MAKERERE  **UNIVERSITY**

ANIMAL AFRICAN TRYPANOSOMAISIS AND ASSOCIATED CYTOKINE PROFILES IN NATURALLY INFECTED CATTLE IN PAICHO AND LAKWANA SUBCOUNTIES, GULU DISTRICT

BY

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DECLARATION

I, **Katiti Dianah**, hereby do declare that this study is original and has not been submitted for any other degree award to any other University before.

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DEDICATION

I dedicate this report to my parents; Mr. and Mrs. Kalibbala. I really appreciate you for your constant love, encouragement and moral support and I owe my success in life to you; I love you dad and mum.

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LIST OF ABBREVIATIONS/ACRONYMS

AAT	Animal African Trypanosomiasis
BBB	Blood Brain Barrier
CD	Cluster of differentiation
DNA	Deoxyribo Nucleic Acids
ELISA	Enzyme Linked ImmunoSorbent Assay
HAT	Human African Trypanosomiasis
HCT	Hematocrit centrifugation technique
HRP	Horse Raddish Peroxidase
IFN-ϒ	Interferon Gamma
IL	Interleukin
ITS	Internal Transcribed Spacer
Km^2	Kilometres squared
LAMP	Loop mediated isothermal amplification
NASBA	Nucleic acid sequence based amplification
NK cells	Natural Killer cells
NO	Nitric Oxide
OD	Optical Density
PARP	Procyclic Acidic Repetitive Protein

PCR	Polymerase chain reaction
RBC	Red Blood Cells
SRA	Serum Resistance Associated gene
T. brucei	*Trypanosoma brucei*
T. b. brucei	*Trypanosoma brucei brucei*
T. b. gambiense	*Trypanosoma brucei gambiense*
T. b. rhodesiense	*Trypanosoma brucei rhodesiense*
T. congolense	*Trypanosoma congolense*
T. Evansi	*Trypanosoma evansi*
T. simiae	*Trypanosoma simiae*
T. theileri	*Trypanosoma theileri*
T. vivax	*Trypanosoma vivax*
TGF-β	Transformation Growth Factor beta
TgsGP	*Trypanosoma brucei gambiense* surface glycoprotein
TNF-α	Tumor Necrosis Factor alpha
VSG	Variant Surface Glycoprotein
WHO	World Health Organization

ABSTRACT

Trypanosomes are the causative agents of Animal African Trypanosomiasis (AAT) and Human African Trypanosomiasis (HAT), the former affecting domestic animals prevalent in the sub Saharan Africa. The main species causing AAT in cattle are *T. congolense*, *T. vivax* and *T. b. brucei*. Northern Uganda has been politically unstable with no form of vector control in place. The return of displaced inhabitants led to restocking of cattle from AAT endemic areas. It was thus paramount to estimate the burden of trypanosomiasis in the region. The current understanding of cytokine profiles during trypanosome natural infections is limited. Most studies on cytokine profiles in trypanosome infections have been carried out on experimental infections using *T. congolense*. There was a need to investigate cytokine profiles in natural field conditions where animals encounter different species of varying pathogenicity including mixed infections. The major aim of this study was to assess the prevalence of trypanosome infections in cattle and the associated cytokine profiles in Paicho and Lakwana sub counties. In this study, prevalence of trypanosomiasis in cattle was determined using Hematocrit Centrifugation Technique (HCT) that was used to detect trypanosome infections in cattle while Polymerase Chain Reaction (PCR) was used to identify the infecting species and also confirm the infection status of some apparent negative samples by microscopy. To compare differences in the plasma levels of IFN-γ, TNF-α, IL-10 and TGF-β in infected and non infected cattle sandwich ELISA was then carried out. Out of the 1329 cattle screened, the microscopy prevalence of trypanosomes was 8.1% respectively. The IFN-γ and IL-10 were up regulated in infected than non-infected cattle regardless of pathogenicity of infecting species. The results indicated that up regulation of IL-10 and IFN- γ cytokine levels is not dependant on pathogenicity of species and whether it was single or mixed infections. It is thus recommended that appropriate control efforts should be put in place to prevent escalation of trypanosomiasis in the area and detailed study on possible use of IL-10 & IFN- γ as targets for supportive therapy in favor of the host.

CHAPTER ONE INTRODUCTION

1.1 Background

Animal African Trypanosomasis (AAT) affects a wide range of hosts and is caused by several trypanosome species namely; *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma evansi, Trypanosoma simiae and Trypanosoma brucei brucei*. The AAT is found mainly in those regions of Africa where its biological vector, the tsetse fly, exists. The tsetse fly infested region known as the tsetse belt covers 37 African countries including Uganda with an approximation of 10 million square kilometers (O'Gorman et al., 2006). Animal African Trypanosomiasis is the cause of death of over 3 million animals per annum with 50 million animals at risk of infection (Chitanga et al., 2011). The disease is thus a major constraint to livestock productivity that has a significant impact on the livelihood of millions of people in African developing countries, costing several billion US dollars each year (Chitanga et al., 2011).

Uganda has suffered several trypanosomiasis epidemics since the early 20th century thus the country is endemic to both AAT and Human African Trypanosomiasis (HAT) (Bardosh et al., 2013; Berrang-ford, 2007). Internal civil conflicts in the country have also escalated the burden of trypanosomiasis as they lead to increased vector- human exposure and uncontrolled vector proliferation (Berrang-ford, 2007). During political instability there is no form of vector control put in place as the area is deserted which ensures a favorable habitat for the tsetse flies.

Trypanosome infection occurs mostly through bites of infected tsetse flies or other biting flies. The infection is usually chronic and is characterized by: immunosuppression, cachexia, anemia and leukocytopaenia (Lutje et al., 1996; O'Gorman et al., 2009). Infection with African trypanosomiasis is mainly characterized with immuno- suppression which is a crucial factor in the host-parasite relationship. The trypanosomes suppress the ability of hosts to elicit a protective immune response by depressing the antibody, T cell and macrophage responses (Taylor & Mertens, 1999). Anemia is the most prominent and consistent clinical sign of infection and is the main indicator for treatment rather than parasitaemia (Trail et al., 1992).

Some African cattle breeds, like *Bos taurus* are highly tolerant of trypanosome infection, but the more productive zebu breeds are extremely susceptible. Increasing tolerance of zebu cattle

to trypanosomiasis could make a significant impact on the productivity of animals (dairy and beef produce) in addition to crop cultivation (Kristjanson et al., 1999). N'Dama cattle, are tolerant of infection with *T. congolense* and remain apparently healthy. The ability to remain productive while harboring pathogenic trypanosomes is known as trypanotolerance (Murray et al., 1984), which has been evolved as a mechanism of disease resistance possibly through the lengthy co-existence of animals with the parasites. The animals can control parasitaemia and severe anemia through an effective immune response to trypanosomes though the mechanisms are not yet well understood (Lutje et al., 1996; Yoshihara et al., 2007).

It has been observed in mouse models that a balance between pro- and counter-inflammatory cytokines is central to the outcome of the disease (Kennedy, 2008a). Cytokines play different roles in the control of infection, but over production of pro-inflammatory cytokines can lead to aggravated pathological changes (Magez et al., 2004). Some studies have suggested that Interferon Gamma (IFN-γ) stimulates parasite growth (Bakhiet et al., 1996) while Tumor Necrosis Factor alpha (TNF-α) has trypanocidal effects (Baral, 2010; Lucas et al., 1993; Magez et al., 1999). Increased production of regulatory cytokines such as Interleukin 10 (IL-10) is associated with decreased pathology and resistance to infection (Sternberg, 2005).

1.2 Statement of the problem

There is an ongoing cattle restocking program in Northern Uganda taking place following the end of the civil conflict and most of the cattle is obtained from the southern districts, including Dokolo, Lira, Kaberamiado, Serere districts, which are endemic to trypanosomiasis. Furthermore, during the insurgencies there was no form of vector control put in place since the area was deserted which led to uncontrolled vector proliferation. However there is hardly any information about the prevalence of AAT in the region yet cattle production is the major source of livelihood and trypanosomiasis has a significant impact on animal productivity. In addition, the current understanding of animal immune responses especially cytokine profiles displayed during animal trypanosomiasis natural infections is limited. Most research studies on cytokine profiles in trypanosome infections have hitherto been carried out on experimentally infected animals of which majority have been based on *T. congolense* infections. This may not portray

what happens in natural field conditions where animals are infected with different species of varying pathogenicity including mixed infections.

1.3 Justification / significance

Information about prevalence of trypanosomiasis will guide policy makers in setting up relevant anti- trypanosomiasis interventions programs in Gulu district. This research will also enhance understanding of cytokine profiles during trypanosomiasis natural settings which in turn will provide a good insight into immune response associated with disease pathogenesis. This could thus lead to the identification of novel molecules/biomarkers which could offer opportunities for advancement in trypanosomiasis anti-disease control strategy.

1.4 Objectives

1.4.1 General objective

To assess the prevalence of trypanosomiasis in cattle in Gulu district and the associated cytokine profiles.

1.4.2 Specific objectives

a) To determine the prevalence of trypanosome infections in cattle in Paicho and Lakwana sub counties.
b) To determine the plasma levels of IL-10, IFN-γ, TNF-α and TGF-β among the trypanosome infected cattle.

1.5 Research questions

a) What is the prevalence of Animal African Trypanosomiasis in cattle in Paicho and Lakwana sub counties?
b) What is the cytokine profile of trypanosome infected cattle?

CHAPTER TWO LITERATURE REVIEW

2.1 Introduction

Animal African Trypanosomiasis (AAT) is also called Nagana, dura, tsetse fly disease or the wasting disease depending on the host or the region (Lopes et al., 2010). The disease affects both wild and domestic animals including; cattle, sheep, dogs, pigs, horses, camels and others. Infection can become fatal if left untreated. Trypanosomasis in cattle is caused by; *T. congolense*, *T. vivax* and to a lesser extent, *T. b. brucei*, (O'Gorman et al., 2006, 2009). There are several clinical signs of the disease which include; fever, listlessness, emaciation, hair loss, discharge from the eyes, edema, anemia, paralysis hence weakening of the animal (Steverding, 2008). This decreases the productivity of the animals for meat and dairy produce, transportation and traction. Following death of the animal, enlarged liver and spleen, excessive fluid in the body cavities and petechial hemorrhages are observed during post mortem. Severe myocarditis is common in animals that die during the chronic phase; the lymphoid organs are usually no longer enlarged. These clinical and the post-mortem signs are pathognomonic (Losos & Ikede, 1970).

2.2 Distribution of African trypanosomiasis

The distribution of the African trypanosomiasis parallels the patchy distribution of tsetse flies (Fig 1) and comprises of an area approximately 10 million Km^2 between 14^0 North and 20^0 South (Chappuis et al., 2005; O'Gorman et al., 2006). The distribution of trypanosomes of veterinary importance varies with locality and depends on the interaction between tsetse flies, domestic and wild animals (Simukoko et al., 2007). The trypanosome species are vectored by different *Glossina* species. *Trypanosoma congolense* is preferentially vectored by *Glossina pallidipes* found near the drier forest patches while *T. brucei* and *T. vivax* are vectored by *Glossina morsitans or Glossina palpalis* found near river banks and the lakeshore (Abila et al., 2008). However in Uganda *Glossina fuscipes fuscipes* appears to be the most widespread vector and obviously transmits all these species (Abila et al., 2008).

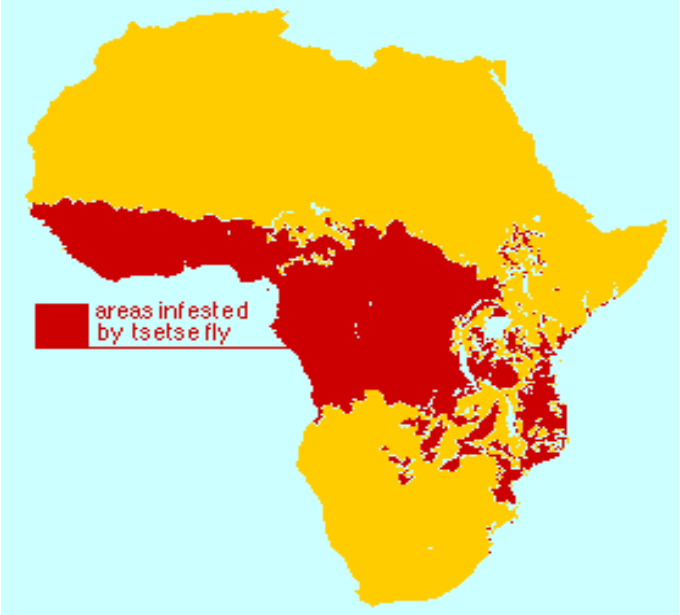

Figure 1: Map of Africa showing the distribution of tsetse flies. Adapted from FAO (1998).

2.3 Trypanosomiasis in Uganda

Uganda is located within the tsetse belt with over 70% of the country infested with tsetse flies and about one third of livestock is at risk of trypanosome infection (Bardosh et al., 2013) thus trypanosomiasis is a major constraint to rural development. Animal movements generally due to the restocking programs taking place have escalated the burden of trypanosomiasis as they lead to introduction of the disease in naive areas since animals act as reservoirs for parasites (Bardosh et al., 2013). Escalation in the trypanosomiasis cases has been also due to political unrests with in the country which led to increased vector- human exposure and uncontrolled vector proliferation since there was no form of vector control measure in place (Berrang-ford, 2007).

Uganda is the only country where both HAT forms are present with a likely potential geographical (Bardosh et al., 2013; Picozzi et al., 2005). *Trypanosoma b. gambiense* HAT is found in the Western Nile districts (Koboko, Arua, Adjuman, Moyo, Yumbe, Maracha) while *T. b. rhodesiense* HAT is in the Eastern and Southern districts (Soroti, Kaberamaido, Serere, Dokolo, Lira, e.t.c) (Bardosh et al., 2013). The potential overlap of the two HAT forms is being facilitated by movement of infected animals from endemic areas to naïve regions (Bardosh et al., 2013).

2.4 African trypanosomes

Trypanosomes are eukaryotes and share several biochemical properties, live and multiply extracellularly in blood and tissue fluids of their mammalian hosts (Barrett et al., 2003). African trypanosomes are of genus *Trypanosoma:* cause disease both in animals (AAT) and humans (HAT) of similar etiology and epidemiology. The trypanosomes are transmitted mainly by the tsetse flies of the genus *Glossina.* Some trypanosomes can also be transmitted mechanically by the blood sucking flies like the tabanids, horse flies and stomoxys (Baral, 2010). The species transmitted by the tsetse flies include; *T. brucei* sub species, *T. congolense* and *T. vivax* and undergo development in the tsetse fly vector,(Welburn & Maudlin, 1999). *Trypanosoma evansi* is transmitted mechanically and thus the infection can be got outside the tsetse belt region (Baral, 2010). Trypanosomes that are transmitted by the tsetse flies can be grouped as haematic *(T. vivax, T. congolense, T. simiae)* and cause anemia, or humoral (T. *brucei* sub species) and are associated with inflammation and tissue (Losos & Ikede, 1972).

2.4.1 Trypanosoma congolense

Trypanosoma congolense is the major cause of AAT in livestock (McDermott et al., 2003). During experimental infection with *T. congolense* in mice and cattle it was suggested that the parasites activate the complement system in presence of anti-VSG antibodies (Tabel et al., 2000). The main symptoms of *T. congolense* AAT are; low body weight, pancytopenia i.e anemia, neutropenia, lymphopenia, thrombocytopenia as well as increasing host susceptibility to secondary infections (Tabel et al., 2000).

2.4.2 Trypanosoma vivax

Trypanosoma vivax is a heteroxenous parasite that can be transmitted cyclically by tsetse flies (*Glossina*) and also mechanically by hematophagous flies (Gardiner, 1989). It's hosts include; domestic animals like the bovines, ovines where it is pathogenic and at times causes a hemorrhagic disease (Thumbi et al., 2010). The severity of *T. vivax* infection varies with host resistance and the parasite strains. The clinical signs of the infection are consistent with those of other trypanosome infections which include; anemia, edema, abortions, nose discharge and progressive weight loss (CFSPH, 2009). Cattle infected with *T. vivax* have a higher parasitaemia and a more acute disease than those infected with *T. congolense* (Taylor et al., 1996).

2.4.3 Trypanosoma brucei brucei

This is a *T. brucei* subspecies and widely distributed within the tsetse-belt region due to the distribution of the *Glossina* vector (Barrett et al., 2003). The parasite is restricted within the sub-Saharan Africa and causes a mild infection in cattle. Unlike the other subspecies of *T. brucei, T. b. brucei* is not human infective because it is susceptible to trypanosome lytic factors associated with high density lipoproteins like the human apolipoprotein L1 in human blood (Vanhamme et al., 2003).

2.4.4 Trypanosoma simiae

Trypanosoma simiae is tsetse- transmitted and belongs to the subgenus Nannomonas. It is not known to cause disease in cattle but is severely pathogenic in pigs, goats, camels and equines (Kaufmann,1996).

2.4.5 Trypanosoma theileri

Trypanosoma theileri is easily distinguished from other trypanosomes by its size. It's size ranges from 10 to 130 mm in length (Kaufmann, 1996). The parasite is generally non-pathogenic in cattle (Farrar & Klei, 1990) although patent infections may do occur. The infected cattle become anaemic but generally recover (Koch, 1904). This could probably be due to an effective control by the host's immune system.

2.4.6 Trypanosoma evansi

Trypanosoma evansi belongs to the trypanozoon group and is morphologically indistinguishable from *T. brucei* sub species. It is the causative agent of surra which affects camels, horses, cattle, pigs, buffaloes and dogs (Baral, 2010). It is transmitted mechanically by the blood sucking flies like the tabanids, stomoxys thus can be got outside the tsetse fly belt region. The parasite cannot undergo cyclical development in the vector. Symptoms of surra include; abortion, central nervous system disorders and productivity of animals is diminished (Baral, 2010). *Trypanosoma evansi* can also cause disease in man as confirmed by WHO, (2005) and Joshi et al., (2005).

2.5 Life cycle of African trypanosomes

The life cycle of trypanosomes involves differentiation into several development stages in both the tsetse vector and the mammalian host (Fig 2). Following a blood meal the tsetse fly infects the mammalian host with metacyclic trypomastigotes into the skin tissue which multiply at the site of infection before invading blood and lymph node tissues. On invading the host's blood tissues the metacyclic trypomastigotes transform into long slender bloodstream forms that are covered by the Variant Surface Glycoprotein (VSG) coat (Baral, 2010). The bloodstream forms continue invasion of other tissues like the lymph tissues. The metacyclic trypomastigotes keep on replicating to differentiate into intermediate forms and stumpy forms which are covered by the VSG, though some trypanosome species are monomorphic (Baral, 2010).

A non infected tsetse fly gets infection during a blood meal on an infected host through sucking bloodstream trypomastigotes. Once inside the tsetse fly, initial infection is established in the mid gut. The bloodstream forms undergo a series of morphological and biochemical changes where the VSG coat is shed off and replaced with coat of procyclic acidic repetitive protein (PARP) or procyclin (Baral, 2010) before transformation into procyclic trypomastigotes. The procyclic trypomastigotes which are long and slender multiply by binary fission before leaving the midgut to the foregut.

The procyclic trypomastigotes once inside the foregut transform into elongated and asymmetrically dividing epimastigotes (Baral, 2010). These multiply actively in the proboscis and then move to the salivary glands where the final transformation takes place. Inside the salivary glands the epimastigotes continue to multiply thus generating short epimastigotes that attach to the salivary gland epithelium. The attached epimastigotes differentiate into VSG-coated metacyclic trypomastigotes that are suited for mammalian bloodstream invasion. The cycle takes about 3 weeks and the fly remains infective for life (Chappuis et al., 2005).

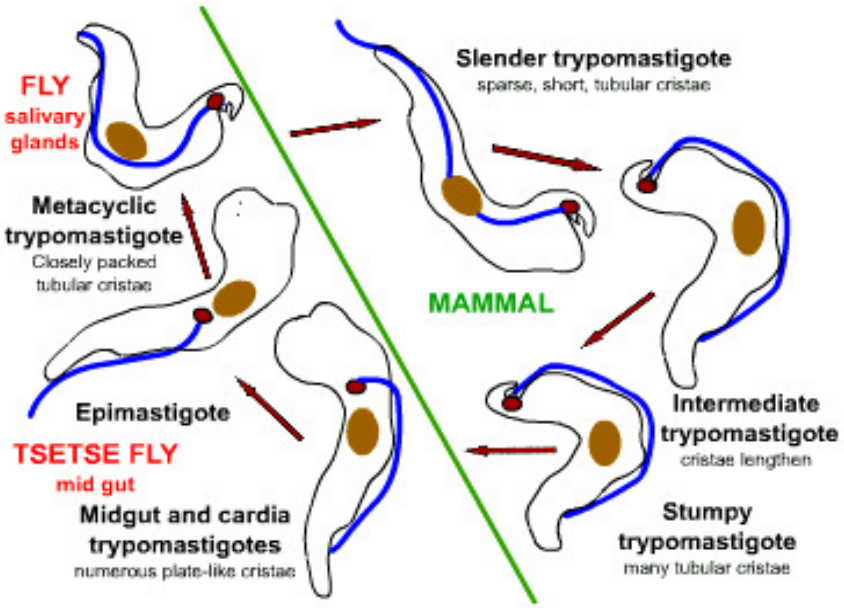

Figure 2: The life cycle of trypanosomes. Adapted from Vickerman (1985).

2.6 Diagnosis of trypanosomes

The diagnosis of trypanosomiasis requires a high degree of training and expertise where the parasite must be detected in humans before treatment can be given. This relies on conventional techniques such as lymph node puncture, blood film examination or more elaborate techniques to concentrate parasites in the blood like Hematocrit concentration technique (HCT), (Woo, 1971). The concentration of trypanosomes in the blood rise and fall often decreasing below detection levels especially in *T. b. gambiense* HAT thus examinations are usually repeated so as to demonstrate the parasite in body fluids. The different techniques used for diagnosis of trypanosome infection include; use of clinical signs, parasitological diagnosis, serological and molecular techniques.

2.6.1 Clinical diagnosis

Diagnosis of both HAT and AAT based on clinical signs alone is complicated due to lack of specific clinical signs of the disease (Nantulya et al., 1987); the clinical symptoms (persistent headache, high fevers, general body weakness and weight loss) can mimic those of other diseases like malaria, filariasis which are endemic in the tropical region.

2.6.2 Serological diagnosis

Serological techniques play a big role in the diagnosis and epidemiological assessment of trypanosomiasis especially in HAT; however, most of these methods cannot detect active infection since they rely on the presence of circulating antibodies which may be due to a past infection since antibodies can persist for up to 3 years after cure (Paquet et al., 1992).

2.6.3 Parasitological diagnosis

Traditional parasitological methods are routinely used to demonstrate presence of trypanosomes in body fluids such as blood, CSF and lymph node aspirates. Microscopy has low sensitivity; it leaves a big portion of trypanosome infections undetected (Majiwa et al., 1994) due to the fluctuating nature of parasitaemia and also it cannot detect parasites sequestered in other organ tissues (Doyle, 1977). Parasitological tools are continually relied on in the field as they can be carried out directly and give results as well as being practical in resource poor settings (Cox et al., 2010). Parasitological methods involve demonstration of trypanosomes in wet, thin and thick blood films in addition to cerebrospinal (CSF), chancre and lymph node aspirates. Detection of trypanosomes in cerebrospinal fluid confirms the involvement of the central nervous system and this differentiates between the haemolymphatic (early) stage and mengo-encephalitic (late) stage which is essential for the correct choice of treatment for HAT.

2.6.4 Molecular diagnosis

Molecular diagnostic tools have been developed with high sensitivity and specificity (Cox et al., 2005) can detect trypanosomes even in cases of low parasitaemia. Up till now molecular techniques were not easily applicable to field conditions owing to the amount of time required to process samples by conventional methods like the phenol chloroform extraction method prior to analysis and the equipments needed. This has been overcome by improved extraction kits available and preserving of DNA which makes sample collection in the field with a view of diagnosis by molecular methods simpler (Picozzi et al., 2002). The molecular tools include; Polymerase Chain Reaction (PCR), PCR ELISA, PCR hybridization, Loop Mediated Isothermal Amplification method (LAMP) and Nucleic Acid sequence based amplification assay (NASBA).

2.6.4.1 Internal Transcribed Spacer Polymerase chain reaction (ITS- PCR)

The ITS- PCR amplifies the internal transcribed spacers (ITS) regions that are located in the ribosomal genes. The ITS regions have inter-species length variations and a high copy number which are desirable qualities for molecular markers in identification and differentiation of trypanosome species (Cox et al., 2005). The nested ITS- PCR amplifies both ITS regions of the ribosomal RNA gene. Using two sets of primers (Cox et al., 2005) the PCR can detect Trypanozoon group, *T. congolense, T. vivax, T. simiae* and *T. theileri* and also mixed infections in a sample.

2.6.4.2 *Trypanosoma brucei gambiense*- surface Glycoproteins PCR

Trypanosoma brucei gambiense- surface Glycoprotein PCR amplifies the TgsGP gene which codes the *T. b. gambiense* specific flagellar pocket glycoprotein which is specific to *T. b. gambiense* (Radwanska, Claes, et al., 2002). This PCR is used to differentiate *T. b. gambiense* from *T. brucei* subspecies since TgsGP gene is specific to this sub species. It is a nested PCR using two sets of primers (inner and outer) (Maina et al., 2007; Radwanska, Claes, et al., 2002)

2.6.4.3 Serum resistance associated gene PCR

Serum Resistance Associated- PCR is based on the serum resistance associated gene which confers resistance to *T. b. rhodesiense* strains from lysis by normal human (Radwanska, Chamekh, et al., 2002). The SRA gene is specific to *T. b. rhodesiense* which distinguishes it from the other *T. brucei* sub species. It is a nested PCR using two sets of primers (Maina et al., 2007; Radwanska, Chamekh, et al., 2002).

2.7 Immune response in trypanosome infection

Several studies on trypanosmiasis that have been carried out in experimental animal models give conflicting results about the factors responsible for disease severity or tolerance (Inoue et al., 1999). Nevertheless, research carried out by Kennedy, (2008a), showed that the host immune response is essential in inhibiting trypanosome proliferation. In attempt by the host to resolve infection; VSG-specific B- and T-cell responses and macrophages are enlisted, (Baral et al., 2007; Magez et al., 2006).

O'Gorman et al., (2006) reported that changes in the cytokine production from pro-inflammatory to anti- inflammatory cytokine profiles, controls the outcome of the disease. Nevertheless, the exact role of individual cytokines is still unclear and possibly depends on genetic variations of the host or parasite and the environmental factors (Baral, 2010). Classically activated macrophages secreting pro-inflammatory cytokine are the host's first immune response during infection (Duxbury et al., 1972). Secretion of pro-inflammatory cytokines is also induced by interaction of macrophages with the GPI anchor of VSG (Coller et al., 2003; Magez et al., 2002). Over production of the pro-inflammatory molecules is detrimental to the host and can promote pathology thus it is important to down regulate the pro-inflammatory cytokines (Baral, 2010).

Studies have indicated that resistance to trypanosomiasis is conferred by the Th1 cytokine responses through limitation of parasite growth during the early stage of infection (Magez et al., 1999; Uzonna et al., 1998). Tolerance of infection relies on the host producing anti-inflammatory cytokines which induce macrophages to become anti-inflammatory (Uzonna et al., 1998). On the other hand, Th2 cytokines improve resistance to infection in cattle (Mertens et al.,1999) and mic*e* (Namangala et al., 2000; 2001).

2.8 Trypanotolerance

Research has shown that different mammalian hosts respond differently to trypanosome infection. Some hosts are genetically resistant to trypanosome infection both in natural and experimental settings compared to their similar counterparts. The BALB/c and C57B1/6 mice have varying susceptibilities to trypanosome infection. The C57B1/6 mice are resistant to trypanosomiasis compared to the BALB/c mice which are susceptible (Kaushik et al., 2000; Yoshihara et al., 2007). This occurrence has also been observed in some cattle breeds like the *Bos taurus* (N'Dama breed) found in West Africa which is relatively resistant compared to the *Bos indicus* (zebu breed). N'Dama remains healthy and productive during infection and perhaps acquired resistance to trypanosomiasis through their long coexistence with the trypanosomes. They have evolved a superior mechanism that controls trypanosome proliferation and anemia (Yoshihara et al., 2007).

The mechanism by which these hosts are able to tolerate African trypanosomiasis is complex thus not well understood (Yoshihara et al., 2007). It is thought to involve a strong immune response towards the parasite antigens as well as cytokine synthesis with lethal effects on trypanosome proliferation (Lutje et al., 1996). Several studies report that there is a difference in cytokine production between resistant and susceptible hosts (Lutje et al., 1996; Namangala et al., 2009b). Studies (Baral et al., 2007; Magez et al., 2006; Taylor et al., 1996) show that trypanotorelant hosts elicit a stronger antibody production than the susceptible ones. In trypanotorelant mice there is an increased production of interleukin (IL) 10 and IL-14 coupled with a stronger antibody response to trypanosome VSGs during infection (Namangala et al., 2009b).

2.9 Cytokines

These are low molecular weight polypeptides secreted by numerous cells which regulate and mediate the immune system, also referred to as immunodulating agents (Dinarello, 2000). Cytokines act via cell surface receptors to control the magnitude and extent of the immune-inflammatory responses. This is through activation of macrophages, controlling growth and differentiation of T and B cells. Many cytokines are pleiotropic in nature and influence synthesis and action of other cytokines. Cytokines are thus immuno- regulatory and can be classified according to their immunological role; anti-inflammatory and pro-inflammatory cytokines.

Pro-inflammatory cytokines enhance inflammation and thus their major role is to enhance systemic inflammation through attraction of inflammatory cells in addition to stimulating increased production of secondary mediators. They are also known as Th 1 cytokines and examples include; interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), Interleukins (IL)-8, IL-11, IL-12, IL-17, IL-18. Anti-inflammatory or Th 2 cytokines counteract role of inflammatory cells thus regulate the extent of inflammation during infection. They include; IL-4, IL-10, IL-13, IL-16, IFN-alpha, TGF-beta (Dinarello, 2000).

2.9.1 Tumor necrosis factor-alpha (TNF-α)

The TNF-α is a pro-inflammatory cytokine produced mainly by macrophages and other several cell types. The TNF-α initiate a surge of cytokines, macrophages and neutrophils to the site of

infection during local inflammation as it plays a major role in inhibiting profileration of intracellular parasites (Yoshihara et al., 2007). African trypanosomiasis studies carried out in mice have shown that TNF-α is associated with immune dysfunction and neuropathogenes (Magez et al., 1999; Quan et al., 2003). It was proposed that TNF-α initiates the late stage through facilitating trypanosomes to cross the blood brain barrier. This concept is supported by Maclean et al., (2004) who suggested that high levels of TNF-α could have been involved in the rapid progression to late stage HAT in Ugandan patients.

2.9.2 Interferon gamma (IFN-γ)

This is a type II interferon which is produced by activated Th1-cells (CD4+), cytotoxic T cells (CD8+) and NK cells. IFN-γ is also produced by activation of T-cells by the parasites in mice infected with trypanosomes (Mansfield, 1994; Taylor et al., 1996). The IFN-γ regulates immune and inflammatory responses as well as activation, growth and differentiation of NK cells, T-cells, B-cells, and macrophages. It also enhances antibody production in response to antigens administered simultaneously with Interferon alpha. During infection with *T. b. brucei* or *T. b. rhodesiense* in mice, IFN-γ stimulates secretion of prostaglandins and macrophage-derived nitric oxide (NO) in the peritoneal cavity and spleen (Taylor & Mertens, 1999).

2.9.3 Interleukin-10 (IL-10)

It is an anti-inflammatory cytokine which is produced by a wide range of cell populations (Asadullah et al., 2003). The IL-10 controls functions of other cells like the natural killer cells, lymphocytes, dendritic cells and monocytes (Asadullah et al., 2003). The major functions of IL-10 are to inhibit differentiation of T cells, B cells, Nk cells, mast cells and granulocytes (Asadullah et al., 2003). Discontinuing of macrophage functions is through reduction of MHC class II molecules' expression and their co-stimulators.

During trypanosomiasis IL-10 regulates production of nitric oxide, TNF-α and other pro-inflammatory cytokines regardless of continued immune stimulation by trypanosomes (Sternberg, 2005). IL-10 levels are reported to be high in susceptible mice compared to the tolerant ones (Kaushik et al., 1999). An experimental *T. brucei* infection in mice demonstrated that IL-10 has

regulatory activities that control pathogenic systemic inflammatory responses (Namangala et al., 2001).

2.9.4 Transformation Growth Factor-β (TGF-β)

It is a pleiotropic cytokine with both pro-inflammatory and anti-inflammatory properties depending on its concentration and environment (Namangala et al., 2007; Wahl et al., 1989). During high concentrations, TGF-β is an anti-inflammatory cytokine and is involved in production of IFN-γ and TNF-α by the NK cells, in addition to secretion of nitric oxide (Bellone et al., 1995; Espevik et al., 1987). The TGF-β also has pro-inflammatory functions during the early stage of infection where it elicits T cells, monocytes and neutrophilis to sites of inflammation (Wahl et al., 1989).

CHAPTER THREE MATERIALS AND METHODS

3.1 Study design

This was a cross sectional study to assess the prevalence of trypanosomiasis in cattle and the associated cytokine profiles. It was carried out in May 2012.

3.2 Study area

The study was conducted in Paicho and Lakwana sub counties, Gulu district (fig 3). The main occupation in this region is subsistence farming. Paicho sub county covers 59,307 hectares with an estimated human population of 33,159 while Lakwana sub county covers 15,615 hectares with a population of 17,768 (www.LCMT.org.).

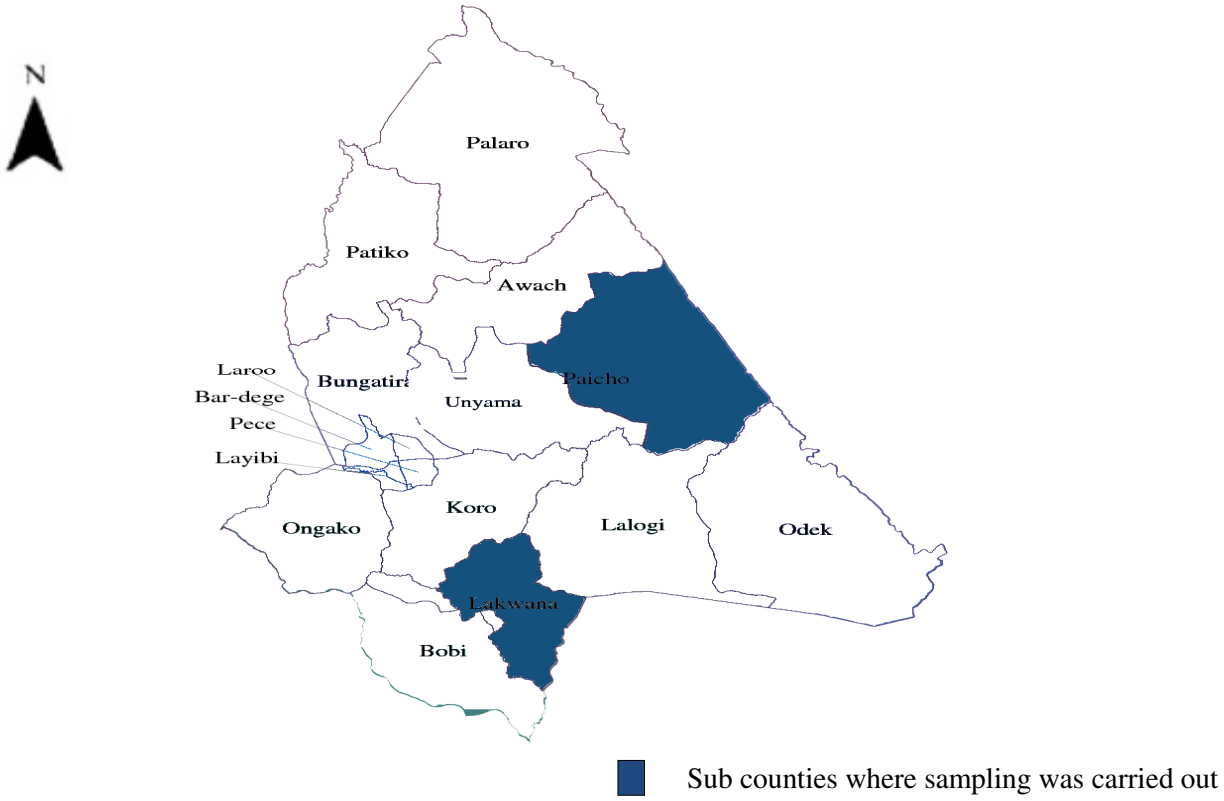

Figure 3; Map of Gulu district showing Paicho and Lakwana sub counties. Adapted from www. LCMT.org

3.3 Study population

The study population was the indigenous cattle found in the region at the time of study. All cattle presented by the farmers were screened irrespective of age or sex.

3.4 Sample size

A total of 1329 local cattle were tested for trypanosome infection using HCT as presented by the local farmers. Sample size for cytokine analysis was calculated from GraphPad StatMate 2.00 (Graphpad software Inc.) using significant level (alpha) of 0.05 and 95% power of detecting means (Maclean et al., 2007) between the cytokine assays. The calculation gave a total of 80 samples to be used in cytokine quantification analysis. Eighty two samples of whole blood and plasma were randomly selected for cytokine analysis.

3.5 Samples collection

About 5ml venous blood from the jugular vein was collected in EDTA vacutainers. The samples were immediately screened for presence of trypanosome parasites using the Hematocrit centrifugation technique which was conveniently performed onsite in the field. Up to 82 samples (30 HCT positive and 52 HCT negative) were randomly selected, processed and saved for cytokine assays and molecular analysis. Up to 500µl of whole blood was aliquoted into a 1.5ml eppendorff tube and frozen immediately in liquid nitrogen. The remaining portion (4mls) of the blood was centrifuged for 10 minutes at 3000g to obtain platelet depleted plasma (1ml) which was frozen in Liquid Nitrogen immediately. Whole blood and plasma aliquots collected were transported to the Molecular Biology Laboratory, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University.

3.6 Hematocrit centrifugation technique

A capillary tube was filled to three-quarters full with blood and one end sealed with plasticine. Basing on the Hematocrit centrifugation test (HCT) (Woo test, 1970), the capillary tubes were centrifuged at 13,000 rpm for 5 minutes to concentrate white blood cells between plasma and the RBCs. The tubes were examined under a light microscope at a magnification of ×40 with oil immersion to check for presence or absence of trypanosomes.

3.7 DNA extraction using Quick-gDNA MiniPrep kit

DNA was extracted from whole blood using the Quick gDNA Minniprep kit (Zymo Research Corp.). Genomic lysis buffer was added to 100µl of thawed whole blood (4:1) and mixed thoroughly by vortexing. The mixture was allowed to stand for 10 minutes at room temperature. There after it was transferred to a Zymo-spin column in a collection tube and centrifuged for 1 minute at 10,000g. The Zymo-spin column was then transferred to a new collection tube; the DNA pre-wash buffer was added followed by centrifugation for 1 minute at 10,000g. This was followed by addition of g-DNA wash buffer to the spin column and centrifugation for 1 minute at 10,000g. Lastly the spin column was transferred to a clean microcentrifuge tube, 50µl of DNA elution buffer added and the column incubated for 5 minutes at room temperature before centrifuging at 13,000g for 30 seconds to elute the DNA. Extracted DNA was stored at -20^0C until use.

3.8 Trypanosome species identification

Internal Transcribed Spacer- PCR (Cox et al., 2005) was used to identify and differentiate the infecting trypanosome species basing on the size of their ITS region. For cases where the ITS PCR results indicated a Trypanozoon group: Serum Resistance Associated gene (SRA) PCR and *Trypanosoma brucei gambiense* surface GlycoProteins (TgsGP) PCR were performed to rule out human infective species (Maina et al., 2007; Radwanska, Claes, et al., 2002).

3.8.1 Internal Transcribed Spacer (ITS) PCR

The nested ITS PCR used in this study targeted the ITS regions in ribosomal RNA genes and thereby produced a unique size of PCR product for each trypanosome species because of the inter-specific length variation (Table 1; fig 4). The first PCR used ITS 1and 2 primer set while ITS 3 and 4 primer set was for the second PCR run, the primer sequences were designed by (Cox et al., 2005), shown in Table 2. The 25µl reaction volume constituted of; 1X Phusion HF buffer, 0.02U Phusion DNA polymerase, 200µM dNTPs, 0.5µM of each primer and 3µl of template. The cycling conditions used; initial denaturation at 98°C for 1 min, denaturation at 98°C for 5 secs, annealing at 64°C for 30 secs, extension at 72°C for 30 secs (25 cycles) and the final extension for 72^0 C for 10 mins. The PCR conditions and cycling conditions were the same for both the 1^{st} and 2^{nd} PCR runs. A 2720 Thermal cycler (Applied Biosystems) was used and the

amplified PCR products were run on a 2% agarose gel, stained in 0.5µg/ml Ethidium bromide solution and viewed using a UV illuminator (Wag tech International) and documented.

Table 1: Differentiation of trypanosome species by ITS- PCR depends on band sizes of amplicons

Trypanosome Species	Expected band size (NCBI database) bp
T. congolense (Forest)	1513
T. congolense (Kilifi)	1422
T. congolense (Savannah)	1413
T. congolense (Tsavo)	954
Trypanozoon	1207-1224
T. simiae	850
T. vivax	611
T. theileri	988

Expected band sizes for amplification using nested ITS primers (Cox *et al.,* 2005) as they were calculated from the sequences present in the NCBI database.

3.8.2 *Trypanosoma brucei gambiense*-specific Glycoproteins (TgsGP) PCR

The TgsGP PCR was used to determine if samples identified as Trypanozoon by ITS-PCR were *T. b. gambiense.* The TbSGP primers used were designed by Maina et al., (2007). It was a nested PCR where the first PCR used the outer primer set while the second PCR used the inner primer set (sequences in Table 2). The reaction was carried out in 25µl reaction volume which was composed of: 1X Phusion HF buffer, 0.02U Phusion DNA polymerase, 200µM dNTPs, 0.5µM of each primer and 5µl of template. The product from the first PCR reaction (5µl) was used as template for the second PCR reaction. The following cycling conditions were used for both PCR runs; One cycle of initial denaturation at 94°C for 5min, 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min and then 1 cycle of final extension for 72^0 C for 5 mins, using the 2720 Thermal cycler (Applied Biosystems). 10µl of

second PCR products were run on a 2% agarose gel stained in 2µg/ml Ethidium bromide solution and viewed using a U.V illuminator (Wag tech International) and documented.

3.8.3 Serum Resistance Associated gene (SRA) PCR

The SRA- PCR was used to establish if any of the samples identified as Trypanozoon group had *T. b. rhodesiense* infection. The primers used were designed by Maina et al., (2007): the first PCR used the outer set while the inner set was for the second PCR run (Table 2). The 25µl reaction volume was made up of: 1X Phusion HF buffer, 0.02U Phusion DNA polymerase, 200µM dNTPs, 0.5µM of each primer and 3µl of template. The product (3µl) from the first PCR reaction was used as template for the second reaction. The following cycling conditions were used; I cycle of initial denaturation at 98°C for 1min, 25 cycles of denaturation at 98°C for 5 secs, annealing at 64°C for 30 secs and extension at 72°C for 30 secs and then 1 cycle of final extension for 72^0 C for 10 mins, using the 2720 Thermal cycler (Applied Biosystems). The first and the second PCRs used the same cycling conditions. The second PCR products (10µl) were run on a 2% agarose gel stained in 2ug/ml Ethidium bromide solution and viewed using a U.V illuminator (Wag tech International) and documented.

Table 2; sequences of ITS, TbSGP and SRA primers and sizes of the expected amplicons

Name	Primer sequence 5' – 3'	Expected size	Reference
ITS 1	TTG CTG CGT TCT TCA ACG AA		
ITS 2	CCG GAA GTT CAC CGA TAT TG	Refer to	Cox et
ITS 3	GGA AGC AAA AGT CGT AAC AAG	table 1	al 2005
ITS 4	TGT TTT CTT TTC CTC CGC TG		
TbSGP outer S	GCG TAT GCG ATA CCG CAG TAA		
TbSGP outer AS	5'GCT TCA ACC GCC GCT GCT TCT A		Maina et
TbSGP inner S	GCT GCT GTG TTC GGA GAG	308 bp	al., 2007
TbSGP inner AS	GCC ATC GTG CTT GCC GCT C		
SRA outer S	GAT AAA ACA AGT ATC GGC AGC AA		
SRA outer AS	CGG TGA CCA ATT CAT CTG CTG CTG TT	284 bp	Maina et
SRA inner S	ATA GTG ACA TGC GTA CTC AAC GC		al., 2007
SRA inner AS	AAT GTG TTC GAC TAC TTC GGT CAC GCT		

3.9 Quantification of cytokine concentrations in cattle plasma using ELISA

Plasma concentrations of IFN-γ, TNF-α, TGF-β and IL-10 were measured using commercially available sandwich bovine cytokine ELISA kits (TSZ ELISA^{TM} Framingham, MA) supplied as pre-coated micro plates. The solid-phase antibody was prepared by purified bovine cytokine antibody being analyzed which was pre-coated onto a micro plate. Sample diluent (40µl) was added to the micro plate wells before adding the plasma samples (10µl) to have a 5X final sample dilution. The samples and the assay standards were added in duplicate to appropriate wells and incubated for 30 minutes at 37^0C. This was followed by washing away any unbound substances. Subsequently a HRP-labeled cytokine antibody was added and incubated at 37^0C for 30 minutes so as to form a complex of antibody-antigen-enzyme labeled antibody. The plate was

again washed to remove any unbound antibody-enzyme reagent. Afterwards tetramethylbenzidine (TMB) substrate was added and plate incubated at 37^0C for 30 minutes in the dark. Lastly the Enzyme-substrate reaction was terminated by addition of Sulphuric acid solution and the absorbance read at 450nm using the ELISA reader (BioTek®) model ELX800™.

The average of the duplicate optical density (ODs) reading of the samples and standard controls was subtracted from the average of the zero standard OD. A graph showing concentration of the assay standards was drawn and the standard curve plotted. The standard curve was used in calculating the cytokine concentration of each sample. The calculated value was further multiplied by the dilution factor (X5).

3.10 Data analysis, management and presentation

The data obtained was presented in form of tables and graphs in the results section (Chapter 4). Concentrations of cytokines (ng/ml) were transferred to Excel spreadsheet and mean values computed. Statistical analysis was done using Graphpad 5.01 statistical package (Graphpad software Inc.). Plasma levels of cytokines were compared between different groups and tested for statistical significance using the Mann-Whitney test or Kruskal-Wallis test at significant level of $P < 0.05$. The different groups were:

a) Trypanosome infected
   - Pathogenic trypanosome infections
   - Non- pathogenic trypanosome infections
   - Single trypanosome infection
   - Mixed trypanosome infections

b) Non- infected cattle

CHAPTER FOUR RESULTS

4.1 Prevalence of trypanosome infections in cattle

A total of 1,329 cattle from Paicho and Lakwana sub counties were screened. Of these, 107 cattle were positive for trypanosomes by hematocrit centrifugation technique (HCT), (Table 3). There was a variation in the sample size between the two sub counties because of the cattle populations per sub county.

Table 3: Trypanosome infections of cattle detected by HCT from each sub county

Sub county	Number of cattle Screened	HCT Positives (% positives)
Paicho	400	18 (4.5%)
Lakwana	929	89 (9.5%)
Total	**1329**	**107 (8.1%)**

The prevalence of trypanosome infection was calculated based on the formula below:

$$\text{Prevalence of trypanosomiasis} = \frac{\text{Number of infected animals in study area}}{\text{Total number of animals at risk}} \times 100$$

4.2 Trypanosome species identification

Eighty two (30 HCT positive and 52 HCT negative) samples of purified DNA were subjected to ITS- PCR and those that showed trypanozoon group were later subjected to TgsGP- and SRA-PCRs in order rule out if they had human infective species.

4.2.1 Internal Transcribed Spacer PCR

In this study out of the 82 (30 HCT positives and 52 HCT negatives) randomly selected purified DNA samples; ITS- PCR detected 49 samples as AAT positives including 19 samples that were

apparent negative by HCT. These included: *T. simiae* (41%), Trypanozoon group (30.8%), *T. theileri* (17.7%), *T. congolense* (7.7%), *T. vivax* (2.6%) and (20.4%) mixed infections (table 4; fig 3).

Table 4: Percentage number of trypanosome species responsible for infections

Trypanosome species	**Number of infections (%)**
Single infection (n= 39)	
Trypanozoon	12 (30.8%)
T. congolense	3 (7.7%)
T. vivax	1(2.6%)
T. theileri	7 (17.7%)
T. simiae	16 (41%)
Mixed infections (n =10)	
Trypanozoon *& T. congolense*	1 (10%)
Trypanozoon *& T. simiae*	5 (50)
Trypanozoon & T. vivax	2 (20%)
T. simiae & T. congolense	1 (10%)
T. vivax & T. congolense	1 (10%)

See Appendix 1 for summary of HCT and PCR results.

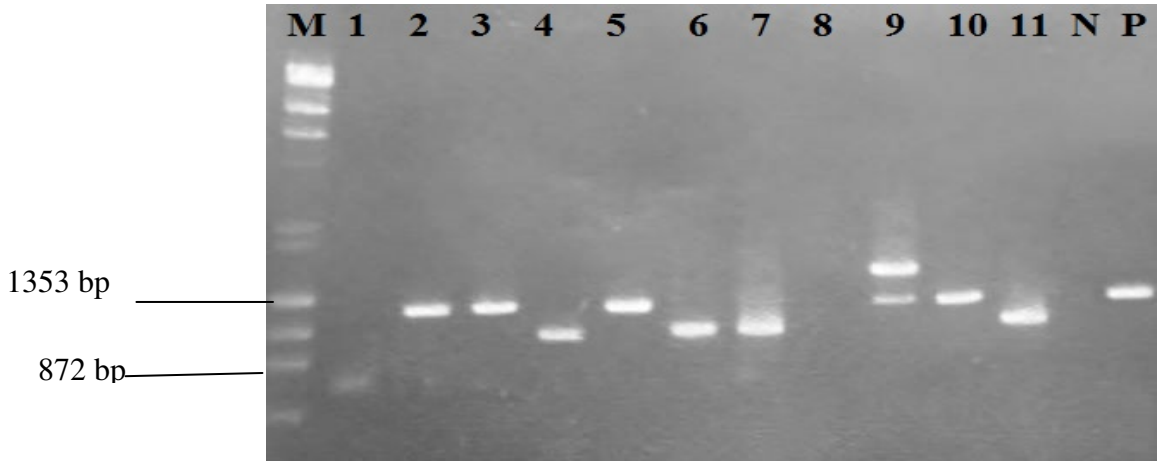

Figure 4: Nested ITS- PCR products. Lane M: Molecular marker (Finn enzymes), Lane 1: *T. vivax*, Lane 2, 3, 5 & 10 : Trypanozoon group, Lane 4, 6 , 7 & 11: *T. theileri*, Lane 8: negative sample, Lane 9: *T. congolense* & Trypanozoon mixed infection, Lane N: Negative control (PCR water), Lane 10: Positive control (*T. b.b GVR 35* genomic DNA).

4.2.2 *Trypanosoma brucei gambiense*-specific GlycoProteins PCR

The TgsGP PCR was used to differentiate *T. b. gambiense* from other *T. brucei* subspecies. All the trypanozoon samples that were detected by ITS- PCR were negative for *T. b. gambiense* when subjected to TgsGP- PCR (Figure 5). This has shown that there was no infection with *T. b. gambiense* in the trypanozoon samples.

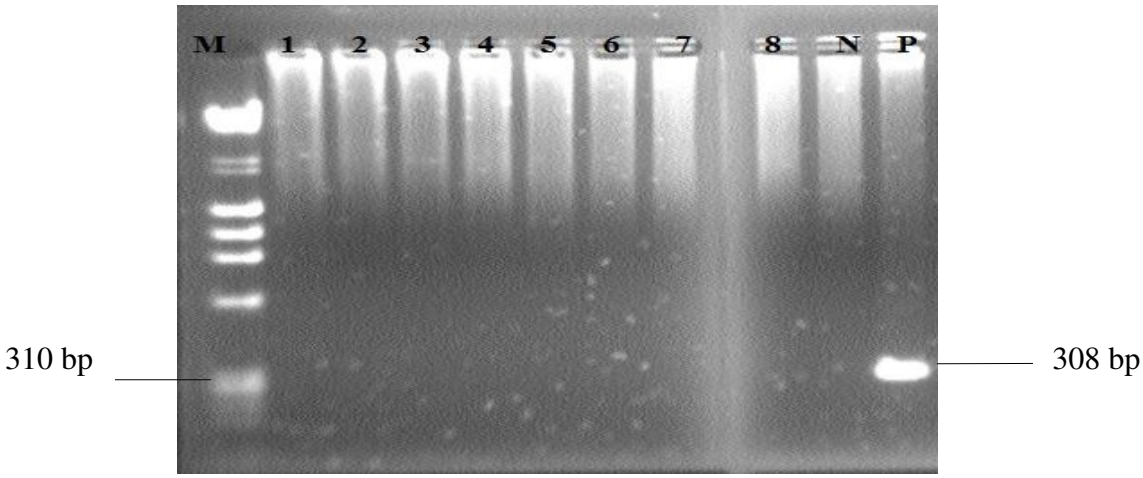

Figure 5: TgSGP PCR products. Lane M; Molecular marker (Finnzymes), Lanes 1 to 8: Trypanozoon group positive samples. Lane N: Negative control (PCR water). P: Positive control *T. b. gambiense* genomic DNA.

4.2.3 Serum Resistance Associated gene PCR

SRA- PCR was used to differentiate *T. b. rhodesiense* from other *T. brucei* subspecies. Twenty (20) samples that were trypanozoon positive (table 4) were subjected to SRA PCR. No sample

was positive for *T. b. rhodesiense* (shown in Figure 6). This indicated that there was no infection with the human infective *T. b. rhodesiense* with the trypanozoon samples.

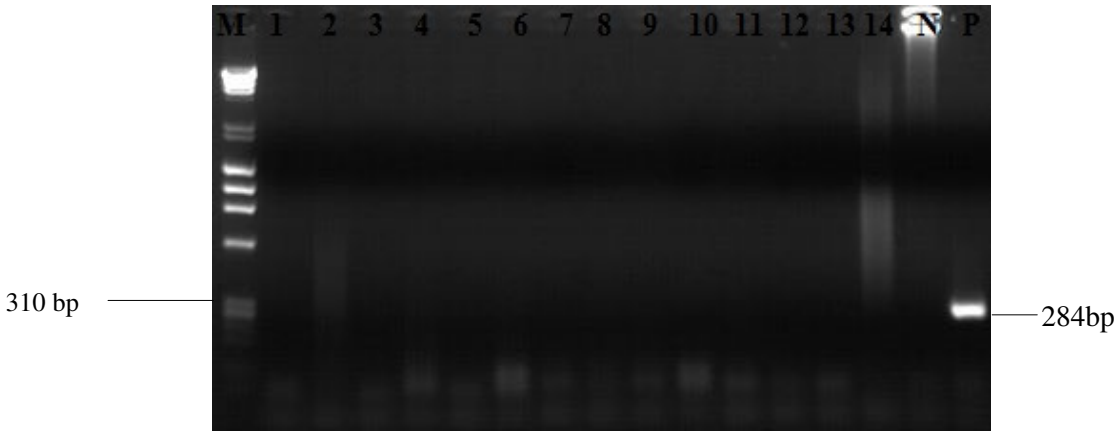

Figure 6; SRA PCR products. Lane M: molecular marker (Finn enzymes). Lane P: Positive control (Genomic DNA extracted from *T. b. rhodesiense*). Lane N: Negative control (PCR water). Lanes 1 to 16: cattle samples confirmed *Trypanozoon* positive by ITS-PCR.

4.3 Plasma concentration of cytokines

Plasma concentration of IFN-γ, TNF-α, TGF-β and IL-10 in the 82 plasma samples (49 PCR positive and 33 PCR negative) was measured using Sandwich ELISA method. Data was categorized according to non-infected, infected, pathogenic, non-pathogenic, single and mixed trypanosome infections. Comparison of plasma cytokine levels between the variable groups was tested for statistical significance using the Mann-whitney test or Kruskal-Wallis test at significant level of $P < 0.05$ with GraphPad Prism 5.01 software (Graphpad software Inc.).

4.3.1 Cytokine levels in trypanosome infected vs non- infected cattle

In trypanosome infections, the plasma concentrations of IL-10 and IFN-γ were significantly elevated (medians, 17.88ng/ml and 63.15ng/ml) over the non- infected cattle (13.62ng/ml; $P = 0.001$ and 47.40ng/ml; $P = 0.0004$ respectively). There was however no significant difference in the TGF-β and TNF-α plasma concentrations in trypanosome infected vs the non- infected cattle (table 5; figure 7). The cattle were grouped into trypanosome infected (n = 49) or non- infected (n = 33) basing on the ITS- PCR results (section 4.3).

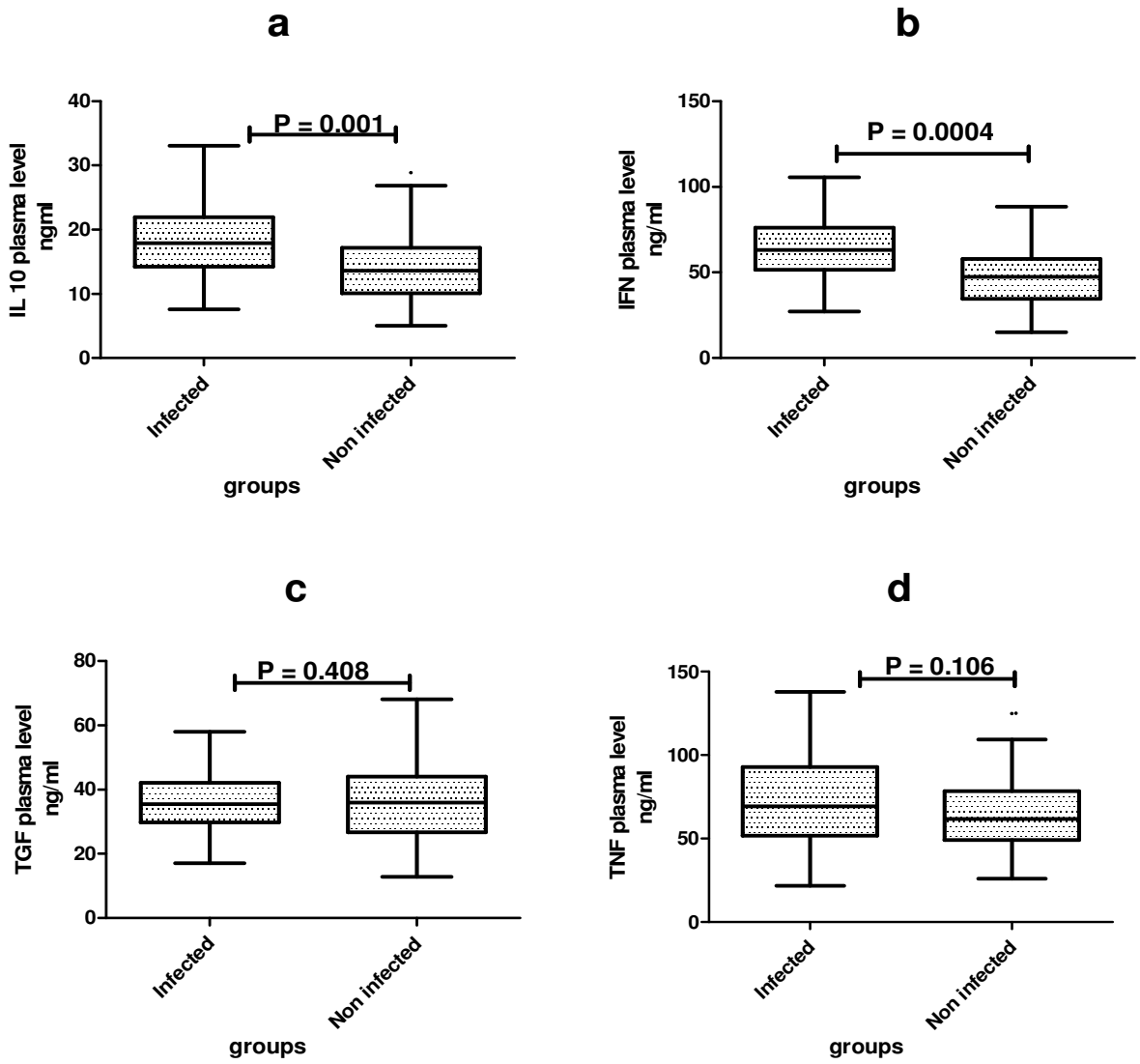

Figure 7; Plasma concentrations of IL-10, IFN-γ, TGF-β and TNF-α between trypanosome infected vs non- infected cattle. a: IL-10 plasma concentration, b: IFN-γ plasma concentration, c: TGF- β plasma concentration and d: TNF-α plasma concentration. (Mann Whitney test $P \leq 0.05$)

4.3.2 Cytokine levels in pathogenic and non- pathogenic trypanosome infections

Plasma concentrations of IL- 10 and IFN-γ were significantly elevated in pathogenic (medians, 17.29ng/ml and 62.71ng/ml) and non- pathogenic (medians, 17.88ng/ml and 59.02ng/ml) trypanosome infections over the non- infected (median, 13.62ng/ml: P = 0.007 and median, 47.4ng/ml: P = 0.004 respectively). In both the pathogenic and non- pathogenic trypanosome infections, plasma TGF-β and TNF-α were however not significantly different from the non-infected cattle (fig 8: table 5).

In pathogenic vs non- pathogenic trypanosome infections, there was no significant difference of IL- 10 and IFN-γ, TGF-β and TNF-α in both groups (fig 8: table 5). The trypanosome pathogenic species (n =16) include; *T. congolense, T. vivax* and *T. b. brucei,* while the non- pathogenic trypanosome species (n = 23) are; *T. theileri* and *T. simiae*

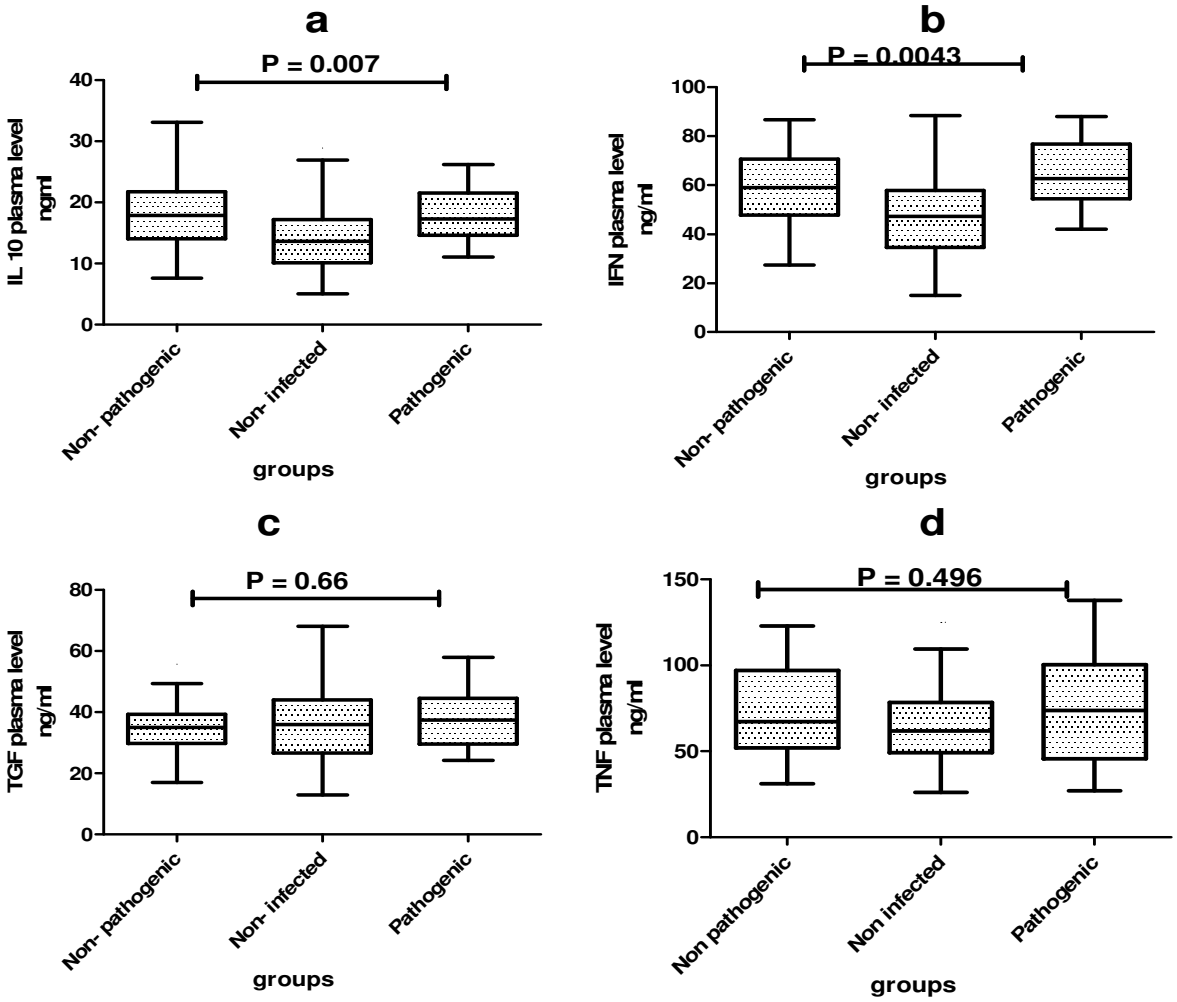

Figure 8; Plasma concentration of IL-10, IFN-γ, TGF-β and TNF-α in pathogenic and non-pathogenic trypanosome infections vs non- infected. a: IL-10 plasma concentration, b: shows IFN-γ plasma concentration, c: TGF- β plasma concentration and d: TNF-α plasma concentration. (Kruskal-Wallis test, $P \leq 0.05$)

4.3.3 Cytokine levels in single vs mixed trypanosome infections

The plasma concentrations of IL-10, IFN-γ, TGF-β and TNF-α were not significantly differently in single vs mixed trypanosome infections (table 5; fig 9). The prevalence of trypanosome mixed

infections was 20.4% being primarily due to *Trypanozoon* and *T. simiae* (50%) as shown in Table 4.

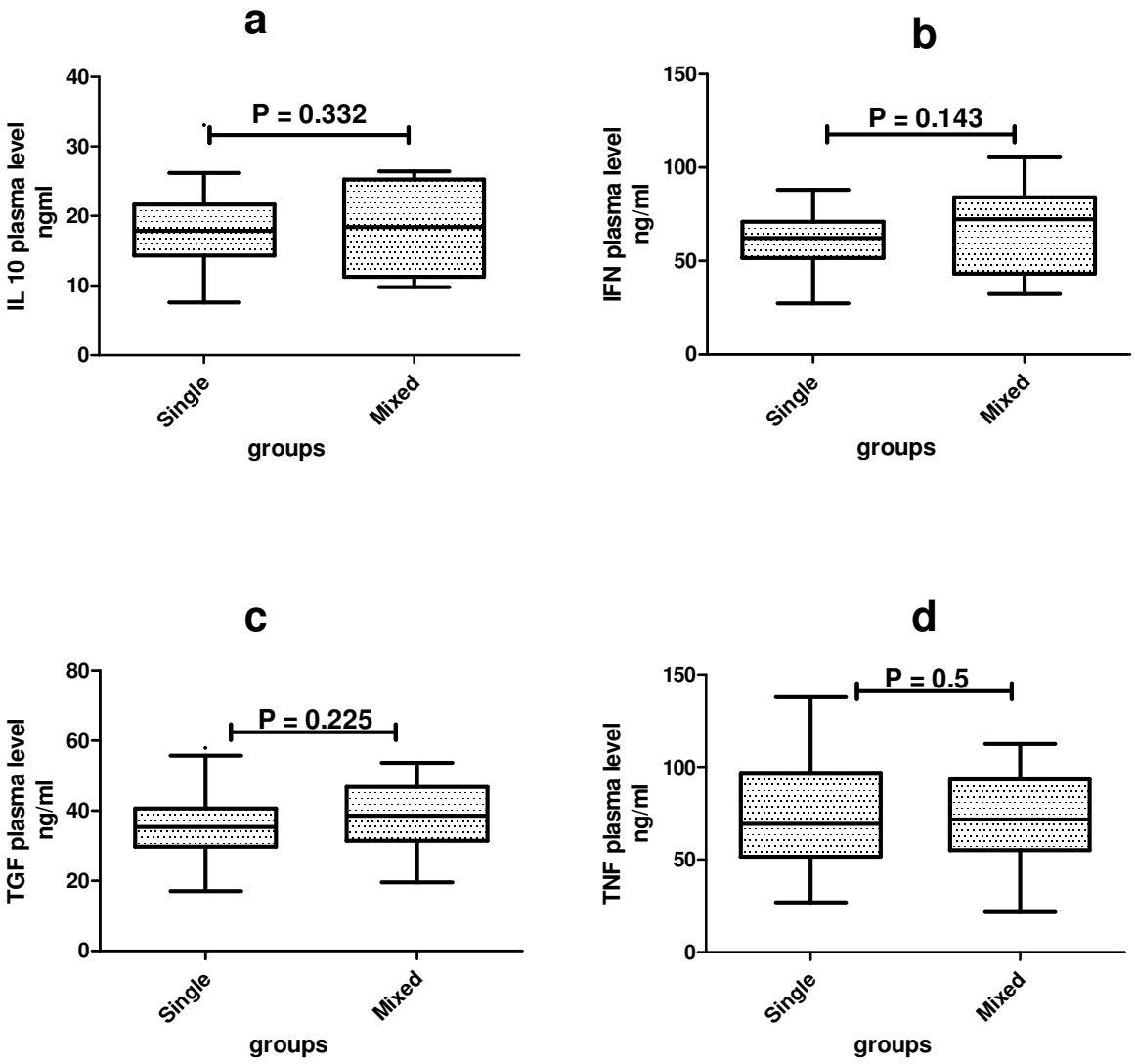

Figure 9; Plasma concentration of IL-10, IFN-γ, TGF-β and TNF-α in single vs mixed trypanosome infections. a: IL-10 plasma concentration, b: shows IFN-γ plasma concentration, c: TGF- β plasma concentration and d: TNF-α plasma concentration. (Mann Whitney test $P \leq 0.05$)

Table 5; Summary of plasma concentrations of IL-10, IFN-γ, TGF-β and TNF-α between the different variables

CYTOKINES	VARRIABLES	MEDIAN (I.Q.R)	P-VALUE
Interleukin 10 (IL 10)	Infected vs Non- Infected	17.88 (14.2 - 21.94) 13.62 (10.12 - 17.17)	**0.001***
	Non- Pathogenic vs Non- Infected	17.88 (14.07 - 21.74) 13.62 (10.12 - 17.17)	**0.007***
	Pathogenic vs Non- Pathogenic	17.29 (14.63 - 21.51) 17.88 (14.07 - 21.74)	0.472
	Single vs Mixed Infection	17.88 (14.34 -21.69) 18.42(11.29 – 25.26)	0.332
Interferon gamma (IFN-γ)	Infected vs Non- Infected	63.15 (51.53 -76.24) 47.4 (34.59 - 57.89)	**0.0004***
	Non- Pathogenic vs Non- Infected	59.02 (47.75 - 70.69) 47.40 (34.59 - 57.89)	**0.0043***
	Pathogenic vs Non- Pathogenic	62.71 (52.50 -76.75) 59.02 (47.75 -70.69)	0.196
	Single vs Mixed Infection	62.27 (51.53 -71.10) 72.19 (43.19- 84.10)	0.143
Transformation growth factor- beta (TGF-β)	Infected vs Non- Infected	35.50 (29.85 - 42.19) 35.97 (26.7 - 44.0)	0.408
	Non- Pathogenic vs Non- Infected	35.12 (29.8 - 39.36) 35.97 (26.7 - 44.0)	0.66
	Pathogenic vs Non- Pathogenic	37.48 (29.56 - 40.61) 35.12 (29.8 - 39.36)	0.192
	Single vs Mixed Infection	35.46 (29.80 - 40.61) 38.61 (31.44- 46.91)	0.225
Tumor necrosis factor-alpha (TNF-α)	Infected vs Non- Infected	69.34 (51.82 - 92.96) 61.66 (49.22 - 78.40)	0.106
	Non- Pathogenic vs Non- Infected	67.27 (51.9 - 96.98) 61.66 (49.22 - 78.4)	0.496
	Pathogenic vs Non- Pathogenic	73.78 (45.58 - 100.5) 67.27 (51.9 - 96.98)	0.494
	Single vs Mixed Infection	69.34 (51.75 – 96.98) 71.71 (55.23 – 93.32)	0.5

* P- value is significant
IQR – Inter quartile range

CHAPTER FIVE: DISCUSSION

The major aim of this study was to determine the prevalence of trypanosomiasis and investigate cytokine profiles in naturally infected cattle in Paicho and Lakwana sub counties, Gulu district. Understanding the prevalence of trypanosomiasis is useful for making informed decisions and putting relevant control measures in place (Cox et al., 2010; Welburn et al., 2001). Lakwana and Paicho sub counties were chosen as study areas because of their location in a region that had insurgencies for about two decades during which no form of vector control program/measure was in place. Since the area was deserted, this favored uncontrolled proliferation of the vector (tsetse flies) in the favorable habitat that ensued. In addition the return of displaced inhabitants following peace was accompanied by restocking of cattle mainly obtained from the southern districts where trypanosomiasis is endemic. It was thus paramount that this study was undertaken to estimate the burden of trypanosomiasis in the area.

The parasitological prevalence of trypanosome infections in cattle in Lakwana and Paicho sub counties was 9.6% and 4.5% respectively (section 4.1). There was a variation in the parasitological prevalence of trypanosome infections between Lakwana and Paicho sub counties. The high prevalence of trypanosomiasis in Lakwana sub county could be due to high tsetse-animal exposure or poor animal management systems in the area (Balyeidhusa et al., 2012). It is also important to note that Lakwana sub county borders Lira district in the south which is endemic for trypanosomiasis (Bardosh et al., 2013) thus some of the cattle screened could have come from Lira district. The overall microscopy prevalence of trypanosome infections in cattle in both sub counties was 8.1% which is comparatively high compared to other areas. This is because Balyeidhusa et al., (2012) reported an overall prevalence of trypanosome infections in cattle as 7% in 4 counties in West Nile region which is a known trypanosomiasis endemic area (Bardosh et al., 2013).

Microscopy is still the most common and suitable methods in rural African settings due to low cost and also because of poor infrastructures in the region (Cox et al., 2010; Picozzi et al., 2002). Nevertheless, microscopy has a low sensitivity and leaves a portion of trypanosome infection cases undetected (Majiwa et al., 1994). The molecular tool (nested ITS PCR) used to identify the infecting trypanosomes detected 19 samples that were apparent negative by HCT perhaps due to

low parasitaemia. Balyeidhusa et al., (2012) also reported a discrepancy between the parasitological and molecular methods as the PCR detected more infections compared to HCT. Microscopy may thus give a wrong impression of the actual AAT magnitude since it lowers the true prevalence of the disease (Nakayima et al., 2012).

Molecular diagnostic methods like PCRs were developed with high sensitivity and specificity (Cox et al., 2005) and can detect trypanosomes even in cases of low parasitaemia. The ITS- PCR identified and differentiated the trypanosomes species basing on the sizes of their ITS regions producing a unique PCR product size for each species. The ITS- PCR therefore distinguished: trypanozoon group, *T. vivax, T. congolense, T. simiae* and *T. theileri*. Moreover mixed infections were also detected, a feature which the classical parasitology can hardly achieve.

Prior studies have established *T. congolense* or *T. vivax* as the principal prevalent trypanosome species in cattle in various parts of Africa (McDermott et al., 2003; Thumbi et al., 2010). It was notable that in this study *T. simiae* (41%) was the most prevalent species in the samples subjected to ITS- PCR since its preferential hosts are pigs, sheep or goats (Kaufmann, 1996). The high prevalence of *T. simiae* detected in cattle is possibly because of the low population of pigs in the study area (Personal communication from DVO, Gulu district). *Trypanosoma vivax* had the lowest prevalence (2.6%) which could possibly be due to the relative insensitivity of ITS-PCR in detection of *T. vivax* DNA from field samples (Cox et al., 2005). But noteworthy is that the hemorrhagic syndrome that is usually exhibited by *T. vivax* infected cattle was not commonly observed in the study area. The ITS- PCR detected mixed infections in some trypanosome infected cattle. These infections are expected given that cattle are frequently bitten by infected tsetse flies that carry a range of species resulting into infections with more than one trypanosome species (Balyeidhusa et al., 2012; Nakayima et al., 2012).

In a *T. brucei* endemic area, having identified the Trypanozoon group by ITS- PCR, it was important to identify the infecting trypanosome subspecies involved (Gibson, 2001; Nakayima et al., 2012), since cattle can act as reservoirs for human infective species especially *T. b. rhodesiense*. In this study, sub-species specific PCRs (TgsGP- and SRA-PCRs) were therefore carried out to determine whether *T. b. gambiense* or *T. b. rhodesiense* were among the infecting species. All the trypanozoon group samples as detected by ITS- PCR were negative following

TgsGP- and SRA- PCRs results therefore suggesting they were all *T. b. brucei* infections, neither are there reports to date of HAT cases detected in the two sub counties.

Previous studies have stressed the importance of cytokines in regulating immune responses and outcome of the disease (Uzonna et al., 1998). Work done by Maclean et al., (2004, 2007) has indicated that the plasma levels of some cytokines increase during trypanosomiasis. This indicates that inflammation induced by these cytokines is involved in the pathology of the disease. Investigating the cytokine profile in trypanosome infected cattle would thus provide a good insight into animal immune responses during natural infections. This would be a starting point for alternative interventions such as supportive treatment to incline the immune response in favour of the host if lethal cytokines are identified that can counter trypanosome proliferation. Most of the immunology studies carried out on trypanosome infections have been based on experimental infections: less research has been done in natural infections (Guilliams et al., 2009; Namangala et al., 2000). This current study analysed the differences in plasma level of IL-10, IFN-γ, TNF-α and TGF-β in natural trypanosome infections and the non-infected cattle from the same area.

Cytokines IFN-γ and IL-10 were up regulated in trypanosome infections regardless of pathogenicity of the infecting species compared to the non infected cattle. Interleukin 10 and IFN-γ are considered to have critical roles during trypanosomiasis (Maclean, et al., 2001). Interleukin 10 is suggested to be controlling pathogenesis and immunosuppression (Kaushik et al., 1999). The probable role of IL-10 is to counteract the inflammatory surge associated with infection and also facilitate host survival by reducing the pathogenic immune effector molecules like TNF-α (Guilliams et al., 2009; Namangala et al., 2000, 2001). Increased IL-10 has also been reported in *T. b. gambiense* and *T. b. rhodesiense* infections (Maclean et al., 2001; Mertens et al., 1999). The high plasma levels of IL- 10 suggest that it has a crucial regulatory role during trypanosomiasis since it is up regulated in both AAT and HAT infections.

Interferon- γ was earlier shown to be involved in parasite control in mice trypanosomiasis studies, (Namangala et al., 2000). Later studies have suggested that IFN-γ to be involved in progression of disease to late stage through facilitating trypanosomes to penetrate the blood brain barrier (Maclean et al., 2007). Elevated levels of IFN-γ were also observed in animals during

clinical and experimental trypanosome infections, (Maclean et al., 2007; Mabbott et al., 1994). In addition there was reportedly a significant increase of IFN-γ in plasma of humans with *T. b. rhodesiense* HAT, (Maclean et al., 2007). High IFN-γ levels could be simulated by presence of parasite factors like the trypanosome lymphocyte triggering factors (Bakhiet et al., 1996).

There was no significant difference in the plasma levels of TNF-α and TGF-β in trypanosome infected cattle regardless of pathogenicity versus the non- infected. There was no up regulation of TNF-α and TGF-β in the infected cattle probably because the cytokines had less crucial roles during the infection at this stage (Liu et al., 1999). On comparison of cattle with single and mixed trypanosome infections: there was no significant difference in the plasma concentration of IFN-γ, TNF-α, IL-10 and TGF-β between the two groups. This could suggest that the immune response does not depend on the different trypanosomes species. There is also hardly any information about cytokine profiles in animals with mixed infections.

From this study we can conclude that Lakwana and Paicho sub counties had high trypanosome prevalence in cattle. In addition, cytokines IFN-γ and IL-10 are involved in pathology of the trypanosomiasis; the high plasma of cytokine levels was not dependent on the pathogenicity of infecting species and whether it was single or mixed infection. It is also worth mentioning that Gulu district is in the tropics thus the animals may have many other infections simultaneously. This may cause immune responses that interfere with that elicited purely by trypanosomes. The cytokine profiles displayed by the animals may thus be due to other diseases like the tick borne diseases or helminth infections which are common in the region.

CHAPTER SIX: CONCLUSION AND RECOMMEDATIONS

6.1 Conclusion

There was a high prevalence of trypanosomes in cattle in Lakwana and Paicho sub counties given that it is a non- endemic trypanosomiasis area. In addition, trypanosome infected cattle had high plasma concentrations of IFN-γ and IL-10 compared to the non- infected but there was no significant difference with TNF-α and TGF-β. High plasma level of cytokines was not dependent on the pathogenicity of infecting species and whether it was single or mixed infection.

6.2 Recommendations

- Appropriate control efforts should be put in place to prevent escalation of trypanosomiasis in this area. Particular attention should be put on restocking programs to prevent introduction of human infective *T. b. rhodesiense*.

- Detailed study on possible use of IL-10 & IFN- γ as targets for supportive therapy in favor of the host or biomarkers in diagnosis.

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APPENDICES

Appendix I: summary of HCT, ITS- TgsGP- and SRA- PCR results carried out on randomly selected cattle samples for cytokine quantification

Sample Code	HCT	ITS PCR						SRA PCR	TgsGP PCR
		ITS	TB	TC	TS	TT	TV		
GU 03	1	1		1	1				
GU 11	1	1			1				
GU 24	0	0							
GU 28	0	1	1		1			0	0
GU 31	1	1		1			1		
GU 34	0	0							
GU 38	0	0							
GU 53	0	0							
GU 76	1	1	1					0	0
GU 103	0	0							
GU 113	1	1	1				1	0	0
GU 135	0	1			1				
GU 124	0	0							
GU 126	0	0							
GU 166	1	1			1				
GU 186	0	1			1				
GU 197	0	0							
GU 201	0	0							
GU 211	1	1	1					0	0
GU 212	1	1				1			
GU 213	0	1			1				
GU 215	0	1			1				
GU 217	0	1			1				
GU 228	0	0							
GU 240	0	0							

GU 242	0	0							
GU 246	0	1			1				
GU 248	0	1	1					0	0
GU 254	0	1			1				
GU 260	0	0							
GU 263	0	1	1		1			0	0
GU 276	0	1	1					0	0
GU 278	1	1	1				1	0	0
GU 285	0	0							
GU 289	0	0							
GU 299	1	1	1					0	0
GU 315	1	1					1		
GU 317	0	0							
GU 319	1	1			1				
GU 340	0	0							
GU 348	0	0							
GU 358	0	0							
GU 380	1	1				1			
GU 430	1	1	1	1				0	0
GU 456	0	0							
GU 461	1	1	1					0	0
GU 470	1	1			1				
GU 484	1	1	1					0	0
GU 488	0	0							
GU 510	0	1			1				
GU 530	0	1			1				
GU 538	0	0							
GU 545	1	1	1					0	0
GU 631	1	1			1				
GU 644	1	1	1		1			0	0
GU 671	1	1	1					0	0

GU 704	0	1	1		1			0	0
GU 719	0	0							
GU 726	1	1	1					0	0
GU 738	0	0							
GU 750	0	0							
GU 767	1	1		1					
GU 780	0	0							
GU 796	0	0		1					
GU 815	1	1				1			
GU 817	0	1				1			
GU 846	0	0							
GU 855	1	1				1			
GU 862	0	1							
GU 882	0	1	1		1				
GU 891	1	1			1				
GU 900	0	1		1					
GU 923	0	0							
GU 965	1	1	1					0	0
GU 1024	0	0							
GU 1070	1	1				1			
GU 1217	1	1				1			
GU 1240	0	1	1					0	0
GU 1249	0	0							
GU 1257	1	1			1				
GU 1271	0	0							
GU 1291	0	0							
TOTAL	**30**	**49**	**20**	**6**	**22**	**7**	**4**	**0**	**0**

Appendix II: concentrations of IL-10, IFN-γ TNF-α and TGF-β (pg/ml) in the plasma of the randomly selected cattle samples

Sample Code	HCT	PCR	TNF- α (Pg/ml)	IL-10 (Pg/ml)	TGF- β (Pg/ml)	IFN- γ (Pg/ml)
GU 03	1	1	59.22	10.58	41.12	78.88
GU 11	1	1	55.36	14.34	31.56	58.88
GU 24	0	0	65.315	13.615	46.8	15.06
GU 28	0	1	88.915	26.045	45.675	85.645
GU 31	1	1	21.645	15.565	19.575	43.2
GU 34	0	0	72.465	19.145	36.56	65.5
GU 38	0	0	89.41	8.63	43.2	52.45
GU 53	0	0	125.1	28.9	49.72	88.44
GU 76	1	1	87.765	17.695	39.5	63.145
GU 103	0	0	34.95	10.6	22.5	28.075
GU 113	1	1	79.54	26.405	45.53	79.175
GU 124	0	0	65.925	13.73	42.7	49.38
GU 126	0	0	40.075	9.605	25.9	22.455
GU 135	0	1	108.98	21.735	35.16	79.175
GU 166	1	1	51.9	9.135	29.8	35.475
GU 186	0	1	53.365	9.37	29.9	37.99
GU 197	0	0	58.855	14.265	32.3	47.9
GU 201	0	0	98.49	18.185	68.2	69.495
GU 211	1	1	62.145	22.55	40.09	45.09
GU 212	1	1	78.555	20.51	39.355	63.735
GU 213	0	1	98.945	19.33	40.015	73.735
GU 215	0	1	61.295	19.45	55.8	70.975
GU 217	0	1	67.27	12.995	35.12	52.93
GU 228	0	0	65.075	12.73	34.5	45.68

GU 240	0	0	53.975	13.26	40.8	48.785
GU 242	0	0	67.755	12.61	46.15	45.09
GU 246	0	1	79.705	21.69	40.605	68.59
GU 248	0	1	104.705	21.55	45.015	88
GU 254	0	1	31.05	7.57	21.9	27.335
GU 260	0	0	52.88	9.99	34.6	40.8
GU 263	0	1	106.515	11.52	36.1	43.165
GU 276	0	1	69.34	19.1	43.25	77.265
GU 278	1	1	59.475	19.285	34.575	62.265
GU 285	0	0	85.925	20.455	47	82.22
GU 289	0	0	61.17	14.145	32.9	62.545
GU 299	1	1	108.32	21.37	49.94	81.97
GU 315	1	1	82.995	18.785	34.575	71.095
GU 317	0	0	61.66	16.21	32.4	66.095
GU 319	1	1	63.585	17.875	34.645	70.06
GU 340	0	0	50.805	10.6	28.2	40.5
GU 348	0	0	31.9	5.065	12.9	18.46
GU 358	0	0	49.34	7.63	24.5	37.69
GU 380	1	0	51.745	17.195	25.38	51.53
GU 430	1	1	112.435	24.185	53.695	105.5
GU 456	0	0	26.05	7.01	17.56	20.09
GU 461	1	1	111.445	26.18	57.995	75.205
GU 470	1	1	122.96	22.64	40.675	81.235
GU 484	1	1	26.91	13.25	24.575	42.12
GU 488	0	0	124.935	19.555	42.3	74.62
GU 510	0	1	104.87	33.075	49.355	86.675
GU 530	0	1	88.95	24.7	47.94	70.69
GU 538	0	0	63.61	22.665	45.2	41.54
GU 545	1	1	41.055	14.565	25.31	53.59
GU 631	1	1	84.145	25.135	35.455	64.03
GU 644	1	1	78.225	17.56	33.62	65.5

GU 671	1	1	54.163	11.063	33.49	62.265
GU 704	0	1	65.195	25	50.605	83.59
GU 719	0	0	44.34	10.255	26.5	31.185
GU 726	1	1	79.375	16.515	37.735	77.56
GU 738	0	0	49.585	8.425	40.4	31.48
GU 750	0	0	54.465	12.375	31.5	48.345
GU 767	1	1	47.305	13.795	28.25	51.53
GU 780	0	0	49.1	11.995	35.97	45.09
GU 796	0	1	137.765	22.14	48.84	57.22
GU 815	1	0	97.96	20.37	35.5	58.897
GU 817	0	1	46.66	9.515	27.1	40.06
GU 846	0	0	109.475	13.615	41.485	80.205
GU 855	1	1	62.145	14.53	38.9	47.75
GU 862	0	1	66.295	15.68	40.8	53.225
GU 882	0	1	43.245	9.78	24.9	32.35
GU 891	1	1	74.375	18.425	27.222	59.021
GU 900	0	1	78.225	16.88	37.22	64.67
GU 923	0	0	84.34	18.125	46	45.535
GU 965	1	1	45	14.835	24.28	58.59
GU 1024	0	0	28.975	7.16	21.5	17.575
GU 1070	1	1	44.67	16.245	31.19	43.165
GU 1217	1	1	41.38	14.065	17.1	58.88
GU 1240	0	1	34.34	16.695	34.575	60.06
GU 1249	0	0	72.27	14.675	44.8	48.635
GU 1257	1	1	96.975	22.28	36.56	63.735
GU 1271	0	0	101.085	14.265	24.355	52.93
GU 1291	0	0	34.705	26.9	26.9	47.4