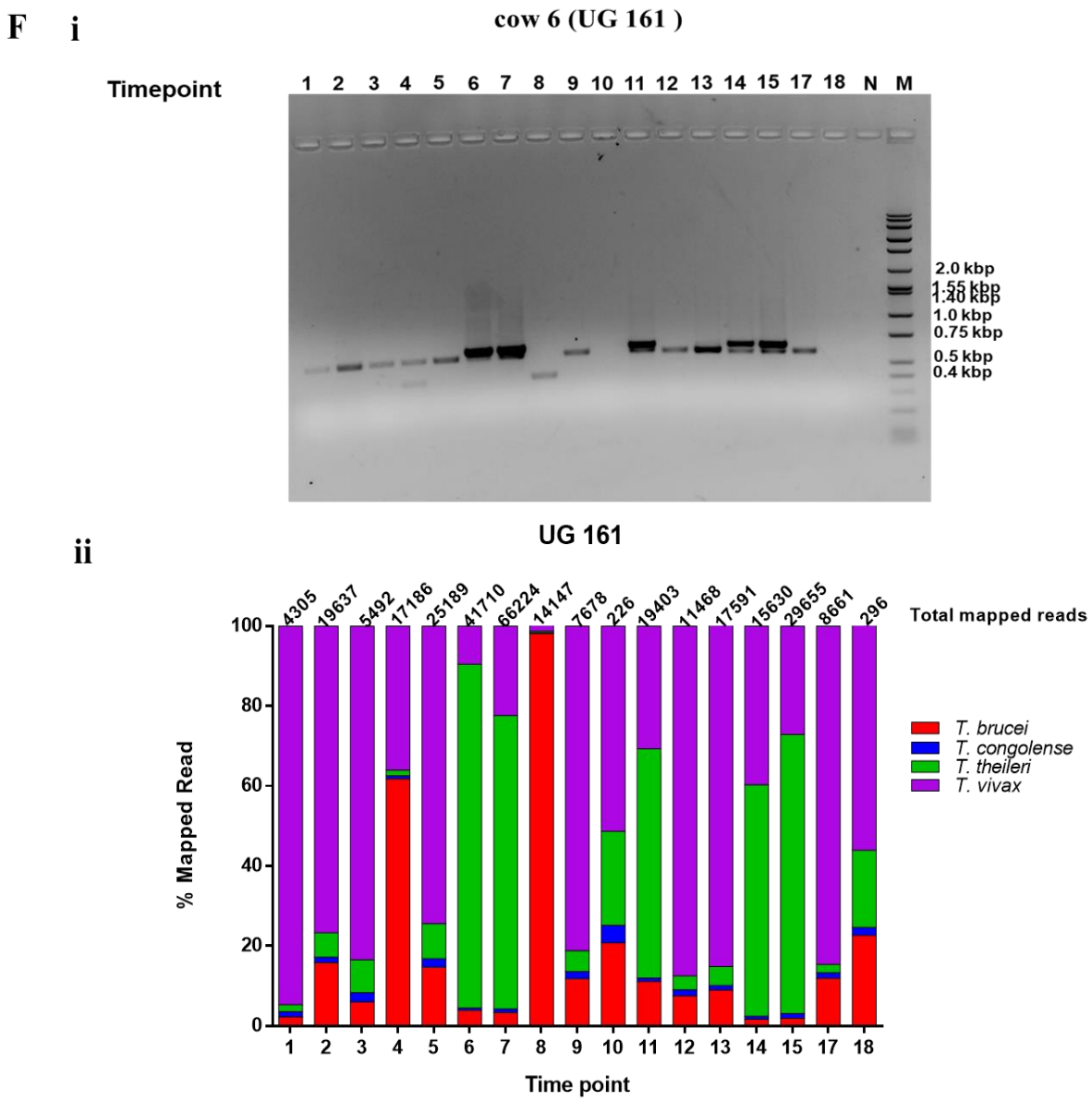
Appendix 2C: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



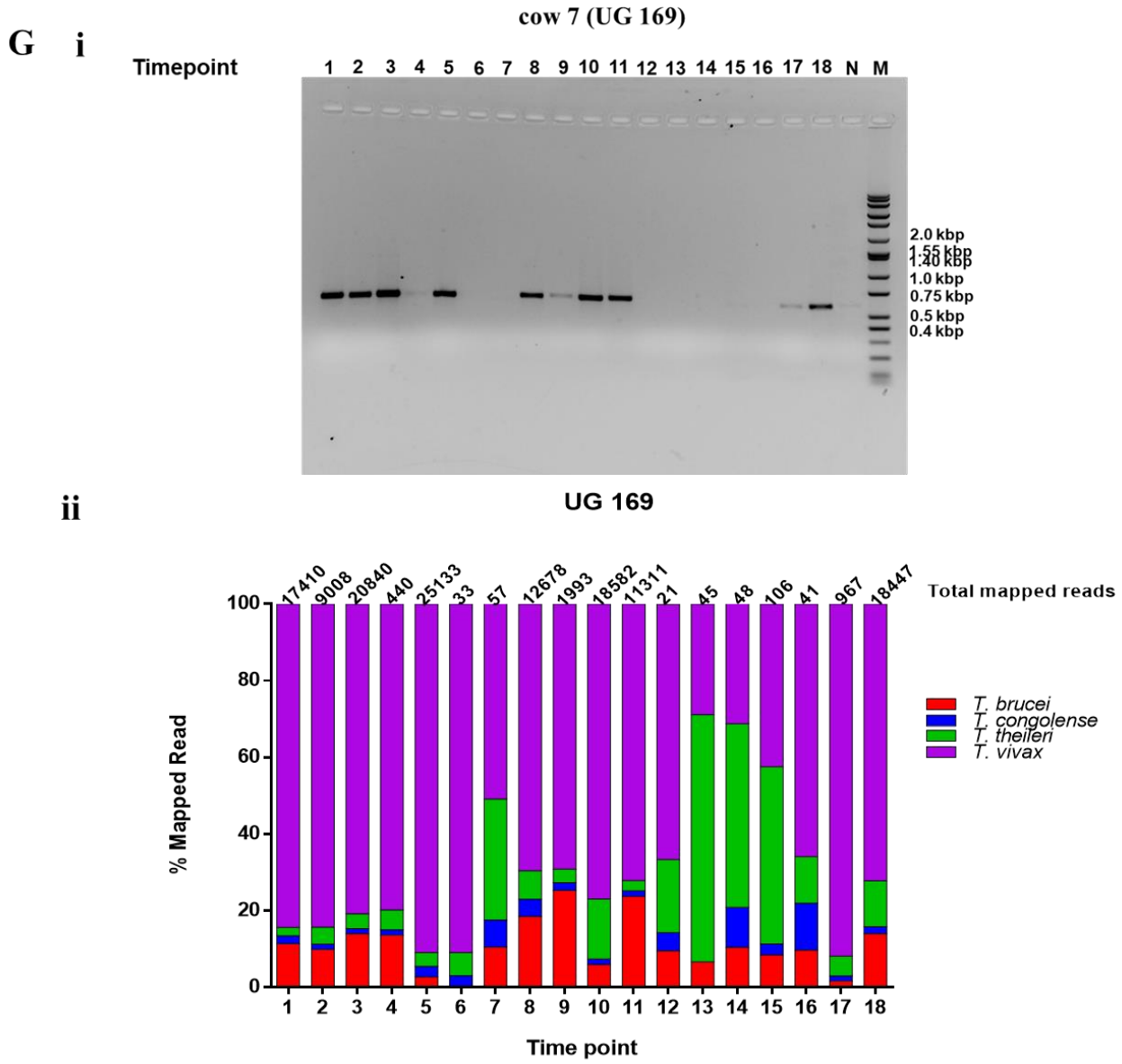
Appendix 2D: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



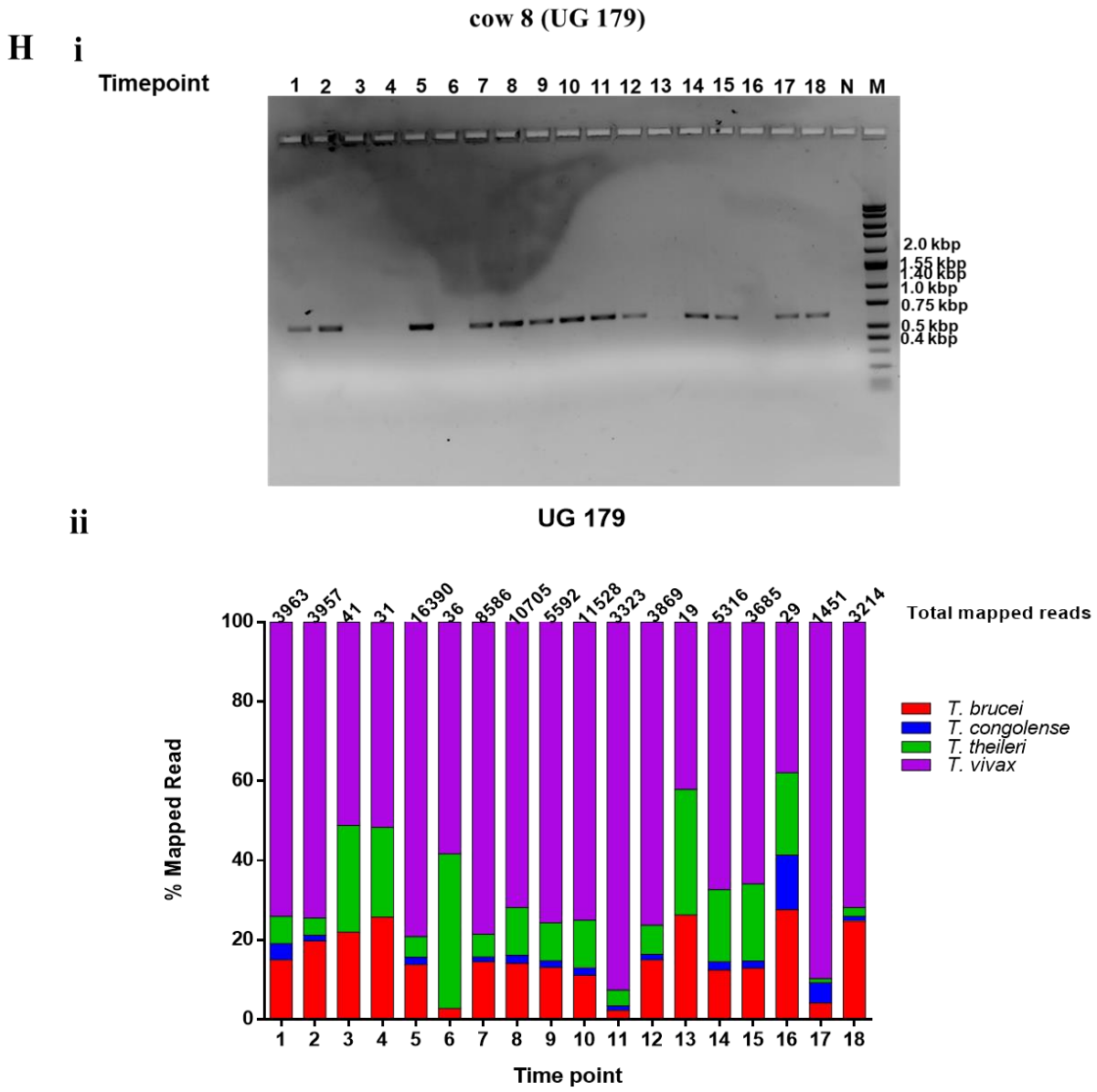
Appendix 2E: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



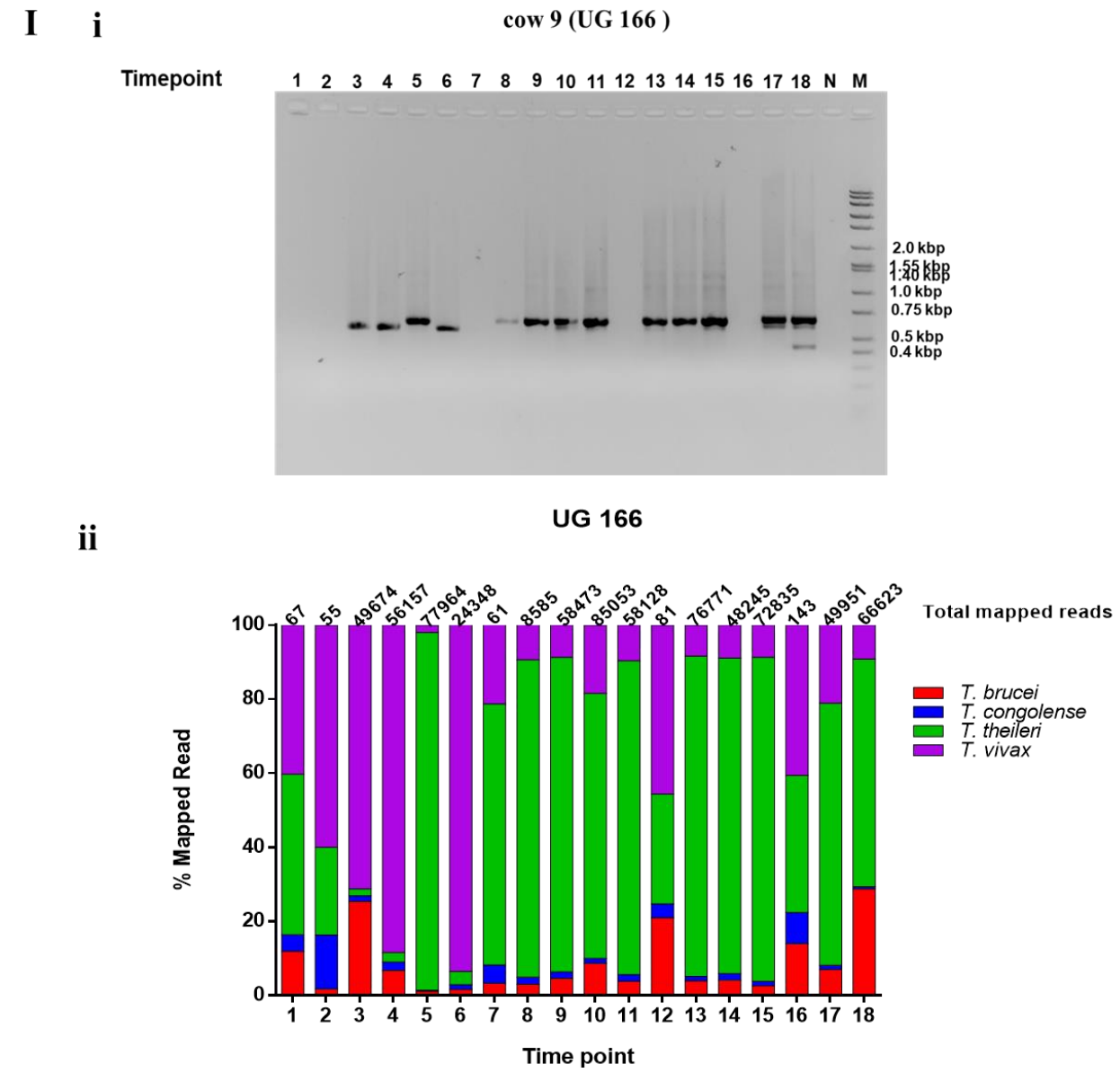
Appendix 2F: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



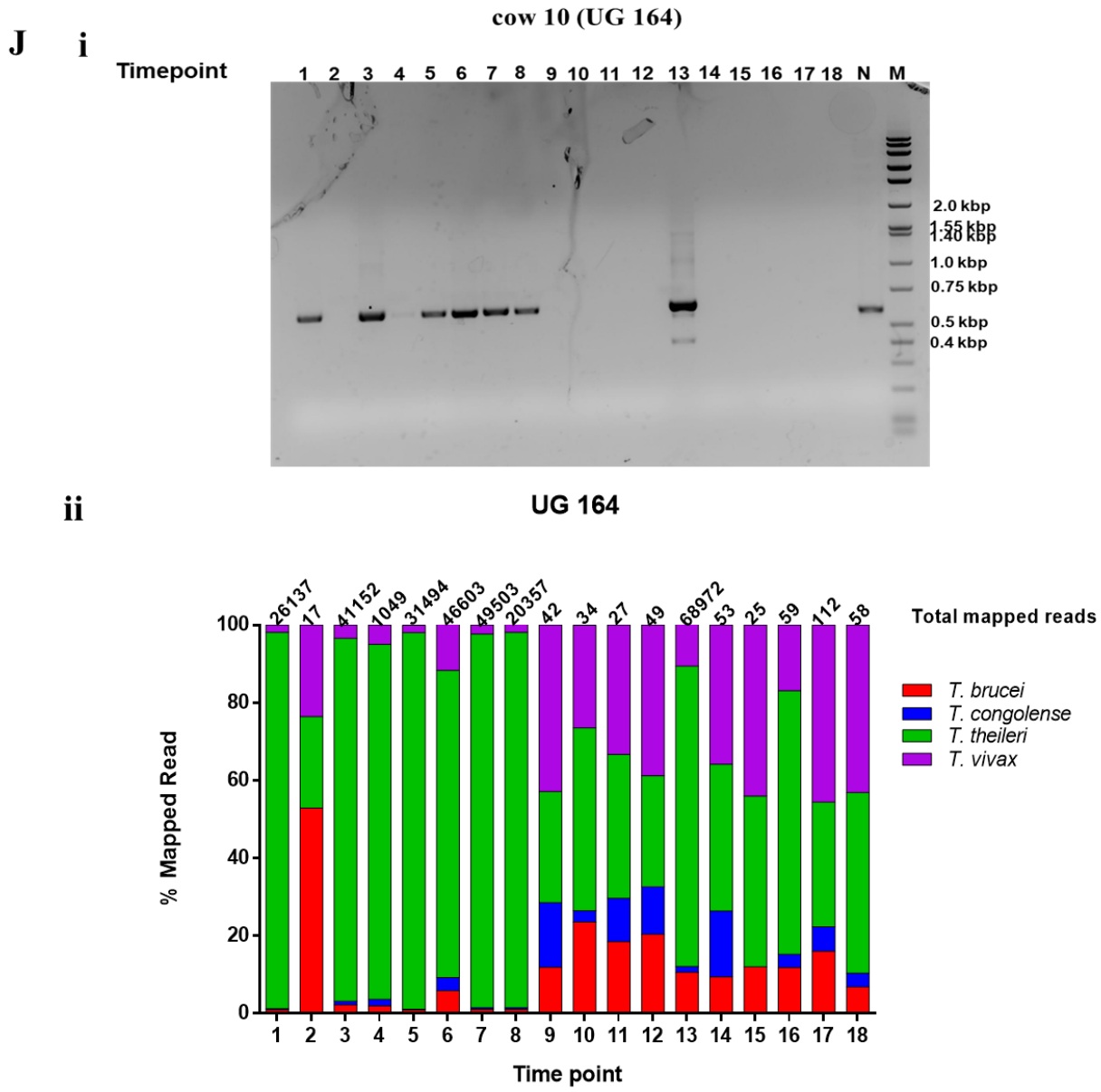
Appendix 2G: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



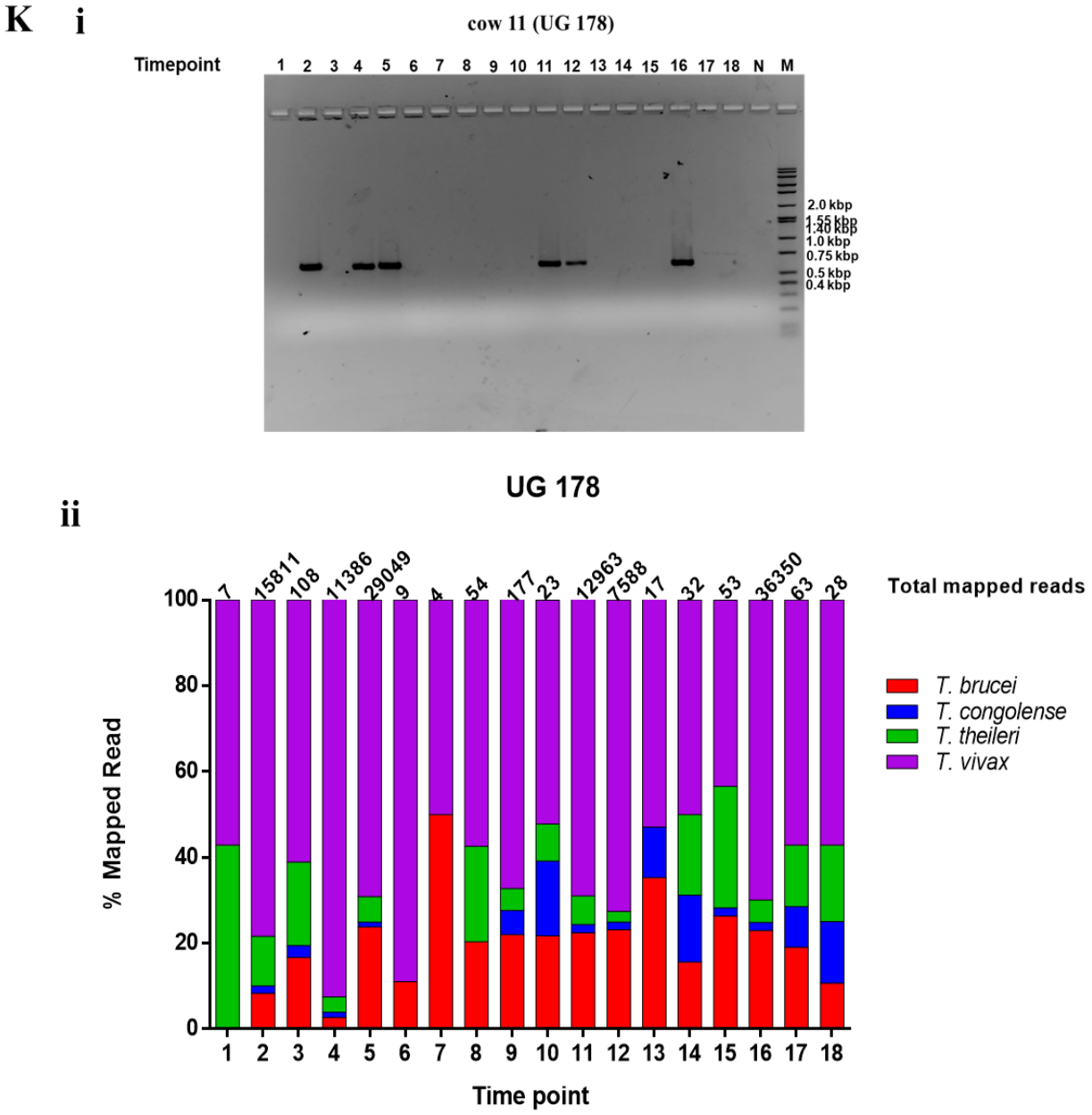
Appendix 2H: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



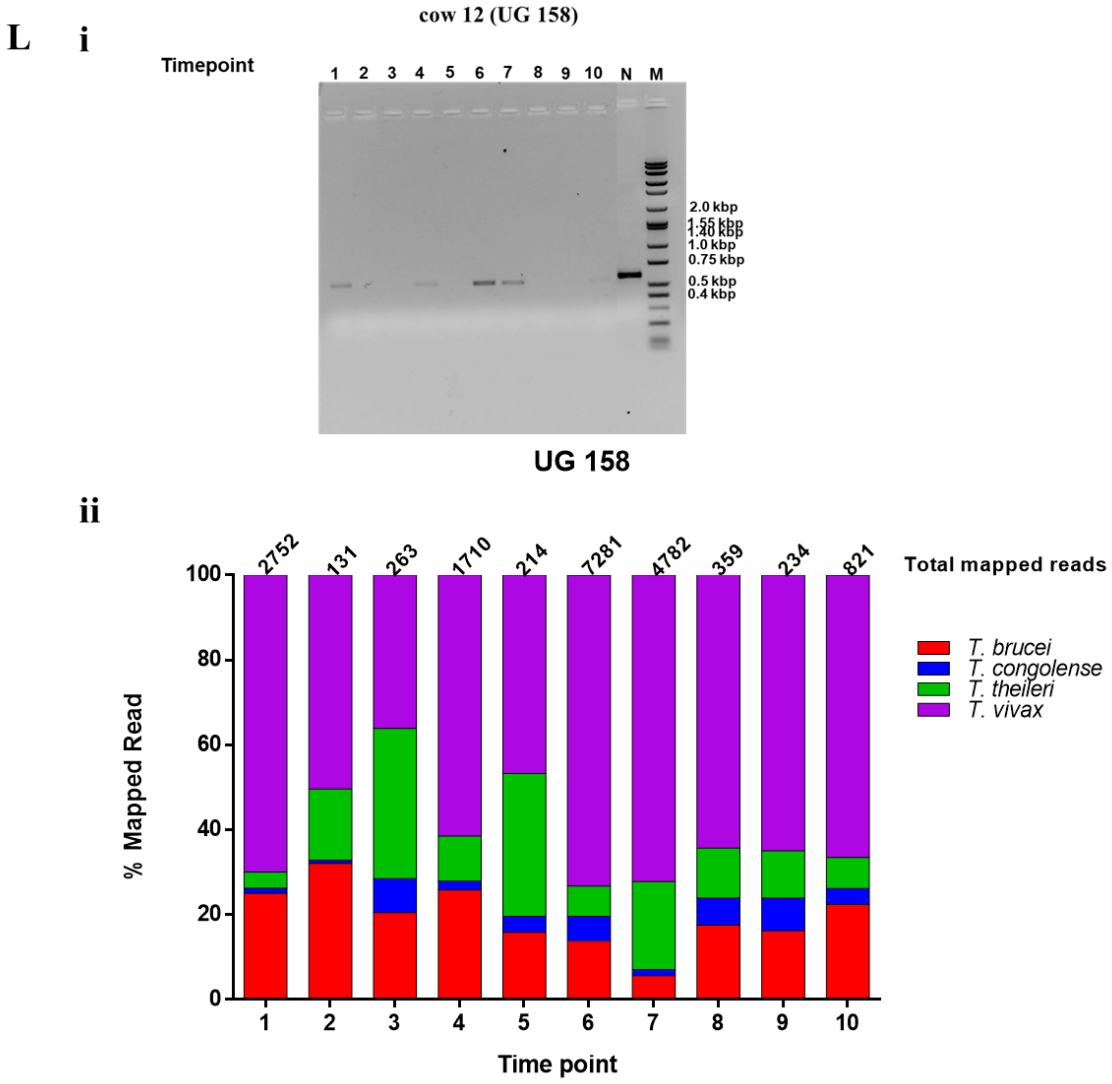
Appendix 2I: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



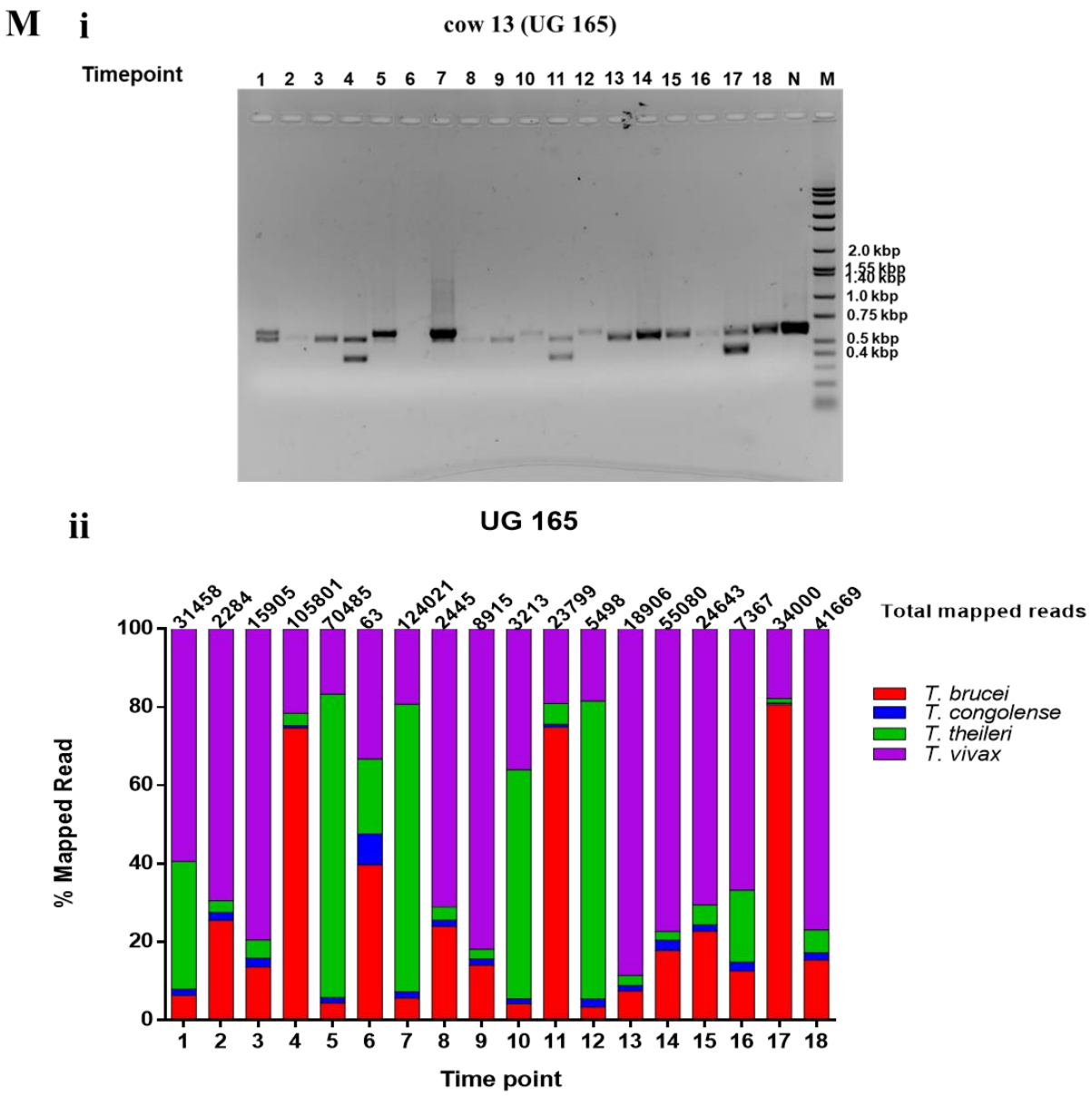
Appendix 2J: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



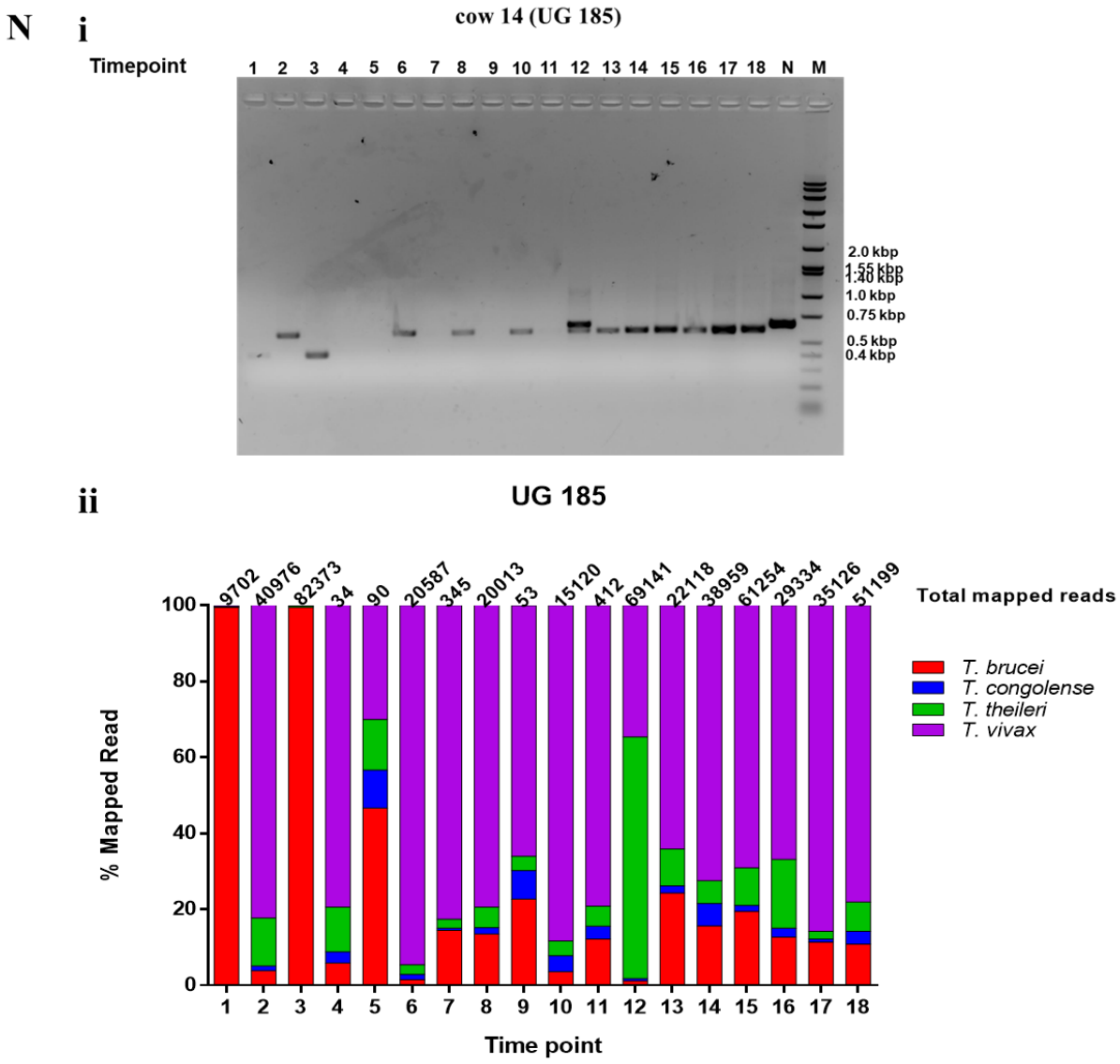
Appendix 2K: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



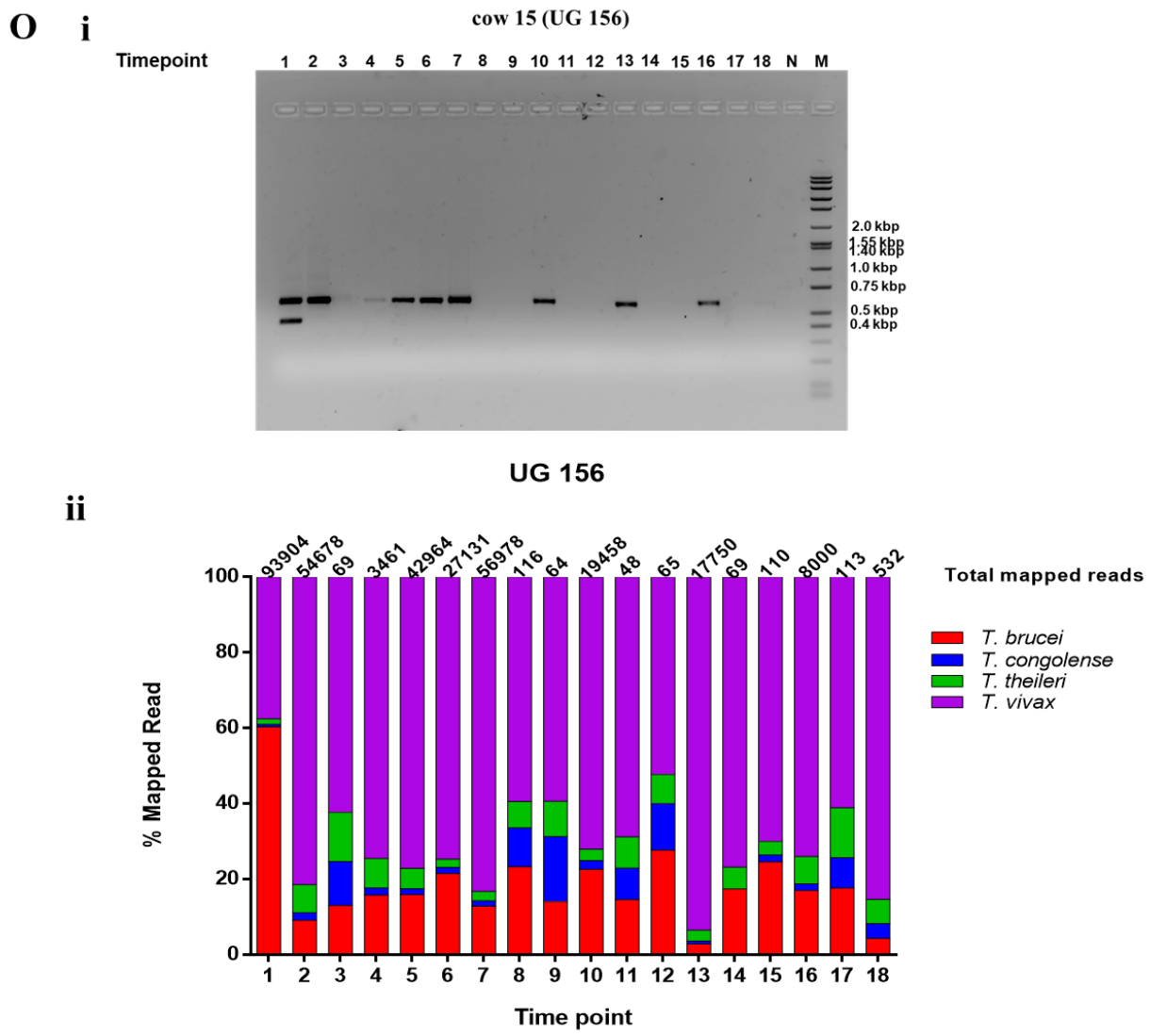
Appendix 2L: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



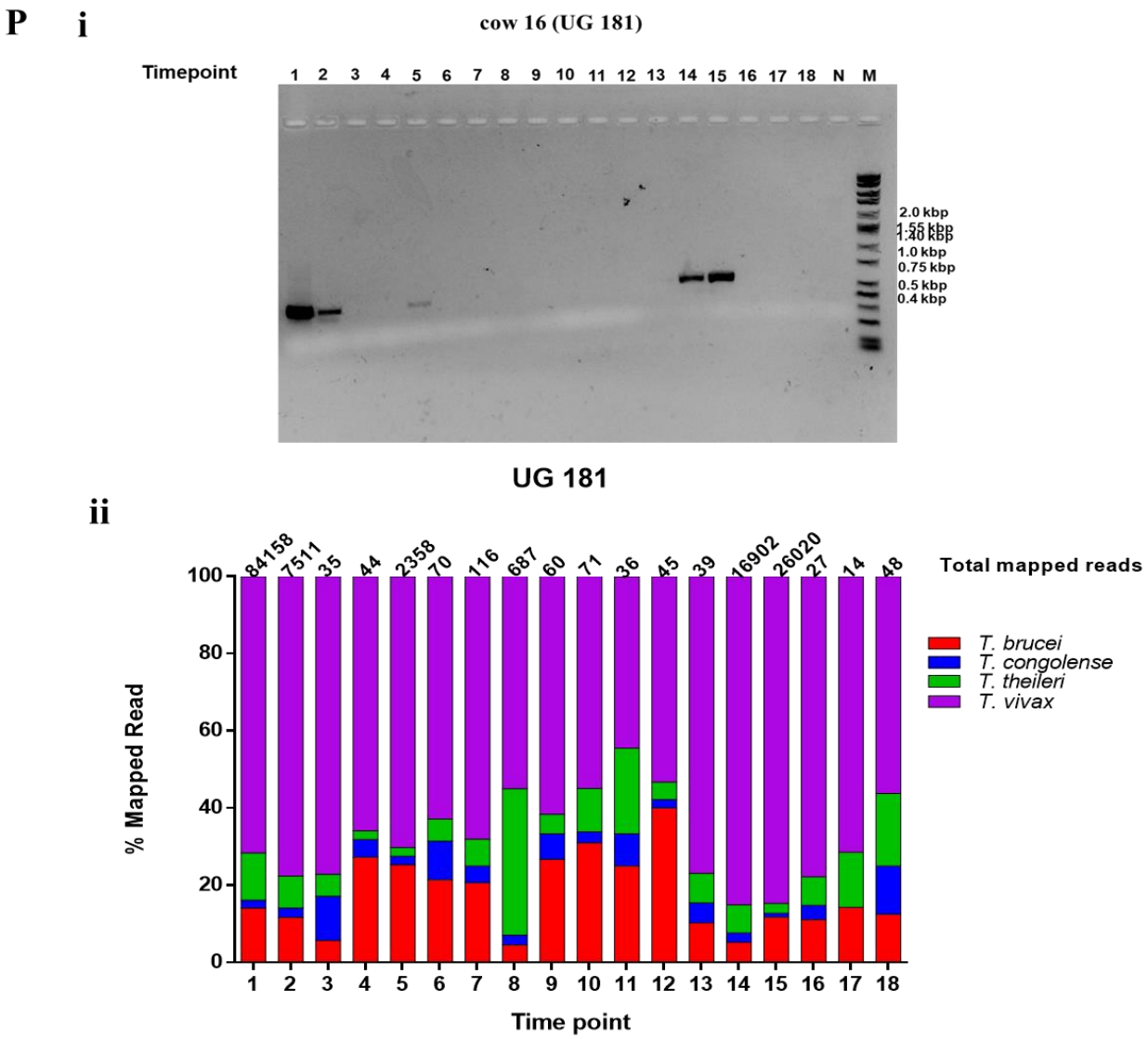
Appendix 2M: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



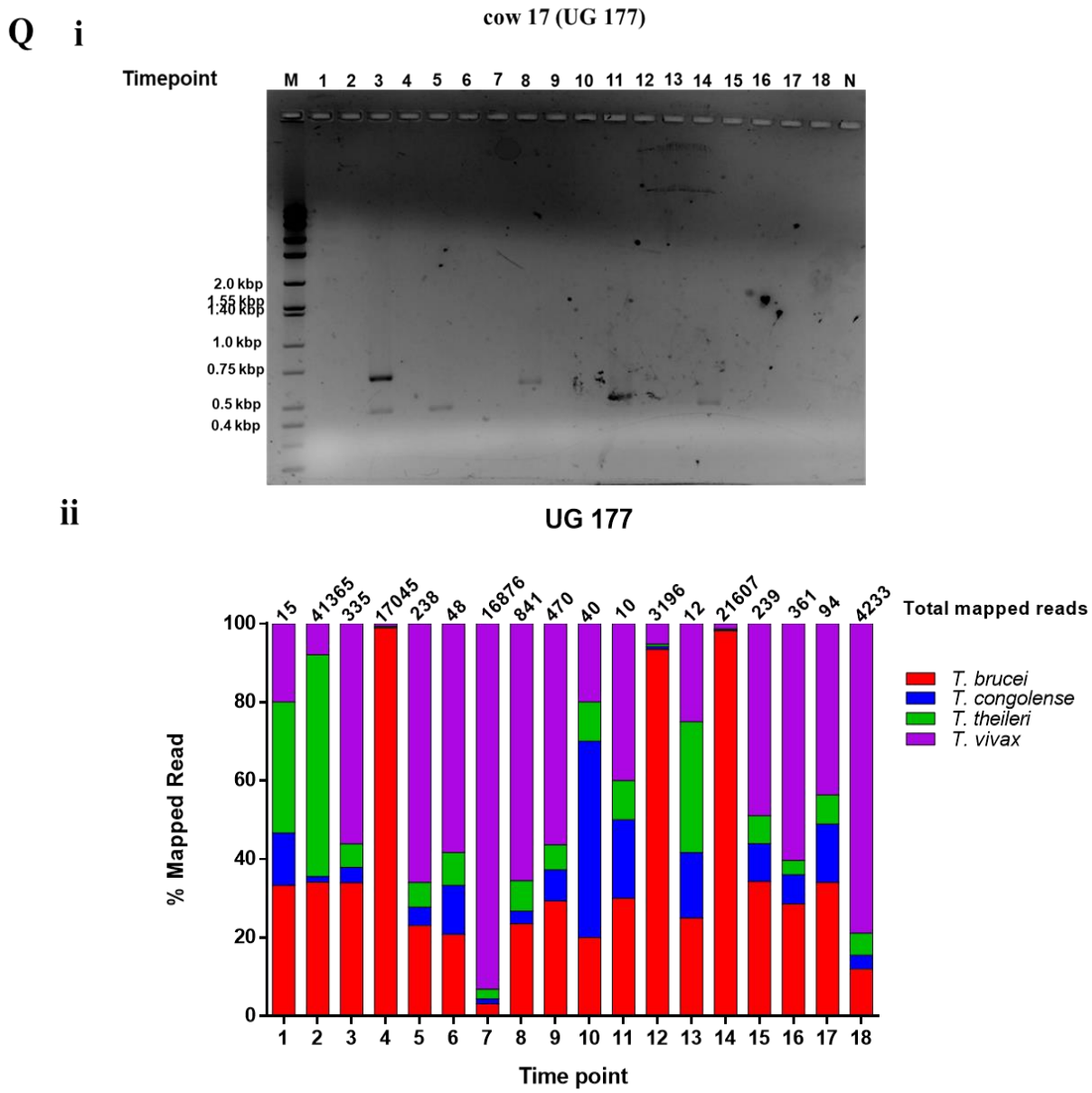
Appendix 2N: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



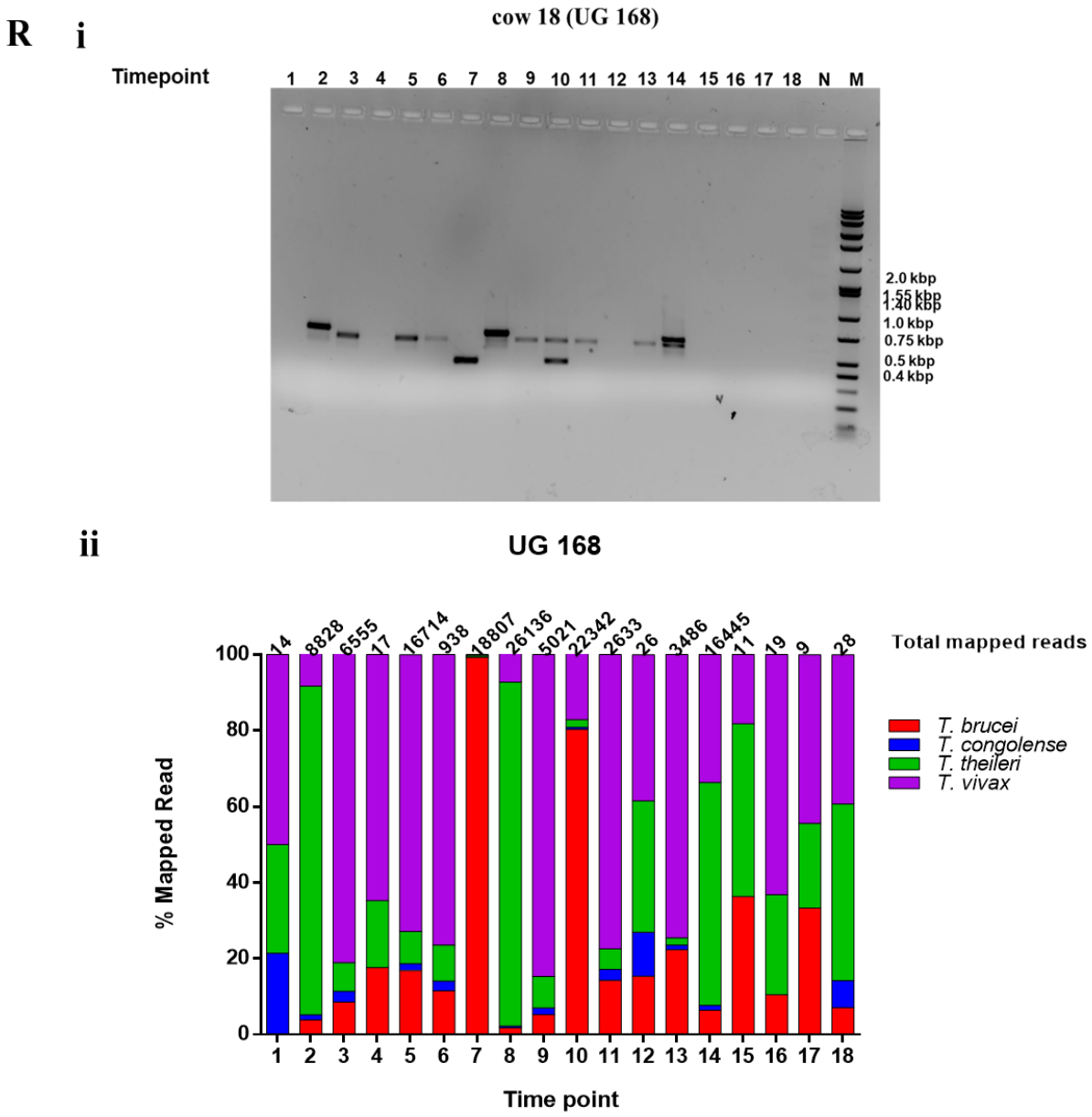
Appendix 2O: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



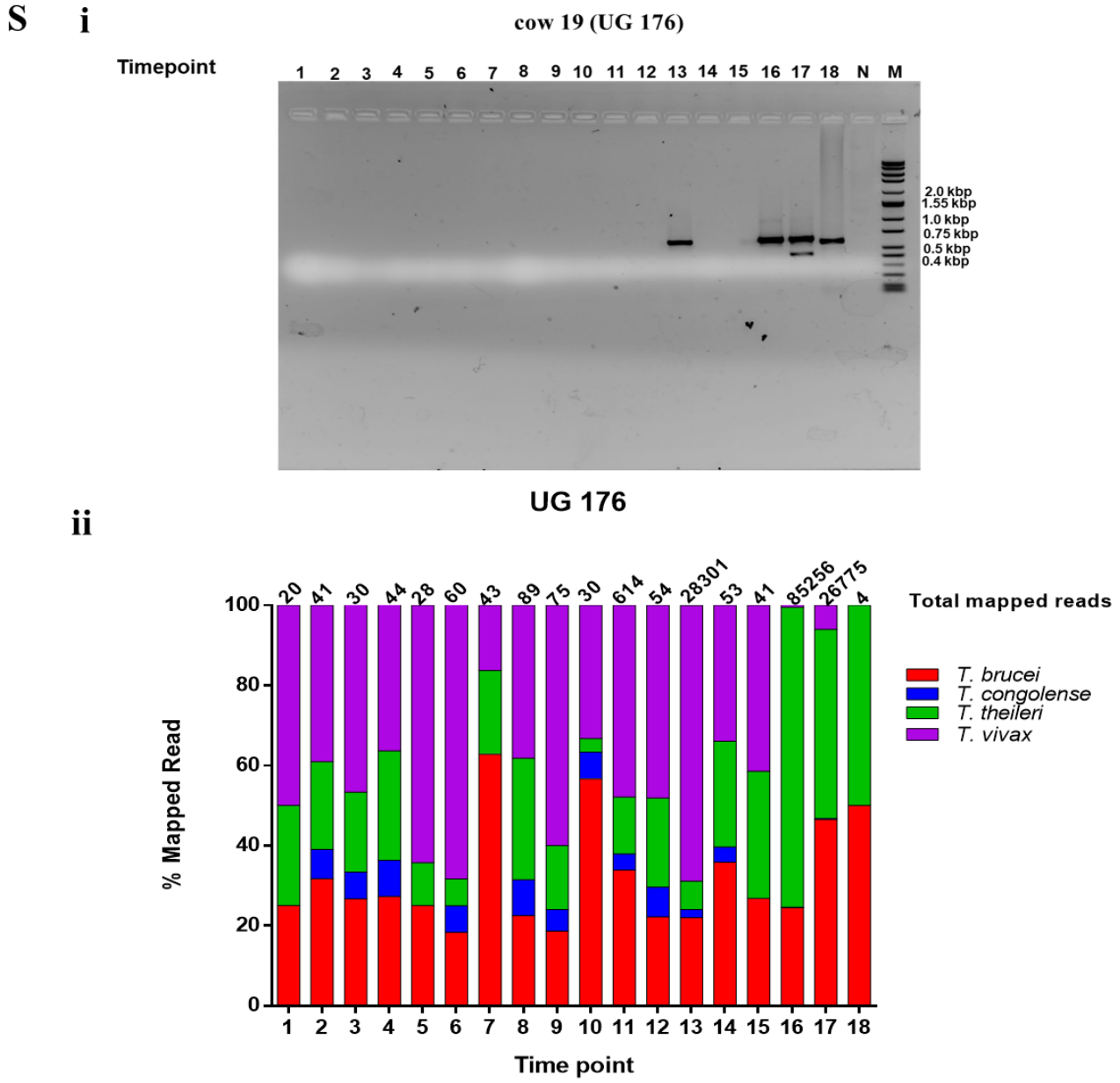
Appendix 2P: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



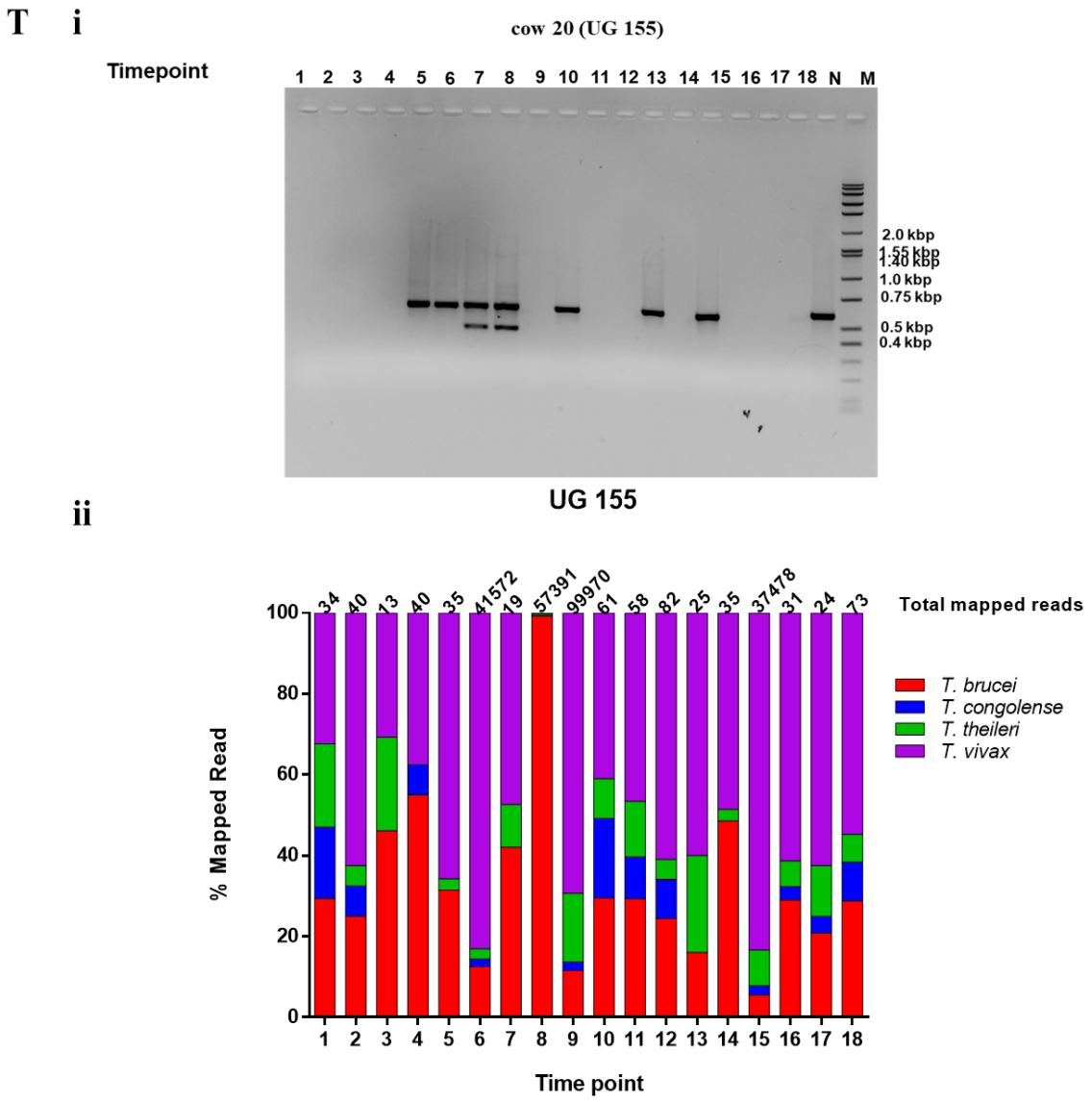
Appendix 2Q: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.



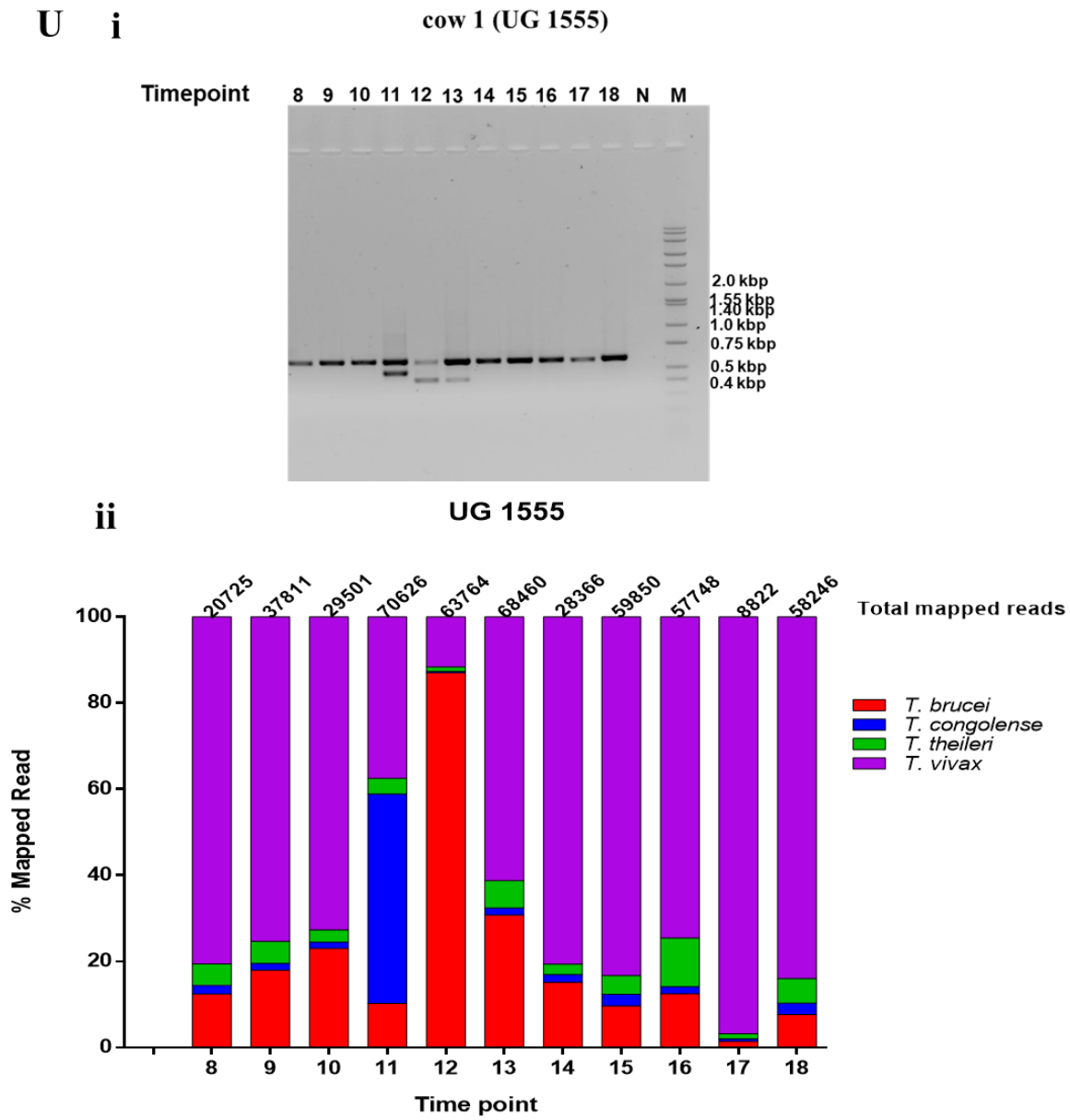
Appendix 2R: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



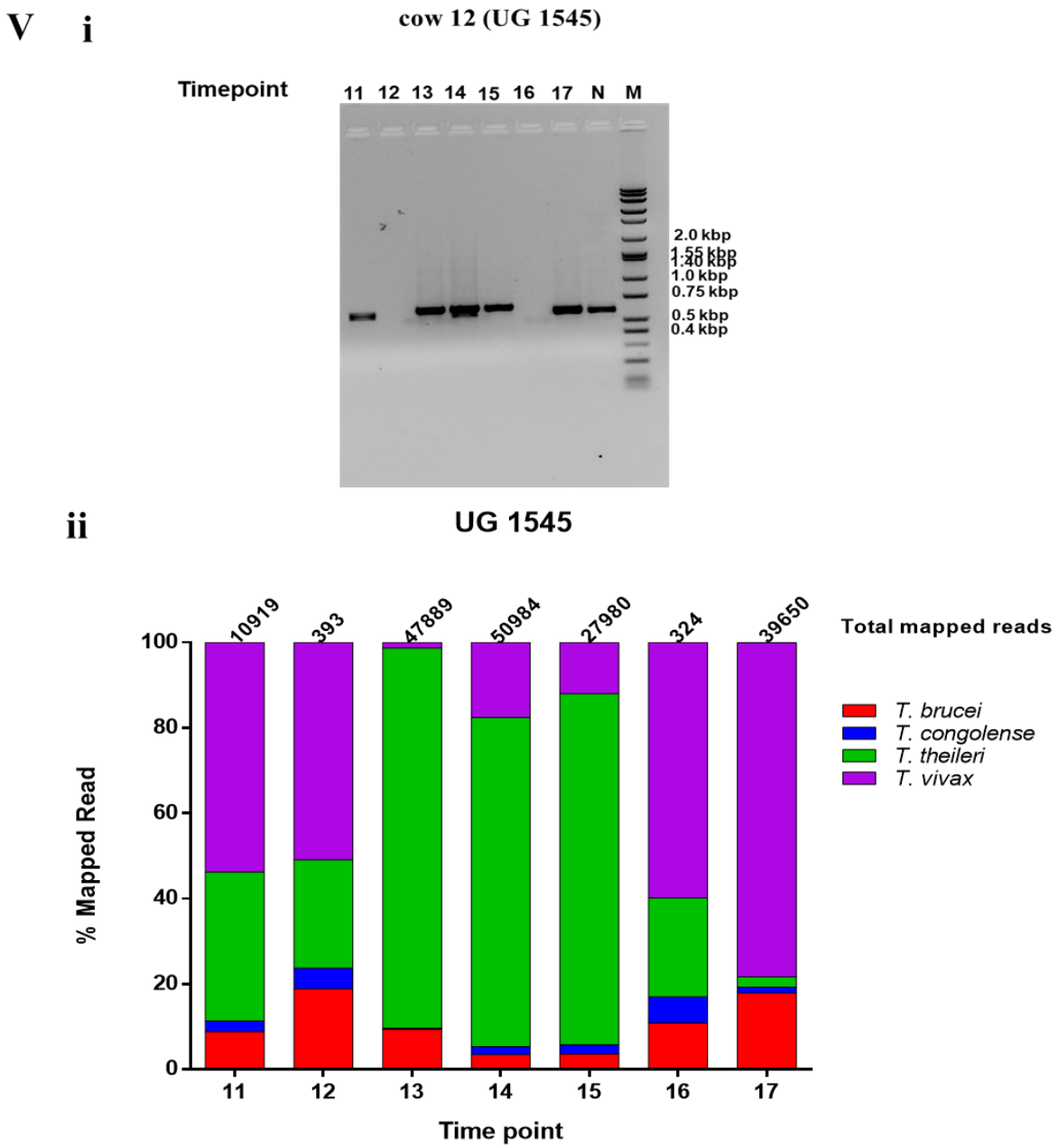
Appendix 2S: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



Appendix 2T: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



Appendix 2U: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp),* "*T. congolense" (456 bp)* and "*T. vivax" (586 bp).*



Appendix 2V: Characterization of trypanosome lifetime infections in individual cattle at Accra study site using (i) nested PCR and (ii) next generation sequencing. Expected band sizes by nested PCR for "*T. brucei" (424 bp)*, "*T. congolense" (456 bp)* and "*T. vivax" (586 bp)*.

Reagents Preparation

Reconstitution of reagents

Protease for DNA extraction

Reconstitute in 5.0 ml of double distilled water (stire in the refrigerator).

Primers

Stock concentration: 100 µM

Working concentration (10 µM): make 100 µL of a 1 in 10 dilution of each primer with PCR water

dNTPs

Stock concentration: 100 mM

Working concentration (10 mM): make 1 in 10 dilution of dNTPs by adding 10 µL of dNTPs to 90 µL of PCR water

Preparation of buffers and solutions

10X TAE buffer (Running buffer for agarose electrophoresis)

Dissolve the following in distilled water and top up to 1 L.

48.4 g of Tris base

11.4 ml of glacial acetic acid

3.7 g of EDTA

Store at room temperature.

Diluted to 1X before use

6X DNA loading dye

3 ml glycerol (30%)

25 mg bromophenol blue (0.25%)

Top up with distilled water to 10 ml.

Adjust to pH 4.6

Preparation of 500 mL 2X HMI-9 (Hirumi's Modified Iscoves medium 9)-Stock solution

Dissolve the following in 250 mL double distilled water

18.14 g HMI-9 powder

3.02 g $NaHCO_3$

14.3 μL β-mercaptoethanol

Top up with 250 mL double distilled water

Adjust pH to 7.5 and filter sterilize

Preparation of 500 mL 1X HMI-9- Working solution

250 mL double distilled water

250 mL 2X HMI-9

5 mL 100X penicillin/Streptomycin solution

50 mL Fetal calf serum

Preparation of 10 mM Tris, pH 7.2

0.06055 g Tris base (mw: 121.14 g/mol)

Add 30 mL distilled water

Adjust pH to 7.2

Top up to 50 mL with distilled water

Preparation of 10X Running buffer (polyacrylamide gel electrophoresis)

30.0 g of Tris base

144.0 g of glycine

10.0 g of SDS

Dissolved in 1000 ml of distilled H_2O.

The pH of the buffer should be 8.3 and no pH adjustment is required.

Store at room temperature

Diluted to 1X before use.